

Theoretical Analysis of the Basis of Collagen Stability

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Abstract: Collagen is the most abundant protein in vertebrates. Collagen fibrils have great tensile strength and thermal stability. Recently a form of collagen was developed with greater thermal stability than the wildtype form. By investigating *ab initio* results we have determined that the gauche effect and steric interactions selectively stabilize the conformation of the Proline derivative present in the collagen helices. We have also found evidence to show that the inductive effect results in strengthening of the inter-strand Hydrogen bonds. These three phenomena work together to produce the observed stability of collagen.

Introduction

Collagen is the most abundant protein in the human body – as well as in the bodies of all other vertebrates.¹ It exists as three polypeptides in a tight right-handed triple helix.² These triple helices are, in turn, organized into fibrils that make up connective tissue such as bone, tendon, cartilage, ligament, skin, blood vessels and teeth. These fibrils have great tensile strength and thermal stability.^{3,4} Abnormalities in the structure of collagen have been linked to a variety of human diseases including arthritis and rheumatism.^{5,6,7,8,9}

The polypeptides that make up each helix contain approximately 300 repeats of the sequence X-Y-Gly, where X is commonly a proline (Pro) residue and Y is commonly a 4(R)-hydroxyproline (Hyp) residue.¹⁰ It has been shown *in vitro* that collagen's thermal stability is strongly dependent upon both the

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content and position of the hydroxyl groups.^{11,12} Collagen that is deficient in hydroxyl groups – having Pro residues in place of Hyp residues – can form triple helices, but these helices are unstable at room temperature.^{13,14}

Several models have been proposed to explain this Hyp-mediated stability by arguing that the Hyp residues utilize hydrogen bonding to orient water molecules into inter-strand bridges.^{15,16} Although there is evidence for the existence of such bridges in the form of high-resolution X-ray diffraction analysis revealing their presence,^{17,18} recent studies have found data inconsistent with such models.¹⁹ For instance, it has been shown that Hyp also confers additional stability to collagen triple-helices in the anhydrous environments of methanol or propane-1,2-diol²⁰. These data, along with the reasoning that the entropic cost of building such bridges would outweigh any stabilization that would result from their existence, casts doubt on the validity of these models.

Recently, a new form of collagen was developed that has a thermal stability even greater than the normal Hyp-containing form. In this protein, Hyp residues were replaced by 4(R)-fluoroproline (Flp) residues which are not able to direct hydrogen bonded water-bridges like Hyp can.²¹ With the addition of this new research, the evidence against the water-bridge model is very strong. Thus, a new model to explain the stability of collagen is required. This model must also account for the data obtained by studying another derivative of proline – 4(S)-fluoroproline (flp). It was shown that collagen-mimics made with flp in place of Hyp or Flp resulted in a form of collagen that was drastically *less* stable than

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any of the other previously studied collagen mimics, including that in which all Hyp residues had been replaced with Pro residues.²²

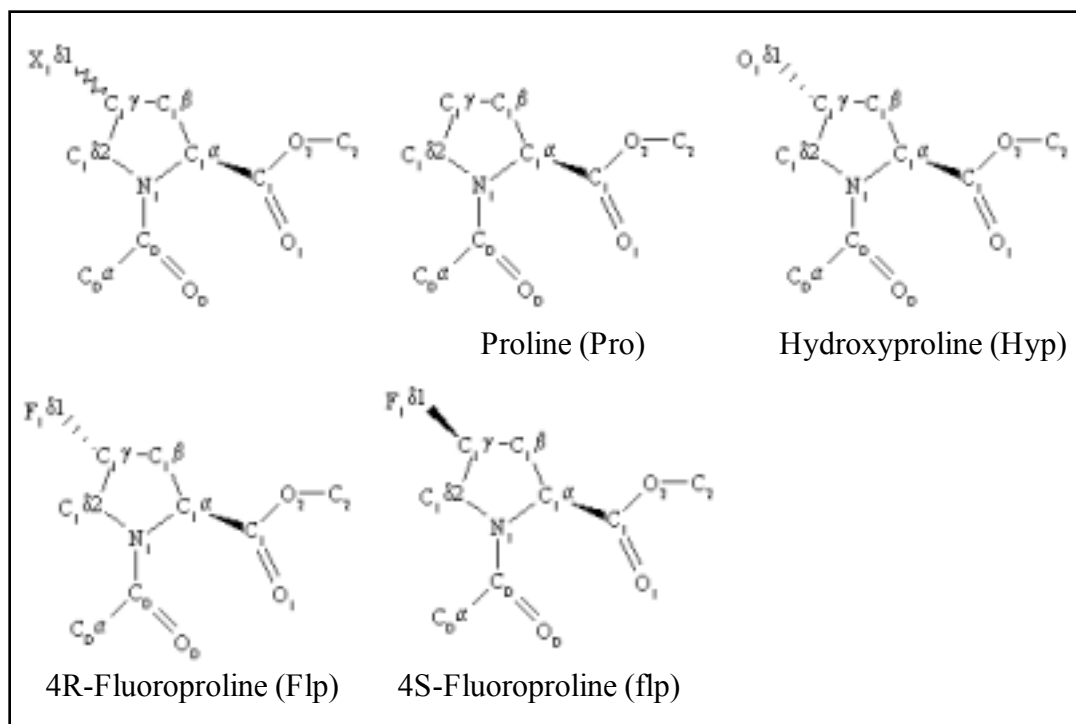


Figure 1 – Labeling of nonhydrogen atoms in the proline derivatives used in these investigations.

When investigating collagen stability, one must bear in mind that Pro and Hyp residues make up 25% of the residues in a typical collagen molecule and thus the stability of collagen is most likely highly dependent upon the properties of these residues. The objective of this study is to propose a new model for the stability of collagen. In constructing this model, we will investigate the role that the gauche effect, the anomeric effect and the inductive effect, as they occur in proline residue derivatives, play in the overall stability of a typical collagen molecule. The ultimate goal of this work is the discovery of a model that takes into account all of the research done on the stability of collagen.

The gauche effect is defined as “the tendency for a molecule to adopt that structure which has the

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maximum number of gauche interactions between adjacent electron pairs and/or polar bonds.²³ In collagen, we are most interested in the gauche interactions that involve a Y-C-C-Z fragment – where Y and Z are highly electronegative atoms. We find this fragment pattern twice in Hyp, Flp and flp residues and only once in Pro residues. According to the gauche effect, the gauche conformation of this fragment should be favored over the trans conformation.^{24,25} Similarly, the anomeric effect is a preference for gauche conformations in the system X-C-Y-C where X and Y are heteroatoms having nonbonding electron pairs. This interaction occurs along the prolyl peptide bond of the Proline derivatives. These two effects are due to the delocalization of electron density from filled orbitals into adjacent empty non-bonding orbitals.²⁶

The inductive effect, unlike the gauche and anomeric effects, occurs when charge is transmitted through a chain of atoms by electrostatic induction.²⁷ The implications of the inductive effect in collagen stability have previously been examined from a kinetic standpoint and it was determined that the presence of an electron-withdrawing substituent at the 4(R) position leads to increased pyramidylization on the prolyl nitrogen, decreased nitrogen pK_a, and reduction in the energetic barrier to isomerization.²⁸ We are particularly interested, not only in how the inductive effect changes the prolyl peptide bond, but also in how it affects the inter-strand hydrogen bonds. The breakdown of collagen occurs, *in vivo*, when the strands that make up the triple helix pull apart – exposing them to degradative enzymes that break them down completely. Thus, it is important to look both at the stability of the hydrogen bonds holding the strands together and the conformation of the individual residues. Rotation about the prolyl peptide bonds could result in steric hindrances within the molecule or stress on the inter-strand hydrogen bonds. If this rotation leads to a weakening of the inter-strand hydrogen bonds, it would facilitate the breakdown of the

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molecule.

Results and Discussion

In order to investigate the importance of the anomeric effect on the stability of collagen, it was necessary to study the rotation about the prolyl peptide bond – the only X-C-Y-C pattern found in the proline derivatives. This rotation was quantified in the form of the $C_1^\alpha-N_1-C_0-O_0$ dihedral angle (θ). These results are shown in Table 1.

θ Angles (Degrees)	Trans Isomer	Cis Isomer
Pro (exo ring pucker)	-1.4	-178.9
Pro (endo ring pucker)	-1.4	-177.9
Hyp (exo ring pucker)	1.4	-177.0
Hyp (endo ring pucker)	-1.2	-178.9
Flp (exo ring pucker)	0.5	-177.6
Flp (endo ring pucker)	-2.3	179.1
Flp (exo ring pucker)	-2.0	-179.6
Flp (endo ring pucker)	-4.2	-179.5

Table 1 – Rotation about the Prolyl Peptide bond measured as the $C_1^\alpha-N_1-C_0-O_0$ dihedral angle. All measurements are rounded to the nearest 1/10 of a degree.

As we can see from the results in Table 1, none of the stable conformations of the Proline derivatives studied here display the gauche conformation typical of stabilization due to the anomeric effect. In fact, we see that, in each of these conformations, the Oxygen is *Trans* to either C_1^α or $C_1^{\beta 2}$. Thus, based upon this information, we can say that the anomeric effect does not play any significant role in the stabilization of the prolyl peptide bond rotation.

A common method for describing the rotational configuration of a residue is using its ψ and ϕ angles to create a Ramachandran plot. The values of ψ and ϕ for each conformation of the Proline residues were determined and can be found in Tables 2 and 3. The corresponding Ramachandran plot can be found in Figure 2 with a magnified version in Figure 3.

ψ Angles (Degrees)	Trans Isomer	Cis Isomer
Pro (exo ring pucker)	143.0	156.0
Pro (endo ring pucker)	152.1	160.6
Hyp (exo ring pucker)	141.4	152.9
Hyp (endo ring pucker)	149.9	157.4
Flp (exo ring pucker)	140.8	150.5
Flp (endo ring pucker)	149.8	153.9
Flp (exo ring pucker)	145.0	161.8
Flp (endo ring pucker)	171.9	-174.7

Table 2 – ψ Angles measured as the $O_2-C_1-C_1^\alpha-N_1$ dihedral angles. All angles are rounded to the nearest 1/10 of a degree.

ϕ Angles (Degrees)	Trans Isomer	Cis Isomer
Pro (exo ring pucker)	-58.6	-64.2
Pro (endo ring pucker)	-70.0	-78.3
Hyp (exo ring pucker)	-59.9	-63.4
Hyp (endo ring pucker)	-68.7	-74.9
Flp (exo ring pucker)	-59.2	-63.5
Flp (endo ring pucker)	-68.8	-76.7
flp (exo ring pucker)	-59.8	-66.5
flp (endo ring pucker)	-76.4	-79.3

Table 3 – ϕ Angles measured as the $C_1-C_1^\alpha-N_1-C_0$ dihedral angles. All angles are rounded to the nearest 1/10 of a degree.

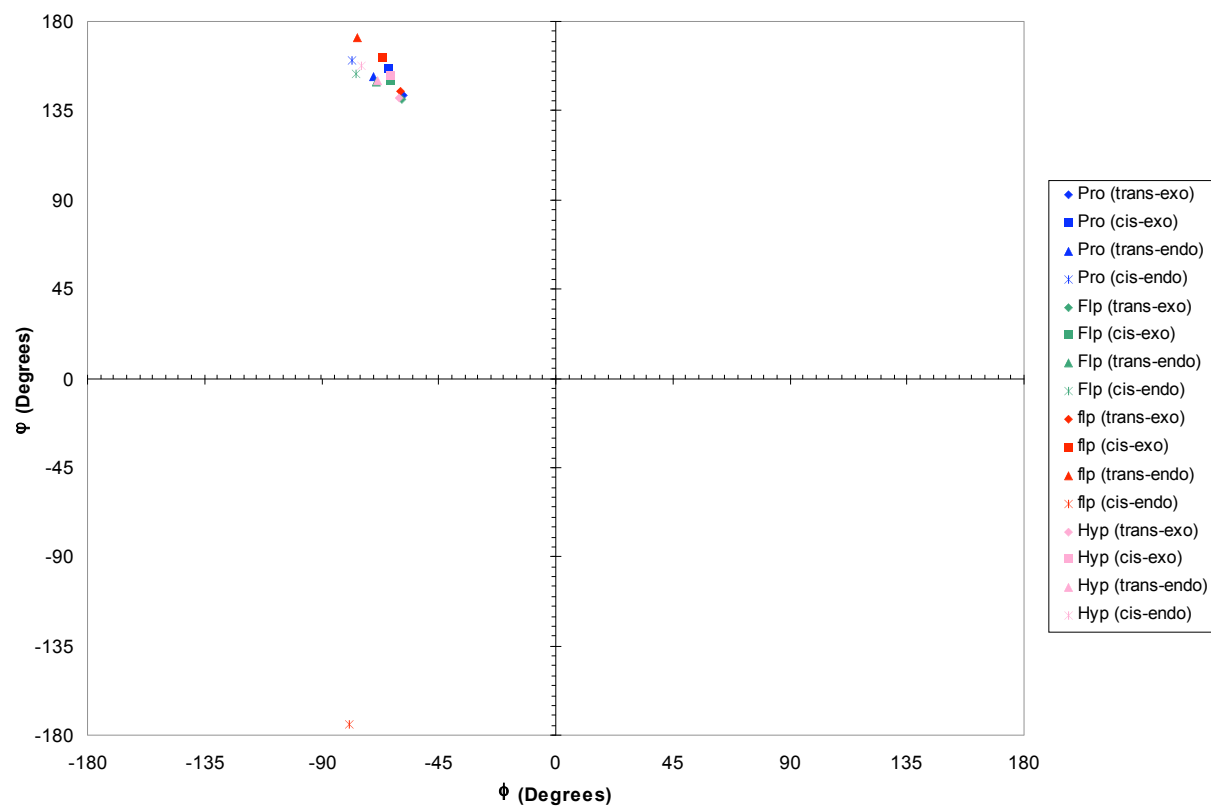


Figure 2 – Ramachandran Plot for the substituted proline residues.

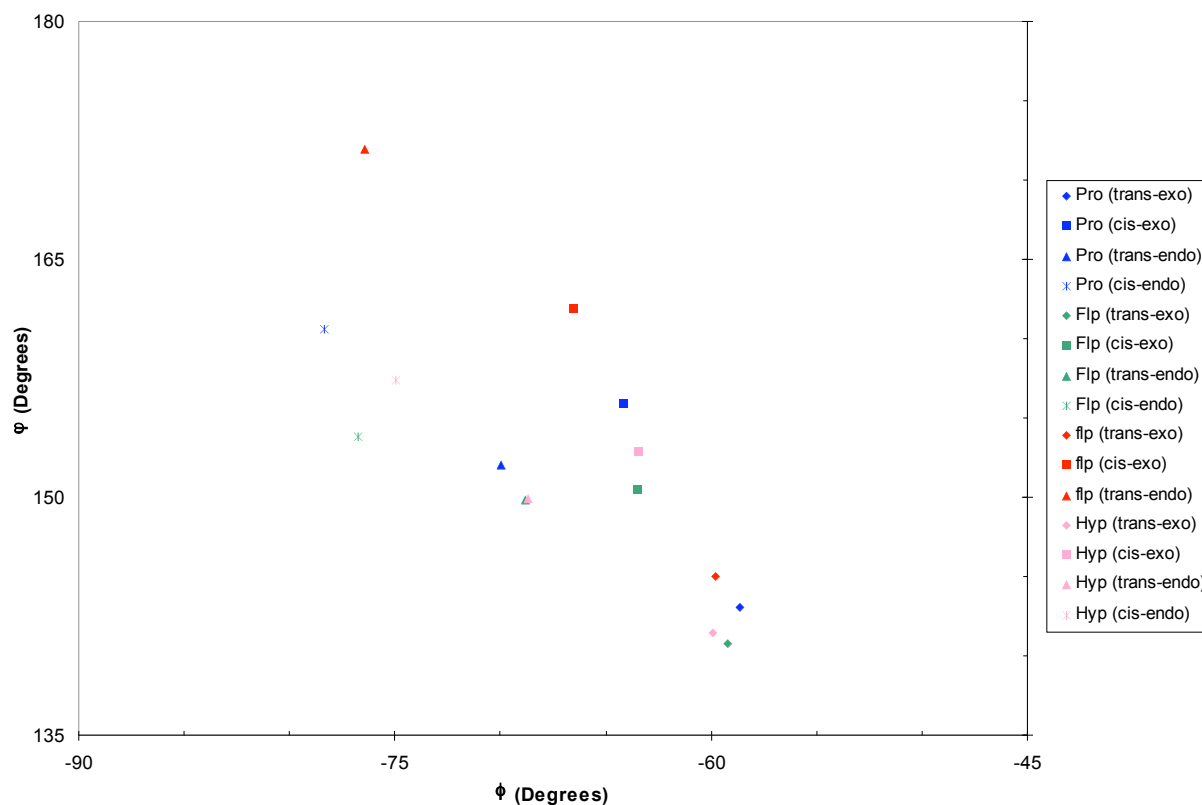


Figure 3 – Ramachandran plot using the same data as Figure 2 but with a different range on the axes.

All of the points in the Ramachandran plot shown in Figures 2 and 3 lie within the expected region for amino acids within collagen molecules. It is interesting to note that, within each conformation, those derivatives with a smaller ψ angle are those which most stabilize collagen.

It is also interesting to note that the ψ angle corresponds to the $O_2-C_1-C_1^\alpha-N_1$ dihedral angle which displays the Y-C-C-Z pattern consistent with the gauche effect. However, based upon the data in Table 2, we find that the same *Trans* stabilization that we found in the prolyl peptide bond. Thus, it seems as though gauche stabilization does not play a role in determining the ψ angle.

However, the rotation about the $C_1-C_1^\alpha$ bond could also be measured by the $O_1-C_1-C_1^\alpha-N_1$ dihedral angle which also displays the Y-C-C-Z pattern consistent with the gauche effect. The

values of this angle, which we will refer to as ψ' , can be found in Table 4 and in Figure 4. It should also be noted that there is one more instance of the Y-C-C-Z pattern to be found in the Proline derivatives. This final instance is found in all of the derivatives except Pro. This angle, which we will refer to as ξ , is calculated as the $X_1^{\delta 1}-C_1^{\gamma}-C_1^{\delta 2}-N_1$ dihedral angle. In Proline, $X_1^{\delta 1}$ is simply a Hydrogen atom, but in all of the other derivatives, this pattern satisfies the gauche interaction. The values that this angle takes on in the stable configurations studied can be found in Table 5 and Figure 5.

ψ' Angle (Degrees)	Trans Isomer	Cis Isomer
Pro (exo ring pucker)	-40.7	-26.4
Pro (endo ring pucker)	-30.9	-21.1
Hyp (exo ring pucker)	-42.3	-29.7
Hyp (endo ring pucker)	-33.3	-24.5
Flp (exo ring pucker)	-42.9	-32.2
Flp (endo ring pucker)	-33.3	-28.3
flp (exo ring pucker)	-38.6	-20.1
flp (endo ring pucker)	-8.1	7.8

Table 4 – Like the ψ angle, the ψ' angle is measured about the prolyl peptide bond, however, it is measured as the $O_1-C_1-C_1^{\alpha}-N_1$ dihedral angle. All angles are rounded to the nearest 1/10 of a degree.

ξ Angles (Degrees)	Trans Isomer	Cis Isomer
Hyp (exo ring pucker)	86.4	85.8
Hyp (endo ring pucker)	147.3	145.6
Flp (exo ring pucker)	85.2	84.9
Flp (endo ring pucker)	145.7	142.4
Flp (exo ring pucker)	-155.8	85.9
Flp (endo ring pucker)	-101.2	-96.3

Table 5 – The ξ angles measure the role that the electronegative substituents on Hyp, Flp and flp play in gauche stabilization of the ring. These angles are measured as the $X_1^{\delta 1}-C_1^{\gamma}-C_1^{\delta 2}-N_1$ dihedral angles. All angles are rounded to the nearest 1/10 of a degree.

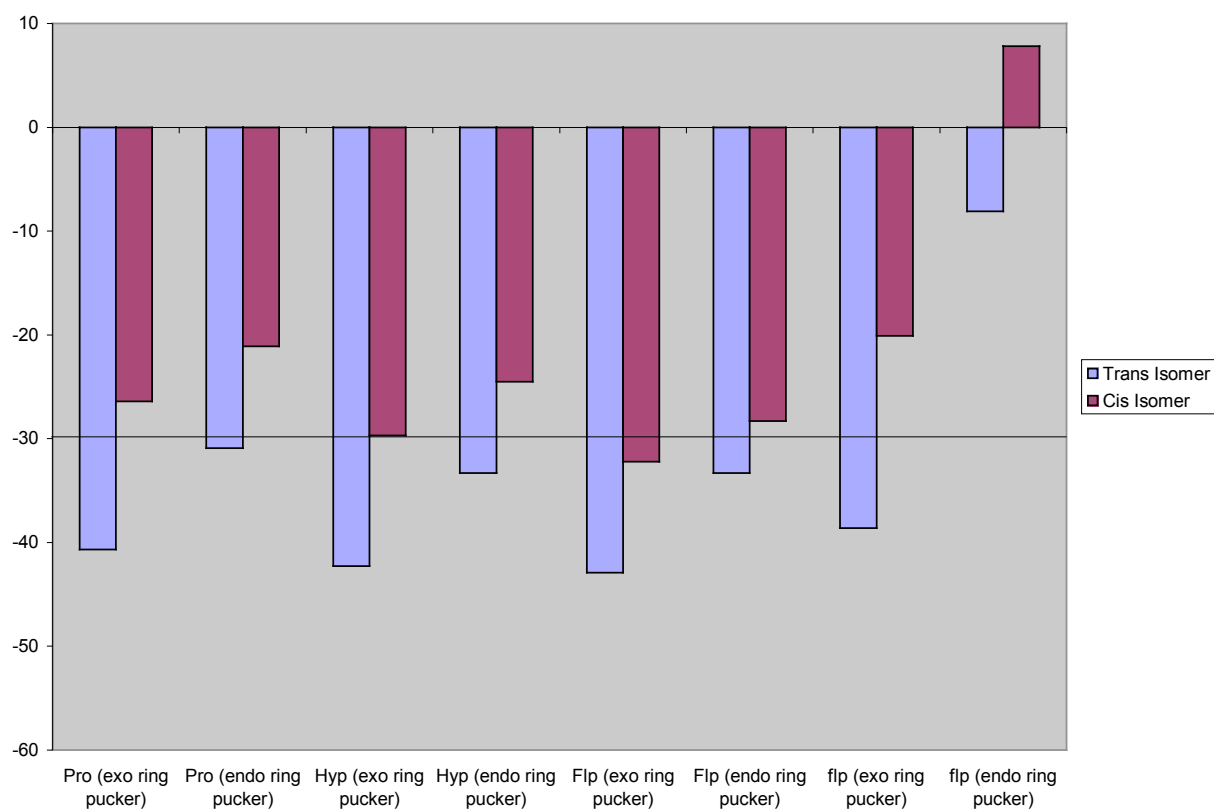


Figure 4 – The values of the ψ' angles for each of the conformations of the four Proline derivatives.

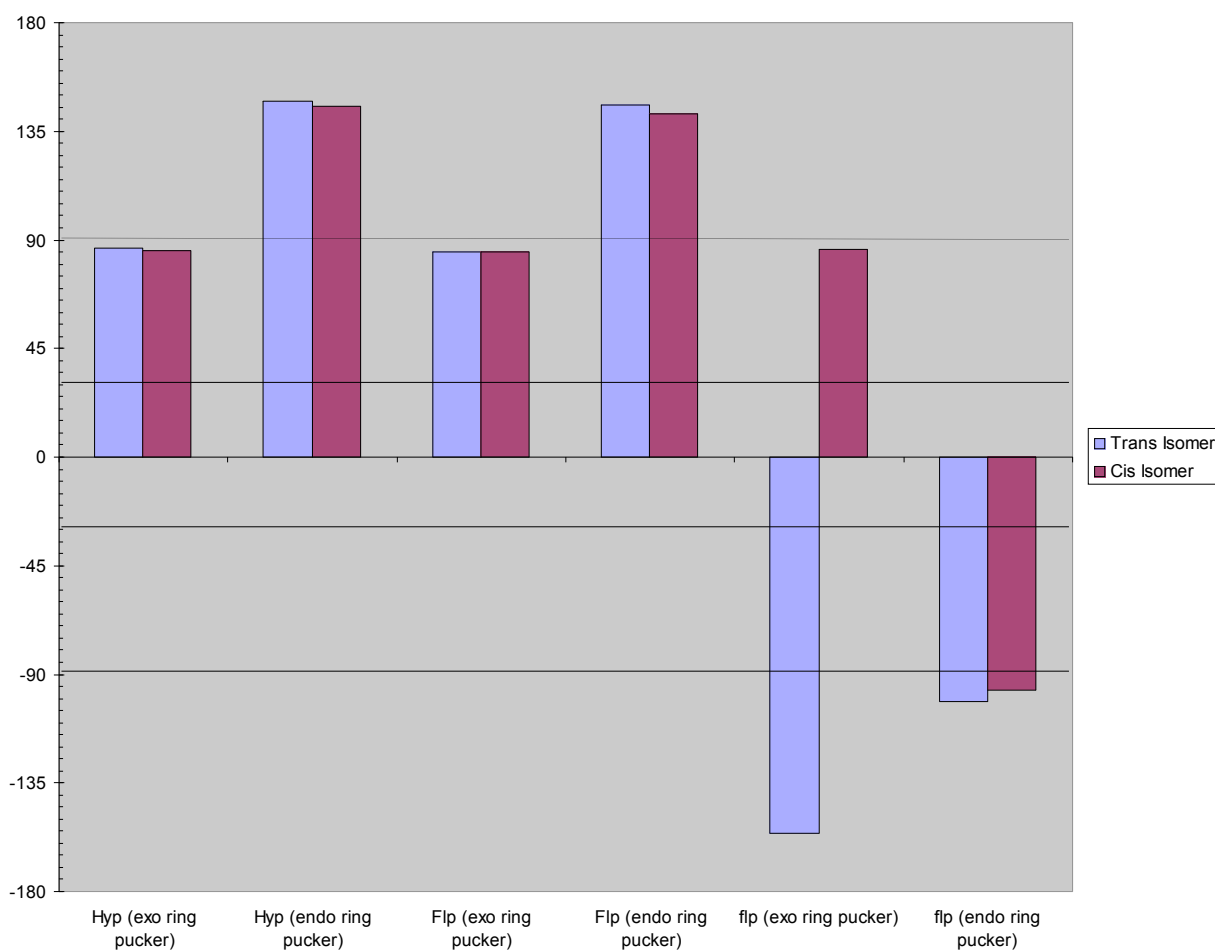


Figure 5 – The values of the ξ angles for each of the conformations of the four Proline derivatives.

Let us first focus on the ψ' angle. The Trans-Exo conformation is most stabilized by the gauche effect in each of the four Proline derivatives and the Cis-Exo conformation is slightly stabilized in Pro, Hyp and Flp. However, we must remember that the ψ' angle is already stabilized by the Trans interaction of the ψ angle, so it is quite possible that the ψ' angle plays no role in stabilization.

If we now look at the ξ angles, we see that for Hyp and Flp there is a strong preference for the Exo conformation without regard for the Trans/Cis state, whereas for flp, the Cis-Exo conformation is the only one that is stabilized by gauche interactions. Thus, based solely upon

investigating the gauche interactions present in these Proline derivatives, we would expect to find Hyp and Flp strongly favor the Exo conformation, and flp strongly favor the Cis-Exo conformation.

We will now turn our attention to steric hindrance and whether or not this can explain the stability of collagen. In these model peptides, C_0^α represents the C_1^α atom in the next residue (a Proline), therefore, not only can steric hindrance occur between O_1 and O_0 and between O_1 and C_0^α , but it can also occur between O_1 and the ring of the Proline residue that C_0^α is a part of. We will investigate this steric hindrance by looking at the distance between O_1 and the closest Hydrogen atom bonded to C_0^α . The results of these investigations can be found in Table 6.

Distance Between Atoms	O_1 and O_0	O_1 and C_0^α	O_1 and H- C_0^α
Pro (trans-exo)	3.291015	4.444279	4.389606
Pro (cis-exo)	4.330422	3.414473	2.800966
Pro (trans-endo)	3.474184	4.441334	4.267548
Pro (cis-endo)	4.393682	3.722619	3.049145
Hyp (trans-exo)	3.30952	4.505125	4.473318
Hyp (cis-exo)	4.372896	3.4092	2.812979
Hyp (trans-endo)	3.458433	4.447838	4.281075
Hyp (cis-endo)	4.378072	3.68337	3.014384
Flp (trans-exo)	3.309411	4.492661	4.453433
Flp (cis-exo)	4.381447	3.452935	2.864467
Flp (trans-endo)	3.47195	4.429363	4.249468
Flp (cis-endo)	4.395435	3.814578	3.169367
flp (trans-exo)	3.293799	4.422666	4.352397
flp (cis-exo)	4.286217	3.403351	2.780119
flp (trans-endo)	3.420419	4.308861	4.111469
flp (cis-endo)	4.232151	3.4417	2.720877

Table 6 – Distance between O_1 and O_0 , C_0^α and the Hydrogen, bonded to C_0^α , that is closest to O_1 .

Based upon the data in Table 6, we expect that all four of the Proline derivatives will prefer the Trans conformation. This is because the Trans conformation minimizes the distance between O_1 and the large ring structure represented by the $-CH_3$ group at C_0^α . These results,

combined with the results from our analysis of the ξ angle, conclude that we would most likely find Pro, Hyp and Flp in the trans-exo configuration. It is supported by previous findings that the Hyp and Flp residues are normally found in the Trans-Exo conformation.²⁹ However, in those same studies, Pro residues were found in the Cis-Endo conformation. In dealing with flp residues, on the other hand, there is a problem. Sterically, flp would be more stabilized by adopting a Trans conformation. However, due to gauche stabilization, flp would prefer to adopt a Cis-Exo conformation. I believe that it is these opposing forces that cause the instability seen in flp-containing collagen – due to gauche interactions, flp attempts to adopt a Cis-Exo conformation, but the resulting steric strain breaks the collagen molecule apart.

Is this combination of gauche interactions and steric strain enough to explain the differences in stability that we see in these collagen helices? No, unfortunately, these methods do not distinguish well between Hyp and Flp, even though it has been shown that the resulting collagen is much more stable when made with Flp than when made with Hyp.³⁰ Thus, we will investigate one more phenomenon in our attempt to explain this complex system.

During this investigation, we also sought to follow up on the suggestion raised by N. Panasik that the electron withdrawal by a substituent in the 4-position of a proline residue would lead to changes in nitrogen pyramidalization and an altering of the electron distribution in the prolyl peptide bond.³¹ This phenomenon was studied using the NBO software package and the data for the “Exo” conformation is provided in Table 7 and Figures 6, 7 and 8.

29 Eberhardt, E. S.; Panasik, N. Jr.; Raines, R. T. *Journal of the American Chemical Society* **1996**, 118, 12261-12266.

30 Holmgren, S. K.; Taylor, K. M.; Bretscher, L. E.; Raines, R. T. *Nature* **1998**, 392, 666-667.

31 Panasik, N., Jr.; Eberhardt, E. S.; Edison, A. S.; Powell, D. R.; Raines, R. T. *International Journal of Peptide & Protein Research* **1994**, 44, 262-269.

“Exo” Conformation	Bond Order		Natural Population	
	N-C6	N	C6	
Pro (trans)	1.3551	7.5154	5.2966	
Pro (cis)	1.3428	7.5184	5.2949	
Hyp (trans)	1.3549	7.5115	5.2947	
Hyp (cis)	1.3336	7.5113	5.3094	
Flp (trans)	1.3511	7.5147	5.2943	
Flp (cis)	1.3392	7.5173	5.2927	
flp (trans-exo)	1.3475	7.5173	5.2942	
flp (cis-exo)	1.3352	7.5201	5.2926	

Table 7 – The results of Natural Bond Order and Natural Population analysis on the prolyl-peptide bond in the substituted proline residues. Results are shown for the exo conformation only.

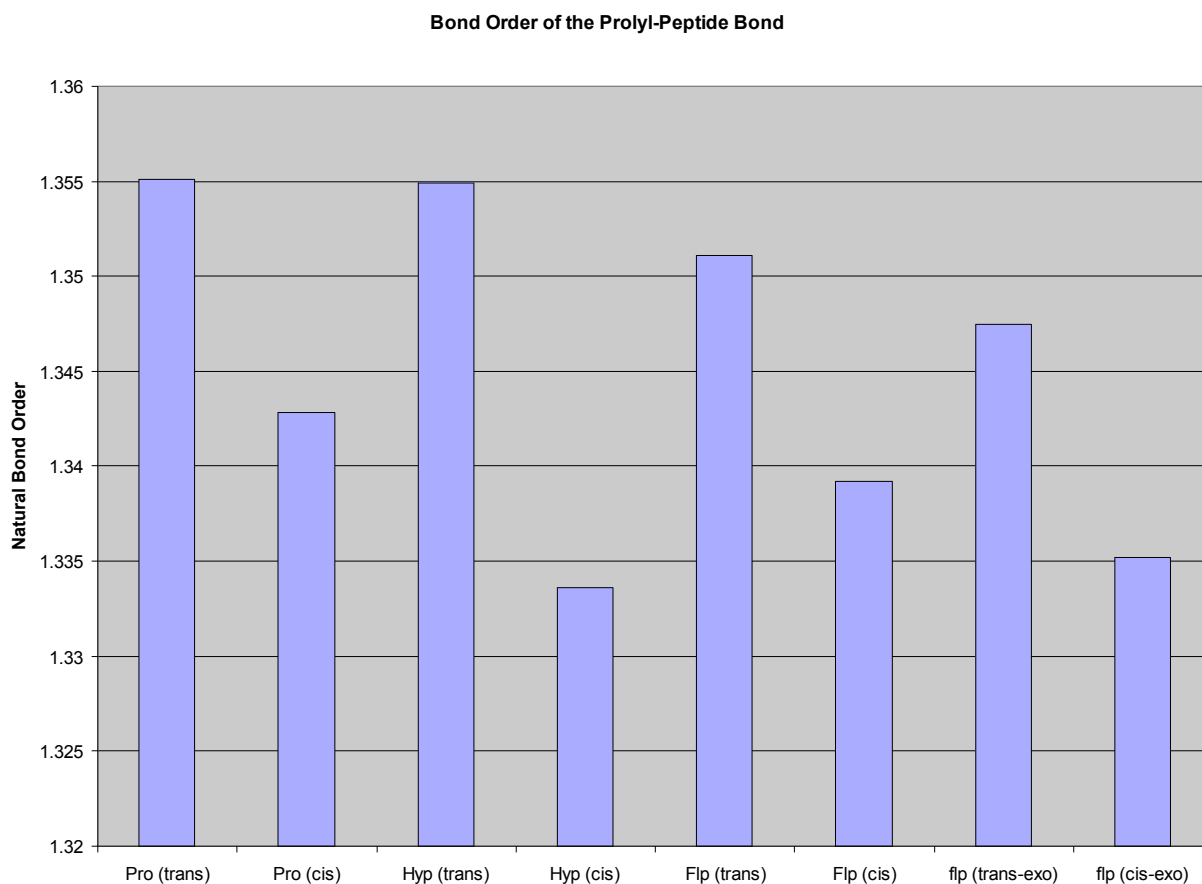


Figure 6 – Bond Order of the Prolyl-Peptide Bond (C_0-N_1) for the “Exo” conformation.

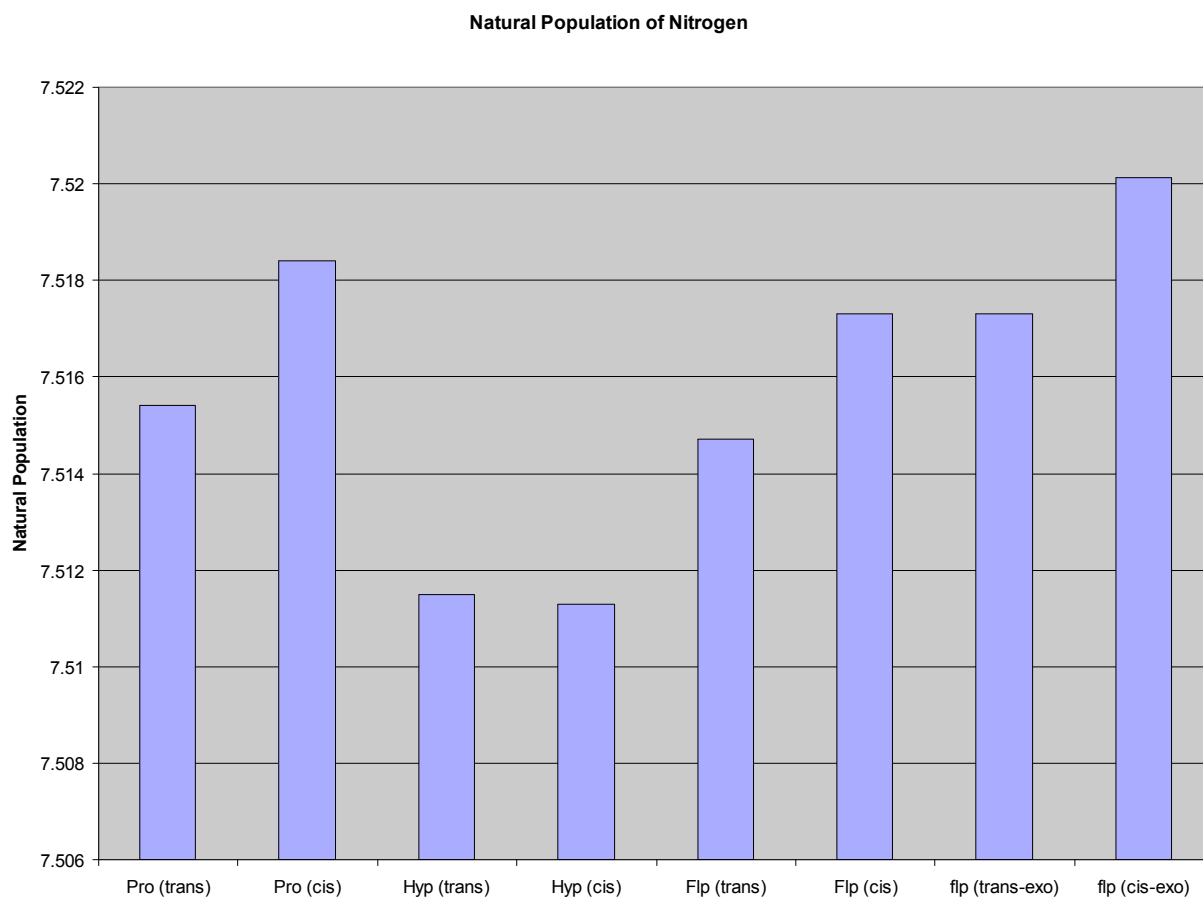


Figure 7 – Natural Population of the Nitrogen atom (N₁) in the prolyl-peptide bond for the “Exo” conformation.

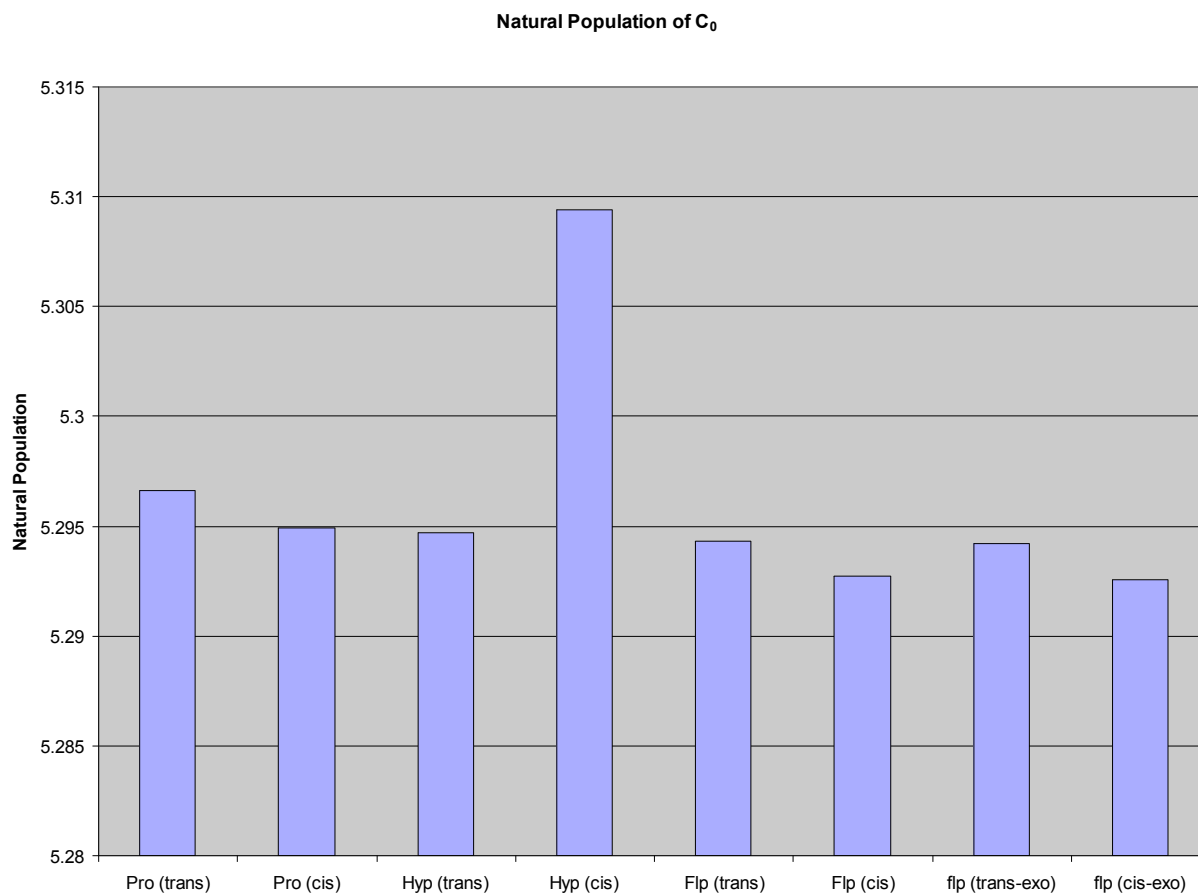


Figure 8 – Natural Population of the Carbon atom (C₀) in the prolyl-peptide bond for the “Exo” conformation.

However, an even more interesting bond to investigate is the C₁-O₁ bond in these Proline derivatives. This is a particularly interesting Oxygen because it is the Oxygen that participates in the inter-strand hydrogen bonding that holds the collagen helix together. When these inter-strand hydrogen bonds weaken, the strands fall apart and are broken down. Thus, the strength of these hydrogen bonds play a key role in the stability of the collagen triple helix. In order to study the inter-strand hydrogen bond strength indirectly, the natural bond order of the C₁-O₁ bond can be found in Table 8 and Figure 9.

Bond Order	C ₁ -O ₁
Pro (trans-exo)	1.9543
Hyp (trans-exo)	1.9536
Flp (trans-exo)	1.9426
flp (cis-exo)	1.9547

Table 8 – The results of Natural Bond Order analysis of the C₁-O₁ bond in substituted proline residues.

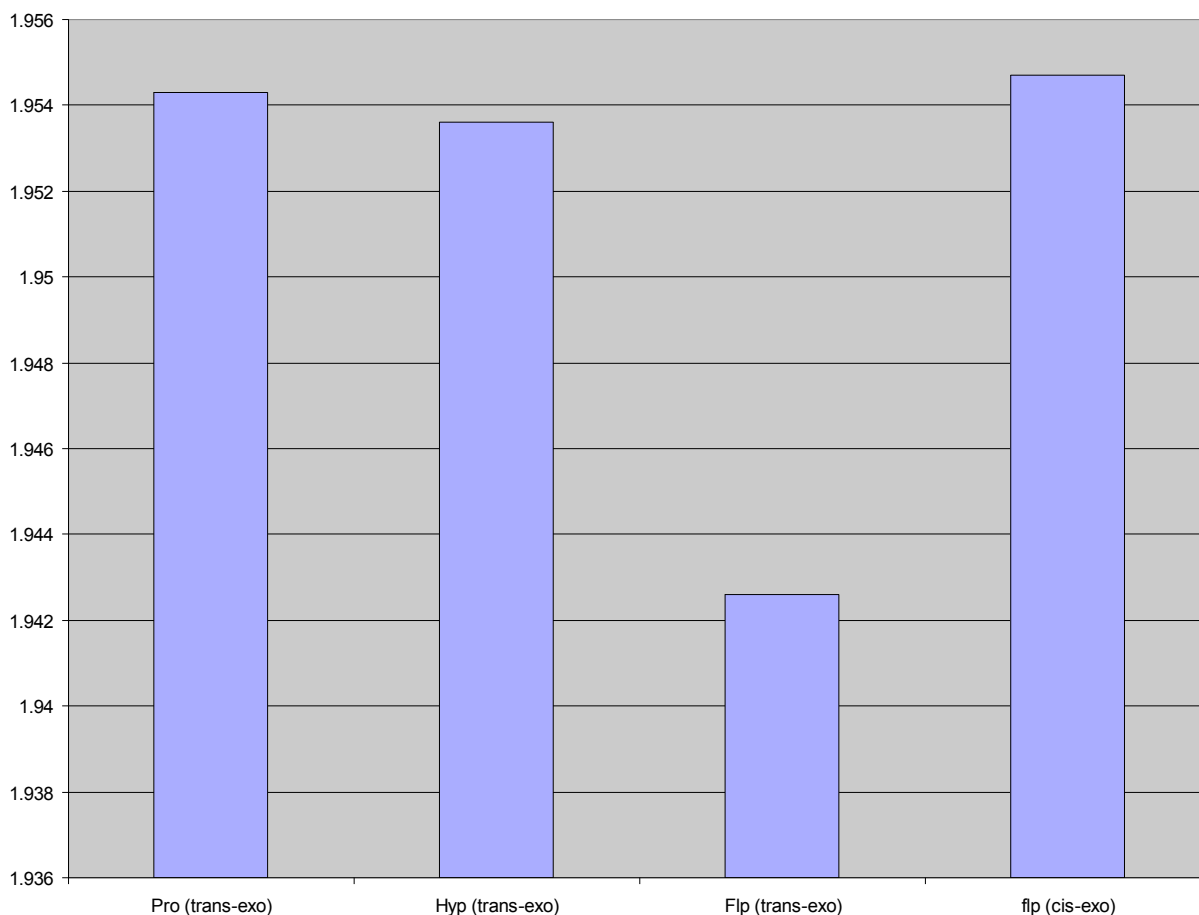


Figure 9 – The results of Natural Bond Order analysis of the C₁-O₁ bond in substituted Proline residues.

The results of studying this bond are very interesting because it gives the most direct way of measuring inter-strand Hydrogen bond strength. As the C₁-O₁ bond reduces in bond order, the highly electronegative Oxygen will strengthen the only other bond that it is currently involved in – a Hydrogen bond with another strand. Thus, the electrons involved in bonding with O₁ become

more delocalized and the strength of the bond between the strands increases in strength.

Conclusions

Due to the gauche effect and steric interactions, Hyp and Flp residues will be stabilized in the Trans-Exo configuration whereas flp residues will prefer the Cis-Exo configuration due to gauche interactions, but this configuration will lead to destabilization due to steric strain. The inductive effect results in an increase in the strength of the inter-strand hydrogen bonds in Hyp over Pro and a much larger increase in Flp over Hyp. Whereas this same effect has the opposite effect in flp residues. These three phenomena work together to produce the observed stability of collagen helices.

Experimental Section

The calculations were carried out using the Gaussian 94³², Gaussian 98³³ and NBO 4.0³⁴ software packages. All geometries were fully optimized and frequency calculations were carried out using the B3LYP method at the 6-31+G* level of theory. Natural Bond Order calculations were done using the B3LYP method and the 6-311+G(2d,2p) level of theory. – a

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level well suited to systems like the ones that we are working with.³⁵ The calculations were carried out at the University of Wisconsin on SGI R10000 and R8000 workstations.

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