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Selective Assembly of a High Stability AAB Collagen Heterotrimer

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> How collagen is able to obtain control of helix composition and register is poorly understood yet is critical for determining the structure and properties of the most abundant protein structure in the human body. In humans there are 28 known types of collagen that can form homotrimeric (AAA) or heterotrimeric (AAB and ABC) compositions. Additionally, because of a single amino acid offset between peptide chains in the triple helix, distinct heterotrimers of different registers can be formed. In this communication we describe an AAB collagen heterotrimer with controlled composition and register. This is the first report of a collagen heterotrimer whose thermal stability is greater than that of any of its component parts and therefore is the dominant species in solution. In a 2:1 mixture of (EOGPOG)₅ with (PRG)₁₀, the AAB heterotrimer melts 10 °C higher in temperature than the next most stable species.

> Over the past decade, research has focused on forming high-stability collagen mimetic triple helices² utilizing amino acid propensity, ³⁻⁵ electrostatics, ^{4,6-9} hydrophobicity, ^{5,10} cysteine knots,11 and natural collagen sequences 12 to drive the formation of triple helical peptide assemblies, but most of these reports focus on homotrimers and little light has been shed on control over composition and register. Recently we introduced a method of peptide assembly which utilizes electrostatics to form high stability triple helices. Here we apply that method to the formation of an AAB heterotrimer. The design concept is simple: combination of peptides who follow the canonical (X-Y-Gly)_n amino acid repeat in a 2:1 ratio in which the more abundant peptide has a charge 1/2 and opposite of the other should result in the formation of an AAB heterotrimeric collagen helix. This will be the dominant species because it is neutral (zwitterionic) while homotrimers should be destabilized because of charge repulsion.

> The thermal stability of each system was analyzed at a total peptide concentration of 0.2mM in 10mM Tris at a pH of 7.0 using circular dichroism (CD). Detailed CD melting profiles for all peptides and their mixtures are available in the supporting information. The peptide mixtures were either melted after an overnight incubation at 10 °C (the non-annealed sample) or preheated for 15 minutes at 85 °C then cooled down and incubated at 10 °C overnight before running a melting experiment (the annealed sample). 9 The annealing process is designed to unfold any homotrimeric or kinetically trapped species present in the sample and to drive the formation of the most thermodynamically stable species. To determine whether a heterotrimer is formed, the non-annealed and annealed samples are compared to the homotrimer data and new thermal transitions seen can be tentatively assigned to heterotrimeric species.

> Two peptides were prepared, (PRG)₁₀ and (EOGPOG)₅. In both cases the N- and C- termini were acetylated or amidated to limit charged groups to the side chains of arginine and glutamic acid. In Tris buffer, (PRG)₁₀ shows no cooperative thermal transition (see supporting information) while (EOGPOG)₅ shows a thermal transition characteristic of a collagen triple

helix at 46 °C. When a 2:1 mixture of $(EOGPOG)_5$ and $(PRG)_{10}$ is examined by CD (Figure 1) a heterotrimer is clearly indicated with a melting temperature of 56 °C - higher than the $(EOGPOG)_5$ homotrimer by 10 °C. This represents the first synthetic peptide system that selectively forms a collagen-like heterotrimer with a thermal stability higher than any of its homotrimeric components. As a triple helix with an AAB composition, this is particularly relevant for collagens of type I, IV, V and VIII.

To further analyze the $(EOGPOG)_5 / (PRG)_{10}$ helix, DSC and NMR experiments were performed. The DSC scans, shown in supporting information, were used to assess the reversibility of the melting profile for the system. The first peptide scan showed a minor peak at 32 °C, that disappeared in all subsequent scans, and a major peak at 56 °C that agrees with the CD melting data. The minor transition at 32 °C may be either $(PRG)_{10}$ homotrimer (see DSC experiments available in supporting information) or a different peptide register of the AAB heterotrimer. In either case, this component is not detectable by CD and does not refold during the relatively rapid DSC cycle time. The reproducibility of multiple DSC scans is consistent with previous reports that heterotrimeric species stabilized by electrostatic interactions have a shorter refolding half-life than neutral homotrimers and thus can be analyzed repeatedly after only a short refolding interval. Solution NMR experiments were run to 1) confirm the triple helical topology of the molecular assembly giving rise to the cooperative thermal transition, 2) to confirm the AAB composition of the helix, 3) to determine how the arginine and glutamic acid side chains are interacting and 4) to assess the register, or relative stagger, between peptide chains within the triple helix.

Nuclear Overhauser effect spectroscopy (NOESY) and total correlated spectroscopy (TOCSY) experiments were recorded at 25 °C to sequence the spin systems. The experiments showed that a 2:1 mixture of the (EOGPOG)₅ and (PRG)₁₀ peptides contains several species including monomeric forms of (EOGPOG)₅ and (PRG)₁₀, and a small quantity of (EOGPOG)₅ homotrimer while the major component corresponds to an AAB heterotrimer. The AAB assembly shows typical NOEs expected from a triple helix, such as the glycine packing interactions at the core of the helix. Using these peaks in conjunction with Arg-Glu backbone cross-peaks it was possible to determine the register of the dominant species to be (PRG)₁₀• (EOGPOG)₅•(EOGPOG)₅. A secondary heterotrimeric spin system with weaker peaks was identified but could not be unambiguously sequenced due to spectral overlap. This spin system may arise from less ordered regions at the termini of the triple helices or from a different peptide register. In either case it is a minor component.

Using the information from the intra-residue and inter-chain NOEs of the Arg and Glu sidechains, a set of conformational restraints was built and a model of the AAB triple helix minimized using experimental constraints was made and is described in the supporting information. The results (Fig 2a) show two main arginine side chain conformers with fixed dihedrals, leading to a unique chemical shift for all diastereotopic methylene protons along the side-chain. While the arginine generates NOE contacts with the glutamic acid amide proton, the predominant configuration does not appear to include direct hydrogen bonded contacts between the charged moieties. Instead, the guanidinium groups are positioned to form optimal hydrogen bonds with the hydroxyproline carbonyls of (EOGPOG)5 which is positioned in the second register (Fig 2b). The glutamic acid side chains are positioned between alternate arginines to screen the positive charges. Furthermore, the γ 2 dihedral of these glutamic acids adopts a dynamic conformation, as evidenced by the chemical shift equivalence of both γ protons. This observation suggests the formation of a weak salt bridge between the glutamate and the arginine 2 conformer, although this cannot be confirmed due to lack of information on the $\chi 4$ dihedral of the arginine side-chain. This conformation differs from the aspartic acidlysine salt bridges previously determined via NMR in an ABC heterotrimer,8 but is not completely unexpected as there are crystal structures of triple helical peptides that show a

similar interaction.¹³ Interestingly, the (EOGPOG)₅ in the third peptide register is chemically distinct with its glutamates oriented away from arginine side chains.

In conclusion, we have identified a collagen mimetic peptide system that selectively forms a high stability AAB heterotrimer with a thermal melting temperature higher than that of any of its homotrimers analyzed via CD and DSC unfolding studies. 2D NMR experiments confirmed the triple helical nature of the system and identified the dominant species to consist of a single register (PRG)₁₀• (EOGPOG)₅• (EOGPOG)₅. The success of this system is likely due to the high content of arginine in the triple helix which in other studies has been shown to form high stability triple helices in host-guest studies due to the ability of the arginine side chain to hydrogen bond with backbone carbonyls of other peptide chains.³ Many collagens, such as type I, are AAB heterotrimers and our AAB collagen mimetic system lays the groundwork for further understanding of the effects that molecular changes within the collagen sequence can have on the helical nature and packing into fibrils as well as for use as flanking regions to study sequences of native collagen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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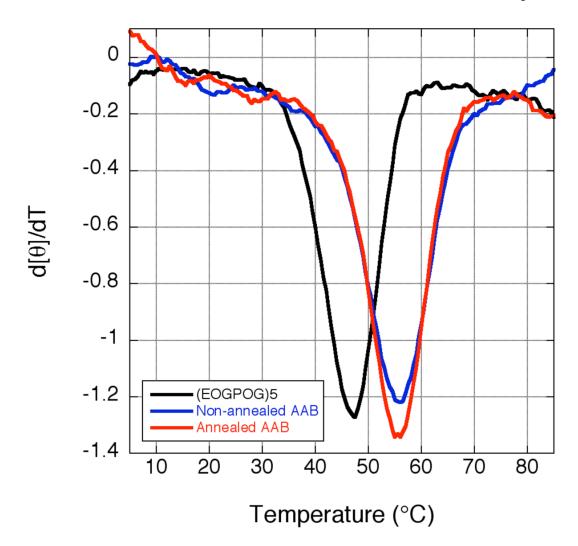


Figure 1. CD data for the first derivative of the melting curve for (EOGPOG)₅ homotrimer (black), non-annealed (blue) and annealed (red) samples of the 2:1 mixture of (EOGPOG)₅ and (PRG)₁₀.

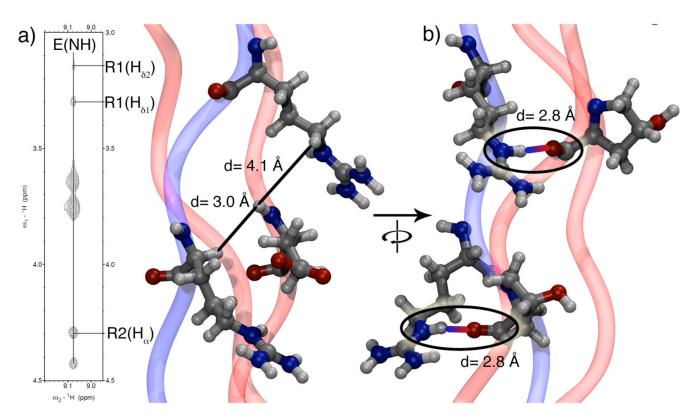


Figure 2.

(a) NOESY spectrum and molecular model highlighting the cross-peaks between the arginine and glutamic acid side-chains and the atoms giving rise to the NOEs, sequential and intraresidue peaks are not labeled for clarity (b) Alternate view of the model highlighting the hydrogen bonds between the guanidium groups and hydroxyproline backbone carbonyls using colored arrows.