The stereoelectronic basis of collagen stability

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Introduction

The helix is the most common conformation assumed by the chains of native biopolymers. The α-helix of Pauling and the DNA double helix of Watson and Crick are the foundation for much of modern biochemistry and biology. The forces that hold these single and double helices together have been revealed in numerous illuminating investigations. By comparison, little is known about the fundamental basis for the conformational stability of the most prevalent triple-helical biopolymer—collagen.

Collagen is the most abundant protein in animals. Vertebrates produce at least 19 different types of collagen. In each type, the polypeptide chains are composed of approximately 300 repeats of the sequence: XaaYaaGly, where Xaa is often an L-proline (Pro) residue and Yaa is often a 4(*R*)-hydroxy-L-proline (Hyp) residue. These chains are wound in tight triple helices, which are organized into fibrils of great tensile strength [1].

The hydroxyl groups of Hyp residues have an important role. Hyp residues are not incorporated into collagen by ribosomes. Rather, the hydroxylation of Pro residues occurs after translation but before three chains fold into a triple helix. In 1973, seminal work by Prockop and coworkers demonstrated that the hydroxyl group of Hyp residues dramatically increases the thermal stability of triple-helical collagen [2].

What is the basis for the stability conferred by the hydroxyl group of Hyp residues? Several models have been proposed in which one or more water molecules form a bridge between the hydroxyl group and a main-chain oxygen. In 1994, the first high-resolution three-dimensional structure of triple-helical collagen was determined by X-ray diffraction analysis [3]. In this structure, the Hyp residues do indeed have water molecules bound to their hydroxyl groups. Individual Hyp residues bond most often to two water molecules, forming an interchain link to the amide oxygen of another Hyp residue.

Are water bridges responsible for collagen stability? We doubted this explanation for several reasons. First, triple helices of $(ProProGly)_{10}$ and $(ProHypGly)_{10}$ are stable in either methanol or propane-1,2-diol, and the Hyp residues confer additional stability in these anhydrous conditions [4]. Second, immobilizing two water molecules for each Hyp residue would evoke an enormous entropic cost. Hyp comprises approximately 10% of the residues in most forms of collagen. Immobilizing two water molecules per Hyp residue would require that >500 water molecules be immobilized to stabilize a single molecule of triple-helical collagen. Third, because water molecules contribute approximately half of the weight to typical protein crystals, we suspected that the water bridges observed in crystalline collagen are artifactual rather than meaningful. Afterall, "[a picture of a horse] does not necessarily tell us how fast it can run" [5]. We sought an alternative explanation for the contribution of Hyp residues to collagen stability.

Results and Discussion

Electron-withdrawing groups can alter the preferred conformation of molecules. To distinguish between the contributions of hydrogen bonding and inductive effects to

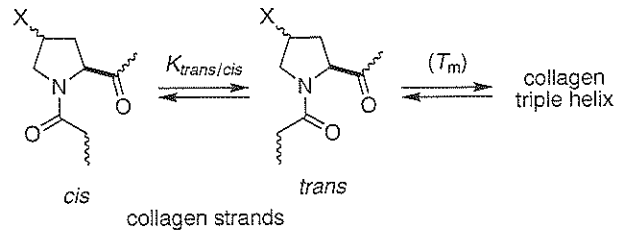

Fig. 1. Relationship between cis–trans prolyl peptide bond isomerization in collagen strands and the thermal stability of the collagen triple helix, which contains only trans peptide bonds.

collagen stability, we used chemical synthesis to replace the hydroxyl groups in Hyp residues with fluorine atoms. We chose fluorine because it is the most electronegative atom and so elicits a large inductive effect, and because organic fluorine does not form hydrogen bonds [6]. By monitoring thermal denaturation with circular dichroism spectroscopy, we found that Flp residues greatly enhance triple helix stability. In both 50 mM acetic acid and phosphate-buffered saline, the values of T_m (which is the temperature at the midpoint of the thermal transition) for the three triple helices differ dramatically, increasing in the order: $(ProProGly)_{10} < (ProHypGly)_{10} < (ProFlpGly)_{10}$ [7,8]. This order is inconsistent with collagen stability arising largely from bridging water molecules, but it is consistent with the manifestation of an inductive effect from an electron-withdrawing 4(*R*) substituent.

How does an inductive effect stablize collagen? All of the peptide bonds in triple-helical collagen are in the *trans* conformation [3]. This requirement suggested to us that the electronegative 4(*R*) substituent could enhance collagen stability by favoring the *trans* conformation, thereby preorganizing individual strands to resemble more closely the strands in a triple helix (Fig. 1). To test this hypothesis, we synthesized AcYaaOMe (Yaa = Pro, Hyp, Flp) as a mimic of the Yaa residues in collagen strands. We then measured $K_{trans/cis}$ in ^2H_2O at 25 °C by NMR spectroscopy. To probe for a role of stereochemistry in $K_{trans/cis}$, we also prepared AcYaaOMe in which Yaa is a diastereomer of Flp, 4(*S*)-fluoro-L-proline (flp). As listed in Table 1, the data from this experiment indicate that both the electron-withdrawing ability *and* the stereochemistry of the 4 substituent have a significant effect on $K_{trans/cis}$.

Table 1. Values of $K_{trans/cis}$ for collagen-related residues.

Yaa in AcYaaOMe	$K_{trans/cis}$
4(R)-fluoro-L-proline (Flp)	6.7
4(R)-hydroxy-L-proline (Hyp)	6.1
proline (Pro)	4.6
4(S)-fluoro-L-proline (flp)	2.5

If the value of $K_{trans/cis}$ does have a significant impact on collagen stability, then flp residues should destabilize triple-helical collagen. To test this supposition, we synthesized diastereomeric collagen strands containing either Flp or flp residues. We found that a $(ProFlpGly)_7$ triple helix has a T_m of 45 °C in 50 mM acetic acid. In contrast, a $(ProflpGly)_7$ triple helix has a T_m of <2 °C under the same conditions. Thus, simply changing the stereochemistry of an electron-withdrawing 4 substituent has a marked effect on the value of T_m. In other words, the conformational stability of triple-helical collagen is strongly dependent on stereoelectronics.

Conclusions

We conclude that collagen stability does not rely on bridging water molecules. Rather, stereoelectronic effects preorganize collagen strands. Specifically, an electronegative substituent in the 4(*R*) position of a proline residue favors a *trans* peptide bond, as is necessary for triple helix formation.

Acknowledgments

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