Electrostatic Interactions in Collagen-like Triple-Helical Peptides†

Manju Grover Venugopal,‡ John A. M. Ramshaw,§ Emory Braswell,‖ Dan Zhu,‖ Barbara Brodsky*,‡

Department of Biochemistry, UMDNJ-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854, CSIRO Division of Biomolecular Engineering, 343 Royal Parade, Parkville, 3052, Australia, and National Analytical Ultracentrifugation Facility and Department of Molecular and Cell Biology, The University of Connecticut, Storrs, Connecticut 06269

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ABSTRACT: Collagen-like peptides with potential for ion pair formation were studied to investigate the role of electrostatic interactions in the triple-helix conformation. Three peptides—$(POG)_{10}$, the EK-containing peptide $(POG)_4EKG(POG)_5$, and T3–487, a peptide with 18 residues of type III collagen and a C-terminal $(GPO)_4$ tail—all form stable triple helices in aqueous solution, with melting temperatures of 58, 46, and 26 °C, respectively, at neutral pH. The thermal stabilities of these peptides correlate with their imino acid content, which is 66%, 60%, and 41%, respectively. Variation of pH over the range of 1–13 led to 8–9 °C changes in the T_m of the EK-containing peptide and peptide T3–487, with the greatest stability seen at pH values where both acidic and basic residues are ionized. Equilibrium ultracentrifugation shows these peptides are largely trimeric at low temperature, with no hexamers or larger aggregates, indicating that the pH-dependent stability arises from intramolecular interaction. Computer modeling indicates both intrachain ion pairs and interchain ion pairs can form and stabilize the triple helix. Studies of the pH dependence of the thermal stability of $(POG)_{10}$ and the N-terminal acetylated form of T3–487 indicate that repulsion of the three charged N-terminal or C-terminal ends has a destabilizing effect. Taking into account these end effects, the energy contribution of two oppositely charged residues in a triple helix which are sterically capable of participating in ion pairs and backbone hydrogen bonding is 0.5–1 kcal/mol ion pair. It is possible that the stabilizing influence of ion pairs arises indirectly, through elimination of like charge repulsion, formation of ion pairs in the single chain form, or solvent effects.

The triple-helical conformation is one of the basic structural motifs found in proteins and is the defining structural element of all collagens. An uninterrupted triple-helical domain of about 1000 amino acids is present in the family of D-periodic fibril forming collagens, which consists of the five homologous types I, II, III, V, and XI. There are also at least 13 other collagens types, denoted as nonfibrillar collagens, which contain triple-helical domains of varying lengths (van der Rest & Garrone, 1991; Linsenmayer, 1991). For example, type IV collagen, the major collagen in basement membranes, associates as a three-dimensional network rather than fibrils and has a 1300 residue triple-helical region. The triple helix is also a structural element in a number of proteins with host defense functions, including the macrophage scavenger receptor, C1q, pulmonary surfactant apoprotein, and mannose binding protein (Kodama et al., 1990; Reid, 1993). The basic conformation of the triple helix has been deduced from high-angle X-ray fiber diffraction studies on collagen in tendon (Rich & Crick, 1961; Ramachandran, 1967; Fraser & MacRae, 1973). Each of the three polypeptide chains in the molecule forms an extended left-handed polyproline II-type helix, which is stabilized by a high imino acid content. The three chains are staggered by one residue relative to each other and are supercoiled about a common axis in a right-handed manner to form the triple helix. Every third residue of each chain is close to the central axis, and the close packing and hydrogen bonding between the three chains requires only glycine residues at this position. Thus, conformational requirements dictate that the amino acid sequence of a triple helix can be represented as $(Gly\text{-}X\text{-}Y)_n$, where a large proportion of X residues are proline and a large proportion of Y residues are hydroxyproline. In collagen, sequences of Gly-Pro-Hyp are the most common tripeptides (about 12%), while sequences of the form Gly-Pro-Y and Gly-X-Hyp represent about 44% of tripeptides, and Gly-X-Y triplets with no imino acids constitute the remaining 44% (Fietzek & Kuhn, 1975).

Charged residues are thought to play an important role in triple-helix interactions, as indicated by their large proportion, their high degree of conservation, and their asymmetric distribution (Li et al., 1975; Trus & Piez, 1976; Katz & David, 1990, 1992). The rod-like nature of the triple helix allows a larger ratio of charged to hydrophobic residues than seen for globular proteins, as has been noted previously for rod-like coiled coil structures (Cohen & Parry, 1986). The ionizable residues Lys, Arg, Glu, and Asp constitute 15–20% of all residues in fibrillar collagens, and about 40% of all Gly-X-Y triplets contain at least one charged residue. There is a net excess of basic over acidic residues, giving collagens a basic isoelectric point. An asymmetric distribution of charged residues is found along the $(Gly\text{-}X\text{-}Y)_n$ collagen chain. Negatively charged residues are predominantly in the X position, and positively charged residues are largely in the Y position (Fietzek & Kuhn, 1975; Salem & Traub, 1975). For example, in type III collagen, 46 out of 48 Glu residues are in the X position, while 40 out of 48 Arg residues are in the Y position (Ala-Kokko et al., 1989). It has been suggested

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* To whom correspondence and reprint requests should be addressed.
‡ UMDNJ-Robert Wood Johnson Medical School.
§ CSIRO Division of Biomolecular Engineering.
‖ The University of Connecticut.

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that the preferences for X or Y positions reflect hydrogen bonding and electrostatic interactions between residues in the triple helix, as well as steric factors (Salem & Traub, 1975). Charged residues are frequently clustered along the chain, with the majority of the basic residues located within 1 or 2 residues of an acidic residue (Salem & Traub, 1975; Traub & Fietzek, 1976; Jones & Miller, 1991). The clusters of charged residues alternating with more imino acid-rich regions can be visualized by electron microscopy of parallel molecules in SLS aggregates (Bruns & Gross, 1973; Doyle et al., 1974). The SLS patterns, together with the sequence data, indicate that the distribution of charged residues is highly conserved among the members of the fibril forming family, over a broad range of phyla (Bruns & Gross, 1973).

The unique conformation of a triple helix gives a charged residue the potential to be involved in intrachain ion pairs, interchain ion pairs between the three chains of one triple-helical molecule, or interactions with other molecules (Katz & David, 1990, 1992; Trus & Piez, 1976). Modeling indicates that ion pairs can form within a single chain when oppositely charged residues are separated by either one or two residues (Katz & David, 1990). Electrostatic interactions are also sterically possible between the three chains in a molecule when oppositely charged residues are adjacent or separated by one residue along the chain (Salem & Traub, 1975; Traub & Fietzek, 1976; Katz & David, 1992). The thermal stability of collagen shows a pH dependence, with the greatest stability when all side chains are ionized, supporting a role for ion pairs in molecular stability (Barber & MacKay, 1966). In addition to interactions with other residues in the same molecule, the X and Y residues of a given chain are available for interactions with other molecules. All of the non-glycine residues in the triple helix are substantially exposed to solvent, with the X position being more exposed than the Y (Jones & Miller, 1991). Calculations indicate that electrostatic interactions are important in the interactions between neighboring collagen molecules associated in D-periodic fibrils (Hulmes et al., 1973; Trus & Piez, 1976), and these calculations are supported by chemical modification studies, the pH dependence of fibril formation, and ion binding studies (Li et al., 1975). Charged residues have also been implicated in binding of collagen to other extracellular matrix components, such as cell surface integrins (Staatz et al., 1991). These observations suggest a uniquely varied role for charged residues at different levels in the function of collagen. However, the large number of charged residues in a collagen molecule makes it difficult to isolate and define individual interactions.

We have approached the characterization of electrostatic interactions in the triple helix through studies on three synthetic peptides containing a small number of ionizable residues in defined positions. To determine end effects that result from the relatively short length of these peptides, studies were carried out on the stable triple-helical peptide $(Pro\text{-}Hyp\text{-}Gly)_{10}$, whose only ionizable groups are the N- and C-terminal ends. A peptide homologous to $(Pro\text{-}Hyp\text{-}Gly)_{10}$ with the central Pro-Hyp residues replaced by Glu-Lys was used to examine the effect of a pair of adjacent charged residues on triple-helix properties. To place the effect of electrostatic interactions in the context of a collagen sequence, a model triple-helical peptide containing 18 residues from the type III collagen was studied. Our results indicate that although the imino acid content is the dominant determinant of stability, the formation of a variety of intrachain and interchain ion pairs within a triple-helical molecule also make contributions that may be important in function.

MATERIALS AND METHODS

Peptides. The peptide $(Pro\text{-}Hyp\text{-}Gly)_{10}$ [designated as $(POG)_{10}$],[1] was purchased from Peptides International, Louisville, KY. The peptides $(POG)_4EKG(POG)_5$ (designated as EK-containing peptide or EK peptide) and GKOGEOGPKGDAGAOGAO$(GPO)_4$GV (designated as T3–487) and its N-terminal acetylated derivative (designated as AcT3–487) were synthesized on Applied Biosystem 430A peptide synthesizers using a standard t-Boc N-protection strategy on t-Boc-L-Gly-PAM and t-Boc-L-Val-PAM resins, respectively. Side chain protection was benzyl for Hyp, benzyl ester for Glu and Asp, and 2-chlorobenzyloxycarbonyl for Lys. For AcT3–487, acetylation was by acetic anhydride and triethylamine in dimethylformamide. For the EK-containing peptide, all amino acids were double coupled, and cleavage from the resin was by HF. For T3–487 and AcT3–487, cleavage from the resin used a low trifluoromethanesulfonic acid/high HF approach. The purity of all peptides was greater than 95% by reverse-phase HPLC on a Vydac C-18 column. Amino acid analysis using a Waters HPLC system with ninhydrin detection confirmed the compositions of peptides T3–487 and Ac T3–487. The identities of these peptides and the EK-containing peptide were further confirmed by liquid secondary positive-ion mass spectroscopy using a VG Analytical ZAB-T instrument at the Center for Advanced Food Technology at Rutgers University.

Circular Dichroism Spectroscopy. Samples for circular dichroism (CD) spectroscopy were prepared at a concentration of 1.0 mg/mL, in an appropriate buffer adjusted to a pH value ranging from 1 to 13. For pH 1–3, peptides were dissolved in either 0.1 M HCl and 0.1 M acetic acid or a mixture of these. For pH 4–9, peptides were dissolved in either 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 or a mixture of these. For pH 10–13, peptides were dissolved in either 0.1 M Na_2HPO_4 or 0.1 M NaOH, or a mixture of these. There was little change in the pH value with temperature, with the pH at 10 °C within 0.2 pH units of the pH at 90 °C. A small change in ionic strength was observed with the change in the composition of the buffers. The highest ionic strength was 0.3 units, which is present at pH 9, while the lowest ionic strength was 0.1, found at pH values of 4 and below and at 13. For comparisons at different pH conditions at equivalent ionic strength, NaCl was added as required.

CD spectra were recorded on an Aviv Model 62DS spectropolarimeter. All solutions were equilibrated at 4 °C for at least 24 h prior to recording spectra. Cells of path lengths from 0.1 to 1 mm were used, and the temperature in the cell was controlled using a Hewlett Packard Peltier thermoelectric temperature controller. The spectra were recorded from 250 to 185 nm. For wavelength scans, the signal was averaged for 5 s, and data points were collected every 0.5 nm. The wavelength near 225 nm, at which a maximum was observed, was kept constant as the temperature was increased to obtain the thermal transition curve. The

[1] Abbreviations: CD, circular dichroism; NMR, nuclear magnetic resonance; T_m, melting temperature; standard one-letter and three-letter abbreviations are used for the common amino acids, with hydroxyproline denoted by O (one-letter code) and Hyp (three-letter code); thus $(POG)_{10}$ is used for $(Pro\text{-}Hyp\text{-}Gly)_{10}$. EK-containing peptide is used to denote the peptide 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. T3–487 is used to denote the peptide Gly-Lys-Hyp-Gly-Glu-Hyp-Gly-Pro-Lys-Gly-Asp-Ala-Gly-Ala-Hyp-Gly-Ala-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Val, and AcT3–487 is used to denote the N-terminal acetylated derivative of peptide T3–487.

maximum occurred at 225 nm for $(Pro\text{-}Hyp\text{-}Gly)_{10}$ and at 224 nm for EK-containing and T3–487 peptides. For the equilibrium melting transitions, the temperature in the cell was either increased at a constant rate of 0.1 °C/min or was increased in increments of 0.3 °C followed by equilibration for 3 min at each temperature before collecting a data point. The temperature at which the first derivative, $d\theta/dT$, of the transition curve is a minimum indicates the inflection point of the curve and is used as a measure of thermal stability. The T_m obtained after correcting for the sloping monomer baseline (fraction folded = 0.5) is typically 1 °C lower than the value obtained from the first derivative, and its value is sensitive to the slope selected. The T_m obtained from curve fitting data in the thermodynamic analysis is 1.5–2.5 °C higher than the first derivative value, but significant error (at least 10%) arises in fitting the initial and final sloping regions of the curve. Since the errors that arise in using the fraction folded and curve fitting are characteristic of each peptide, the simple derivative of the observed data was used as a measure of thermal stability for the comparison of different peptides.

Calculation of Thermodynamic Parameters. The equilibrium melting curves showed a flat region at low temperatures (trimer region), followed by a sharp transition, followed by a linear change in ellipticity in the monomer region. The fraction folded was calculated from the experimental curve as previously described (Engel et al., 1977; Long et al., 1993). The slope of the linear monomer region was determined by collecting data points every 0.3 °C for more than 10 °C above the trimer to monomer transition, and this slope was used to extrapolate to lower temperature to obtain the monomer ellipticity value. The equilibrium melting curves were fit to a two-state trimer to monomer transition, and the T_m and $\Delta H°$ at 25 °C were determined by curve-fitting as previously described (Engel et al., 1977; Marky & Breslauer, 1987). The $\Delta S°$ and $\Delta G°$ values were calculated using the T_m and $\Delta H°$ values.

Equilibrium Sedimentation. Prior to sedimentation, peptides were dissolved in 0.1 M sodium phosphate buffer, pH 7.0, at a concentration of 1.0 mg/mL, and dialyzed (Spectra/Por, molecular weight cutoff of 1000) against the same buffer for 18 h at 4 °C. Runs were carried out at both 10 and 30 °C in a Beckman Model E analytical ultracentrifuge, at a speed of 52 000 rpm, as previously described (Long et al., 1993). The concentration resulting from the sedimentation ranged from about 10 μg to 1–3 mg/mL.

Data analysis were performed using a nonlinear least-squares program, assuming various models, as previously described (Long et al., 1993; Johnson et al., 1981). The criteria for goodness of fit are that a reasonable value of the monomer molecular weight be produced, that the rms error be small (usually less than 0.02 fringe, which is approximately 10 μg/mL) and that there be little systematic error in the residuals. To calculate molecular weight, values of the specific volume (v) were calculated from the amino acid composition data (Cohn & Edsall, 1943) and were found to be 0.695 and 0.698 for the EK-containing peptides and T3–487 peptides, respectively. The density of the solvent was estimated from density tables at 10 and 30 °C to be 1.014 and 1.010 g/mL, respectively.

Computer Modeling. The coordinates for the starting structure of $(POG)_{10}$ were obtained from X-ray fiber diffraction data (Fraser et al., 1979). The model for the EK-containing peptide conformation was generated from $(POG)_{10}$ by replacing the central Pro-Hyp residues by Glu-Lys, and models for the T3–487 conformation were generated by adding residues GKOGEOGPKGDAGAOGAO to the N-terminus of a $(GPO)_4$ block. The conformation of isolated triple helices of these model peptides was then energy minimized with IMPACT (Kitchen et al., 1990), using the method of steepest descent and conjugated gradient algorithms. A distance-dependent dielectric constant was used to simulate the effect of solvent.

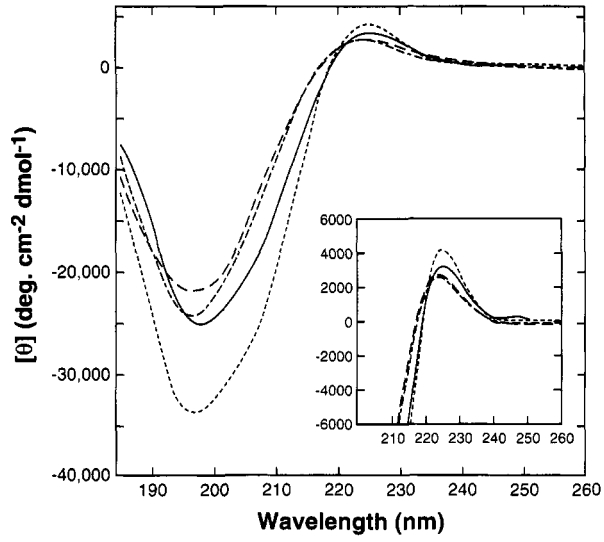

FIGURE 1: Circular dichroism spectra of peptides $(Pro\text{-}Hyp\text{-}Gly)_{10}$ [- - -], EK-containing peptide [—], T3–487 [– – –], and AcT3–487 [– · –] at 1 mg/mL at pH 1, 10 °C, showing mean residue ellipticity as a function of wavelength. The insert includes a magnified scale to show the maximum near 225 nm.

RESULTS

(a) End Effects: Studies of $(Pro\text{-}Hyp\text{-}Gly)_{10}$. The peptide $(POG)_{10}$ has been shown by sedimentation equilibrium to be fully associated as trimers in aqueous solution at 10 °C (Sakikabara et al., 1973; Long et al., 1993). Two-dimensional NMR studies indicate these trimers adopt a closely packed triple-helical structure similar to the model derived from fiber diffraction (Li et al., 1993). The circular dichroism (CD) spectrum of $(POG)_{10}$ has a maximum ellipticity at 225 nm and a minimum at 198 nm which is characteristic of a triple helix (Figure 1). The magnitude of this 225-nm maximum can be followed to monitor triple-helical stability as a function of temperature, and the first derivative of this transition curve gives a measure of its thermal stability (Figure 2).

The thermal stability of $(POG)_{10}$ is dependent on pH (Figure 3). The terminal groups on this peptide are the only ionizable groups, so the effect of pH on its thermal stability can clarify the electrostatic end effects associated with using relatively short peptides as model systems for collagens. The N-terminal imino group is expected to have a pK near 8–9, higher than that of an amino-terminal group (Dawson et al., 1986). The C-terminal carboxyl group is expected to have a pK near 2–3 (Yang et al., 1993). The thermal stability of this peptide is greatest at acidic and basic pH values, with the T_m = 61 °C at pH 1 and T_m = 62 °C at pH 11. The melting temperature, T_m, decreases as the pH values become more neutral, with a minimum T_m of 56 °C at pH 6.0. Thus, a decrease in stability of 4–5 °C is observed for the form where both the N- and C-terminal groups are ionized, compared to the case where only the N-terminal groups or only the C-terminal groups are ionized. The midpoint of the C-terminal carboxyl pK is near 3.5 and the midpoint of the N-terminal imino pK is near 9.

A one-residue stagger arrangement of three parallel chains in the highly ordered $(POG)_{10}$ helix would leave the three

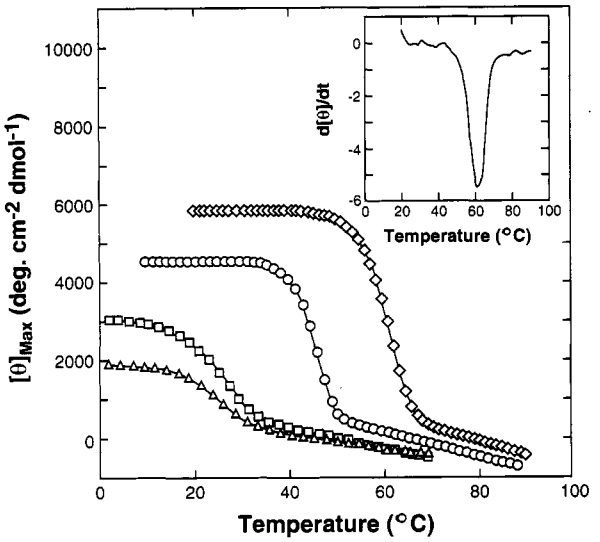

FIGURE 2: Thermal equilibrium transitions of peptides (Pro-Hyp-Gly)$_{10}$ [◇], EK-containing peptide [○], T3–487 [△], and AcT3–487 [□] at acidic pH. The ellipticity of each peptide at the wavelength of its maximum was followed as a function of temperature [225 nm for (POG)$_{10}$ and 224 nm for the other peptides]. All peptides are at a concentration of 1 mg/mL at pH 3. The inset shows the first derivative of the ellipticity as a function of temperature for (Pro-Hyp-Gly)$_{10}$, which was used to obtain the melting temperature.

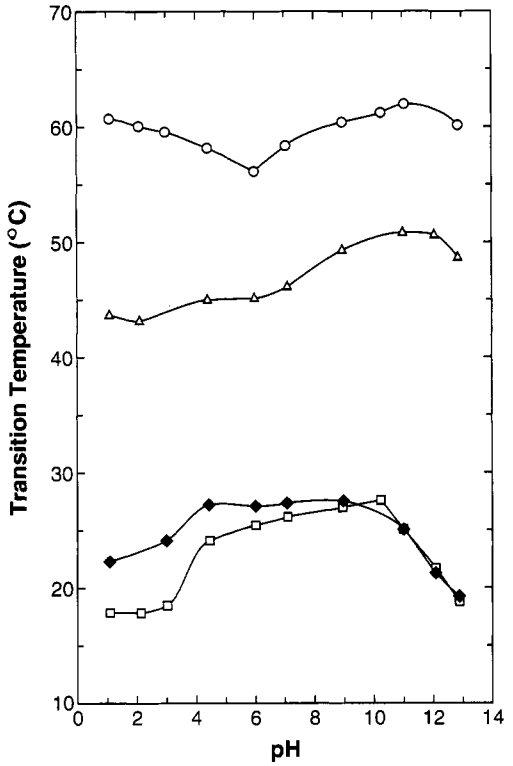

FIGURE 3: Profile of the thermal stability as a function of pH is shown for (Pro-Hyp-Gly)$_{10}$ [○], the EK-containing peptide [△], T3–487 [□], and AcT3–487 [◆].

charged terminal amino and carboxyl groups in close proximity at neutral pH (Figure 4). This could result in increased disordering of the ends of the peptide chains and interruption of interchain hydrogen bonding near the termini. Thermodynamic analysis (Table 1; see next section) suggests that the decreased stability at neutral pH may be enthalpically driven, which would be consistent with the expected disruption of hydrogen bonds. The data show broad titration curves for the end groups, and this breadth is likely be due to the presence of three distinct ionizations occurring at each end. To cover

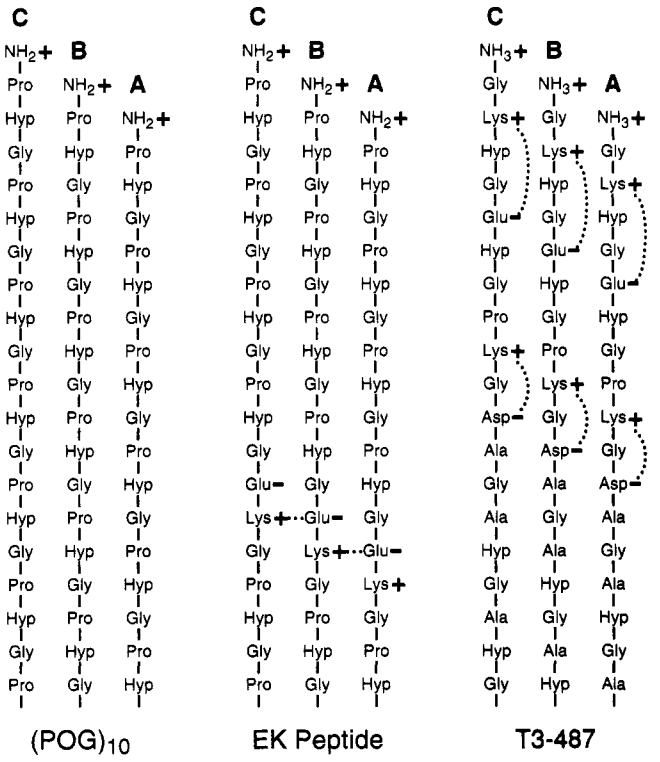

FIGURE 4: Schematic models of the N-terminal sequences of peptides (Pro-Hyp-Gly)$_{10}$, the EK-containing peptide, and T3–487 at neutral pH. A triple-helical form with three chains, A, B, and C, staggered by one residue is assumed, to show the relative positions of the charged residues.

Table 1: Thermodynamic Parameters Calculated for (Pro-Hyp-Gly)$_{10}$ and EK-Containing Peptide at Acidic, Neutral, and Basic pH Values

peptide	pH	T_m[a] (K)	$\Delta H°$ [b] (kcal/mol)	$\Delta S°$ (eu/mol)	$\Delta G°$ (kcal/mol)
(Pro-Hyp-Gly)$_{10}$	1	334	−158	−439	−27.6
	7	331	−156	−436	−26.4
	13	334	−169	−473	−28.6
EK-peptide	1	317	−183	−542	−21.3
	7	319	−176	−517	−22.1
	13	322	−151	−437	−21.5

[a] The T_m listed here is the minimum of the first derivative of the equilibrium melting curve. [b] The enthalpy was determined as a best fit to the data using a two-state model. There is considerable error in the fit, of the order of about 10%.

the wide range of pH values used in these studies, various buffer conditions were used which resulted in small changes in ionic strength of at most 0.2 units. Studies on peptides where ionic strengths were adjusted by the addition of NaCl indicated that the ionic strength changes of this magnitude produce a change of at most 0.4 °C in the melting temperature and are not significant compared to the differences observed as a result of change in pH (data not shown). Repeat equilibrium melting curves (n = 2, 3, or 4) were carried out on independently prepared solutions of selected specimens, to assess the accuracy of the T_m values. These results indicated that repeated T_m measurements had a standard deviation of ±0.3 °C.

(b) Isolated Charged Pair: Studies on a Model Peptide Containing Glu-Lys. To investigate the effect of charged side chains on the thermal stability of the triple helix, a peptide was made containing one charged triplet, (Pro-Hyp-Gly)$_4$-Glu-Lys-Gly-(Pro-Hyp-Gly)$_5$, and this is referred to as the EK-containing peptide. This peptide has a negatively charged

Table 2: Charges Present on Each of the Three Peptides Are Shown at Acid, Neutral, and Basic pH Values, Together with Their Melting Temperatures

peptide	pH	charge distribution	T_m (°C)
$(POG)_{10}$	1	$^+H_2$NPOGPOGPOGPOGPOGPOGPOGPOGPOGPOGCOOH	61
	7	$^+H_2$NPOGPOGPOGPOGPOGPOGPOGPOGPOGPOGCOO$^-$	58
	13	HNPOGPOGPOGPOGPOGPOGPOGPOGPOGPOGCOO$^-$	60
EK-peptide	1	$^+H_2$NPOGPOGPOGPOGE$^+$KGPOGPOGPOGPOGPOGCOOH	44
	7	$^+H_2$NPOGPOGPOGPOGE$^-$K$^+$GPOGPOGPOGPOGPOGCOO$^-$	46
	13	HNPOGPOGPOGPOGE$^-$KGPOGPOGPOGPOGPOGCOO$^-$	49
T3–487	1	$^+H_3$NGK$^+$OGEOGPK$^+$GDAGAOGAOGPOGPOGPOGPOGVCOOH	18
	7	$^+H_3$NGK$^+$OGE$^-$OGPK$^+$GD$^-$AGAOGAOGPOGPOGPOGPOGVCOO$^-$	26
	13	H_2NGK$^-$OGEOGPK$^-$GDAGAOGAOGPOGPOGPOGPOGVCOO$^-$	19

residue in the X position (Glu) and an adjacent positively charged residue in the Y position (Lys) substituted for Pro-Hyp in the middle of the $(POG)_{10}$ peptide. The effects of the terminal groups should be similar to those seen in $(POG)_{10}$, but there is now the possibility of the formation of ion pairs and hydrogen bonding at pH values where both Glu and Lys are ionized, and the possibility of hydrogen bonding with backbone groups or charge repulsion at other pH values.

Equilibrium ultracentrifugation on the EK-containing peptide indicates that this peptide is almost completely in the trimer form at 10 °C, with a molecular weight very close to that expected for a pure trimer. At 30 °C, the peptide is still largely trimeric with a small amount of monomer present. The results indicate that this peptide at neutral pH fits a two-state monomer to trimer model with an equilibrium constant of 15 [units of $K(L/g)^2$] at 10 °C, while at 30 °C the value was 10. There was no detectable hexamer or larger molecular weight form present, even at the highest concentrations (3 mg/mL). At all pH values examined, the peptide has a CD spectrum with the characteristic 224-nm maximum and 198-nm minimum expected for a triple-helical conformation (Figure 1). A sharp thermal transition is observed as the peptide undergoes a trimer to monomer transition (Figure 2). Compared with $(POG)_{10}$, the EK-containing peptide forms a triple helix which is less stable, with a T_m of about 46 °C compared with 58 °C at neutral pH.

The thermal transition of the EK-containing peptide was determined as a function of pH (Figure 3). In contrast to $(POG)_{10}$, the stability of this peptide is lowest at acidic pH values (T_m = 44 °C) and increases, with a plateau between 4.5 and 6, as the pH is increased. The deprotonation of the C-terminal groups as the pH values increase from 1 to 6 must be destabilizing as found for $(POG)_{10}$, but this loss of thermal stability is now compensated for by the ionization of the Glu residues (pK 3.5–4.5), which become capable of forming ion pairs with the charged lysine residues (Table 2). With further increase in pH, the stability rises further and reaches a maximum at pH 11 (T_m = 51 °C). The similarity of the pH profile of the EK-containing peptides to that of $(POG)_{10}$ in the pH 6–11 region suggests that it too is undergoing increased stability due to the deprotonation of the N-terminal group over this pH range. A reduced thermal stability is expected at high pH, as the Lys groups (pK typically about 10.5) become deprotonated and cannot form ion pairs, and a small decrease is observed as the pH is raised from 11 to 13. Very broad titration curves are seen for this peptide, as expected again because each ionizable group will have three distinct transitions in a trimer molecule.

Thermodynamic analyses were carried out on the equilibrium melting curves described above. All thermal transitions were carried out at rates of change slow enough to ensure that there was no longer any rate dependence of the T_m value; this was assumed to represent an equilibrium state. Previous studies on triple-helical peptides such as those studied here indicate that the thermal transition approximates a two-state model. Equilibrium melting curves of $(POG)_{10}$ obtained by two different physical methods, 1D NMR spectroscopy monitoring the protons on side chains and CD spectroscopy monitoring the peptide backbone, gave very similar results (Long et al., 1993; Li, 1993), and the calorimetric enthalpy value for the triple-helical peptide $(Pro\text{-}Pro\text{-}Gly)_{10}$ agreed well with the van't Hoff value (Engel et al., 1977). Comparison of the parameters of $(POG)_{10}$ with the EK-containing peptide at the pH values of 1 and 7 show that the greater negative free energy, and thus greater thermal stability of $(POG)_{10}$, arises from its more favorable entropy term, even though it is less enthalpically favorable than the EK-containing peptide (Table 1). The more favorable enthalpy of the EK-containing peptide relative to $(POG)_{10}$ is expected on the basis of its Glu and Lys residues which can participate in hydrogen bonding and electrostatic interactions. The more favorable entropy of $(POG)_{10}$ can be explained on the basis of a more rigid monomer chain, because of its higher imino acid content (Nemethy et al., 1966). However, at pH 13, $(POG)_{10}$ is stabilized enthalpically relative to the EK-containing peptide, which is not consistent with expectations. Comparison of the EK-containing peptide thermodynamic parameters at different pH values shows enthalpy values which are the same within experimental error, except for pH 13, which has a considerably lower enthalpy. If the stabilization at pH values where both Lys and Glu are ionized is due to the formation of ion pairs, one might expect to see significant enthalpic stabilization at pH 6–9 values compared to pH 1 and 13, which is not observed.

(c) Electrostatic Interactions in Collagen Sequences: Studies of a Model Peptide Containing 18 Residues of Type III Collagen Sequence. A peptide has been synthesized containing 18 residues from the human type III collagen chain, including two basic and two acidic residues. Type III collagen, a member of the fibril forming collagen family, is a homotrimer of three α1(III) chains, and its features in a small region may be modeled by this triple-helical peptide consisting of three identical chains. To promote triple-helix formation, four Gly-Pro-Hyp tripeptide units were added to the C-terminus of the six Gly-X-Y triplets (18 residues) from the α1(III) chain. A C-terminal Gly-Val was included to eliminate diketopiperazine formation.

The peptide, GKOGEOGPKGDAGAOGAO(GPO)$_4$GV, was designated T3–487 since its sequence came from type III collagen, residues 487–505 (Ala-Kokka et al., 1989). It was synthesized both with its N-terminus acetylated (peptide AcT3–487) and in a nonacetylated form (peptide T3–487). Peptide T3–487 contains two Lys residues, one separated by two residues from a Glu and the other separated by one residue from an Asp residue, and thus provides a system where electrostatic interactions can be studied in a collagen-like context.

Both peptides, AcT3–487 and T3–487, formed stable triple-helical molecules. Ultracentrifugation carried out on T3–487 shows that it forms largely trimers with a small amount of monomer at low temperature. However, the best fit to the data is a monomer ↔ dimer ↔ trimer ↔ tetramer model, although the trimers and monomers are always the dominant species. It is possible that the dimer and tetramer forms involve some folding back of one peptide chain so that a triple helix structure can still be formed. Assuming a simple monomer to trimer transition, the association constant ln K_3 has a value of 2.1 at 10 °C and –0.7 at 30 °C. It is evident that this peptide is a weaker trimer former than the EK-containing peptide. No high molecular weight species (e.g., hexamers) were observed, even at the highest concentrations (3 mg/mL), indicating that there is no association of trimers. The characteristic triple-helical CD spectrum was observed for both peptides (Figure 1), and discrete melting transitions were seen, where the peptide undergoes a trimer to monomer transition (Figure 2). At neutral pH, both peptides melted near 26–27 °C, indicating they formed triple helices that were much less stable than either the EK-containing peptide or (POG)$_{10}$. The effect of pH on both peptides was examined. Nonacetylated peptide T3–487 showed the lowest thermal stability at low pH (T_m = 18 °C at pH 1 and 2) and at high pH (T_m = 19 °C at pH 13) (Figure 3). It undergoes a sharp increase in stability at pH 3–4.5 (to 24 °C at pH 4.4), followed by a more gradual increase, reaching a maximum of T_m = 27 °C at pH 9. The stability plateaus between pH 9 and 10 and then undergoes a steady fall with increased pH. At the lowest pH values, the amino-terminal group and the two Lys groups are positively charged (Table 2). As the pH increases, the terminal carboxyl group will become negatively charged (pK about 2–3), presumably leading to a destabilizing effect, as seen for (POG)$_{10}$. However, the thermal stability is observed to increase and not decrease as the pH increases from 2 to 4.5, and this is attributed to the ionization of the Glu and Asp residues, which can then participate in the formation of ion pairs. Stability gained from the ion pairs must be greater than the destabilization caused by carboxyl-terminal repulsion. Between pH 4.5 and 9, the steady increase in stability can be attributed to the deionization of the N-terminal group, as confirmed by the lack of this increase in the acetylated peptide (see below). At pH values higher than 10, deionization of Lys is expected and results in the observed decrease in T_m. The decreased stability could be due directly to the loss of ion pairs, to charge repulsion which might now occur between the three Glu or three Asp residues, or to indirect solvent effects.

The acetylated peptide AcT3–487 is more stable than the nonacetylated peptide at pH values below 9 (Figure 3). The acetylated form is about 4 °C more stable than the nonacetylated form at pH 1 and about 2 °C more stable at pH 4. The difference between the acetylated and nonacetylated forms narrows between pH 5 and 9, and the two curves are superimposable at pH values of 9 and higher. These results confirm the destabilizing, repulsive effect of the charged N-terminus. At very low pH values, there may be intrachain repulsion between the charged amino terminus and Lys at position 2 from the N-terminus, which may be relieved as the Glu at position 5 is ionized and can interact with Lys 2 (Table 2). Thus at low pH the acetylated peptide lacks this charge repulsion as well as the repulsion of the three charged terminal amino groups, leading to a 4 °C difference. At pH values of 4.5 or higher, the interchain repulsion of the N-terminal groups is likely be the only factor leading to stability differences between the acetylated and nonacetylated peptides, and thus the observed 2 °C differential is probably a reasonable estimate of the magnitude of this amino-terminal repulsion. The titration curves appear very broad for both of the T3–487 peptides, as seen for the previous peptides, supporting the idea that the source of these broad curves lies in the presence of three of each type of ionizable group in each molecule. Thermodynamic analyses were not carried out on the equilibrium thermal transitions of AcT3–487 and T3–487, because the presence of small amounts of dimers and tetramers, as well as monomers and trimers, would not allow the application of a 2-state model.

DISCUSSION

Collagen is found as a major structural protein in all multicellular animals, and the thermal stability of the collagen in an organism is close to the upper body temperature of the animal (Rigby, 1971; Mathews, 1975). The thermal stability of collagen depends on its amino acid sequence, i.e., the identity and sequence of the X and Y residues in the (Gly-X-Y)$_n$ sequence. Some of the features important in triple-helix stability have been elucidated. The imino acids, proline and hydroxyproline, are both stabilizing factors, so that the melting temperature of collagen from many animals is proportional to the imino acid content (Josse & Harrington, 1964). Comparison of many different collagens has suggested that hydroxyproline provides an additional stabilizing force (Burjanadze, 1982), apparently through the formation of hydrogen-bonded networks of water (Fraser et al., 1979). Beyond the role of imino acids, it has proved difficult to evaluate the stabilizing effect of residues in the X and Y positions, although it has been hypothesized that electrostatic interactions, close packing of side chains, and hydrogen bonding to peptide backbone atoms may be important (Salem & Traub, 1975; Traub & Fietzek, 1976; Bhatnagar et al., 1988). Recently, conformational energy computations were reported on the contribution of side chain interactions to the stabilization of the triple-helical structure in a collagen-derived sequence (Vitagliano et al., 1993).

The study of model peptides of defined sequence and of CNBr peptides of collagen can be useful in investigating the sequence dependence of stability and quantitating the interactions involved (Heidemann & Roth, 1982; Sutoh & Noda, 1974; Saygin & Heidemann, 1978; Piez & Sherman, 1970). Based on studies of synthetic peptides, an attempt has been made to create a hypothetical scale for the stabilities of different Gly-X-Y triplets (Bachinger & Davis, 1991). Although the basis of the scale has not been defined, this approach demonstrated the usefulness of having such a scale, to compare the relative stability of different regions along a collagen molecule. The stabilizing influence of electrostatic interactions in the triple helix has been approached through computer modeling studies (Trus & Piez, 1976; Katz & David, 1990, 1992), and in this report it is approached through the study of a set of (Gly-X-Y)$_n$ peptides containing a small number of charged residues. The triple-helical conformation

is stable in peptides over a very wide pH range, from pH 1 to 13, which makes it feasible to examine the effects of all ionizable residues. The peptides studied here are about 30 residues in length and have end effects not encountered in larger collagen molecules, which were evaluated by studying $(POG)_{10}$, with only terminal ionizable groups, and the N-terminal acetylated form of one peptide. The decreased stability of $(POG)_{10}$ at neutral pH where both terminal groups are ionized, compared to the extreme pH values where only one of the terminal groups is ionized, supports a destabilizing effect of about 4–5 °C due to charge repulsion at either end of the triple helix. The stabilization (about 2 °C) resulting from the acetylation of the N-terminus of peptide T3–487 supports this conclusion. This supports a parallel arrangement of the three chains in the triple helix, since an antiparallel arrangement would not have like terminal charges in proximity (Berg et al., 1970). In addition, it is consistent with a one-residue stagger of adjacent chains (Figure 4), as observed in NMR studies of triple-helical peptides (Li et al., 1993). These results agree qualitatively with the results on $(Pro\text{-}Pro\text{-}Gly)_{10}$, where the molecule with both ends charged at neutral pH had a thermal stability 10 °C lower than the peptide at either high or low pH (Berg et al., 1970). For both $(PPG)_{10}$ and $(POG)_{10}$, the titration of the ends groups is very broad. The breadth is likely to be due to the presence of three different terminal groups in a molecule staggered with respect to each other, which will have different pK values. Thus, when all three C-terminal ends are charged, the first C-terminus should have a greater tendency to become deionized, with an anomalously high pK value, but subsequent deionization of the other two charged C-termini should show lower pK values.

The EK-containing peptide was designed to study the effect of three pairs of charged residues in a single location in a Pro-Hyp-Gly triple-helical environment. This peptide forms stable trimers, but the substitution of a EKG triplet for one POG resulted in a decreased stability due to entropic destabilization, which overwhelms the enthalpically favorable nature of this substitution. Triplets with a negative charge in the X position and a positive charge in the Y position account for about 6–8% of all triplets in collagen, and the Glu-Lys-Gly triplet is particularly frequent in type IV collagen (about 4–5% of all triplets). The pH profile of the thermal stability of the EK-containing peptide is consistent with the formation of an ion pair at pH values where both Glu and Lys are ionized. As the pH goes from 2 to 6, a 5 °C destabilization due to C-terminal repulsion is expected, but instead a 2 °C increase in stability is observed; this leads to an estimate of 7 °C stability due to ion pair formation (about 2 kcal/mol of peptide). Ultracentrifugation indicates that the peptide is a trimer, with no hexamers or higher aggregates. Thus the stabilization observed when Glu and Lys residues are ionized arises from intramolecular interactions. These could include interactions between Glu and Lys in the same chain or interactions between Glu and Lys residues of the three chains that make up one trimer. Earlier modeling studies showed the Glu in the X position of one chain is in a very favorable position to interact with the Lys in the Y position of an adjacent chain, which is staggered by one residue (Figure 4; Salem & Traub, 1975; Traub & Fietzek, 1976). Recent computer modeling studies on this type of sequence support the formation of "tripoles", consisting of the Glu and Lys from one chain, together with either a Lys or Glu from an adjacent chain, and such "tripoles" would position the charge residues for intermolecular interactions (Katz & David, 1992). Our molecular modeling and energy minimization studies using the EK-containing peptide

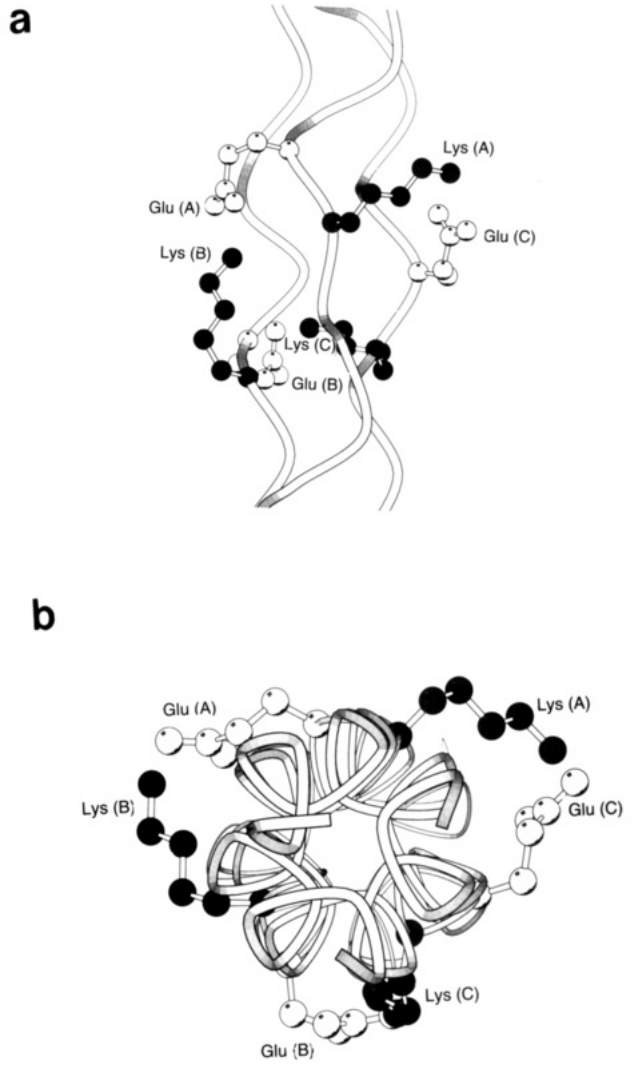

FIGURE 5: Molecular models of EK-containing peptide (a) axial view and (b) cross section, showing the region near the Lys and Glu residues of chains A, B, and C. Molscript was used to obtain these drawings (Kraulis, 1991). Potential ion pair interactions between Lys(B) and Glu(A), Lys(C) and Glu(B), and Lys(A) and Glu(C) are indicated; the latter interaction requires slight distortion of several residues from standard helical parameters. The calculations were carried out for a single triple helix using a distance-dependent dielectric constant.

sequence suggest that interactions between adjacent Glu and Lys side chains in the same polypeptide chain are not sterically favorable, but that two strong ion pairs could be formed between the three chains [Lys(B)···Glu(A) and Lys(C)···Glu-(B)], and that one weaker interaction [Lys(A)···Glu(C)] is possible if the chain is slightly distorted in the vicinity of the Glu and Lys residues (Figures 4 and 5).

To approach the electrostatic interactions present in the real sequence environment of collagen, peptide T3–487 containing 18 residues from type III collagen was synthesized. In this case, a $(GPO)_4GV$ cap was placed at the C-terminus for stabilization. Type III collagen has been ascribed a role in triggering the adhesion of platelets (Morton et al., 1989). The region included in peptide T3–487 has been implicated in platelet binding, and the charged residues were shown to be involved (Erikson et al., 1992). The relatively lower imino acid content of this peptide (near 40%) is closer to that found in some regions of collagen, compared with the very high imino acid content of the other peptides in this study. Peptide T3–487 formed a triple helix but had a stability markedly decreased compared to the other more imino acid-rich peptides.

As seen for the EK-containing peptide, the ionization of both the Lys and the Glu and Asp side chains led to an increased stability of the triple helix. Most strikingly, the pH profile closely resembles that seen for collagen in the range recorded (Barber & Mackay, 1966), suggesting that this peptide is a useful model for collagen. The formation of ion pairs leads to an observed increase of 5 °C, and since this must be compensating for the destabilizing effect of having charges at the C-terminus (about 3–5 °C), a better estimate would be 8–10 °C (3–4 kcal/mol of peptide). A variety of interactions could be occurring in peptide T3–487 that could lead to the observed electrostatic stabilization. The Lys(2) separated by two residues from Glu(5) can participate in intrachain ion pairs together with hydrogen bonding to the backbone (Katz & David, 1990), and model building indicates such intrachain pairs are possible in this case. The Lys(9)-Gly(10)-Asp(11) sequence can also form good intrachain ion pairs with hydrogen bonding to the backbone (Katz & David, 1990) but could also participate in interchain ion pairs within one trimer (Figure 4). Although intermolecular interactions are a theoretical possibility which could influence stability, no higher aggregates were seen by ultracentrifugation.

The design of peptides of defined sequence with the potential for forming ion pairs provides a basis for experimentally investigating electrostatic interactions in the triple-helical conformation. It is worth noting that the dipole moment of a triple helix is near zero (Wada, 1976), so studies in this conformation should reflect the interactions of more isolated groups of ion pairs than is possible in other contexts, such as an α-helix. The pH dependence of the thermal stability of the EK-containing peptide and T3–487 are consistent with a stabilizing effect of ion pair formation, and the ultracentrifugation studies indicate that these are all within one triple-helical molecule. Modeling suggests that two strong and perhaps one weaker *interchain* ion pairs are formed for the EK-containing peptide, giving rise to a free energy decrease of about 2 kcal/mol of peptide, while for T3–487 there could be two sets of three *intrachain* ion pairs or one set of three intrachain pairs plus a set of two interchain pairs, resulting in a free energy decrease of about 4 kcal/mol of peptide. These results give a rough approximation of the energy changes resulting from ion pair formation in a triple helix of about 0.5–1 kcal/mol ion pair, and these estimates are markedly less than those suggested from computer modeling calculations (Katz & David, 1990, 1992). The results also suggest that intrachain ion pairs may be of roughly the same magnitude as interchain pairs and that the different kinds of pairs (interchain GEK; intrachain KOGE; intrachain KGD) confer stabilizing energies of about the same value. Although a stabilization is observed at pH values where both the Lys and negatively charged groups are ionized, and it is energetically and sterically feasible for ion pairs to form, the favorable energy factors need not come directly from the ion pairs themselves. It is also possible that the stabilization is an indirect effect resulting from a reduction in repulsive interactions between unpaired charged residues, the formation of charged pairs in monomers (Katz & David, 1990), or perturbations of solvent structure.

A comparison of the three peptides studied here showed that the amino acid sequence markedly affects thermal stability, with $(POG)_{10}$ being the most stable, followed by the EK-containing peptide, and then the less stable T3–487 peptides. The differences in stability seen between the three sets of peptides were much greater than the smaller variations seen as a function of pH for each peptide (Figure 3). This indicates that factors such as imino acid content and positions of imino acids are dominant ones dictating stability, while the stability is modulated over a smaller range by electrostatic interactions. It is perhaps surprising that the triple-helical peptides containing charged residues did not aggregate, since such charged interactions were thought to form an important basis of molecular association in fibrils (Trus & Piez, 1976; Katz & David, 1992), but these peptides do not contain hydrophobic residues which are also known to be important in association between molecules. Studies on globular proteins suggest that ion pairs contribute 1–3 kcal/mol to stability, which may confer specificity on interactions, while the dominant driving forces in folding and association lie in hydrophobic interactions (Alber, 1989). Perhaps, as seen for globular proteins, the electrostatic interactions are not the driving forces for collagen association but are involved in specificity, and that peptides with only imino acids and charged residues lack the energetically favorable interactions of hydrophobic residues which are required for association. The availability of stable, soluble triple-helical peptides with a small number of ion pairs makes them good candidates for NMR and X-ray crystallography studies to confirm the nature of the ion pairs and their steric arrangements, and such studies are in progress.

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