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Communication

Threonine Phosphorylation Is a Bioreversible Surrogate of Proline Hydroxylation in a Collagen Triple Helix

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ABSTRACT: Collagen, the most abundant protein in the human body and structural foundation of connective tissue, derives its remarkable stability from repeating (Xaa-Yaa-Gly)$_n$ sequences rich in (2*S*)-proline (Pro) and (2*S*,4*R*)-4-hydroxyproline (Hyp), the product of a post-translational modification. Here, we demonstrate that another such modification, phosphorylation, yields a bioreversible mimic of Hyp. Using synthetic collagen-mimetic peptides, we substituted the Xaa or Yaa position of a central Pro-Hyp-Gly triplet with (2*S*)-serine (Ser), (2*S*,3*R*)-threonine (Thr), or their *O*-phosphorylated derivatives, ^{P}Ser or ^{P}Thr. All substitutions were destabilizing—except ^{P}Thr in the Yaa position, which preserved the triple-helical stability conferred by Hyp. Moreover, ^{P}Thr within a collagen triple helix is a substrate for a mammalian secretory phosphatase. Notably, Thr is enriched at the Yaa position in mammalian fibrillar collagens, consistent with ^{P}Thr being a linchpin of collagen remodeling in the extracellular matrix. Thus, the phosphorylation of threonine (which is bioreversible) mimics the hydroxylation of proline (which is not), enabling dynamic modulation of collagen architecture in development and disease.

Collagen is the primary component of the extracellular matrix (ECM) in animals.[1,2] Its remarkable mechanical properties arise from a hierarchical structure, culminating in a right-handed triple helix composed of three left-handed, polyproline type-II (PPII) chains. Each chain has the sequence (XaaYaaGly)$_n$, in which glycine (Gly or G) occupies every third position, Xaa is typically (2*S*)-proline (Pro or P), and Yaa is typically (2*S*,4*R*)-4-hydroxyproline (Hyp or O), though these residues can vary. This motif is characteristic of all collagens, though interruptions occur in nonfibrillar subtypes.[3]

The abundance of proline-derived amino acids in the collagen sequence is critical for triple-helix formation. Their conformational rigidity preorganizes the main-chain torsion angles for PPII helix formation and minimizes the entropic penalty for folding.[4] These torsion angles correlate with the pyrrolidine ring pucker. Specifically, a C$^{\gamma}$-endo and C$^{\gamma}$-exo pucker yield ϕ angles that align with those in the Xaa and Yaa positions of a collagen strand, respectively. Unmodified proline favors a C$^{\gamma}$-endo pucker, which is optimal for the Xaa position (Figure 1A), whereas the Yaa position prefers the ϕ angle generated by a C$^{\gamma}$-exo pucker (Figure 1B).[5−7] Indeed, peptides with the sequence (ProProGly)$_7$ do not form stable triple helices, as they lack sufficient preorganization.[8,9]

Animals have evolved a post-translational modification (PTM) of Pro that enforces a C$^{\gamma}$-exo pyrrolidine ring pucker. Catalysis by collagen prolyl-4-hydroxylase installs a hydroxy group in the *R* configuration at C$^{\gamma}$.[10,11] The stereochemistry here is critical, as a N_i−$C^{\delta}{}_i$−$C^{\gamma}{}_i$−$O^{\delta}{}_i$ gauche effect elicits the C$^{\gamma}$-exo ring pucker (Figure 1B).[12−15] This PTM increases triple-helical stability[16] and is essential for animal life.[17,18]

Collagen acquires other PTMs.[1,2] For example, *O*-phosphorylation is well known to occur on its (2*S*)-serine (Ser) and (2*S*,3*R*)-threonine (Thr) residues.[19−22] Recently, Zondlo and co-workers noted that Thr phosphorylation in globular proteins leads to a cyclic structure that resembles proline (Figure 1C).[23] In this structure, a P−O···H−N hydrogen bond links the side chain to the main chain.[23] Hyperconjugation from this hydrogen bond enforces an n→π* interaction that is a hallmark of the PPII-type structure adopted by collagen strands.[26,27] In addition, *O*-phospho-(2*S*,3*R*)-threonine (^{P}Thr) prefers to adopt a ϕ angle that is near −60°,[23] which aligns better with the ϕ angle of Hyp rather than Pro in a collagen triple helix (Figures 1A and 1B). By contrast, *O*-phospho-(2*S*)-serine (^{P}Ser), which lacks the 3*R*-methyl group of ^{P}Thr, exhibits much greater conformational flexibility.[23,28]

Prior studies examined phosphorylation either at the Xaa position[29] or in native sequences, without regard to structural context.[22] To isolate the contribution of each phosphorylated residue, we synthesized a series of host−guest collagen-mimetic peptides (CMPs) with single amino acid substitutions within a defined (POG)$_n$ framework.

Peptides were synthesized by microwave-assisted Fmoc/*t*Bu solid-phase peptide synthesis, purified by HPLC, and characterized by MALDI-TOF MS. Each phosphopeptide had the sequence Ac-(POG)$_3$XYG(POG)$_2$(PO)-NH$_2$, where the central Xaa or Yaa residue was substituted with Ser, ^{P}Ser, Thr, or ^{P}Thr. A control peptide was synthesized with the sequence Ac-(POG)$_6$PO-NH$_2$. All nine peptides were capped with terminal amides to avoid interstrand Coulombic

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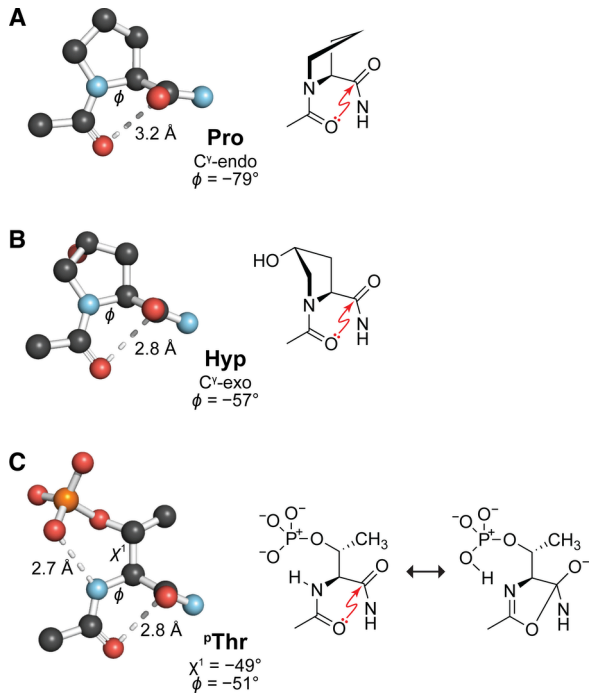

Figure 1. Structures of Pro (A) and Hyp (B) residues in a collagen triple helix and a ^{P}Thr (C) residue in a globular protein. C'_{i-1}═O_{i-1}···C'_i═O_i n→π^* interactions and a P−O···H−N hydrogen bond are indicated, as is an ensuing hyperconjugative resonance form of ^{P}Thr. ϕ refers to C'_{i-1}−N_i−C^{α}_i−C'_i; χ^1 refers to N_i−C^{α}_i−C^{β}_i−$O^{\gamma 1}_i$. Pro and Hyp are from PDB entry 1v7h;[24] ^{P}Thr is from PDB entry 3a8x.[25]

repulsion, and their sequences begin and end with ProHyp to avoid the instability of a terminal Gly, as described by Wennemers and co-workers.[30]

Peptide samples were prepared in buffer, heated to 65 °C, cooled to 4 °C, and equilibrated at 4 °C for ≥48 h. Circular dichroism (CD) spectroscopy revealed that each peptide adopted a collagen triple helix, as indicated by a maximum molar ellipticity at 225 nm (Figures 2 and S10). To evaluate the stability of each triple helix, we monitored the molar ellipticity at 225 nm as the samples were heated slowly from 4 to 65 °C. The temperature-dependent decreases in signal were consistent with cooperative denaturation of the triple helices (Figures 2 and S11). The value of T_m (which is the temperature at the midpoint of the thermal transition) was quantified for each peptide by fitting the data using a four-parameter Hill equation.

Several trends emerged (Figure 3). First and foremost, the phosphorylation of Thr in the Yaa position increased the T_m by 10.5 °C, nearly matching the stabilizing effect of Hyp. In contrast, Ser phosphorylation in the same position decreased T_m by 2.1 °C. Remarkably, ^{P}Thr conferred this stability despite Coulombic repulsion invoked by the introduction of three dianionic residues in the triple helix—underscoring the powerful structural contribution of ^{P}Thr (Figure 1C). In the Xaa position, the phosphorylation effects were more modest: ^{P}Thr and ^{P}Ser increased the T_m by 6.9 and 5.9 °C, respectively.

The position-dependent effect of ^{P}Thr mirrors the positional preference of Hyp itself and can be understood in the same terms. In the Yaa position, the collagen triple helix demands a backbone torsion angle ($\phi \approx -60°$) that is enforced by the C^{γ}-exo pucker of Hyp through a gauche effect. The cyclic,

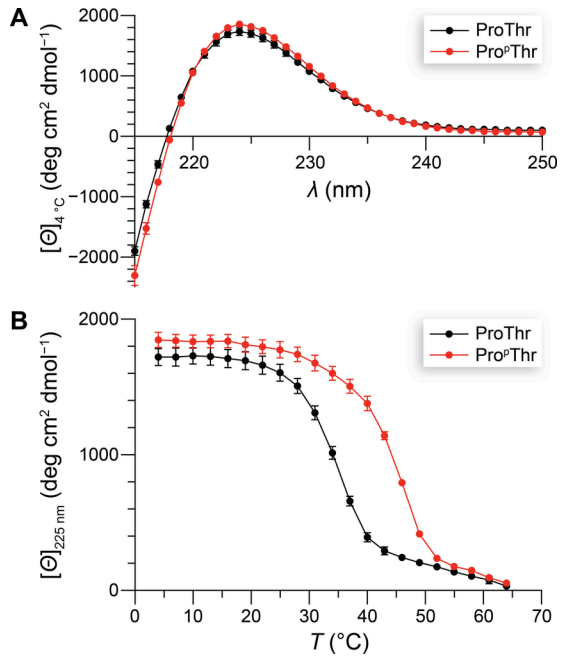

Figure 2. Conformational analyses of triple-helical Ac-$(POG)_3$XYG-$(POG)_2$(PO)-NH_2 peptides containing ProThr or Pro^{P}Thr as XY. (A) CD spectra of peptides at 4 °C in 0.4 mM in 10 mM sodium phosphate buffer, pH 7.4. (B) Effect of temperature on the molar ellipticity at 225 nm. T_m values: ProThr, 35.0 °C; Pro^{P}Thr, 45.5 °C. Values are the mean ± SD from 3 experiments. For additional spectra and thermal denaturation curves, see Figures S10 and S11.

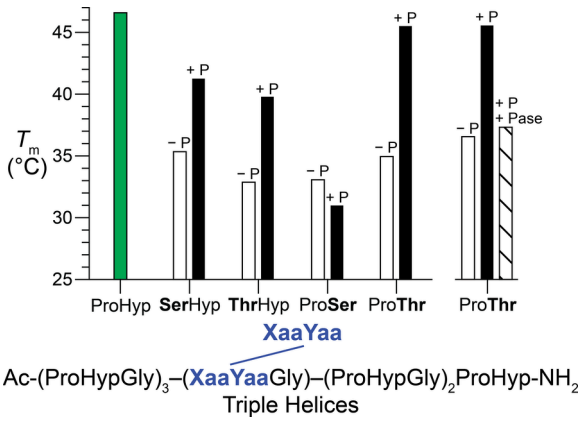

Figure 3. Bar graph of the T_m values of triple-helical peptides containing a central Ser (−P), ^{P}Ser (+P), Thr (−P), or ^{P}Thr (+P) at pH 7.4. In separate measurements at pH 7.9, which is optimal for enzymatic catalysis, an XaaYaa = Pro^{P}Thr triple helix was treated with a mammalian secretory phosphatase ("Pase") prior to analysis. Values were determined with CD spectroscopy. Thermal denaturation curves are shown in Figures S11 and S12B, and values of T_m are listed in Table S2.

hydrogen-bonded structure of ^{P}Thr (Figure 1C) likewise constrains ϕ to approximately −60°,[23] thereby mimicking the conformational role of Hyp at Yaa. In the Xaa position, by contrast, the required torsion angle ($\phi \approx -75°$) is a poorer match for the preferred ^{P}Thr geometry, and both ^{P}Thr and ^{P}Ser confer similar, modest stabilization, consistent with a nonspecific effect (e.g., Coulombic or solvation) rather than the specific conformational preorganization that distinguishes ^{P}Thr at Yaa. The absence of the 3*R*-methyl group in ^{P}Ser

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relaxes the conformational restriction,[23] explaining why Ser phosphorylation fails to replicate the Hyp-like stabilization at Yaa. Thus, just as Hyp is overwhelmingly concentrated in the Yaa position of natural collagens, the stabilizing power of ^{P}Thr is likewise Yaa-specific.

A key distinction between Thr phosphorylation and Pro hydroxylation lies in their reversibility. Whereas the hydroxylation of Pro residues is a permanent PTM,[31] animal cells secrete both kinases and phosphatases.[20,32] Accordingly, ^{P}Thr could act as a regulatory linchpin for collagen remodeling in the ECM. To test this hypothesis, we asked whether ^{P}Thr within a triple helix is a substrate for a mammalian phosphatase. We treated a CMP bearing a ^{P}Thr in the Yaa position with calf intestinal phosphatase (EC 3.1.3.1; UniProtKB P19111), a secretory enzyme. We found that the enzyme catalyzed the removal of the phosphoryl group within the triple helix (Figure S12). This dephosphorylation reduced the T_m to nearly that of the unphosphorylated Thr-containing triple helix (Figure 3).

To explore the biological relevance of these findings, we analyzed the distribution of Ser and Thr residues within the XaaYaaGly triplets of natural collagen triple-helical domains (THDs). Sequence analysis of abundant mammalian fibrillar collagens revealed a striking enrichment of Thr in the Yaa position (Yaa > Xaa; Figure 4, Table S3). No such enrichment was observed for Ser, which is distributed more evenly across the Xaa and Yaa positions (Yaa ≈ Xaa). Mass spectrometry-based phosphoproteomics has confirmed phosphorylation on collagen chains from multiple species, including types I, II, III, IV, and XVII.[19−22] Current proteomics databases, however, do not provide sufficient positional resolution to systematically determine whether Thr phosphorylation preferentially occurs at Yaa versus Xaa within collagen triple-helical domains. This gap reflects the technical challenges of collagen phosphoproteomics: the repetitive collagen sequence hampers unique peptide identification, its extensive post-translational processing (e.g., hydroxylation, glycosylation, oxidation, and cross-linking) complicates spectral interpretation, and extracellular phosphorylation events can be labile during sample preparation.[20,21] Nonetheless, the pronounced enrichment of Thr specifically at Yaa in fibrillar collagens (Figure 4)—where its phosphorylation would confer maximal stabilization (Figure 3)—is a striking coincidence that is difficult to rationalize on the basis of the unphosphorylated residue alone, given the negligible stability difference between Thr and Ser in the unphosphorylated state. Advances in enrichment strategies for extracellular phosphopeptides[20,21] should enable a direct test of this hypothesis.

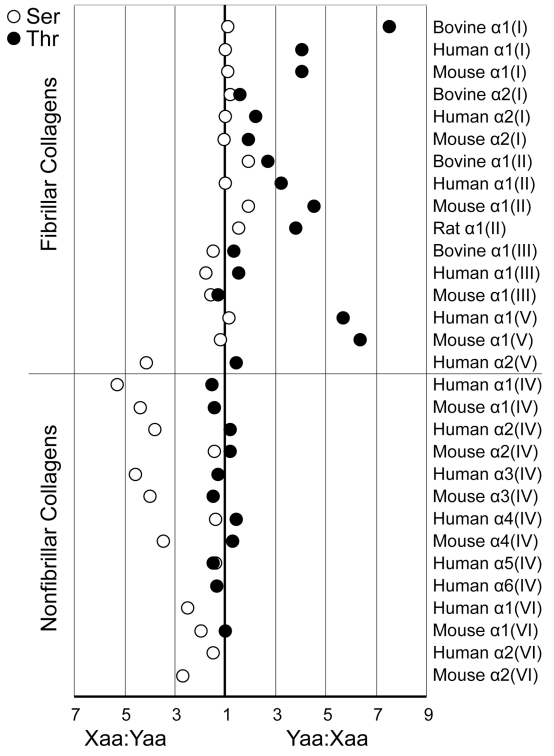

Figure 4. Relative abundance of Ser (○) and Thr (●) in the Xaa and Yaa positions of the THDs in 30 mammalian collagen chains. Human α1(VI), human α2(VI), and mouse α2(VI) do not contain Thr in the Yaa position. Data are listed in Table S3.

Unlike fibrillar collagens, nonfibrillar collagens such as collagen IV and collagen VI are not enriched in Thr in the Yaa versus Xaa position (Figure 4 and Table S3). In these collagens, which have THDs that are much shorter than those of fibrillar collagens and can contain interruptions, Ser was more prevalent in the Xaa than the Yaa position. In the human proteome, Ser or Thr followed by Pro is more likely to be phosphorylated than other Ser or Thr residues.[33−36] The phosphorylation of either of these residues in SerHyp and ThrHyp sequences confers significant stability to a collagen triple helix (Figure 3) and could have biological relevance.

Phosphorylation could provide a dynamic means of tuning the thermostability of collagen in vivo. The ECM undergoes constant remodeling through cycles of synthesis and proteolysis, often mediated by matrix metalloproteinases (MMPs) and cathepsin K.[37] For example, MMP-1 recognizes a motif beginning with Gly865 (Gly775 in the THD) of human collagen α2(I)[38] and locally unwinds the triple helix to access the scissile bond.[39] Phosphorylation of nearby Thr860 could enhance local triple-helix stability, thereby modulating susceptibility to enzymatic cleavage during development or repair. Moreover, pathological conditions such as inflammation,[40,41] ischemia,[42] and cancer[43] elevate extracellular ATP levels, enabling kinase activity that might influence collagen dynamics through phosphorylation.

Hyp was discovered in 1902,[44] and phosphorylated amino acids were described in 1906.[45] Our findings conceptually link these two historical landmarks: Thr phosphorylation mimics Pro hydroxylation both structurally and functionally in the collagen triple helix. This mimicry, unlike hydroxylation, is reversible, allowing the dynamic modulation of collagen architecture. These insights open the door to future studies on upstream signaling pathways that regulate collagen phosphorylation and its downstream consequences on ECM biology. We also anticipate the creation of artificial collagens whose triple-helical stability can be toggled by phosphorylation and dephosphorylation, enabling dynamic, stimulus-responsive biomaterials.

■ ASSOCIATED CONTENT

Supporting Information

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Peptide synthesis, purification, and characterization protocols; analytical procedures and data; Tables S1−S3; and Figures S1−S12 (PDF)

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Notes

The authors declare no competing financial interest.

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