Helical Structures

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Investigation of pH-Dependent Collagen Triple-Helix Formation**

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Collagen is a ubiquitous biomaterial that forms the supporting structures in skin, bone, tendons, cartilage, and blood vessels. Numerous types of collagen have been identified, and the tertiary structure of each shares the common structural motif of the collagen triple helix (CTH).[1] The CTH motif is composed of three chains, each of which adopts a left-handed type-II polyproline helix, that come together to form a right-handed superhelix.[2] Repeating units of GlyXaaYaa are common within the different types of collagen, and a tendency for X to be proline and Y to be hydroxyproline (Hyp, O) has been observed.[3] Minimal peptide sequences based on this idealized sequence have provided a wealth of information concerning the structural and sequence requirements for triple-helix stability.[4] The ability to control triple-helix formation fundamentally would be useful for a range of collagen-based biomaterial applications, such as tissue engineering and drug delivery.[5] Herein we disclose modifications to collagen peptides that lead to the formation of triple helices on demand through environmental control.

Our plan for the design of a pH-responsive CTH was to include carboxylate moieties along the collagen peptide.[6] Under neutral conditions, interstrand electrostatic repulsion would disfavor triple-helix formation. However, when the carboxylate groups are protonated under acidic conditions, a stable triple helix should form, as long as steric repulsion from the appended groups is not a factor. This design is complementary to the use of electrostatic interactions to promote the stabilization of CTHs.[7] As we wished to minimize alteration to the $(POG)_n$ helical structure, our design strategy was to incorporate a carboxylate functionality within the Hyp residue by O-alkylation[8] (to give a P_E residue; Figure 1). Molecular modeling of a CTH with the inclusion of P_E suggested that this nonnatural amino acid should be accommodated well with minimal interstrand steric interactions (Figure 1 a).

Five peptides, therefore, formed the basis of this study: two control peptides containing seven repeating units of POG or PEG (**POG-7** and **PEG-7**, respectively), two host–guest peptides containing one or three central PP_EG units (**PP_EG-1** and **PP_EG-3**, respectively), and **PP_EG-7**, which contains seven repeating units of PP_EG (Figure 1 b). The host–guest peptide

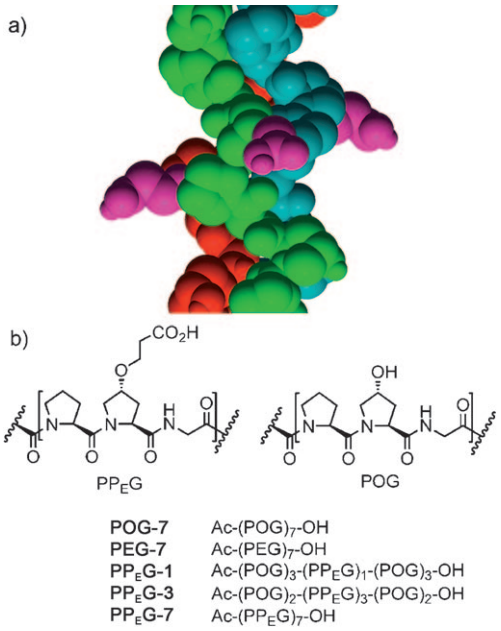
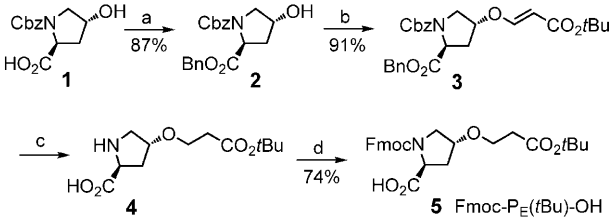


Figure 1. a) Model of **PP_EG-1** showing the incorporation of a single carboxy-modified (pink) hydroxyproline residue, P_E, into each strand of a collagen triple helix. b) Structures of the peptides used in this study.

PP_EG-1 would enable us to evaluate the destabilizing effect of a single P_E residue per strand on a CTH by comparison with the results of previous host–guest studies.[4c,d] We envisioned that the inclusion of increasing numbers of P_E residues in place of Hyp residues, as in **PP_EG-3** and **PP_EG-7**, would enable us to determine the degree of modification necessary for environmental control.

A protected version of P_E, Fmoc-P_E(*t*Bu)-OH, was synthesized from *N*-(carbobenzyloxy)hydroxyproline (Scheme 1). After benzyl protection of the carboxylic acid to yield **2**, a DMAP-promoted Michael addition to *tert*-butyl propiolate gave compound **3**. The treatment of **3** with hydrogen over Pd/C led to the removal of the Cbz and



Scheme 1. Synthesis of the protected nonnatural amino acid: a) BnBr, Cs_2CO_3, DMF; b) *tert*-butyl propiolate, DMAP, CH_2Cl_2; c) H_2, Pd/C, MeOH; d) Fmoc-Cl, Na_2CO_3, water/dioxane. Bn = benzyl, Cbz = carbobenzyloxy, DMAP = 4-dimethylaminopyridine, DMF = *N*,*N*-dimethylformamide, Fmoc = 9-fluorenylmethoxycarbonyl.

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benzyl protecting groups and the simultaneous reduction of the double bond. The resulting amine, compound **4**, was treated with 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) to provide the desired compound Fmoc-P_E(*t*Bu)-OH (**5**). The tripeptide Fmoc-Pro-P_E(*t*Bu)-Gly-OH was synthesized on the 2-chlorotrityl resin by using standard Fmoc-based solid-phase chemistry (see the Supporting Information). Fmoc-Pro-Hyp-Gly-OH was synthesized as previously reported.[9] Peptides (Figure 1b) were synthesized by coupling units of Fmoc-Pro-P_E(*t*Bu)-Gly-OH or Fmoc-Pro-Hyp-Gly-OH on the PAL–PEG Rink amide resin (PAL = Peptide Amide Linker, PEG = poly(ethylene glycol)), followed by cleavage with a TFA cocktail (TFA = trifluoroacetic acid). These peptides were purified to homogeneity by reversed-phase HPLC and characterized by MALDI-TOF mass spectrometry.

Circular dichroism (CD) studies were performed to investigate the conformation of the peptides at neutral (7.2) and acidic (2.7) pH values. For these studies, solutions of the peptides (400 μM) were incubated at 4 °C for 24 h to enable complete folding. At both pH values, **POG-7**, **PP_EG-1**, and **PP_EG-3** displayed a polyproline type-II helical profile with a maximum positive band at 225 nm, as is typically observed for a CTH (see the Supporting Information, Figures S3 and S4).[10] Thermal unfolding experiments were carried out with these peptides at both pH values (Figure 2a,b). All three peptides displayed cooperative thermal transitions, with T_m values of approximately 37, 35, and 31 °C for **POG-7**, **PP_EG-1**, and **PP_EG-3**, respectively (Table 1). The T_m value for **POG-7** is consistent with previously reported values for

Table 1: Thermal-transition data derived from CD for collagen peptides as a function of pH value.

Peptide	T_m [°C]	
	pH 2.7	pH 7.2
POG-7	37	37
PP_EG-1	35	35
PP_EG-3	31	31
PP_EG-7	17	no CTH
PEG-7	no CTH	no CTH

triple-helical peptides with a heptameric repeat.[4e] Two results are of particular significance: First, the presence of the nonnatural amino acid P_E within **PP_EG-1** and **PP_EG-3** had only a slight effect on CTH stability relative to that of **POG-7**, with a 2° decrease in T_m observed for the incorporation of each P_E unit. Furthermore, the stability of the **PP_EG-1** and **PP_EG-3** triple helix was not influenced by the pH value, as the same T_m value was observed at both pH values for each peptide. We may conclude that the nonnatural amino acid, in either protonation state, is accommodated well by the CTH structure. This result is interesting, as the replacement of even a single Hyp unit with a Glu residue in a CTH peptide has been reported to cause a significant loss in thermal stability.[4d] We may also conclude that the incorporation of one or three P_E residues per strand does not introduce a sufficient level of electrostatic repulsion at a neutral pH value to create a pH switch for CTH formation.

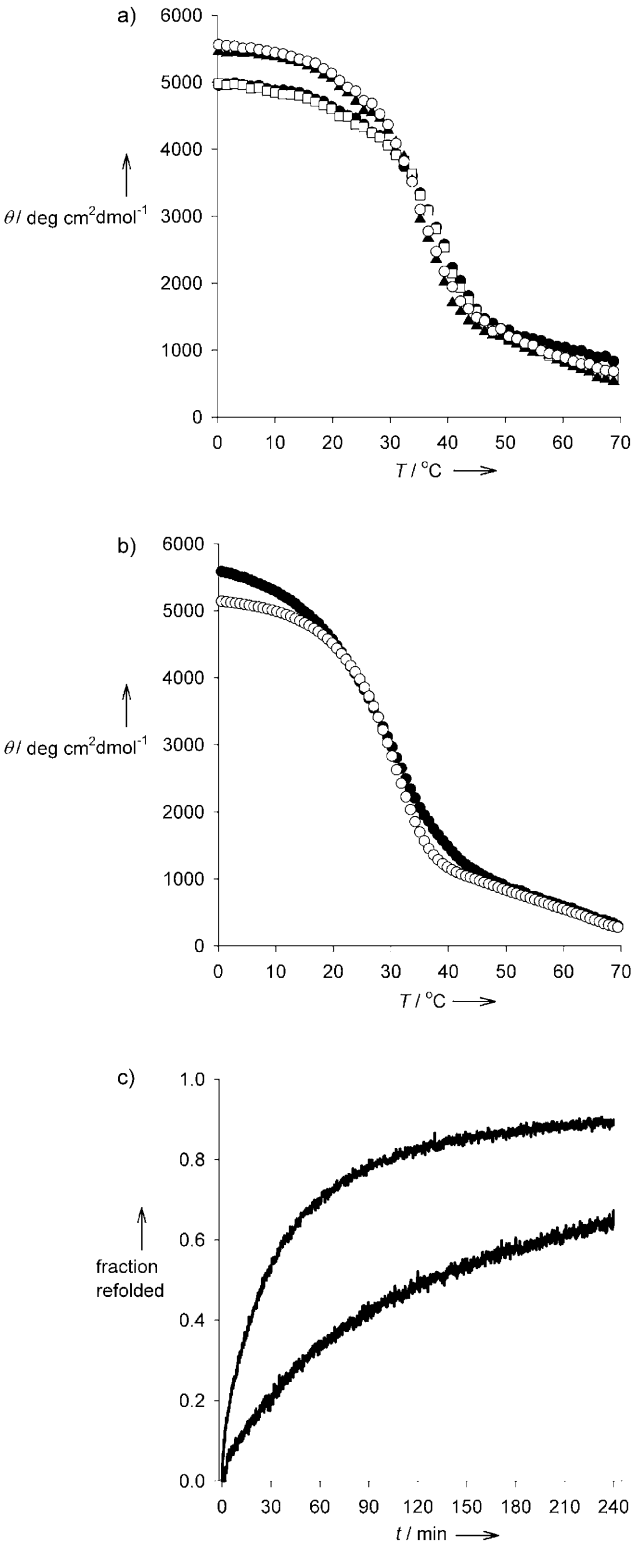

Figure 2. a) CD thermal-transition curves for **POG-7** (● pH 7.2, □ pH 2.7) and **PP_EG-1** (▲ pH 7.2, ○ pH 2.7). b) CD thermal-transition curves for **PP_EG-3** (○ pH 7.2, ● pH 2.7). c) Rate of refolding of **PP_EG-3** (top: pH 2.7, bottom: pH 7.2) monitored at 225 nm after a rapid transition from 90 to 4 °C according to previously reported refolding studies.[11]

It was surprising that a stable triple helix formed at the neutral pH value even after the replacement of three of the seven Hyp residues with P_E. Therefore, we investigated the folding characteristics of **PP_EG-3** further with thermal refolding studies at both pH values. Interestingly, the rate of refolding of **PP_EG-3** was found to be pH-dependent: Whereas refolding experiments performed at pH 2.7 demonstrated 80% assembly after 100 min, only 45% refolding was observed at a pH value of 7.2 (Figure 2c). These results indicate that the assembly of **PP_EG-3** into a triple helix is pH-dependent, even though the thermal stability at both pH values is identical.

We next evaluated the structure of **PP_EG-7**, which contains seven P_E residues, by CD. A polyproline type-II helical conformation was also identified for this peptide at neutral and acidic pH values (Figure 3a). By contrast, a control peptide, **PEG-7**, which contains seven Glu residues in place of Hyp, was found by CD not to adopt a polyproline helix, even under acidic conditions[7c] (Figure 3a). This result confirms the importance of the more rigid P_E residue in our design. Temperature-dependent CD studies with **PP_EG-7** indicated significantly different thermal melting profiles over a range of pH values (Figure 3b). At pH values of 2.7 and 4.0, **PP_EG-7** (400 μM) exhibited a cooperative thermal transition, which indicates that the peptide forms a stable triple helix at low temperature, with T_m values of 17 and 15 °C, respectively. This trend was also observed at lower concentrations of **PP_EG-7** (100 and 200 μM) at pH 2.7 (see the Supporting Information, Figures S5 and S6). However, a linear decrease in ellipticity was observed at pH 5.5 and 7.2 with increasing temperature, an effect characteristic of the unfolding of a single polypeptide chain.[4g] These data indicate that it is difficult to form a stable triple helix at these pH values, presumably as a result of interhelical electrostatic repulsion between negatively charged carboxylate groups. We may therefore conclude that **PP_EG-7** does indeed form a collagen triple-helical structure at low pH values and low temperatures, and that the peptide adopts a monomeric, polyproline II helical conformation at pH values of 5.5 and higher. These data for **PP_EG-7** confirm the existence of a pH switch for collagen triple-helix formation.

The introduction of certain kosmotropic salts, such as Na_2SO_4, has been shown to induce the formation of a coiled-coil structure in peptides containing a number of Glu residues under neutral conditions.[12] We therefore evaluated the effect of Na_2SO_4 on the triple-helix formation and stability of **PP_EG-7**. We anticipated that the addition of the kosmotrope Na_2SO_4 (0.75 M) might enable **PP_EG-7** to form a stable triple helix under neutral conditions. **PP_EG-7** was found by CD to undergo a cooperative thermal transition at pH 7.2 with a T_m value of 33 °C, which suggests the formation of a stable CTH (Figure 3c). By comparison, the addition of NaCl up to a concentration 0.5 M had no effect on the triple-helix stability of **PP_EG-7** (see the Supporting Information, Figure S7).

In conclusion, we have engineered a pH-responsive CTH peptide. The introduction of one or three units of a non-natural amino acid, P_E, into a collagen peptide had little effect on triple-helix stability at acidic and neutral pH values, although differential folding rates were observed. However, the replacement of the Hyp residues within **POG-7** with P_E resulted in the designed pH control. By elongating the **PP_EG-7** sequence, it may be possible to obtain collagen triple helices on demand at higher temperatures, and we are currently

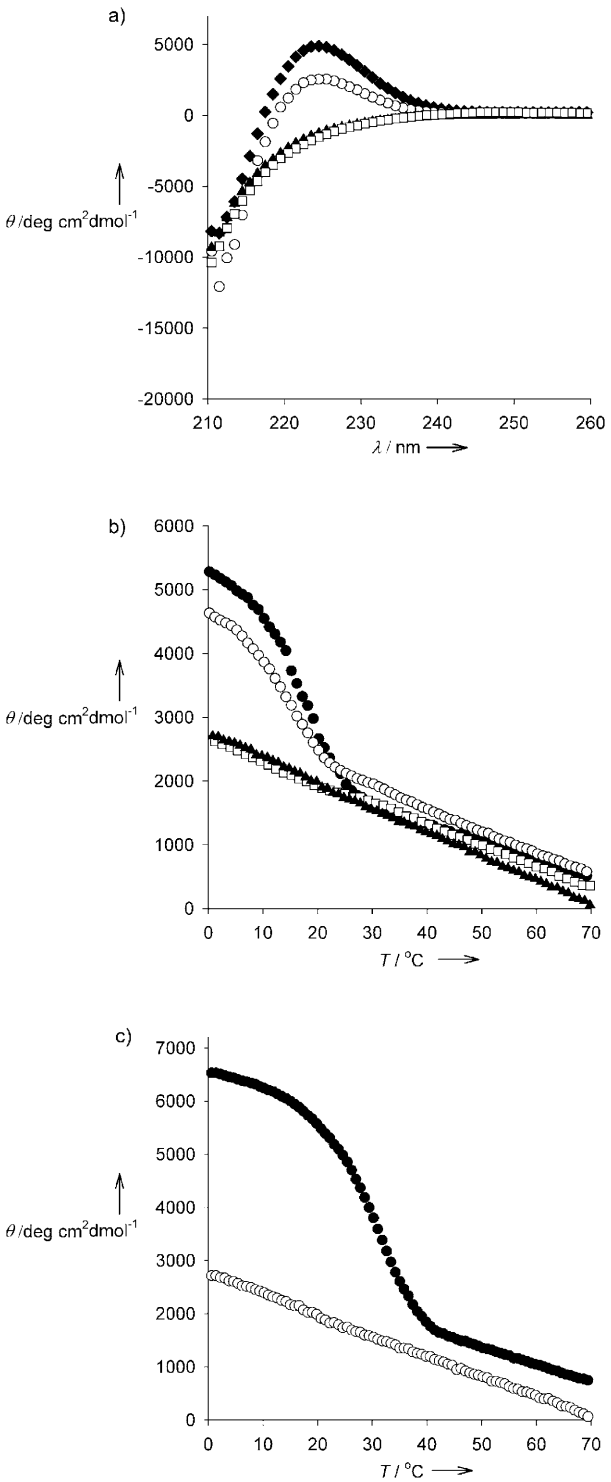

Figure 3. a) CD spectra of **PP_EG-7** (○ pH 7.2, ◆ pH 2.7) and **PEG-7** (▲ pH 7.2, □ pH 2.7) at 400 μM and 4 °C. b) CD thermal-transition curves for **PP_EG-7** at various pH values (● pH 2.7, ○ pH 4.0, □ pH 5.5, ▲ pH 7.2). c) CD thermal-transition curves for **PP_EG-7** in the presence (●) and absence (○) of Na_2SO_4 (0.75 M) at pH 7.2.

evaluating longer sequences and alternative designs for environmental control.

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