The Collagen-like Peptide (GER)$_{15}$GPCCCG Forms pH-dependent Covalently Linked Triple Helical Trimers* 

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A collagen-like peptide with the sequence (GER)$_{15}$GPCCCG was synthesized to study the formation of a triple helix in the absence of proline residues. This peptide can form a triple helix at acidic and basic pH, but is insoluble around neutral pH. The formation of a triple helix can be used to covalently oxidize the cysteine residues into a disulfide knot. Three disulfide bonds are formed between the three chains as has been found at the carboxyl-terminal end of the type III collagen triple helix. This is a new method to covalently link collagen-like peptides with a stereochemistry that occurs in nature. The peptide undergoes a reversible, cooperative triple helix → coil transition with a transition midpoint ($T_m$) of 17 to 20 °C at acidic pH and 32 to 37 °C at basic pH. At acidic pH there was little influence of the $T_m$ on the salt concentration of the buffer. At basic pH increasing the salt concentration reduced the $T_m$ to values comparable to the stability at acidic pH. These experiments show that the tripeptide unit GER which occurs frequently in collagen sequences can form a triple helical structure in the absence of more typical collagen-like tripeptide units and that charge-charge interactions play a role in the stabilization of the triple helix of this peptide.

The extracellular matrix protein collagen is an important component in the architectural framework of tissues. At present count, 19 different types of vertebrate collagens have been identified which share the defining structural element of the triple helix. The triple helical conformation consists of three left-handed polyproline II helices that twist around each other to form a right-handed superhelix (1, 2). Each chain is staggered by one residue relative to each other to allow hydrogen bond formation between glycine of one chain with a residue following glycine in a neighboring chain (3). Steric restraints necessitate that every third residue be glycine and the amino acid sequence of a collagenous domain can be represented as (G-X-Y)$_n$, where X and Y can be any residue but are frequently proline and hydroxyproline (Z), respectively. In type I collagen, 12% of the tripeptide units are G-P-Z making it the most frequently occurring tripeptide unit. G-P-Y and G-X-Z together account for 44% of the tripeptide units, while G-X-Y tripeptide units with no imino acid constitute the remaining 44%. It had been postulated that imino acids transmit significant stability to the structure of the triple helix by conformational constraint and via inter- and intrachain hydrogen bonds involving water molecules (4–6). The high-resolution crystal structures of collagen-like peptides indeed indicate a high level of hydration with water molecules ordered around the Y position hydroxyprolines (7–10), but the role of water in the stabilization of collagen was questioned (10–13).

Several methods have been used to cross-link synthetic collagen-like peptides. Initially dilysine cross-links were used (14–16). More recently Goodman’s group (17) showed that Kemp's triacid is a useful cross-linker for collagen-like peptides. However, in both cases the stereochemistry of the covalently linked peptide does not coincide with the natural staggering of the three chains. Here we introduce a cysteine cross-link that is found at the carboxyl-terminal end of the triple helix of type III collagen (18). This cross-link allows all potential hydrogen bonds to form between the chains without creating flexible loops or strain between the chains. The cross-link stabilizes the triple helical conformation substantially and makes the triple helix = coil transition a monomolecular reaction.

Electrostatic interactions between the ionizable residues have been proposed to provide additional stabilizing forces for the collagen triple helix (19, 20). Approximately 40% of all tripeptide units contain at least one charged residue, especially the positively charged residues lysine and arginine. These charged residues are asymmetrically distributed with glutamic acid and aspartic acid predominately in the X position and arginine and lysine most often found in the Y position (21–23). All the residues in the X and Y position are partially exposed to solvent (24) and can form intra-chain or inter-chain interactions or interact with solvent molecules. We have tested the role of electrostatic interactions in determining the stability of the collagen triple helix by synthesizing a peptide composed of 15 repeating units of G-E-R. We chose this tripeptide unit specifically because it is the fifth most frequently occurring tripeptide sequence in collagens I, II, III, V, and XI (25). Cross-linked ([GER]$_{15}$GPCCCG)$_x$ is shown to adopt a pH-dependent triple helical conformation and exhibits salt dependent stability at alkaline pH.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification—Peptides were synthesized using N$_2$-9-fluorenylmethoxycarbonyl (Fmoc) chemistry on a Milligen Biosearch 9050 peptide synthesizer. Cleavage and deprotection were performed in trifluoroacetic acid, thioanisole, ethanedithiol, and anisole (90:5:3:2 volume %, 12 h, room temperature) which was thoroughly degassed with nitrogen before mixing with the peptide resin to ensure that cysteine residues remained reduced after removal of the trityl protection groups. The cleaved peptide was separated from the resin by filtration through a medium sintered glass filter. The filtrate was precipitated overnight in ethyl ether at 4 °C and recovered by centrifugation for 10 min at 3,000 × g. The soft pellet was dissolved in 20 volumes of water, degassed, and lyophilized. The dried peptide was
suspended in 1% trifluoroacetic acid and purified on a Vydac C-18 reversed-phase high performance liquid chromatography column (22 mm × 25 cm) with a mobile phase of 0.1% trifluoroacetic acid and a linear 60-min gradient of 0 to 75% acetonitrile. A typical initial chromatogram yielded a major peak elution at 20% acetonitrile with 14 subsequent shoulders indicating incomplete deprotection of the 4-methoxy-2,3,6-trimethylbenzenesulfonyl group off the arginine. These peaks were pooled, re-deprotected in the same reagent for 12 h, and rechromatographed. Peptide eluting at the correct position was dissolved (2 mg/ml) in 50 mM N-ethylmorpholine acetate, pH 8.3, the cysteine residues were reduced with 50 mM dithiothreitol for 2 h at 55 °C under nitrogen, and the peptide was rechromatographed and characterized by amino acid analysis, Edman sequencing, and mass spectrometry. From a theoretical yield of 500 mg, approximately 100 mg of peptide was pure and utilized for stability studies.

Solubility Studies—100 mM buffers ranging from pH 1 to 13 were prepared using appropriate combinations of hydrochloric acid, acetic acid, sodium phosphate, and sodium hydroxide. Once the pH range of solubility was established, different buffer compositions were tried in these pH ranges and the relative amount of triple helix formation was analyzed by circular dichroism.

Triple Helix Formation—Fresly reduced peptide was suspended in degassed 50 mM glycine, pH 8.6, at 4 °C under N2 in a Hydrazoid Cabinet (air Control). Upon any exposure to oxygen, soluble peptide at this pH quickly formed a visible precipitate. This precipitate also formed in buffers around pH 5, but over a period of days rather than minutes.

Disulfide Bond Formation—Disulfide bonds were formed via a multitude of strategies in an effort to optimize the yield of cross-linked association products. After triple helix formation under N2, the sample was removed from the Hydrazoid cabinet, exposed to atmospheric O2, and the pH was raised to 8.5 with saturated Tris. Alternatively, attempts were made to mimic natural oxidation mechanisms with the addition freshly prepared stock solutions of reduced and oxidized glutathione to catalyze the formation of correct disulfides.

Trimer Purification—The formation of disulfide bonded trimers was monitored and the products separated by a semi-preparative Vydac C-18 column (10 mm × 25 cm). To increase resolution of oxidation reaction products, the gradient was expanded over time between 10 and 25% acetonitrile.

SDS-Polyacrylamide Gel Electrophoresis—The reaction mixtures and fractions of chromatography runs were run on 10% Tricine SDS-PAGE1 gels (Novex) and stained with 0.02% Coomassie Blue (Serva). and fractions of chromatography runs were run on 16% Tricine SDS-PAGE gels (Novex) and stained with 0.02% Coomassie Blue (Serva). Molecular weight markers are shown on the left and the corresponding molecular weights are indicated.

RESULTS

The synthetic collagen model peptide (GER)15GPCCG is able to form a pH-dependent triple helix. The CD spectrum of this peptide is typical for collagen-like structures. A triple helix is usually indicated by a positive maximum at 221 nm and a negative minimum at 198 nm. The spectra of [(GER)15GPCCG]3 shows a positive maximum at a slightly lower wavelength of 217 nm (∆ε = 5 M−1 cm−1 at pH 4.0; ∆ε = 3 M−1 cm−1 at pH 9.0) and a negative minimum at 192 nm (∆ε = −12 M−1 cm−1 at pH 9.0).

FIG. 1. SDS-polyacrylamide gel electrophoresis of the covalently linked peptide [(GER)15GPCCG]3. Reduced and nonreduced samples were run on a 16% gel and stained with Coomassie Blue. Molecular weight markers are shown on the left and the corresponding molecular weights are indicated.

above this range. The chemical composition of the buffer as well as the pH seemed to influence the extent of triple helical formation. At pH 8.5–9.0 several buffer systems were utilized with the most stabilizing being 50 mM glycine/HCl, pH 8.6. Less stabilizing environments were Tricine, sodium borate, and sodium phosphate buffers.

The formation and purification of the cross-linked species was monitored by Tricine SDS-PAGE. Fig. 1 shows reduced [(GER)15GPCCG]3 and reduced (GER)15GPCCG. The cross-linked species migrates to a position of about 17 kDa while the reduced monomer migrates to 6 kDa. The molecular weight of the monomer calculated from the sequence is 5569.85 while the reduced monomer migrates to 6 kDa. The chemical composition of the buffer as well as the pH seemed to influence the extent of triple helical formation. At pH 8.5–9.0 several buffer systems were utilized with the most stabilizing being 50 mM glycine/HCl, pH 8.6. Less stabilizing environments were Tricine, sodium borate, and sodium phosphate buffers.

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Fig. 3 shows the thermal unfolding transition of reduced and non-reduced [(GER)15GPCCG]3 in 50 mM glycine/HCl buffer, pH 8.6, and in 100 mM HAc/NaPO4, pH 4.0. The increase in thermal stability due to cross-linking is quite large (15 °C) and the Tm increases with increasing pH. The sigmoidal shape of

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; CHAPS, 3-[3-cholamidopropyl]dimethy lammonio]-1-propanesulfonic acid.
the thermal transition indicates that the peptide undergoes a cooperative unfolding (27). This transition is reversible with 100% refolding obtained upon a 10 °C/h decrease of the temperature and concentration independent in a concentration range from 0.3 to 3.5 mg/ml. The thermal transition is also reproducible in 50 mM glycine/HCl buffer, pH 8.6, however, with high temperature and increasing pH the sample deteriorates via base hydrolysis and disulfide exchange.

The relative thermal stability of [(GER)\textsubscript{15}GPCCG]\textsubscript{3} as a function of pH is given in Table I. At acidic pH the thermal stability of [(GER)\textsubscript{15}GPCCG]\textsubscript{3} is marginal with a \(T_m\) of 17–20 °C whereas at alkaline pH the \(T_m\) rises to 32–37 °C.

Ionic strength was varied from 0 to 2 M NaCl in both high and low pH environments. Triple helix formation occurs in 75 mM acetic acid pH 3.5 over the entire range of ionic strength, however, it is only able to form in 50 mM glycine, pH 8.6, at ionic strengths up to 600 mM. In salt concentrations above 600 mM the peptide becomes insoluble. Fig. 4 show the salt dependence of the triple helix formed by [(GER)\textsubscript{15}GPCCG]\textsubscript{3} in these buffers. At pH 3.5 the \(T_m\) is 21 °C without any salt and lowers slightly to 17 °C with 2 mM NaCl. A more dramatic effect is seen at pH 8.6 where the thermal stability of [(GER)\textsubscript{15}GPCCG]\textsubscript{3} decreases from 37 to 20 °C when the NaCl concentration is increased from 0 to 600 mM.

The effect of urea and guanidinium hydrochloride on the formation of the triple helix of [(GER)\textsubscript{15}GPCCG]\textsubscript{3} was studied. A triple helix was able to form in 2 mM guanidinium hydrochloride but was unstable with a \(T_m\) of 10 °C (data not shown). No triple helical structure could be obtained at higher concentrations of guanidinium hydrochloride or at any concentration of urea.

**DISCUSSION**

It is well known that the presence of prolines and hydroxyprolines in the X and Y positions, respectively, serve to stabilize the triple helical structure. The present results with a substantially proline- and hydroxyproline-free peptide, however, show that imino acids are not necessary for the formation of a triple helix. We have successfully shown that (GER)\textsubscript{15}GPCCG can form a stable triple helix which indicates that charged amino acids can effectively confer the triple helical structure. It is possible that the nature of the forces which

**TABLE I**

| Solvent                  | pH  | \(T_m\) °C |
|--------------------------|-----|-----------|
| 100 mM AcOH/PO\textsubscript{4}/NaOH | 1.1 | 17        |
|                          | 2.0 | 15        |
|                          | 3.0 | 17        |
|                          | 4.0 | 19        |
|                          | 10.0| 33        |
|                          | 11.0| 33        |
|                          | 12.0| 34        |
|                          | 13.0| 32        |
| 50 mM Glycine/HCl        | 8.6 | 37        |
| 50 mM Tricine/HCl        | 8.0 | 21        |
| 50 mM Tricine/HCl, 1 M NaCl | 8.0 | 22        |
| 50 mM CHAPS              | 9.0 | 36        |
| 50 mM CHAPS, 1 M NaCl    | 9.0 | 20        |
serve to stabilize the triple helix formed by [(GER)15GPCCG]3 differ from that of collagen model peptides containing a high content of imino acids which have a higher thermal stability. These studies of [(GER)15GPCCG]3 are of significance because they help clarify the nature of the minor contribution made by charged residues to the composite stability of a native collagen molecule.

The ability of (GER)15GPCCG to solubilize and the stability of its triple helix varies with pH. The thermal stability of naturally occurring collagen also shows a pH dependence with the greatest stability seen when all side chains are ionized (28). Collagen has a basic isoelectric point with a net excess of basic over acidic residues and forms fibrils at neutral pH. (GER)15GPCCG precipitates at neutral pH and this could be a result of reciprocal cancellation of the positive and negative charges contributed by the arginine and glutamic acid. Synthetic collagen model peptides are known to trimerize in a parallel rather than an anti-parallel manner (29) even though this manner of association brings together like-charge end chain repulsion. It could be that before (GER)15GPCCG could reach the free energy minimum of parallel trimerization, it either self-associated in an anti-parallel manner, end to end, or maybe in a ordered three-dimensional array. Once associated in any structure other than a triple helix, if the pH was above 7.5, disulfide bonds between any cysteine in proximity with each other will rapidly form and thus prohibit any subsequent trimerization opportunities.

{(GER)15GPCCG} is soluble and triple helical in both an acidic and basic environment but shows more stability at high pH. The carboxyl group on the glutamic acid has a pKₐ of around 4.5 therefore the triple helix formed in the pH range of 1–4 consists of positively ionized glutamic acid and arginine. Although the secondary amine of arginine has strong ionization capabilities, the three carbon linker between the peptide backbone and the reactive amine undoubtedly modulates its behavior in solution. In contrast to proline, the arginine side chain has significant rotational flexibility because of the aliphatic nature of the carbon chain and does not provide stabilizing energy by steric constraints. Side chains may therefore form hydrophobic interactions between the long aliphatic side chains and hydrogen bond between the guanidine groups. At low pH self-repulsion of positive charges may be minimized in the triple helical conformation in which the side chains point outward from the central axis. At high pH ionic interactions of arginine provide some increase in stability that can be shielded by increasing salt concentrations in the buffer.

Previous studies on (GERGPP)₉ showed similar results (30). This peptide was soluble and random coiled at pH 7.2, but was able to form a triple helix at pH 2.5 and 9.5. In a more recent study on positional preferences of ionizable residues in the G-X-Y tripeptide repeat, it was shown that Ac-(G-P-Z)₉-G-E-R-(G-P-Z)₉-G-NH₂ is more stable at pH 12.2 with a T�� of 39.1 °C than at pH 2.7 with a T�� of 37.3 °C (31). At neutral pH this peptide had a T�� of 40.4 °C which is the highest value of the eight guest triplet combinations of oppositely charged residues. The relative position of the glutamic acid and arginine in the triplet seems also to be important in determining the stability because the peptide with the guest triplet G-E-R was 6 °C more stable that the peptide with G-R-E.

The relatively small dependence of stability on pH and ionic strength support only minor contributions of salt bridges at acid and neutral pH. Such interactions become somewhat stronger at basic pH. This was also observed for charged groups in coiled-coil structures (32–34).

The existence of GER tripeptide units in many collagen sequences seems to be a compromise of using charged residues that allow the triple helical conformation with the need of charged residues required in interactions between individual collagen molecules in fibrils. The formation of fibrils was shown to be much more sensitive to pH and ionic interactions than the stability of individual collagen molecules (35, 36).

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