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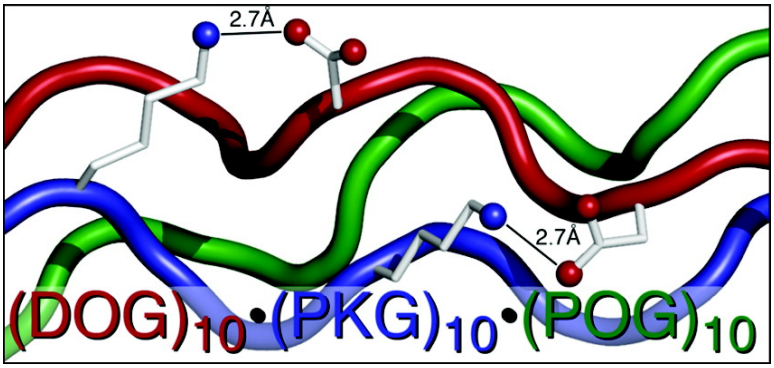

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Surprisingly High Stability of Collagen ABC Heterotrimer: Evaluation of Side Chain Charge Pairs

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Abstract: Type I collagen is a major component of skin, tendon, and ligament and forms more than 90% of bone mass. It is an AAB heterotrimer assembled from two identical α1 and one α2 chains. However, the majority of studies on the effects of amino acid substitution on triple helix stability have been performed on collagen homotrimeric helices. In a homotrimer, it is impossible to determine whether the contribution to stability is from the polyproline II helix propensity of the amino acids or from interhelix amino acid interactions. The presence of amino acids in all three chains further exaggerates their contribution. In contrast, in a heterotrimer, the individual chains may be tailored in order to have the substitution in one, two, or all three chains. Therefore, a heterotrimer can divulge specific information about any interaction based upon the substitutions in individual chains. In this paper, we evaluate the contribution of electrostatic interactions between side chain charge pairs on the stability of heterotrimers. We synthesize and analyze the stability of four AAB and four ABC heterotrimers including a surprisingly stable ABC heterotrimer composed of $(DOG)_{10}$, $(PKG)_{10}$, and $(POG)_{10}$ chains (O = hydroxyproline). This heterotrimer has a stability comparable to that of a $(POG)_{10}$ homotrimer even though D and K occur 20 times in the heterotrimeric helix and have been previously shown to significantly destabilize the triple helix compared to the P and O imino acids. These results show that the stability of heterotrimers cannot be directly determined from the analysis of charge pairs in homotrimers. Because collagen heterotrimers can be designed to have substitution in one, two, or three chains, it gives us the ability to decode cross-strand interactions in collagen in a similar fashion to α-helical coiled-coil interactions and DNA duplex hydrogen bonding.

Introduction

Understanding the effect of amino acid substitution on collagen triple helix stability is crucial in analyzing the structural, mechanical, and biological changes observed in mutant collagens which lead to diseases such as *Osteogenesis Imperfecta* (OI) and other deleterious sequence variations.[1,2] OI is a brittle bone disease which is caused by mutations in type I collagen, an AAB heterotrimer consisting of two identical α1(I) and one α2(I) chains.[2] Other naturally occurring heterotrimeric collagens, such as AAB type IV and VIII and ABC type V, VI, and IX, are involved in various diseases including Alport Syndrome, Ehlers-Danlos Syndrome, and Bethlem Myopathy.[1,2] Collagen homotrimers have been extensively used to study amino acid substitutions,[3−7] their polyproline II helix propensity,[4] their effect on stabilization by water-mediated hydrogen bonding[8,9] or steric and stereoelectronic effects,[10,11] and the interhelix amino acid interactions.[8,9] There are many good reviews which discuss the structure and biochemistry of collagen.[12] Several high-resolution crystal structures which have been solved are of particular interest.[13,14] However, most natural collagens, including the most abundant type I collagen, are heterotrimers, not homotrimers, and diseases caused by mutations in these sequences affect only

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one or two of the three chains which make up the helix. A number of studies have prepared collagen heterotrimers by cysteine-knot strategy;[15] however, only a few have shown noncovalent assemblies that form heterotrimers.[10,16,17] Because of the importance of heterotrimers in natural collagens, it is important to design systems that have the potential to study these variations in their native form, whether they are AAB heterotrimers or ABC heterotrimers. The synthesis of extracellular matrix mimics based upon collagen-like peptides has completely relied upon homotrimers.[18] Collagen-like heterotrimers, with their ability to assemble multiple functional groups close to each other and thereby increase chemical diversity, can lead to the synthesis of more sophisticated biomaterials which more closely mimic extracellular matrix when compared to those based on homotrimers. Furthermore, the effect of amino acid substitution, for example, glycine mutations, could be assessed in one, two, or all three strands of the triple helix. In this paper, we demonstrate the synthesis, characterize the stability of four ABC and four AAB collagen heterotrimers, and illustrate how results from the analysis of homotrimers cannot be directly applied to heterotrimers. We conclude that to more fully understand the structural implications of collagen structural mutants these molecules must be prepared as heterotrimers and not as homotrimers.

Collagen is characterized by a unique right-handed triple helical structure composed of three left-handed polyproline type II helices.[9,19] The primary sequence of collagen has a X−Y−Gly repeating motif, where X and Y are generally P and O, respectively. The presence of G at every third position allows the formation of a tightly packed triple helix which, along with an extensive network of $CO_{(X)}-NH_{(Gly)}$ hydrogen bonds, imparts stability to the triple helix. Side chains of amino acids in the X and Y positions are exposed to solvent to varying degrees[20] and can take part in intra- and intermolecular interactions.[14,21] Substitution of imino acids (P and O) by amino acids has been shown to cause destabilization of the triple helix.[4] Charge pair interactions have also been studied in contiguous triplets,[5,7] and they follow a pattern with GER causing the least destabilization followed by GDR, GEK, and GDK when compared to parent polypeptide containing GPO. KGE and KGD triplets[6] have also been shown to stabilize the triple helical structure as much as the parent OGP triplet and are found abundantly in natural collagens. These results can be used to predict the stability of charge pairs for homotrimers. However, the effect observed on stability will be a mixture of attractive and repulsive interactions between various charges, along with the polyproline II helix propensity of every substituted amino acid. Heterotrimers assembled by charge pair interactions may lead to a better estimate of the attractive interactions between the opposite charges because these interactions can be studied in isolation from other complicating interactions. We recently[17] reported the formation of an ABC heterotrimeric system that consisted of negatively charged, positively charged, and neutral polypeptide with the sequence $(EOG)_{10}$•$(PRG)_{10}$•$(POG)_{10}$. Extending this strategy, we can assess the magnitude of attractive interactions between E−R, D−R, E−K, and D−K in heterotrimeric collagen helices.

Table 1. Sequence of Peptides Studied[a]

#	sequence	abbreviation
1	$(POG)_{10}$	***O***
2	$(EOG)_{10}$	***E***
3	$(DOG)_{10}$	***D***
4	$(PRG)_{10}$	***R***
5	$(PKG)_{10}$	***K***

[a] The N- and C-terminals of all peptides are acetylated and amidated, respectively.

The polypeptides prepared are abbreviated as ***O***, ***E***, ***D***, ***R***, and ***K*** throughout the remainder of this paper, as shown in Table 1. All of the peptides used in the study are N-terminal acetylated and C-terminal amidated to avoid charge interactions between the termini.[7] The triple helices are abbreviated, for example, ***E•R•O*** for $(EOG)_{10}$•$(PRG)_{10}$•$(POG)_{10}$.

We report the formation of an ABC heterotrimer with stability comparable to that of ***O•O•O*** homotrimer. This extremely stable ABC heterotrimer consists of ***D•K•O*** as its triple helix and thus contains 20 imino to amino acid substitutions. The attractive interactions between the D and K side chains are strong enough to overcome the low polyproline II helix propensity[4] of both these amino acids and are able to stabilize the heterotrimer to within 2.5 °C of the ***O•O•O*** homotrimer. This work is a step closer to understanding of thermal stability of collagen heterotrimers and lays the foundation for the detailed study of amino acid sequence−structure relationship in heterotrimers and the synthesis of extracellular matrix mimics.[18]

Experimental Section

Peptide Synthesis and Purification. All of the peptides were synthesized on an Advanced ChemTech 396 multipeptide automated synthesizer using Fmoc solid-phase chemistry based on a 0.15 mmol scale. The synthesis, purification by reverse phase HPLC, and analysis by MALDI are the same as described previously.[17] HPLC and MALDI-TOF spectra can be found in the Supporting Information.

Circular Dichroism Spectroscopy. CD measurements were performed with a Jasco J-810 spectropolarimeter, equipped with a Peltier temperature control system, using quartz cells with a path length of 0.1 cm. Thermal unfolding curves were obtained by monitoring the

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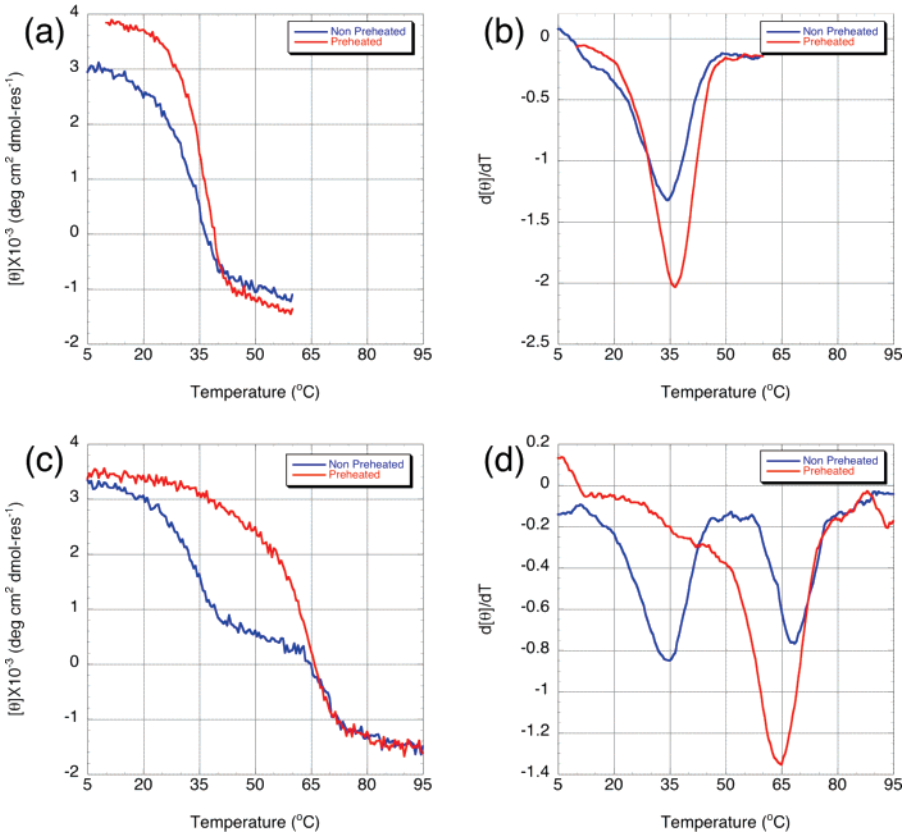

Figure 1. Circular dichroism for AAB and ABC heterotrimeric helices formed by mixing ***D***, ***K***, and ***O***. (a) Thermal analysis shows cooperative unfolding for the ***D***/***K*** mixture in a 1:1 ratio for the non-preheated and preheated case. (b) The first derivative of unfolding versus temperature. (c) Cooperative unfolding for the ***D***/***K***/***O*** mixture in a 1:1:1 ratio for non-preheated and preheated case. (d) The first derivative of unfolding shows that the ***D***/***K*** AAB heterotrimer and ***O•O•O*** homotrimer are observed without preheating, and these merge to a single transition corresponding to the ***D•K•O*** ABC heterotrimer after preheating.

decrease in ellipticity in a desired temperature range at a wavelength where the CD spectra show a positive maximum. The temperature ranged from 5 to 95 °C at a heating rate of 10 °C/h, depending on the peptide mixture studied. Fraction folded was calculated for the melting curves according to the equation:

$$F_T = \frac{[\theta]_T - [\theta]_{\text{unfolded}}}{[\theta]_{\text{folded}} - [\theta]_{\text{unfolded}}} \quad (1)$$

where F_T is the fraction folded at temperature T, $[\theta]_T$ is the molar residue ellipticity (MRE) at temperature T, $[\theta]_{\text{folded}}$ and $[\theta]_{\text{unfolded}}$ are the MRE of the maximally folded and unfolded forms, respectively, calculated as follows: $[\theta] = (\theta \times m)/(10 \times c \times l \times n_r)$, where θ is the ellipticity in mdeg, m is molecular weight in g/mol, c is concentration in mg/mL, l is path length of the cuvette in cm, and n_r is the number of amino acid residues in the peptide.

Peptide solutions were mixed in desired ratios in such a way that the final total peptide concentration was 0.2 mM, and a neutral pH was maintained by using 10 mM phosphate buffer solution. Unfolding studies were performed with and without preheating. For preheating studies, peptides were mixed in desired ratios, heated to 85 °C, and incubated for 15 min. The peptide solution was then slowly cooled to 25 °C at a rate of 1 °C/min and then incubated overnight at room temperature before performing the unfolding studies. For non-preheating studies, peptides were mixed in the desired ratios, and the unfolding studies were performed immediately. The minimum of the derivative of the fraction folded plot indicates the steepest slope of the unfolding process and is used in this paper to indicate the melting temperature (T_m) under the conditions described above. This was calculated using the Jasco Spectra Manager software. All experiments were repeated at least once, and T_m values were found within ±1 °C or less.

Thermodynamic Analysis. Melting curves were analyzed assuming a two-state reversible model[22,23]

$$3M \rightleftharpoons T_3 \quad (2)$$

where M and T_3 denote the monomer (unfolded) and triple-helical trimer (folded) state, respectively. It has been reported[23] that the experimental conditions for CD studies similar to those used in this article (c = 0.2 mM; heating rate = 10 °C/h) do not result in equilibrium, and the melting transitions observed are kinetic. However, a two-state kinetic model can still be used in comparative studies. Ideally, a much slower heating rate is desirable so that equilibrium is established at every data collection point. However, in practice, an optimal heating rate and concentration similar to the one reported here have been shown[23] to follow a two-state model. If the same heating rate and concentration is used for all the experiments, a comparative analysis can be made.

If the molar peptide concentration is $c_o = [M] + 3[T_3]$ and the fraction of folded peptide is $F_T = 3[T_3]/c_o$, then the concentration of folded and unfolded molecules is given by $[T_3] = (F_T \times c_o)/3$ and $[M] = c_o(1 - F_T)$, respectively. The equilibrium constant can be written as

$$K(T) = \frac{[T_3]}{[M]^3} = \frac{F_T}{3c_o^2(1 - F_T)^3} \quad (3)$$

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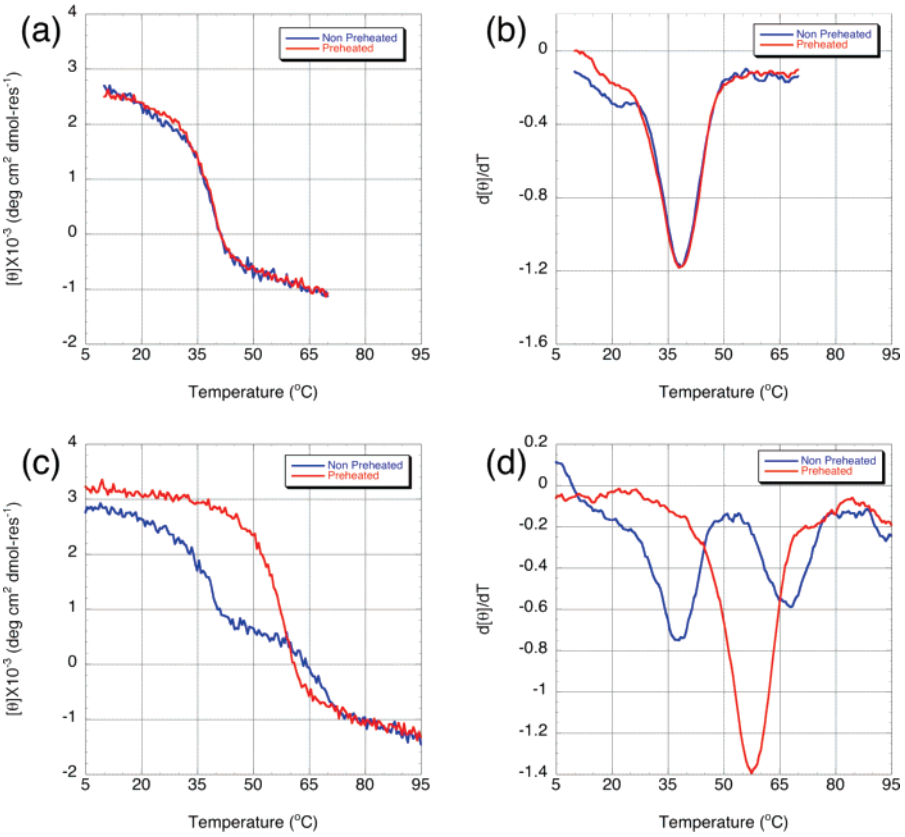

Figure 2. Circular dichroism for AAB and ABC heterotrimeric helices formed by mixing ***E***, ***K***, and ***O***. (a) MRE versus temperature for the ***E***/***K*** mixture in a 1:1 ratio. (b) The first derivative of unfolding versus temperature for (a). (c) MRE versus temperature for the ***E***/***K***/***O*** mixture in a 1:1:1 ratio. (d) The first derivative of unfolding versus temperature for (c).

The equilibrium constant can be related to $\Delta H°$ by using the following equation

$$logK(T) = -\frac{\Delta H°}{2.303R}\left(\frac{1}{T}\right) + const \qquad (4)$$

An estimate of $\Delta H°$ value can be made from the slope of log $K(T)$ versus $(1/T)$ plot. The value of $\Delta H°$ can then be used to estimate the value of $\Delta S°$ by curve fitting to the experimental data using the following relationship:

$$3c_o^2 e^{\frac{\Delta H - T\Delta S}{-RT}} = \frac{F_t}{(1-F_T)^3} \qquad (5)$$

After obtaining the values of $\Delta H°$ and $\Delta S°$, standard Gibbs free energy can be calculated at $T = 298$ K using the equation

$$\Delta G = \Delta H - T\Delta S \qquad (6)$$

Molecular Modeling. Molecular model for the ***D•K•O*** heterotrimer was based on the atomic coordinates of $[(Pro\text{-}Hyp\text{-}Gly)_4(Leu\text{-}Hyp\text{-}Gly)_1\text{-}(Pro\text{-}Hyp\text{-}Gly)_5]_3$ (http://www.rcsb.org/pdb, PDB Code: 2DRT).[24] The model was built using HyperChem 1.0, and molecular mechanics calculations were done using Amber 99. In one chain, Asp substituted all Pro and Leu. For the second chain, the central Leu was substituted by Pro, and all Hyp were substituted by Lys. Only the central Leu was substituted by Pro in the third chain so as to generate an ABC heterotrimer with $(DOG)_{10}$, $(PKG)_{10}$, and $(POG)_{10}$ chains. The backbone geometry was fixed, and the amino acid side chain conformation was energy minimized using the Polak−Ribiere conjugate gradient until the rms gradient was 0.01 kcal/Å·mol. The conformer with the minimum energy was used for further analysis, and the distance between the nitrogen of the K side chain and the closest oxygen of the D side chain was measured.

Results and Discussion

Mixing a positive, negative, and neutral peptide together can theoretically lead to 10^{25} possible collagen-like triple helices: three AAA homotrimers, six AAB-type heterotrimers, and one ABC heterotrimer. To differentiate between these possible outcomes, the potential formation of homotrimers (AAA) and heterotrimers (AAB and ABC) was assessed separately by thermal unfolding experiments. Eliminating some of the possible outcomes makes analysis of the complete mixture easier.

Our design utilizes the simple idea that like charges will repel each other while opposite charges will attract each other. Because of charge repulsion, only one homotrimer (***O•O•O***) is able to form. This reduces the analysis of any A/B/C mixture to eight possible triple helices. Mixing all possible pairs of peptides allowed us to assess the potential of AAB heterotrimer formation. Results from all the unfolding experiments on AAB heterotrimers show that only pairs of oppositely charged peptides lead to a helix formation, and these triple helices are of rather low stability. Therefore, four AAB heterotrimers composed of charged and neutral peptides can also be eliminated. This leaves

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(25) In fact, there are 27 possible combinations for mixing peptides A, B, and C if one differentiates between the different registers controlled by glycine packing that A, B, and C may be in. These include single peptide homotrimers AAA, BBB, and CCC; two peptide heterotrimers AAB, ABA, BAA, ABB, BAB, BBA, AAC, ACA, CAA, ACC, CAC, CCA, BCC, CBC, CCB, BBC, BCB, and CBB; and three peptide heterotrimers ABC, ACB, CAB, CBA, BCA, and BAC.

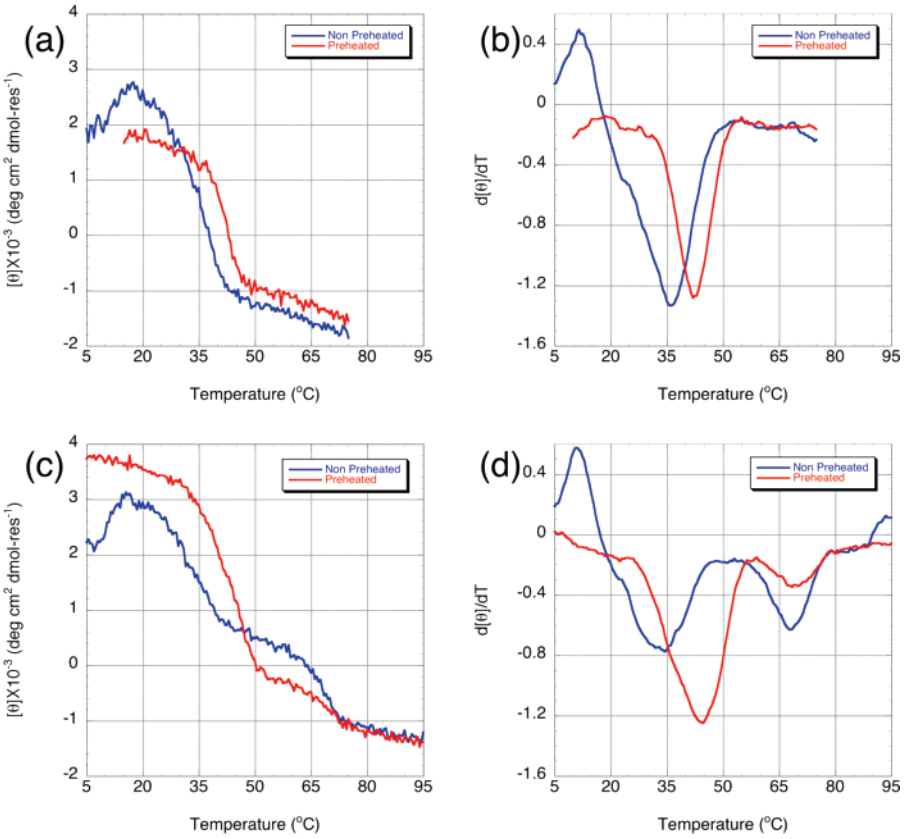

Figure 3. Circular dichroism for AAB and ABC heterotrimeric helices formed by mixing ***D***, ***R***, and ***O***. (a) MRE versus temperature for the ***D***/***R*** mixture in a 1:1 ratio. (b) The first derivative of unfolding versus temperature for (a). (c) MRE versus temperature for the ***D***/***R***/***O*** mixture in a 1:1:1 ratio. (d) The first derivative of unfolding versus temperature for (c).

us with two AAB heterotrimers composed of oppositely charged peptides. Within our experimental system, AAB and ABB triple helices are not distinguishable, thus we combine these two possibilities into one. Finally, we are left with three possible triple helices to consider: The ***O•O•O*** homotrimer with high stability, AAB-type heterotrimers that incorporate a positively charged peptide with a negatively charged peptide and are of relatively low stability, and a novel ABC heterotrimer with unknown stability.

Homotrimers. ***O•O•O***, ***E•E•E***, ***R•R•R***, ***D•D•D***, and ***K•K•K*** homotrimers were studied at a concentration of 0.2 mM in 10 mM phosphate buffer at neutral pH. As expected, only ***O•O•O*** forms a triple helix within the time frame of the experiment. Unfolding studies for ***E•E•E***, ***R•R•R***, ***D•D•D***, and ***K•K•K*** homotrimers show a linear decrease in ellipticity, suggesting that they do not form triple helices. Rather they are present as disordered or weak polyproline II helices.

Therefore, in the assessment of a mixture of three different peptides such as ***D***, ***K***, and ***O*** peptides, the ***D•D•D*** and ***K•K•K*** homotrimers can be eliminated. This reduces the analysis to eight possible triple helices: the ***O•O•O*** homotrimer, six AAB-type heterotrimers, and the ***D•K•O*** ABC heterotrimer. Formation of AAB and ABC heterotrimers was then assessed separately by mixing the corresponding peptides.

AAB Heterotrimers. Oppositely charged peptides were mixed in 1:1 ratio to achieve a final peptide concentration of 0.2 mM in 10 mM phosphate buffer at neutral pH. The mixtures were studied with and without preheating. We previously reported[17] the results for ***E***/***R*** mixture and showed that, although the ***E•E•E*** and ***R•R•R*** homotrimers do not exist, they form an AAB heterotrimer with a T_m = 41 °C upon mixing and preheating at neutral pH.

T_m values of 34.5 and 36 °C were observed for a 1:1 ***D***/***K*** mixture for preheating and non-preheating, respectively (Figure 1a,b). The corresponding values of T_m were 38 and 38.5 °C for the preheated and non-preheated ***E***/***K*** mixture, respectively (Figure 2a,b). ***D***/***R*** mixture showed the corresponding T_m values of 42 and 36 °C for preheating and non-preheating, respectively (Figure 3a,b). T_m values for all the mixtures are summarized in Table 2. AAB heterotrimer formation is observed in all the cases with and without preheating. Furthermore, on lowering the pH of the various mixtures to 3, it was observed that the positive peak decreased in intensity, signaling that the triple helix is destroyed (see Supporting Information Figure S2). At pH 3, R and K side chains have a positive charge but D and E side chains are neutral. Owing to the absence of negative charge on D and E side chains, the ion pairs can no longer form, which leads to destabilization of the triple helix.

It has been reported that the content of imino acids in human fibril-forming collagens [type I (α1 and α2), II (α1), III (α1), V (α1, α2 and α3), and X1 (α1 and α2)] is around 35%, and the content of triplets with E/D in the X position and R/K in the Y position is around 6−8% of all triplets.[4,7] Using peptides with a sequence Ac-$(GPO)_3$-GXY-$(GPO)_4$-GG-$CONH_2$, Brodsky and co-workers reported on the quantification of pairwise interactions in collagen homotrimers.[5,26] For triplets with E/D in the X position and R/K in the Y position, they observed that the GER triplet caused the least amount of destabilization

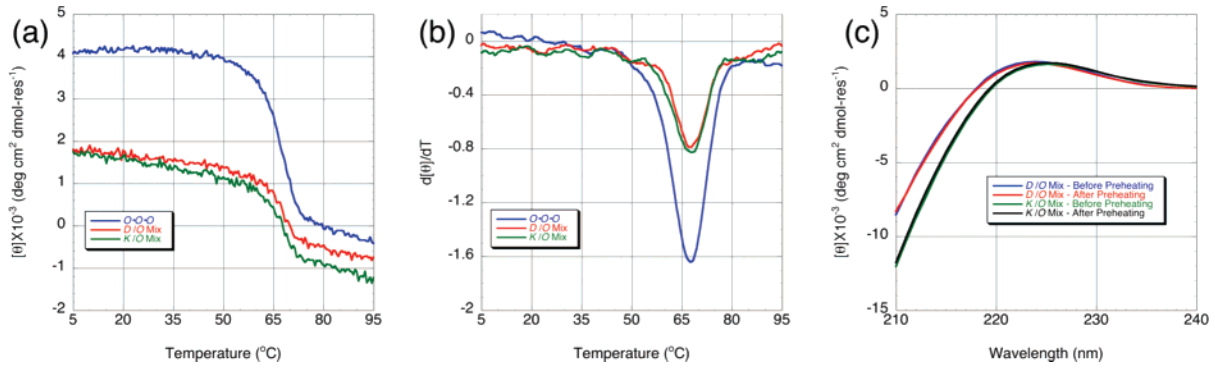

Figure 4. Circular dichroism analysis for ***D***/***O*** and ***K***/***O*** mixtures. (a) MRE versus temperature. (b) The first derivative of unfolding versus temperature for (a). (c) MRE versus wavelength. The corresponding data for ***E***/***O*** and ***R***/***O*** have been shown previously and behave in a similar fashion.[17]

Table 2. Melting Temperatures (°C) of AAB and ABC Heterotrimers

AAB heterotrimer	non-preheated	preheated	ABC heterotrimers	non-preheated	preheated
D/***K***	34.5	36	***D•K•O***	35, 68.5	65
E/***K***	38.5	38	***E•K•O***	38, 68	57.5
E/***R***	9, 41.5	41	***E•R•O***	19, 38.5, 68	54
D/***R***	36	42	***D•R•O***	34.5, 68	44.5

followed by GDR, GEK, and GDK with T_m of 40.4, 37.1, 35, and 30.9 °C, respectively (T_m = 44.5 °C for GPO). In the current study, T_m values observed for various AAB heterotrimers approximately follow the same trend with the corresponding T_m values as follows: ***E***/***R*** mix (41 °C), ***D***/***R*** mix (42 °C), ***E***/***K*** mix (38 °C), ***D***/***K*** mix (36 °C). In both of the previously published homotrimers[5,26] and the AAB heterotrimers reported here, there is a combination of repulsive interactions between the same charges and the attractive interactions between opposite charges, along with the polyproline II helix propensity of amino acids. The combination of amino acid propensity and electrostatic interactions determines the final stability of the triple helix, and charge pairs in AAB heterotrimers closely follow the trend as reported in literature.

Unfolding studies were also performed on ***D***/***O*** and ***K***/***O*** mixtures in a 1:1 ratio to study the interactions between a charged and neutral polypeptide and their ability to form a heterotrimer. The melting curve for both showed a single transition with T_m of 67 and 68 °C for ***D***/***O*** and ***K***/***O*** mixture, respectively, which is indistinguishable from the T_m value of 67.5 °C for the ***O•O•O*** homotrimer (Figure 4b). The MRE value for these mixtures is about one-half that for what is observed for ***O•O•O*** alone (Figure 4a), indicating that only ***O•O•O*** contributes to the MRE of the mixture while neither the ***D•D•D*** and ***K•K•K*** homotrimers nor AAB heterotrimers form. It was also observed that the CD spectra for both ***D***/***O*** and ***K***/***O*** are very similar before and after preheating (Figure 4c). These results indicate that ***D*** and ***K*** peptides do not interact with ***O***. Instead, the ***D*** and ***K*** peptides are present as disordered or weak polyproline II helices. Results for ***E***/***O*** and ***R***/***O*** mixtures are similar.[17]

Considering all the results from the unfolding experiments on AAB heterotrimers reveals that only pairs of oppositely charged peptides lead to helix formation, and these triple helices are of rather low stability. Therefore, in the assessment of a mixture of ***D***, ***K***, and ***O*** peptides, four AAB heterotrimers composed of charged and neutral peptides can be eliminated. This leaves us with two AAB heterotrimers composed of oppositely charged ***D*** and ***K*** peptides. Combining these results with the results from unfolding studies on homotrimers, the analysis can be reduced to three possible triple helices: the highly stable ***O•O•O*** homotrimer, AAB-type heterotrimers that incorporate the positively charged peptide with negatively charged peptide and are of relatively low stability, and a novel ***D•K•O*** ABC heterotrimer with unknown stability.

ABC Heterotrimers. Charged peptides were mixed with ***O*** in a 1:1:1 ratio, and the mixtures were studied with and without preheating. As deduced above, mixing ***D***, ***K***, and ***O*** can lead to the formation of three triple helices: ***O•O•O*** homotrimer, AAB-type heterotrimers that incorporate the positively charged peptide with negatively charged peptide, and a novel ***D•K•O*** ABC heterotrimer. In a 1:1:1 ***D***/***K***/***O*** mixture, under non-preheating conditions, two transitions were observed corresponding to the ***O•O•O*** (68.5 °C) and ***D***/***K*** AAB heterotrimer (35 °C). The transition at 35 °C can be assigned to the ***D***/***K*** AAB heterotrimer as a ***D***/***K*** mixture shows a transition corresponding to 34.5 °C under non-preheating conditions. Both of the transitions observed in a non-preheated ***D***/***K***/***O*** mixture merged to a single transition upon preheating, corresponding to T_m of 65 °C (Figure 1c,d). This transition can only be attributed to the ***D•K•O*** ABC heterotrimer, which is significantly more stable than the ***D***/***K*** AAB heterotrimer (ΔT_m = +29 °C) and only slightly less stable than ***O•O•O*** (ΔT_m = −2.5 °C). If the ***O•O•O*** homotrimer were to form, and not the ***D•K•O*** ABC heterotrimer, it must also result in the formation of less stable ***D***/***K*** AAB heterotrimers or unfolded species. A greater system-wide stability is expected when an ABC heterotrimer is formed as almost all the polypeptides are involved in a stable triple helix formation. This contrasts the case in which both the ***O•O•O*** homotrimer, with stability comparable to that of the ABC heterotrimer, and the ***D***/***K*** AAB heterotrimers, which have relatively low stability,

(26) Chan, V. C.; Ramshaw, J. A. M.; Kirkpatrick, A.; Beck, K.; Brodsky, B. *J. Biol. Chem.* **1997**, *272*, 31441−31446.
(27) Nemethy, G.; Leach, S. J.; Scheraga, H. A. *J. Phys. Chem.* **1966**, *70*, 998−1004.

are present. The same analysis applies to other ABC heterotrimers discussed in this article.

Analysis of the ***E***/***K***/***O*** mixture was similar. Two transitions were observed in the non-preheating case corresponding to the ***O•O•O*** (68 °C) and ***E***/***K*** AAB heterotrimers (38 °C). A T_m of 38.5 °C was observed for the ***E***/***K*** mixture under non-preheating conditions, thus the transition at 38 °C in the ***E***/***K***/***O*** mixture could be assigned to the ***E***/***K*** AAB heterotrimer. Only a single transition corresponding to the ***E•K•O*** ABC heterotrimer with a T_m of 57.5 °C was observed after preheating in the case of the ***E***/***K***/***O*** mixture (Figure 2c,d).

The ***D***/***R***/***O*** mixture behaved in a different way when compared to other mixtures. Under non-preheating conditions, two transitions were observed at T_m = 68 and 34.5 °C corresponding to ***O•O•O*** and ***D***/***R*** AAB heterotrimers, respectively. A T_m of 36 °C was observed for the ***D***/***R*** mixture under non-preheating conditions, thus the transition at 34.5 °C in the ***D***/***R***/***O*** mixture could be assigned to a ***D***/***R*** AAB heterotrimer. When the ***D***/***R***/***O*** mixture was preheated, two transitions were still observed: a weak transition at T_m = 68.5 °C, corresponding to residual ***O•O•O*** and a strong transition at 44.5 °C (Figure 3c,d). The corresponding T_m value for the ***D***/***R*** mixture upon preheating was 42 °C. We believe that, in the case of a preheated ***D***/***R***/***O*** mixture, the transition observed at around 44.5 °C corresponds to a mixture of ***D***/***R*** AAB heterotrimers (probably a minority component based on the intensity of the ***O•O•O*** transition) and the ***D•R•O*** ABC heterotrimer. It seems that the ABC heterotrimer in this case is not much more stable compared to the AAB heterotrimer, leading to the formation of a mixture of both AAB and ABC heterotrimers. This idea is also supported by the fact that a weak transition corresponding to ***O•O•O*** still appears in the preheated ***D***/***R***/***O*** mixture. This mixture acts as a control to the other systems shown in this article as it demonstrates that residual ***O•O•O*** can be observed even after preheating. All other mixtures show only a single transition in the preheated case and do not show the presence of any residual ***O•O•O*** homotrimer. Melting temperatures for all the ABC heterotrimers are summarized in Table 2.

On the basis of the results from the AAB heterotrimers, it was observed that the melting temperature follows a trend in terms of contribution of charge pairs to the stability of triple helix and it is in agreement with the results obtained for charge pairs previously reported.[5,26] T_m values observed for various AAB heterotrimers were as follows: ***E***/***R*** mix = 41 °C, ***D***/***R*** mix = 42 °C, ***E***/***K*** mix = 38 °C, ***D***/***K*** mix = 36 °C. However, this trend is completely reversed when the stability of ABC heterotrimers is assessed. The results show that the ***D•K•O*** heterotrimer is the most stable with T_m = 65 °C, followed by ***E•K•O*** (T_m = 57.5 °C) and ***E•R•O*** (T_m = 54 °C). For the ***D***/***R***/***O*** mixture, it cannot yet be unambiguously identified whether the transition at approximately 44.5 °C corresponds to an AAB heterotrimer, an ABC heterotrimer, or a mixture of both, but it is clearly weaker than the other systems. We believe that the trend observed from the ABC heterotrimers is a true indication of the stabilization caused by interaction between opposite charges, rather than what is observed in AAB heterotrimers or homotrimers. In AAB heterotrimers, as two chains are identical, there is always a net positive or negative charge. Homotrimers can be designed to be neutral overall but, consequently, have no net intermolecular electrostatic interaction. Thus, in both cases, the stabilizing effect of opposite charge pairs is diluted or eliminated. An ABC heterotrimer, however, is composed of one neutral, one negatively charged, and one positively charged chain at neutral pH. Thus, the repulsive electrostatic interactions between peptide chains can be eliminated, and the effect of attractive interactions between oppositely charged chains can be evaluated. We observe that the favorable interactions between D and K side chains in the case of ***D•K•O*** are able to increase its stability to nearly that of ***O•O•O***. ***D•K•O*** is surprisingly stable even though D and K amino acids have significantly less inherent propensity to form a triple helix than P and O imino acids and occur a total of 20 times in the triple helix. ***D•K•O*** has 10 P and O imino acid residues substituted by D and K, respectively, as compared to ***O•O•O***. Therefore, the imino acid content of ***D•K•O*** decreases to approximately 45% when compared to 67% for ***O•O•O***. Molecular modeling of ***D•K•O*** indicates that the side chains of lysine and aspartate can be ideally positioned to form an ionic hydrogen bond with a N−O distance of 2.7 Å (Figure 5). This potential for the formation of this ideally situated hydrogen-bonded charge pair may be the reason for the high stability of the triple helix.

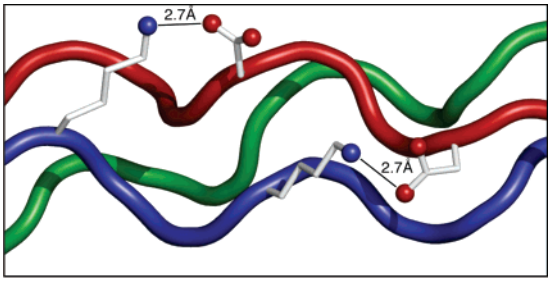

Figure 5. Molecular model for the ***D•K•O*** heterotrimer. Only a portion of the heterotrimer is shown for clarity. ***D***, ***K***, and ***O*** polypeptides are represented by red, blue, and green chains, respectively. Distances between nitrogen (blue) of K side chain and the closest oxygen (red) of D are shown.

Table 3. Thermodynamic Parameters for ABC Heterotrimers[a]

triple helix	T_m (°C)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (kcal/mol/K)	$\Delta G°$ (kcal/mol)
O•O•O	67.5	−110	−0.308	−18.2
D•K•O	65.0	−92	−0.258	−14.9
E•K•O	57.5	−104	−0.299	−14.7
E•R•O	54.0	−97	−0.282	−13.1
D•R•O	44.5	−85	−0.255	−9.5

[a] Only data points in the vicinity of the transition temperature are included for curve-fitting analysis.

Thermodynamic Analysis. Elaborating on the factors stabilizing ***D•K•O*** as well as other ABC heterotrimers, thermodynamic parameters were calculated for all the ABC heterotrimers and ***O•O•O*** homotrimer (Table 3). $\Delta H°$ values were obtained for the triple helices using eq 4. This was then used to estimate $\Delta S°$ by curve fitting using eq 5.[22,23] $\Delta H°$ and $\Delta S°$ values were then used to calculate $\Delta G°$.

For ***O•O•O***, a best fit was obtained for $\Delta H°$ and $\Delta S°$ values of −110 cal/mol and −0.308 cal/mol•K respectively, with the corresponding $\Delta G°$ of −18.2 Kcal/mol. These were the maximal values for all peptides tested. Heterotrimers containing peptide ***E*** shoed a more favorable enthalpy when compared to heterotrimers containing peptide ***D***. When comparing triple helices which both contain the peptide ***E*** or both contain the peptide ***D***, triple helices containing the peptide ***K*** had a more favorable enthalpy than those containing the peptide ***R***. Although the

E•K•O and ***E•R•O*** heterotrimers showed a more favorable enthalpy (−104 Kcal/mol and −97 Kcal/mol), the entropy penalty of these helices was significantly higher than ***D•K•O*** leading to its significantly greater thermal stability. Although there is no clear trend in $\Delta S°$ values when comparing all the heterotrimers, $\Delta G°$ values follow the melting temperature and a more negative value is observed for ***D•K•O*** followed by ***E•K•O***, ***E•R•O*** and ***D•R•O***.

Many factors including the polyproline II helix propensity of amino acids,[3−6] water-mediated hydrogen bonds,[8,9] steric and stereoelectronic effects,[10,11] and the interactions between side chains of neighboring amino acids play a significant role in triple helix stability. Contributions of all these factors have been studied extensively on collagen homotrimers. We examined the formation of four ABC heterotrimers that utilize electrostatic interactions between D, E, K, and R amino acid side chains. ***D•K•O*** consists of polypeptides with 10 P to D substitutions, 10 O to K substitutions, and without any imino acid substitution. Despite these substitutions, the melting temperature of ***D•K•O*** is only 2.5 °C lower than that of ***O•O•O*** (67.5 vs 65.0 °C). This highly stable complex is unexpected based on previous work that examined the effect of amino acid substitutions on the stability of homotrimeric helices and demonstrates the importance of examining helix stability in heterotrimers, particularly, in ABC heterotrimers. The ability to selectively form an ABC triple helix with stability comparable to the most stable collagen triple helix formed from natural amino acids will have substantial implications in the field of collagen-like peptides.

Conclusions

In this paper, we have demonstrated three critical points: (1) Mixing three peptides with individually neutral, negative, and positive charge is a general mechanism for forming specific ABC heterotrimeric collagen helices in high yield. The formation of a triple helix with net-neutral charge is the thermodynamically most favored assembly over all of the other nine possible combinations and will preferentially form when the solution is given the opportunity to come to equilibrium after preheating. (2) An amino acid contribution to the stability of a heterotrimeric collagen helix cannot be extrapolated from similar studies on homotrimeric collagen helices. Previous work with homotrimers demonstrated that substitutions of proline in the X position or hydroxyproline in the Y position with any other natural amino acid led to significant destabilization. In our work, we find that even as many as 20 substitutions of proline and hydroxyproline in a triple helix can still lead to a highly stable helix. (3) Charge pairing between aspartate and lysine leads to the formation of a triple helix of equivalent stability to helices containing exclusively the collagen consensus sequence P−O−G. Construction of such high stability collagen triple helices using solely natural amino acids and without the use of covalent tethers or cross-links will have great impact on our ability to probe heterotrimer structure and stability, particularly, in model peptides which mimic diseases found in naturally occurring heterotrimeric collagens. Additionally, this will allow us to begin designing more sophisticated collagen based constructs both for bioengineering applications as well as for nanoscience applications derived from well-controlled self-assembling architectures.

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Supporting Information Available: Additional CD, HPLC, and mass spectra are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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