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Author(s):

Fiala, Tomas ; Barros, Emilia P.; Heeb, Rahel; Riniker, Sereina ; Wennemers, Helma

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Peptides Hot Paper

Predicting Collagen Triple Helix Stability through Additive Effects of Terminal Residues and Caps

Tomas Fiala⁺, Emilia P. Barros⁺, Rahel Heeb, Sereina Riniker,^{*} and Helma Wennemers^{*}

In memory of Professor François Diederich

Abstract: Collagen model peptides (CMPs) consisting of proline-(2*S*,4*R*)-hydroxyproline-glycine (POG) repeats have provided a breadth of knowledge of the triple helical structure of collagen, the most abundant protein in mammals. Predictive tools for triple helix stability have, however, lagged behind since the effect of CMPs with different frames ([POG]_{*n*}, [OGP]_{*n*}, or [GPO]_{*n*}) and capped or uncapped termini have so far been underestimated. Here, we elucidated the impact of the frame, terminal functional group and its charge on the stability of collagen triple helices. Combined experimental and theoretical studies with frame-shifted, capped and uncapped CMPs revealed that electrostatic interactions, strand preorganization, interstrand H-bonding, and steric repulsion at the termini contribute to triple helix stability. We show that these individual contributions are additive and allow for the prediction of the melting temperatures of CMP trimers.

Introduction

Collagen is the most abundant protein in mammals and the primary component of connective tissue.^[1,2] The basic structure of collagen is a triple helix assembled from three peptide chains with Xaa-Yaa-Gly repeat units (Figure 1a).^[1] Proline (Pro, P) is most common in the Xaa position and (4*R*)-hydroxyproline (Hyp, O) in the Yaa position.^[1-3] Glycine (Gly, G) is key in every third position to allow for a compact packing of the triple helix. The high Pro and Hyp

[*] T. Fiala,⁺ R. Heeb, H. Wennemers
Laboratory of Organic Chemistry, ETH Zürich
Vladimir-Prelog-Weg 3, 8093 Zürich (Switzerland)
E-mail: helma.wennemers@org.chem.ethz.ch
E. P. Barros,⁺ S. Riniker
Laboratory of Physical Chemistry, ETH Zürich
Vladimir-Prelog-Weg 2, 8093 Zürich (Switzerland)
E-mail: sriniker@ethz.ch

[⁺] These authors contributed equally to this work.

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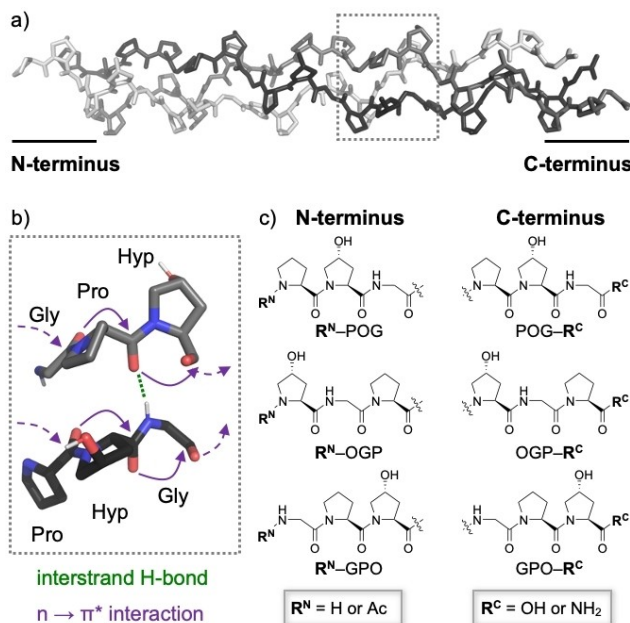


Figure 1. a) Structure of triple-helical collagen; b) H-bonds and $n \rightarrow \pi^*$ interactions within a collagen triple helix; c) N- and C-terminal regions of frame-shifted CMPs covering every possible terminal amino acid and capping group.

content preorganizes the strands into a left-handed polyproline type II (PPII) helix with all-*trans* amide bonds and stabilizing $n \rightarrow \pi^*$ interactions between neighboring amide bonds (Figure 1b).^[1,4-6] The right-handed triple-helical assembly forms from the PPII-helical strands with a one-residue stagger (Figure 1a). Interstrand H-bonds between the N-H groups of Gly and the C=O groups of the Xaa amino acid stabilize the supramolecular assembly (Figure 1b).^[1]

Studying native collagen is challenging due to its heterogeneity and difficult isolation. Collagen model peptides (CMPs) with Pro-Hyp-Gly repeats have hence been used as synthetic surrogates.^[1] These peptides consist typically of 20–40 amino acids and are useful for studying the factors that affect the stability^[7-19] and the dynamics^[20-24] of the collagen triple helix. Such studies revealed, for example, the importance of steric and stereoelectronic effects, interstrand H-bonding, hydrophobicity, and PPII helicity of the individual strands for collagen triple helix stability.^[1,8,16,17,25-32] These insights are useful for the develop-

ment of CMP-based biological probes^[33–36] and synthetic materials.^[37–44]

CMPs can start and end with either of the three repeating amino acids, and thus be synthesized in three different frames—[POG]_n, [OGP]_n, or [GPO]_n (Figure 1c). CMPs with the POG frame have been the most common.^[1,7] Several examples used also the GPO frame,^[45,46] but the OGP frame has rarely been used.^[47] CMPs can also bear different terminal functional groups, either a free base/acid (N-terminal amine, C-terminal carboxylic acid) or caps (N-terminal acetyl, C-terminal carboxamide; Figure 1c).^[48,49] Historically, little attention has been paid to the N- and C-terminal residues and functional groups of CMPs, and, as a result, different types of model systems were used.^[1,2] In addition, the triple helix stabilities were determined in aqueous solutions of different pH, with aqueous acetic acid at pH 3^[8,13,14,17] and phosphate buffer saline at pH 7.0–7.4^[9,13,18,24] as the most common environments. Despite reports of different thermal stabilities of collagen triple helices formed by seemingly identical CMPs, effects of the collagen frame and terminal groups have only been recognized recently.^[48–51] This includes a study of the effect of capped and uncapped termini on the stability of triple helices derived from CMPs with the POG-frame.^[48] Recently, we showed that capped frame-shifted CMPs provide triple helices with vastly different thermal stabilities, with melting temperature (T_m) differences as high as 10 °C between constitutional isomers.^[50] Our data revealed that a Pro-Hyp diad with a high PPII helix propensity acts as a “clamp” and has the largest stabilizing effect at either terminus while a C-terminal Gly destabilizes the triple helix. Furthermore, the C-terminal amino acid affects the triple helix stability significantly more than the N-terminal residue.

Here, we explored the effect of the frame of uncapped CMPs and CMPs bearing only one cap at either the C- or N-terminus on the stability of collagen triple helices. We show that the impact of the terminal group on triple helix stability depends largely on the frame. Studies at different pH values (3.0, 7.4, and 10.8) allowed for dissecting the contribution of charged versus uncharged terminal groups on triple helix stability. Aided by molecular dynamics (MD) simulations, our studies provided a deep understanding of the influence of the frame and the terminal groups on collagen triple helix stability at the molecular level. Furthermore, we show that the observed stability differences are additive and allow for predicting the melting temperatures of collagen triple helices.

Results and Discussion

Stability of frame-shifted collagen triple helices with different capping patterns

Building on our recent discovery that the thermal stability of capped frame-shifted collagen triple helices differs significantly,^[50] we asked whether the stability of uncapped or semi-capped (cap at either the N- or C-terminus) triple

Table 1: Melting temperatures of triple helices derived from frame-shifted CMPs with varying terminal capping patterns at different pH values.

pH	Termini (N/C)	T_m [°C] ^[a]		
		[POG] ₇	[OGP] ₇	[GPO] ₇
3.0 ^[b]	AcN/CONH ₂	42.3	42.2	52.9
	HN/CONH ₂	31.4	35.1	39.4
	AcN/CO ₂ H	48.3	44.1	41.6
	HN/CO ₂ H	36.3	36.8	27.5
7.4 ^[c]	AcN/CONH ₂	42.6 ^[e]	42.3 ^[e]	52.8 ^[e]
	HN/CONH ₂	34.3	40.7	47.1
	AcN/CO ₂ H	40.7	34.8	35.5
	HN/CO ₂ H	31.2	32.5	27.8
10.8 ^[d]	AcN/CONH ₂	42.9	42.5	53.0
	HN/CONH ₂	38.8	37.9	48.1
	AcN/CO ₂ H	40.4	34.7	35.3
	HN/CO ₂ H	36.2	29.9	30.5

[a] Measured by thermal denaturation monitored by CD spectroscopy. Concentration 200 μM, heating rate 1 °C/114 s. $N=2-4$, standard deviation (SD) ≤ 0.4 °C. [b] 50 mM aqueous acetic acid. [c] 1 × phosphate buffer saline (PBS). [d] 50 mM NaHCO₃/Na₂CO₃ buffer. [e] Taken from ref. [50].

helices follows the same trend. We, therefore, evaluated the thermal stability of a series of twelve collagen triple helices formed by every possible combination of CMP frame ([POG]₇, [OGP]₇, and [GPO]₇) and capping pattern (N-acetyl or amino group, C-amide or acid group) at pH 7.4 (Table 1). The stability of each triple helix was determined by thermal denaturation using CD spectroscopic monitoring.^[52] These experiments revealed a strong effect of both the frameshift and the capping pattern on the stability of triple helices (Figure 2).

Further, the data revealed that the stability order of frame-shifted triple helices critically depends on the capping pattern. For example, whereas the GPO-frame CMP with caps at both termini (Figure 2, solid blue circle) forms the

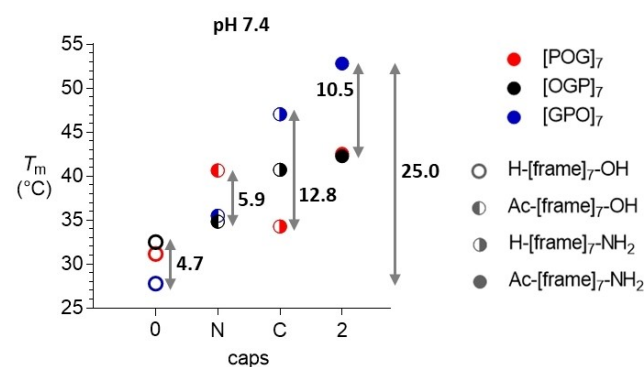


Figure 2. Dependence of the triple helix melting temperature (T_m values as listed in Table 1) of frame-shifted CMPs on terminal capping groups at pH 7.4. The arrow depicts the ΔT_m (in °C) between the most and least stable triple helix within each capping pattern.

most stable triple helix ($T_m=52.8^\circ\text{C}$),^[50] the fully uncapped [GPO]₇ triple helix is the least stable ($T_m=27.8^\circ\text{C}$; Figure 2, open blue circle) of all frames.

Within the semi-capped series, the [GPO]₇ triple helix is the most stable when the C-terminus is capped ($T_m=47.1^\circ\text{C}$; $\Delta T_m=12.8^\circ\text{C}$; Figure 2, semi-filled blue circle on the right) but is, together with the [OGP]₇ trimer, the least stable when the N-terminus is capped ($T_m=35.5^\circ\text{C}$; Figure 2, semi-filled blue circle on the left). Furthermore, the relative stability within one series of frame-shifted collagen triple helices differs significantly. Within the fully uncapped series, the stability difference between the isomeric helices is only 4.7°C but it is pronounced in the fully capped series, $\Delta T_m=10.5^\circ\text{C}$. It is worth noting that the uncapped system with the comparatively small ΔT_m of 4.7°C was the most common in earlier studies.^[9,13,18,24] Between the routinely used H-[POG]₇-OH and H-[GPO]₇-OH, the ΔT_m is even smaller, $\Delta T_m=3.4^\circ\text{C}$. This small stability difference may have created the common belief that frameshifts affect collagen stability only modestly. Our data, however, show that a small ΔT_m is rather an exception. In fact, the stability difference between frame-shifted constitutional isomers is as high as $\Delta T_m=12.8^\circ\text{C}$ in the semi-capped series with a free N-terminal amino group and a C-terminal amide cap. Stability differences of frame-shifted collagen triple helices of the same length on the order of 10°C are striking since they are similar to those of triple helices that differ in length by an entire tripeptide repeat unit.^[53] Thus, the data show a strong influence of the frame and the capping pattern on collagen triple helix stability with an astonishing T_m difference of 25°C between the fully capped and fully uncapped [GPO]₇ triple helices (Figure 2). Such large stability differences between seemingly similar collagen triple helices are surprising and highly relevant for the design of synthetic materials and biological probes.

We, therefore, asked whether the contributions of the terminal capping groups (NH vs. NAc and CO₂H vs. CONH₂) and their charge (NH vs. NH₂⁺ and CO₂H vs. CO₂⁻) to the stability of frame-shifted collagen triple helices can be dissected and whether these effects are additive. To tackle these goals, we determined the T_m values of all twelve triple helices also at pH 3.0 and 10.8 (Table 1, Figure S1).

Contribution of terminal charges to the stability of frame-shifted collagen triple helices

Within a collagen triple helix, the three termini are located close to one another. Repulsion between identically charged terminal groups, therefore, destabilizes the assembly.^[48,49] The charge state of the terminal group at a given pH depends on its pK_a value. The pK_a of the N-terminal amino group of a CMP can be approximated by those of H-(amino acid)-NH₂ ($pK_a \approx 8-9$).^[54] Since the pK_a of proximal amino groups decreases in polyamines, the terminal amino groups in uncapped triple helices are likely partially protonated at pH 7.4. At pH 3.0, the N-terminal amino groups are fully protonated, and at pH 10.8, they are neutral. The pK_a of the C-terminal carboxylic acid groups can be approximated by

those of Ac-(amino acid)-OH ($pK_a \approx 3.5$).^[55-57] Within the triple helices, the terminal carboxylic acid groups are therefore at pH 3.0 in the neutral, protonated state (CO₂H) and at pH 7.4 and 10.8 fully deprotonated (CO₂⁻; Figure 3).

To visualize the individual contributions of the terminal charge states on triple helix stability for each CMP frame, we plotted the dependence of T_m on the pH value (Figure 3). As expected, the stability of the frame-shifted triple helices derived from fully capped CMPs is the same at each pH value (Figure 3a). In contrast, the stability of triple helices with other capping patterns depends strongly on the pH and the frame.

Free N-terminus, capped C-terminus (H-[frame]₇-NH₂): The thermal stability of the H-[POG]₇-NH₂ triple helix was, as observed before,^[48] the lowest at pH 3.0 and increased with pH, by 2°C at pH 7.4 and by another 5°C at pH 10.8 (Figure 3b, red). The stabilities of the OGP and GPO frames were also the lowest at pH 3.0, consistent with the repulsion of positively charged ammonium groups at the N-termini. However, the pH-dependent stability differences varied significantly from those observed for H-[POG]₇-NH₂. The

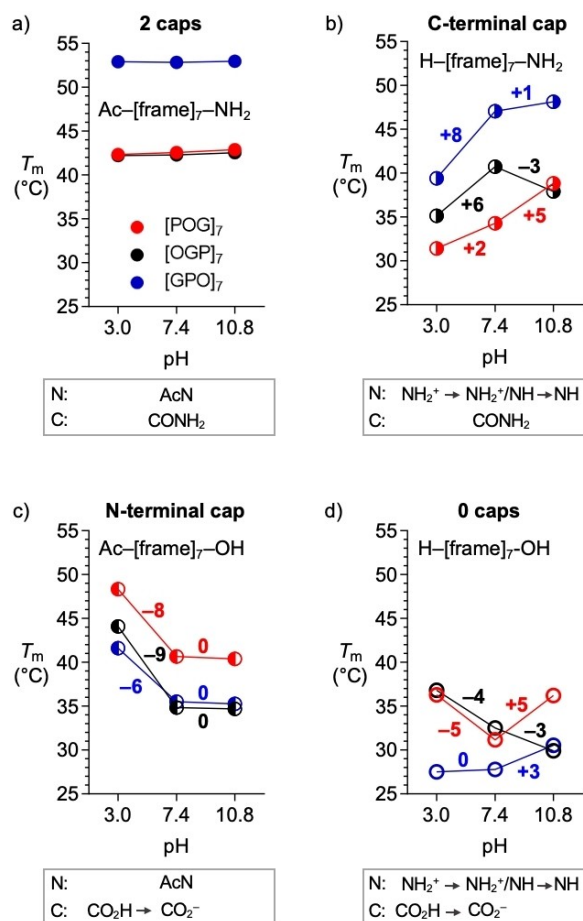


Figure 3. Dependence of the triple helix melting temperature (T_m values as listed in Table 1) of frame-shifted CMPs on pH for each capping pattern: a) N-acetyl, C-amide; b) N-amine, C-amide; c) N-acetyl, C-acid; d) N-amine, C-acid. Numbers next to the lines denote the ΔT_m in $^\circ\text{C}$. The change in protonation state of the terminal functional groups at the three pH values is denoted below the graphs.

T_m of H-[GPO]₇-NH₂ increased by as much as 8 °C from pH 3.0 to pH 7.4, and almost plateaued between pH 7.4 and 10.8 ($\Delta T_m \approx +1$ °C; Figure 3b, blue). The T_m of H-[OGP]₇-NH₂ increased by 6 °C from pH 3.0 to pH 7.4 but decreased by 3 °C from pH 7.4 to pH 10.8 (Figure 3b, black). The overall ΔT_m from pH 3.0 to pH 10.8, which represents the change in triple helix stability upon deprotonation of all three terminal ammonium groups, also varied significantly depending on the frame: +7 °C for POG, +3 °C for OGP, and +9 °C for GPO. The increased stability of the triple helices at pH 7.4 compared to pH 3.0 is in accordance with protonation of one or two but not all amino groups at pH 7.4 and, thus, reduced charge repulsion. The different degrees of stabilization through the release of charge repulsion—with even a stability maximum at pH 7.4 for H-[OGP]₇-NH₂—indicate that the amino/ammonium groups are in markedly different relative positions depending on the collagen frame, see below.

Capped N-terminus, free C-terminus (Ac-[frame]₇-OH): The thermal stability of Ac-[POG]₇-OH decreased by 8 °C from pH 3.0 to pH 7.4 and did not further change to pH 10.8 (Figure 3c, red), consistent with charge repulsion between CO₂⁻ groups at pH 7.4 and 10.8.^[48] For this capping pattern, the stability changes are similar for frame-shifted collagen triple helices ($\Delta T_m \approx -9$ °C, OGP frame; $\Delta T_m \approx -6$ °C, GPO frame, Figure 3c black and blue, respectively).^[58] This finding suggests that the C-terminal carboxylic acid groups are in similar relative positions with respect to each other in the three frame-shifted collagen triple helices.

Free N- and C-termini (H-[frame]₇-OH): The relative stability of the uncapped frame-shifted collagen triple helices differs significantly (Figure 3d). The stability of POG-triple helices is lowest at neutral pH ($T_m = 31$ °C) and highest at acidic and basic pH ($T_m = 36$ °C). The stability of OGP-triple helices decreases with rising pH: $T_m = 37$ °C (pH 3.0), $T_m = 33$ °C (pH 7.4), $T_m = 30$ °C (pH 10.8; Figure 3d black). In contrast, GPO-triple helices are more stable at basic pH ($T_m = 31$ °C) and less stable at neutral and acidic pH ($T_m = 28$ °C; Figure 3d blue). Thus, the frame affects the relative stability of uncapped collagen triple helices significantly. Of note, a comparison of the T_m values of the semi-capped triple helices at different pH values revealed that the effects of charge at the N- and C-termini are additive, i.e., the ΔT_m values in Figure 3d correspond to the sum of those in Figures 3b and 3c within ± 2 °C.

Contribution of the terminal capping group to the stability of frame-shifted collagen triple helices

Next, we analyzed the contribution of the terminal capping groups to the stability of frame-shifted collagen triple helices and whether these contributions are additive. We, thus, compared the T_m values of uncapped, semi-capped, and fully capped collagen triple helices within each frame-shifted series at acidic, neutral, and basic pH. Firstly, we noticed that acetylation of a fully uncapped triple helix had the same effect as acetylation of a C-terminally capped triple helix on the thermal stability. Likewise, C-terminal capping with an

amide had the same effect on uncapped and N-terminally capped triple helices. Thus, parallelograms depict the effect of capping groups on triple helices that share the same amino acid sequence (Figure 4).

Acetylation of the N-terminus: Acetylation of the N-terminus resulted in more stable triple helices compared to the uncapped analog at each pH value and for each collagen frame (Figure 4, solid arrows). The extent of stabilization depended, however, significantly on the pH, the frame, or both. At pH 10.8, acetylation stabilized the collagen triple helices to a similar extent regardless of the frame: $\Delta T_m \approx +4$, POG-frame; $\Delta T_m \approx +5$, OGP-frame; and $\Delta T_m \approx +5$ °C, GPO-frame (Figure 4g–i, solid arrows). Thus, an N-terminal amide group stabilizes collagen triple helices compared to a neutral amino group. At pH 3.0, acetylation stabilizes the triple helices more since charge repulsion is released (Figure 4a–c, solid arrows), with the extent of stabilization depending on the frame. The ΔT_m was significantly larger for the POG and GPO frames ($\Delta T_m \approx +11$ and $+14$ °C, respectively) than for the OGP frame ($\Delta T_m \approx +7$ °C). Qualitatively similar but weaker effects were observed at pH 7.4, where the three N-terminal amino groups are neither all neutral nor all positively charged: $\Delta T_m \approx +9$, $+2$ and $+7$ °C for the POG, OGP and GPO frame, respectively (Figure 4d–f, solid arrows).

Capping of the C-terminus. The effect of amidation of the C-terminus depended significantly on the frame and the pH (Figure 4, dotted arrows). At pH 3, C-terminal amidation destabilized the triple helices with POG and OGP frames ($\Delta T_m \approx -5$ and -2 °C, respectively), but strongly stabilized the GPO-triple helix ($\Delta T_m \approx +12$ °C; Figure 4a–c, dotted arrows). Thus, neutral C-terminal CO₂H and CONH₂ groups have opposite effects depending on the frame. At pH 7.4 and 10.8, amidation of the C-terminal carboxylate (CO₂⁻) stabilized collagen triple helices in all three frames, however, to a different extent. The degree of stabilization mirrored the order seen for amidation of the neutral CO₂H group with ΔT_m values of +3, +8, and +18 °C for the POG, OGP, and GPO frame, respectively (Figure 4d–i, dotted arrows).

Molecular reasons for the effects of termini and frame on collagen triple helix stability: Molecular dynamics simulations

Our previous studies on fully capped frame-shifted CMPs showed that differences in triple helix stability arise from an interplay between the preorganization of the single strands into PPII helices and interstrand H-bonding.^[50] We reasoned that these effects play in concert with electrostatic repulsion in the case of uncapped triple helices and, hence, the relative position of the terminal residues. We used molecular dynamics (MD) simulations to explore these contributions.

For the MD simulations, we used nine of the triple helices derived from 21-mer CMPs that represent the three collagen frames and different capping and charge patterns at the termini:

- Three fully uncapped, zwitterionic triple helices in each frame; H-[frame]₇-OH (frame = POG, OGP, and GPO),

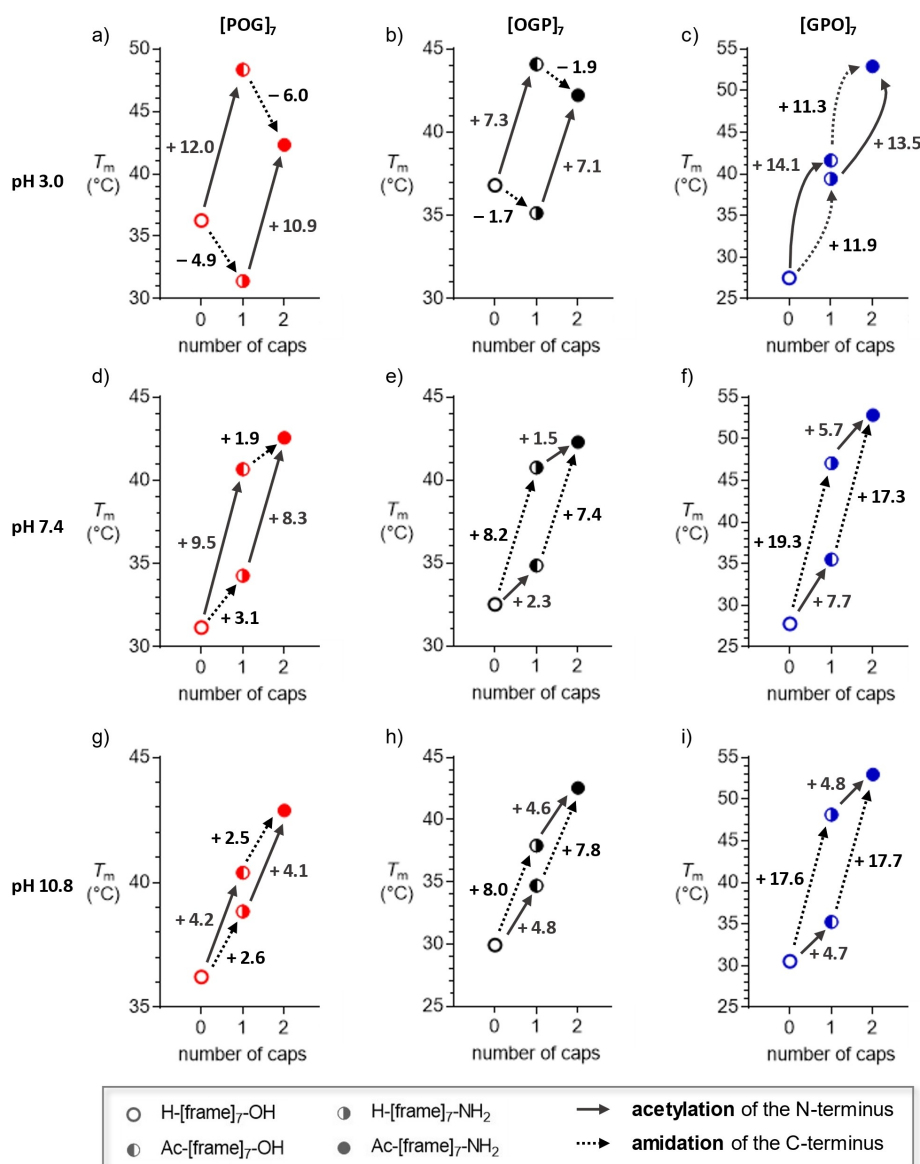


Figure 4. Additive effects of capping terminal residues (N-acetyl, C-amide) on the thermal stability (T_m as listed in Table 1) of collagen triple helices illustrated by parallelograms. The effects depend on the pH value: pH 3.0 (panels a–c), pH 7.4 (panels d–f), and pH 10.8 (panels g–i); as well as on the collagen frame: [POG]₇ (panels a, d and g), [OGP]₇ (panels b, e and h), and [GPO]₇ (panels c, f and i). Numbers next to the arrows denote the ΔT_m in °C. Note that the scales of the y-axes are not identical among the panels.

- b) Three N-terminally capped triple helices in each frame with a neutral C-terminus; Ac-[frame]₇-OH.
 c) Three C-terminally capped triple helices in each frame with a neutral N-terminus; H-[frame]₇-NH₂.

For each triple helix, we conducted MD simulations with water as an explicit solvent for 100 ns. We carried out the simulations at 300 K in five replicates using the GROMOS software package^[59] and the GROMOS 54A7 force field.^[60] The starting structures were based on a crystal structure of collagen (PDB ID: 3B0S).^[61] In addition, we used our previous MD simulations with fully capped CMPs (Ac-[frame]₇-NH₂) that were carried out with the same methods as a reference.^[50]

During the MD simulations, the triple helices with neutral/capped termini largely retained their trimeric structure while the termini of the zwitterionic triple helices unfolded to a certain degree (Figure S3). This result is consistent with the experimentally observed low thermal stability of zwitterionic triple helices regardless of the frame (Table 1 and Figure 4, open circles at pH 7.4).^[62]

Capped versus charged termini. We suspected that an interplay between charge repulsion and PPII helical preorganization at the CMP termini is responsible for the different stabilities of triple helices with charged versus capped termini. The stabilization arising from capping should be largest for (1) triple helices with strong charge-charge repulsion in the uncapped state, and (2) triple helices with

better PPII-helical preorganization when the termini are capped. Analysis of the ϕ and ψ dihedral angle distributions of the MD simulations revealed that ammonium groups at the N-termini and carboxylate groups at the C-termini disorder the terminal regions of each of the frame-shifted triple helices (Figure S4–S6). This disorder propagates all the way to the second triplet (Figure S4–S6). Since charge-charge repulsion increases with vicinity and since the relative positions of Pro, Hyp, and Gly residues within the collagen triple helix are not identical, we analyzed the distances between the terminal ammonium and the carboxylate groups in the leading, middle, and lagging strands (Figure 5). For this analysis, we used frame-shifted models derived from crystal structures of collagen triple helices.

The distances between the N-terminal ammonium groups depend greatly on the frame, being significantly shorter for Gly (3.8 Å) than for Pro and Hyp (5.8 and 6.7 Å, respectively; Figure 5, Figure S7a). In line with these distances, the capping of a charged N-terminal Gly residue boosts the triple helix stability the most (Gly > Pro > Hyp, $\Delta T_m \approx +14$, $+11$, and $+7$ °C, respectively; Figure 4, pH 3, solid lines). The greater stabilization by the capping of an N-terminal Pro compared to a Hyp residue cannot, however, be explained by charge-charge repulsion alone. Here, the MD simulations show that triple helices with a capped terminal Pro exhibit the highest degree of preorganization (Figure S4–S6). This is in agreement with our previous report that a capped Pro-Hyp diad acts as a “clamp” that preorganizes CMPs into PPII helices and thus stabilizes collagen triple helices.^[50]

At the C-terminus, the distances between the carboxylate groups were similar for all frames (4.9 Å, 5.2 Å, and 5.7 Å for terminal Gly, Hyp, and Pro, respectively; Figures 5 and S7b). Yet, the stabilizing effect of capping depends greatly on the C-terminal residue; Gly $\Delta T_m \approx +3$, Pro $\Delta T_m \approx +8$, or Hyp $\Delta T_m \approx +18$ °C (Figure 4, pH 7.4 and 10.8, dotted lines). Therefore, these different degrees of stabilization cannot be ascribed to charge repulsion but arise from PPII-helical preorganization of the termini, which is greatest with the terminal Pro-Hyp clamp.^[50]

Amidated versus neutral amino or carboxylic acid termini. We hypothesized that the stability differences

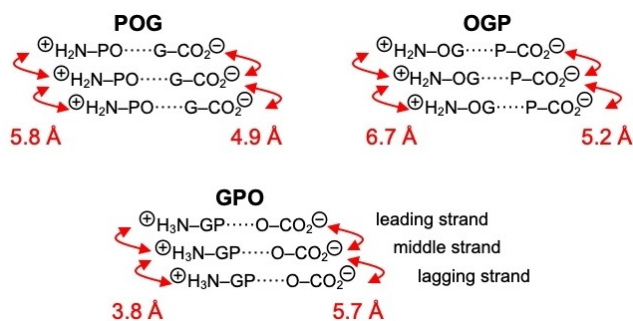


Figure 5. Schematic representation of the distances between N-terminal ammonium groups and C-terminal carboxylate groups in frame-shifted collagen trimers in an ideal triple-helical arrangement. For the corresponding 3D structural models, see Figure S7.

between triple helices with neutral, uncapped termini (NH and CO₂H) and capped termini (NAc and CONH₂) arise from an interplay between PPII helical preorganization through additional $n \rightarrow \pi^*$ interactions and interstrand H-bonds. The MD simulations show increased disorder of neutral uncapped N-termini compared to the capped ones, with propagation beyond the N-terminal residue, as judged by the distributions of ϕ and ψ dihedral angles (Figure S4–S6). The simulations also indicate that the cap C=O groups are involved in $n \rightarrow \pi^*$ interactions^[4–6] and, thus, contribute to PPII helical preorganization as well as the strengthening of the interstrand H-bonds (Figure 6, Figure S8). These effects boost the stability of the frame-shifted triple helices to a similar extent ($\Delta T_m \approx +4$, $+5$, and $+5$ °C for POG, OGP, and GPO, respectively; Figure 4, pH 10.8, solid lines).

At the C-terminus, the effect of a CONH₂ versus a CO₂H group depends greatly on the frame, with ΔT_m values of -5 °C, -2 °C, and $+12$ °C, for the POG, OGP, and GPO frames, respectively (Figure 4, pH 3, dotted lines). The MD simulations predict similar properties for the triple helices with the OGP frame, in agreement with the small influence of a C-terminal CONH₂ versus CO₂H group on thermal stability ($\Delta T_m \approx -2$ °C). For the POG frame, our previous studies suggested that a C-terminal CONH₂-capped Gly residue has a narrow conformational preference that

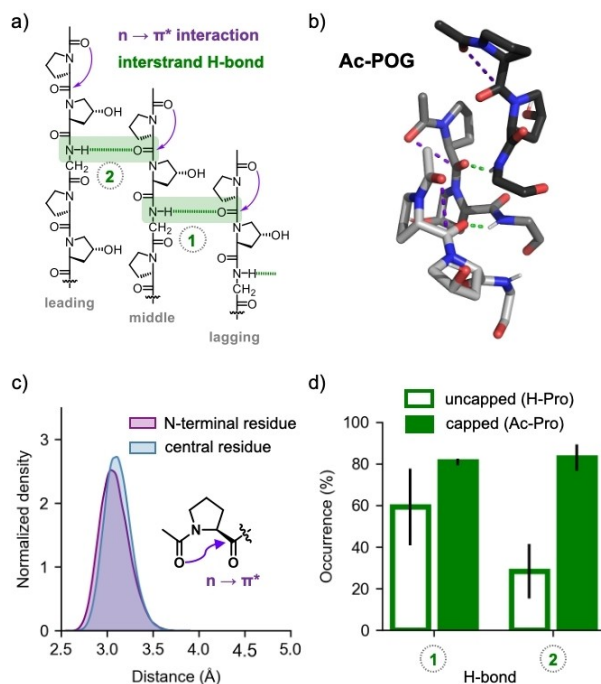


Figure 6. a) Schematic representation and b) 3D model of an N-acetylated collagen triple helix in the POG frame. The $n \rightarrow \pi^*$ interactions involving the capping group are highlighted in purple; the interstrand H-bonds which are strengthened by the cap $n \rightarrow \pi^*$ interactions are highlighted in green. c) Distributions of C=O...C=O distances involving the N-terminal acetyl caps (purple) and distances from the center of the triple helix (blue) from MD simulations of Ac-[POG]₇-NH₂. d) The occurrence (mean \pm SD) of the H-bonds indicated in (a) during the time course of the MD simulations. Empty bars: neutral uncapped CMPs, filled bars: acetyl capped CMPs.

deviates from a PPII helix, and thus destabilizes the triple helix.^[50] The MD simulations of the analogous triple helix with a C-terminal CO₂H revealed higher flexibility which propagates to the second POG triplet from the C-terminus as judged by the dihedral angles ϕ and ψ (Figures S4 and S9). This finding implies that POG-frame triple helices with a C-terminal CO₂H group avoid steric repulsion through greater flexibility and are thus more stable than POG-frame triple helices with a C-terminal CONH₂ group by $\Delta T_m \approx 5^\circ\text{C}$ (Figure S9).

GPO-frame collagen triple helices stand out, with a large stabilization by a C-terminal CONH₂ compared to a CO₂H group of $\Delta T_m \approx +12^\circ\text{C}$. We hypothesized that part of this stabilization arises from the formation of two additional interstrand H-bonds, with the amide cap N–H serving as a surrogate of a Gly residue (Figure 7a). The MD simulations corroborate these H-bonds (Figure 7b). Since one interstrand H-bond corresponds to an approximate increase in T_m of 3–4°C,^[63] the remaining stabilization arises from the “clamping effect” of the C-terminal Pro-Hyp diad that preorganizes the strands into PPII helices and propagates the stabilization along the triple helix. Thus, the seemingly small structural change from a C-terminal Hyp carboxylic acid to an amide has a profound effect on the preorganization of single-stranded CMPs and interstrand H-bonding within the triple helix.

Predictive power of the contributions from terminal functionalities

Finally, we probed whether the determined contributions of terminal residues and functional groups to the stability of collagen triple helices allow for predicting the stability of triple helices from the structure of any CMP that consists of Pro, Hyp, and Gly residues. Table 2 summarizes the set of ΔT_m values for the effect of a structural change at the termini on triple helix stability provided by the experiments with the 21-mer CMPs.

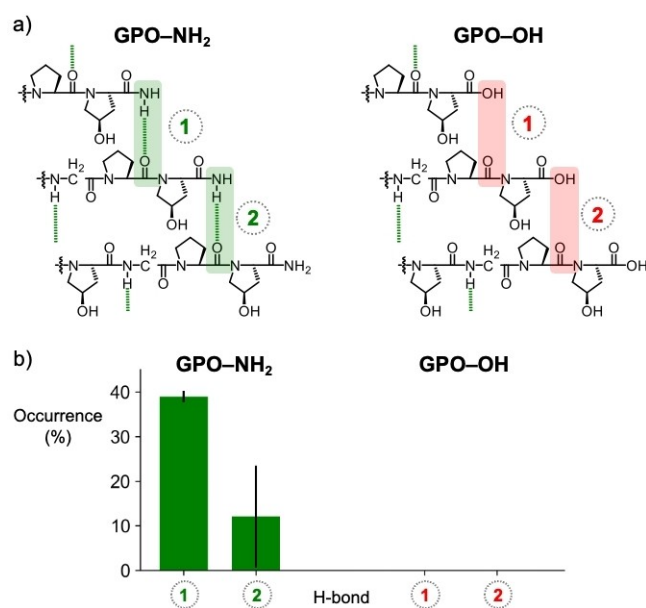


Figure 7. a) C-terminal region of a collagen triple helix in the GPO frame. Left: amide capped C-terminus; interstrand H-bonds involving the amide caps are highlighted in green. Right: non-capped C-terminus; lack of equivalent terminal H-bonds is highlighted in red. b) Occurrence, in per-cent, of interstrand H-bonds involving the C-terminal amide or carboxylic acid group of Ac-[GPO]₇-NH₂ and Ac-[GPO]₇-OH, respectively, from the MD simulations.

Since T_m values depend on the heating rate used in the thermal denaturation experiments due to the slow folding and unfolding of collagen triple helices,^[24] we probed whether also the ΔT_m values change with the heating rate. Reassuringly, thermal denaturation experiments with several frame-shifted collagen triple helices at three different heating rates revealed that the stability difference, ΔT_m , is independent of the heating rate and should, thus, be a universally applicable parameter (Figure S10).^[64]

To test the additivity of the ΔT_m parameters (Table 2), we predicted the T_m values of eighteen non-capped and

Table 2: Changes of the triple helix melting temperatures upon change of protonation state and/or capping pattern at CMP termini.

Terminus	Functional group change	ΔT_m [°C]		
		Terminal amino acid		
		Pro (Xaa)	Hyp (Yaa)	Gly
N	+ H ₂ N → HN ^[a]	+ 7 (+ 5) ^[b]	+ 3 (− 3) ^[b]	+ 9 (+ 1) ^[b]
	HN → AcN ^[c]	+ 4	+ 5	+ 5
	+ H ₂ N → AcN ^[d]	+ 11 (+ 9) ^[b]	+ 7 (+ 2) ^[b]	+ 14 (+ 7) ^[b]
C	CO ₂ [−] → CO ₂ H ^[e]	+ 9	+ 6	+ 8
	CO ₂ H → CONH ₂ ^[f]	− 2	+ 12	− 5
	CO ₂ [−] → CONH ₂ ^[g]	+ 8	+ 18	+ 3

[a] T_m difference at pH 10.8 vs. 3.0 (7.4). [b] The values in parentheses correspond to the change from a partial (rather than full) protonation state of the N-terminal amine at pH 7.4. [c] Average ΔT_m for N-acetylation from Figure 4g–i. [d] Average ΔT_m for N-acetylation from Figure 4a–c (Figure 4d–f). [e] T_m difference at pH 3.0 vs. 10.8. [f] Average ΔT_m for C-amidation from Figure 4a–c. [g] Average ΔT_m for C-amidation from Figure 4g–i.

semi-capped triple helices derived from 20-mer and 22-mer CMPs at pH 3.0, 7.4, and 10.8 (Tables 3 and S1). These peptides differ by one amino acid in length from the 21-mers that provided the parameters and contain different combinations of N- and C-terminal amino acids. As the basis of the predictions, we used the previously reported melting temperatures of the fully capped analogs.^[50]

For example, prediction of the T_m value for H-[POG]₆PO-OH at pH 3.0: Starting from Ac-[POG]₆PO-NH₂ ($T_m = 50^\circ\text{C}$),^[50] subtracting the N-terminal $^+\text{H}_2\text{N} \rightarrow \text{AcN}$ increment for Pro ($\Delta T_m = +11^\circ\text{C}$) and the C-terminal $\text{CO}_2\text{H} \rightarrow \text{CONH}_2$ increment for Hyp ($\Delta T_m = +12^\circ\text{C}$) predicts a T_m value of 27°C for H-[POG]₆PO-OH at pH 3.0 (Table 3, first line).

To evaluate the predicted T_m values, we synthesized the uncapped 20-mers H-[POG]₆PO-OH, H-[OGP]₆OG-OH, and H-[GPO]₆GP-OH as sample CMPs and determined the T_m values of the respective collagen triple helices at pH 3.0, 7.4, and 10.8 (Table 3). Remarkably, the experimentally determined T_m values match the predicted values within $\pm 2^\circ\text{C}$, the margin of the parallelogram deviations. These results corroborate that the determined ΔT_m values for the effects of structural modifications at the N- and C-termini of collagen triple helices are a predictive tool for assessing trimer stability.

Conclusion

This work dissected the influence of the terminal residues and the capping groups at different pH values on the thermal stability of frame-shifted collagen triple helices. Our combined experimental and computational approach unraveled an intricate balance between electrostatic interactions, strand preorganization, interstrand H-bonding, and steric effects that determines triple helix stability. The study revealed significantly different effects of the capping groups

depending on the frame of the triple helix. In the most extreme case, amidation of a C-terminal Hyp carboxylic group leads to a large stabilization of the triple helix ($\Delta T_m = +12^\circ\text{C}$), whereas the same modification at a Gly residue leads to a drop in stability ($\Delta T_m = -5^\circ\text{C}$). The data advise caution when designing collagen triple helices since seemingly subtle changes, such as a CMP frameshift or altering a terminal capping group, can have a profound impact on the triple helix structure and thermal stability. The results also revealed that the individual contributions from each of these structural and environmental factors are additive and allow for predicting the melting temperatures of new triple helices with similar lengths.

We foresee the importance of these findings at several levels:

- (1) Functional CMPs have become valuable as biological probes and therapeutics^[33–36] and as building blocks for the formation of synthetic materials.^[37–44] The properties of these CMPs depend on the stability of the respective collagen triple helices. Our work provides a detailed guide for the choice of the frame, terminal residue, and capping group, as well as the pH of the environment.
- (2) The scientific community has historically used CMP model systems with different frames and terminal groups. Cross-comparisons between different works have therefore been virtually impossible. Our set of parameters allows for recalculating reported T_m data to a common reference peptide and, thus, enables the comparison of results from diverse sources.
- (3) The development of computational models for the prediction of T_m values of collagen triple helices is currently an active area of research.^[65–71] Several of these models succeeded in capturing the influence of inner residues on triple helix stability. These algorithms, however, do not take frameshifts, terminal capping groups and their charge into account. Our work fills this gap. The additive parameters presented herein will

Table 3: Predicted and experimentally verified melting temperatures of selected CMPs.

CMP	pH	T_m [$^\circ\text{C}$]		Deviation [$^\circ\text{C}$] ^[c]
		Predicted ^[a]	Experimental ^[b]	
H-[POG] ₆ PO-OH	3.0 ^[d]	27	25.8	1
	7.4 ^[e]	23	21.9	1
	10.8 ^[f]	28	30.1	-2
H-[OGP] ₆ OG-OH	3.0 ^[d]	32	31.7	0
	7.4 ^[e]	29	28.4	1
	10.8 ^[f]	26	23.8	2
H-[GPO] ₆ GP-OH	3.0 ^[d]	28	27.4	1
	7.4 ^[e]	25	23.8	1
	10.8 ^[f]	27	26.9	0

[a] Predicted using the increments in Table 2 and the T_m of capped peptides reported in ref. [50]: Ac-[POG]₆PO-NH₂ ($T_m = 50^\circ\text{C}$), Ac-[OGP]₆OG-NH₂ ($T_m = 34^\circ\text{C}$) and Ac-[GPO]₆GP-NH₂ ($T_m = 40^\circ\text{C}$). [b] Measured by thermal denaturation monitored by CD spectroscopy. Concentration 200 μM , heating rate $1^\circ\text{C}/114\text{ s}$. $N = 2$, $\text{SD} \leq 0.4^\circ\text{C}$. [c] Deviation = Predicted T_m - experimental T_m . [d] 50 mM aqueous acetic acid. [e] $1 \times \text{PBS}$. [f] 50 mM $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ buffer.

enable the prediction of T_m values of collagen triple helices with different frames as well as different groups and charge states at the termini.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

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