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Carbohydrate Co-Solutes Stabilize Collagen Triple Helices

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Carbohydrates are common co-solutes for the stabilization of proteins. The effect of carbohydrate solutions on the stability of collagen, the most abundant protein in mammals, is, however, underexplored. In this work, we studied the thermal stability of collagen triple helices derived from a molecularly defined collagen model peptide (CMP), Ac-(Pro-Hyp-Gly)$_7$-NH$_2$, in solutions of six common mono- and disaccharides. We show that the carbohydrates stabilize the collagen triple helix in a concentration-dependent manner, with an increase of the melting temperature of up to 17 °C. In addition, we show that the stabilizing effect is similar for all studied sugars, including trehalose, which is otherwise considered a privileged bioprotectant. The results provided insight into the effects of sugar co-solutes on collagen triple helices and can aid the selection of storage environments for collagen-based materials and probes.

Introduction

Collagen is the most abundant biopolymer in mammals. As a structural extracellular matrix protein, collagen provides stability to bone, skin, tendon, cartilage and any connective tissue.[1–2] The physicochemical properties of the collagen triple helix (Figure 1), the basic structural fold of collagen, have been extensively studied using synthetic collagen model peptides (CMPs).[1] These studies probed the influence of the CMP sequence on the thermal stability of the collagen triple helix. Variations of the Xaa-Yaa-Gly repeat sequence of collagen has revealed the fundamental importance of glycine (Gly, G) as every third amino acid and the stabilizing effect of (2*S*)-proline (Pro, P) and (2*S*,4*R*)-4-hydroxyproline (Hyp, O) in the Xaa and Yaa positions, respectively (Figure 1).[1] The incorporation of non-canonical amino acids into CMPs has provided insight into the contributions of interstrand H-bonding, preorganization of the single strands into polyproline type II (PPII)-helices, hydrophobicity, as well as steric and stereoelectronic effects on triple helix stability.[3–19] Recently, we have shown that also the collagen frame[20] and terminal functional groups[21–22] strongly affect the stability of collagen triple helices.

The influence of the environment on the stability of collagen triple helices is less explored. Studies with natural collagen preparations showed that the addition of propan-1-ol, sodium chloride, or urea to aqueous solutions destabilizes the triple helix.[23–24] Recent studies used this destabilizing effect of co-solvents or additives on the triple helix to develop a solvent-switching protocol for targeting damaged collagen in histological samples with collagen hybridizing peptides.[25] Other solvents than water, for example, propane-1,2-diol and octan-1-ol stabilize CMP trimers to a significant extent.[26–27] Our group showed that a local hydrophobic environment created by lipidation stabilizes collagen triple helices in water.[13,28] Brodsky introduced trimethylamine-*N*-oxide (TMAO) as a stabilizing additive,[29] which is commonly used to quantify the stability of triple helices from CMPs with low trimerization propensity.[29–31] Several studies have also shown that aqueous solutions of glycerol and other polyols stabilize collagen triple helices.[23–25,32–34] These include two reports that used carbohydrate solutions as stabilizing environments for natural collagen preparations.[33–34] Wang observed stabilization of natural type I bovine tendon collagen by trehalose[34] and Gekko and Koga stabilization of type III calf skin collagen by various other sugars.[33] Among saccharides, trehalose has been reported to have unique bioprotective properties against freezing, drying, and other environmental stress factors.[35–36] Often, trehalose has been found to be more efficient at preventing protein denaturation than other saccharides.[37–40] We became curious whether trehalose also stabilizes collagen more than other carbohydrates. Whereas the prior studies hint at such an effect,[33–34] the inhomogeneity and variability of collagen

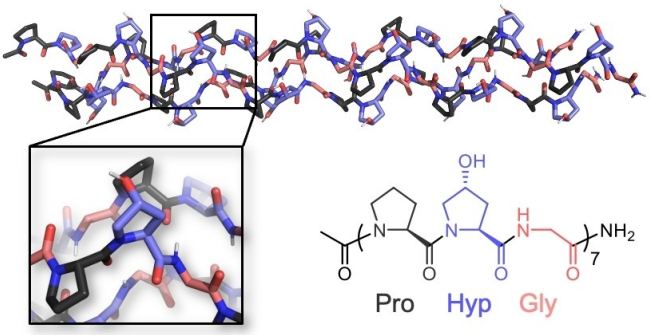

Figure 1. Structure of triple-helical collagen with Pro-Hyp-Gly repeat units and the CMP used in this study.

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preparations from natural sources do not allow for a quantitative comparison of the influence of trehalose versus other carbohydrates on collagen triple helix stability.

In this work, we used a molecularly defined CMP to quantify collagen triple helix stabilities in solutions of six carbohydrates that are widespread in nature: arabinose (Ara), galactose (Gal), glucose (Glc), lactose (Lac), maltose (Mal), and trehalose (Tre; Figure 2).

Results and Discussion

To evaluate the effect of carbohydrates on the stability of collagen triple helices, we used the 21-mer CMP Ac-(Pro-Hyp-Gly)$_7$-NH$_2$ (Ac-(POG)$_7$-NH$_2$), one of the most studied CMPs.[1] Firstly, we prepared solutions of Ac-(POG)$_7$-NH$_2$ in pure water and in aqueous solutions of glucose (2.0 M) and trehalose (1.0 M; equivalent to 2.0 M glucose units). These solutions were heated to 85 °C to dissociate any assembly and subsequently cooled to induce triple helix formation. CD spectra of all samples show a maximum at 225 nm and a minimum close to 200 nm, the signature of PPII helicity (Figure 3a). The spectra were similar, regardless of the sugar and did not change upon increasing the concentration of glucose (Figure 3b, Figure S1).

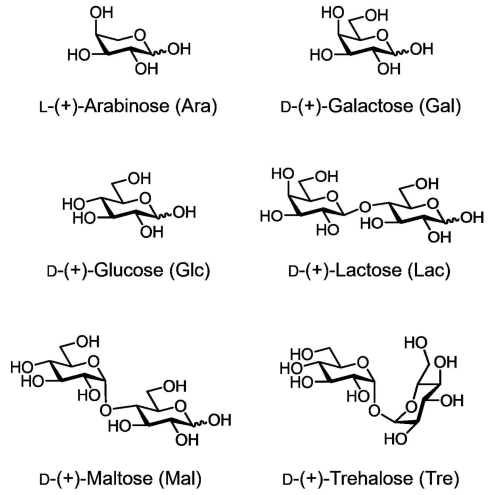

Figure 2. Structures of the carbohydrates used in this study.

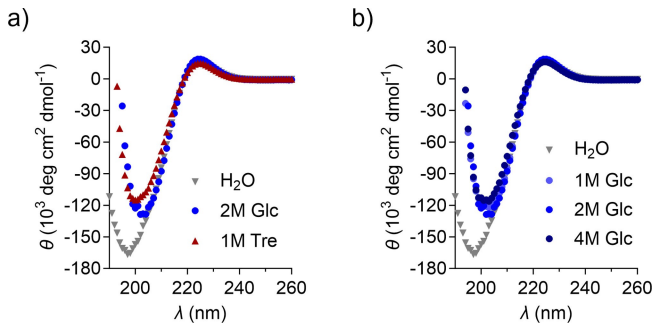

Figure 3. CD spectra of Ac-(POG)$_7$-NH$_2$ (200 μM) in (a) pure water, and aqueous solutions of glucose (2 M), and trehalose (1 M); (b) aqueous solutions with increasing glucose concentration.

These results show that the CMP adopts a PPII-helical conformation in aqueous solutions of common saccharides.

Next, we determined the thermal stability of the triple helix derived from Ac-(POG)$_7$-NH$_2$ in water, 2.0 M glucose, and 1.0 M trehalose by thermal denaturation with CD spectroscopy monitoring. For all three solutions, we observed a sigmoidal decrease in ellipticity typical for collagen triple helix denaturation (Figure 4a). The melting temperature (T_m), the midpoint of the sigmoidal transition, which is a good relative measure of triple helix stability, was more than 7 °C higher in 2.0 M glucose and 1.0 M trehalose (T_m = 50.2 and 50.4 °C, respectively) than in pure water (T_m = 42.8 °C). These results show that carbohydrates stabilize collagen triple helices to a significant extent. Importantly, the triple helix stability in a 2.0 M glucose solution (T_m = 50.2 °C) was the same as in a 1.0 M solution of trehalose (T_m = 50.4 °C). These results indicate that an effective concentration of 2.0 M per glucose unit has the same stabilizing effect on the collagen triple helix regardless of whether in the form of a monosaccharide or a disaccharide linked through an α-1,1′ glycosidic bond.

Experiments in solutions with varied concentrations of glucose (0.1–4.0 M) revealed that the stability of the triple helix derived from Ac-(POG)$_7$-NH$_2$ increases in more concentrated glucose solutions (Figure 4b, Table 1).[41] In a 4.0 M glucose solution, the melting temperature was as high as 59.6 °C, almost 17 °C higher than in pure water (Figure 4b). Further experiments in aqueous solutions of four other common carbohydrates, arabinose (Ara), galactose (Gal), lactose (Lac), and maltose (Mal) (Table 1, Figure S2) further corroborated the concentration-dependent stabilization of the collagen triple helix by carbohydrate solutions (Table 1, Figure 5).

These experiments also revealed that monosaccharide solutions and disaccharide solutions at half the concentration stabilize the collagen triple helix to a similar extent, regardless of the type of saccharide. In 1.0 M monosaccharide and 0.5 M disaccharide solutions, the T_m values are within a ΔT_m range of 1.1 °C (from 45.2 °C for arabinose, to 46.3 °C for galactose; Table 1). This similarity indicates that saccharides stabilize the collagen triple helix by a common mechanism.

These results let us wonder, whether there is a common variable for all carbohydrate solutions with which the triple helix melting temperature scales linearly. We thus plotted the

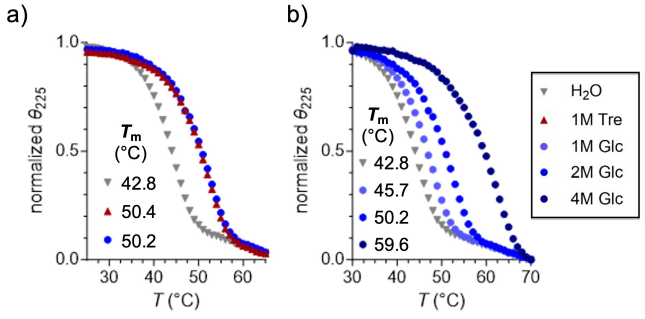

Figure 4. Thermal denaturation curves and T_m values obtained with CD monitoring (225 nm) of Ac-(POG)$_7$-NH$_2$ (200 μM) in (a) pure water, 2 M aq. glucose, and 1 M aq. trehalose; (b) aqueous solutions with increasing glucose concentration. Heating rate: 1 °C/114 s.

Table 1. Melting temperatures (T_m) of triple helices derived from Ac-$(POG)_7$-NH_2 in aqueous solutions of carbohydrates at different concentrations.

c (M)[a]	T_m (°C)[b]					
	Ara	Gal	Glc	Lac	Mal	Tre
0.0				42.8		
0.1	42.8	43.0	42.9	–[c]	–[c]	–[c]
0.2	43.2	43.4	43.3	–[c]	–[c]	–[c]
0.5	–[c]	–[c]	–[c]	45.6	45.8	46.1
1.0	45.2	46.3	45.7	–[c]	49.1	50.4
2.0	48.1	–[c]	50.2	–[c]	–[c]	–[c]
4.0	–[c]	–[c]	59.6	–[c]	–[c]	–[c]

[a] Carbohydrate concentration in mol L^{-1}. [b] Measured by thermal denaturation monitored by CD spectroscopy. CMP concentration 200 μM, heating rate 1 °C/114 s. [c] Not measured.

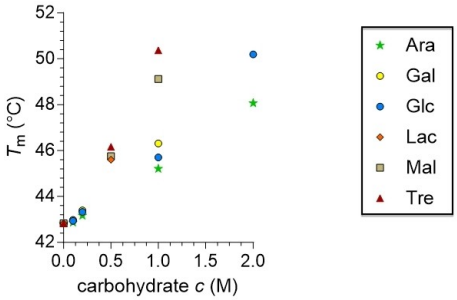

Figure 5. Melting temperatures of the triple helix derived from Ac-$(POG)_7$-NH_2 in aqueous solutions of different carbohydrates at varying molar concentrations. Measured by thermal denaturation monitored by CD spectroscopy. CMP concentration 200 μM, heating rate 1 °C/114 s.

obtained T_m values as a function of the molar concentration of the monosaccharide units (Figure 6a), the mass concentration of the carbohydrates (Figure 6b), the molar concentration of the hydroxy groups (Figure 6c), and the molar concentration of equatorial hydroxy groups (Figure 6d).[42] The best linear fit with $R^2 = 0.981$ resulted from the dependence of the T_m value on the mass concentration of the saccharides (Figure 6b). These results further support that carbohydrate solutions stabilize collagen triple helices but that the specific carbohydrate structure has little influence on the stabilizing effect.

Finally, we asked whether the viscosity of the carbohydrate solutions could explain the observed trends. We, therefore, determined the stability of the collagen triple helix derived from Ac-$(POG)_7$-NH_2 in aqueous solutions of poly(ethylene glycol) (PEG; average $M_n = 4.6$ kDa) at 10, 50, and 100 mM concentrations (4.6%, 23%, and 46% *w/w*, respectively) and obtained T_m values of 43.0, 44.0, and 46.3 °C, respectively (Figure S2). Thus, the PEG solution with the highest concentration (46% *w/w*, 100 mM) increases the stability of the Ac-$(POG)_7$-NH_2 derived triple helix by $\Delta T_m = 3.5$ °C. This solution has a reported viscosity of over 40 mPa·s at 25 °C.[43] In contrast, a glucose solution at the highest investigated concentration (4.0 M) has a lower viscosity (~20 mPa·s)[44] but causes a significantly higher stability increase of nearly $\Delta T_m = 17$ °C (Table 1). These results show that the macroscopic viscosity of the aqueous solution plays only a minor role in the stabilizing effect of carbohydrate solutions on collagen triple helices.

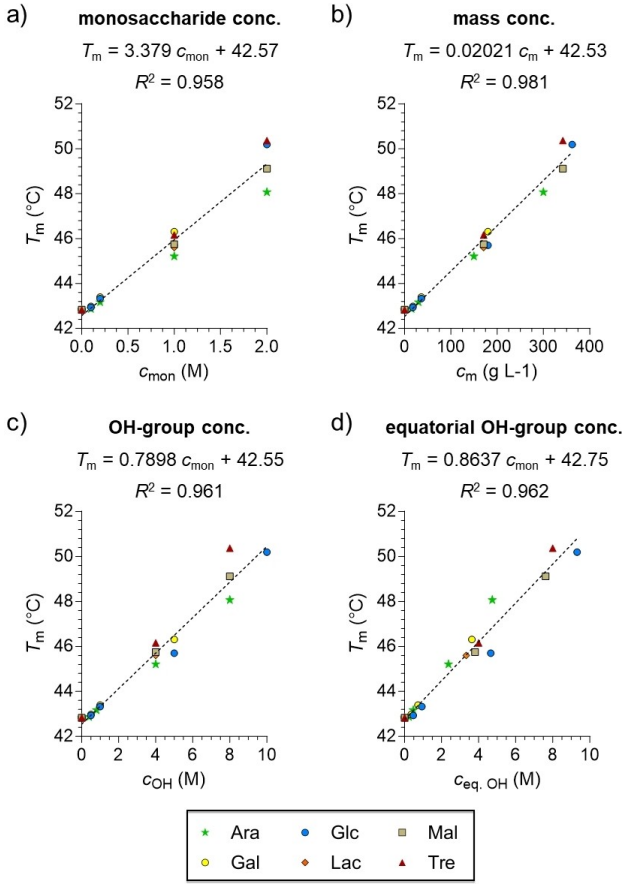

Figure 6. Melting temperatures of triple helices derived from Ac-$(POG)_7$-NH_2 in aqueous solutions of carbohydrates plotted against (a) the effective monosaccharide concentration; (b) the mass concentration of the carbohydrate; (c) the molar concentration of carbohydrate OH-groups; and (d) the molar concentration of equatorial carbohydrate OH-groups. Measured by thermal denaturation monitored by CD spectroscopy. CMP concentration 200 μM, heating rate 1 °C/114 s.

Overall, the results show that a) carbohydrate solutions stabilize collagen triple helices in a concentration-dependent manner and b) the different studied mono- and disaccharides

have a similar stabilizing effect. In particular, our results revealed that trehalose is not special in the context of collagen triple helix stabilization.

This finding is, at first glance, surprising since trehalose exhibited unique protein-stabilizing properties for several α-helical and β-sheet proteins.[37–40] Considering the evolution of trehalose as a bioprotectant and that of collagen as a structural protein, the result is less surprising. Trehalose appeared early in the evolutionary tree and is conserved in prokaryotes, early eukaryotes, plants, and invertebrates.[45] In contrast, collagen, despite being the most abundant protein in mammals, emerged only with the evolution of multicellular animals.[46] Trehalose thus evolved as a bioprotectant in the absence of collagen. In fact, the two biomolecules, with the exception of some invertebrates,[47–48] are not commonly produced by the same organism.

The stabilization of α-helical and β-sheet protein folds by sugar solutions has been well studied, indicating an interplay between protein–sugar volume exclusion complemented by supramolecular and soft interactions as the stabilizing force.[49] In contrast, PPII folds have been largely overlooked in this regard. Our findings indicate that different sugar solutions stabilize collagen triple helices by a common mechanism but that the features of collagen stabilization may differ from those of other protein folds. The work, therefore, presents a case for the inclusion of PPII-helical and triple helical proteins in more detailed studies on the mechanisms of bioprotection by carbohydrate co-solutes.

Conclusions

In this study, we evaluated the effects of six common mono- and disaccharides on the stability of collagen triple helices derived from the molecularly defined CMP Ac-$(POG)_7$-NH_2. We found that the saccharides stabilize the triple helix in a concentration-dependent manner. The triple helix melting temperature increases by as much as 17 °C in a 4.0 M glucose solution compared to pure water. In addition, we found a strong linear correlation between the triple helix melting temperature and the mass concentration of the saccharide, showing that the stabilizing effects of all tested mono- and disaccharides do not significantly differ. Interestingly, trehalose, generally considered to possess unique protein-stabilizing properties, does not provide stronger collagen triple helix stabilization compared to the other tested mono- and disaccharides. Our work provides quantitative fundamental insights into the behavior of PPII-helical assemblies in saccharide solutions and can guide the choice of environments used in the preparation and storage of collagen-based materials[50–56] and probes.[57–60]

Supporting Information

The authors have cited an additional reference within the Supporting Information.[61]

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: collagen · carbohydrates · peptides · proline · trehalose

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