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Understanding the Role of Stereoelectronic Effects in Determining Collagen Stability. 2. A Quantum Mechanical/Molecular Mechanical Study of (Proline-Proline-Glycine)$_n$ Polypeptides

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Abstract: The importance of vicinal and long-range interresidue effects in determining the stability of the collagen triple helix has been investigated by quantum mechanical (QM) and molecular mechanical (MM) computations on suitable model polypeptides, taking into account solvent effects by the polarizable continuum model (PCM). At the QM level, the PII conformation corresponds to an energy minimum for pentapeptide analogues incorporating the sequence Gly-Pro-Pro-Gly, irrespective of the down or up puckering of the pyrrolidine ring. However, our computations indicate that the alternation of down and up prolines characterizing collagen and collagen-like peptides is not due to an intrinsic preference of the Pro-Pro-Gly sequence. This result is confirmed by MM computations of longer polypeptides. Next, MM computations on model triple helices show that a better packing is obtained for specific values of backbone dihedrals, which, in turn, favor the alternation of down and up prolines along each chain.

1. Introduction

Since the first proposals of collagen models,[1] the elucidation of collagen structure and the understanding of the molecular basis of its stability have been the subject of several studies, from both experimental and theoretical points of view.[2−12] Collagen is composed of approximately 300 repeats of the X-Y-Gly sequence, arranged in a PII triple helix ($\phi \approx -70°$, $\psi \approx 160°$), with X and Y positions frequently occupied by proline (Pro) and hydroxyproline (Hyp) iminoacids. (Pro-Pro-Gly)$_n$ model compounds have thus been thoroughly investigated, and their study has been particularly fruitful for the elucidation of the main structural features of collagen.[5−9]

The availability of a high-resolution structure of the (Pro-Pro-Gly)$_{10}$ peptide (hereafter PPG$_{10}$)[6−8] has very recently made it possible to highlight the strong correlation between the position of the iminoacid in the chain and the puckering of the pyrrolidine ring.[8] As a matter of fact, the pyrrolidine ring can adopt two distinct puckerings in which the C$^\beta$ and the C$^\gamma$ atoms are displaced from the mean plane of the ring.[13] The two puckered forms are generally referred to as "exo" and "endo" (more precisely C$^\gamma$-exo and C$^\gamma$-endo) or "up" and "down", respectively. In collagen-like polypeptides, prolines in the X position adopt a down puckering, whereas those in the Y position adopt an up puckering. X-ray structures of (Pro-Hyp-Gly)$_n$ compounds show the same X(down)-Y(up) alternation.[9] These findings, together with the observation of a strong correlation between the adopted puckering and the backbone ϕ dihedrals, suggest that the formation of a collagen triple helix requires the presence of a down iminoacid in the X position and an up iminoacid in the Y position.[8]

In the first part of this study,[11] devoted to dipeptide analogues of proline (ProDA), hydroxyproline (HypDA), and fluoroproline (FlpDA), we have shown that the relative stability of the up puckering increases with the electronegativity of the 4(*R*) substituent. This result, which agrees with previous experimental

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indications,[10] could explain the role of Hyp and, even more, Flp occupying Y positions in stabilizing triple helices of collagen and collagen-like peptides.

However, despite their usefulness for the analysis of intraresidue effects, dipeptide analogues (i.e., amino acidic residues capped with an acetyl group (Ac) at the N-terminus and with a methylamino group (NHMe) at the C-terminus) are too small models for analyzing the role played by interresidue effects. Since these interactions are expected to play a significant role in determining the conformational behavior of the collagen triple helix, in this paper we present a quantum mechanical (QM) study of the pentapeptide analogue Ac-Gly-Pro-Pro-Gly-NHMe (hereafter GPPG) and a molecular mechanical (MM) study of PPG_{10} peptide and of its trimer (3PPG) arranged in a collagen-like triple helix.

The main purpose of our study is to obtain a deeper understanding of the influence of the proline puckering on the conformation of the polypeptide and on the stability of the triple helix. For each compound under study, we have thus analyzed the four possible combinations of ring puckerings, i.e., Pro(X)-down-Pro(Y)down, Pro(X)down-Pro(Y)up, Pro(X)up-Pro(Y)-down, and Pro(X)up-Pro(Y)up.

The natural environment of many proteins is an aqueous solution, and PPG_{10} is strongly hydrated also in the solid state:[8] it is thus very important to take solvent effects into the proper account. To that aim we resorted to the polarizable continuum model (PCM) that has already provided reliable and accurate results when applied to the study of biological systems in the condensed phase, at both QM and MM levels.[14]

2. Methods

QM calculations were carried out by a development version of the Gaussian package,[15] using the standard 6-31G(d) and 6-31+G(d,p) basis sets[16] and taking into account electron correlation effects by means of DFT calculations at the PBE0 level.[17] PBE0 is a parameter-free hybrid Hartree−Fock/Kohn−Sham method, whose exchange-correlation contribution is represented by eq 1,

$$E_{XC}^{PBE0} = E_{XC}^{PBE} + {}^1/_4(E_X^{HF} - E_X^{PBE}) \quad (1)$$

where E_X^{HF} is the Hartree−Fock exchange and E_X^{PBE}, E_{XC}^{PBE} are the exchange and the complete density functionals proposed by Perdew, Burke, and Ernzerhof (PBE).[18] The PBE functional is particularly attractive, since it is based on a number of limiting conditions and does not involve empirical parameters. The inclusion of some HF exchange (see eq 1) increases the reliability of the PBE0 model, especially in the field of conformational studies of biomolecules.[12,19]

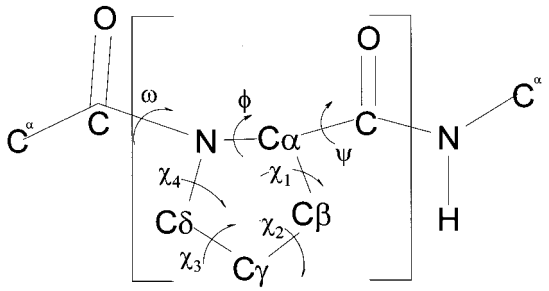

Figure 1. Atom labeling and selected geometric parameters of the proline residue.

MM computations in vacuo have been performed by the AMBER 6 package using the 1994 parameters and atomic charges.[20]

Solvent effects have been taken into account by the PCM.[21] In this method the solvent is represented by an infinite dielectric medium characterized by the relative dielectric constant of the bulk (78.39 for H_2O at 25 °C and 1 atm). A molecular-shaped cavity contains the system under study (the solute), and its surface separates the solute from the surrounding solvent. The cavity including the molecule, defined in terms of interlocking spheres centered on non-hydrogen atoms, is built by a new version of the GePol procedure using the UAHF atomic radii.[22] The free energy of solvation (ΔG_{solv}) includes electrostatic, dispersion/repulsion, and cavitation contributions.

$$\Delta G_{sol_v} = \Delta G_{el} + \Delta G_{dr} + \Delta G_{cav} \quad (2)$$

The cavitation term is determined using the Pierotti scaled particle theory,[23] while ΔG_{dr} is evaluated using semiempirical atom−atom parameters.[24] Finally, ΔG_{el} takes into account the solute−solvent electrostatic interactions: in the quantum mechanical implementation this contribution is obtained by adding a proper operator to the solute Hamiltonian. In this work we used the CPCM[25] variant of PCM that, using conductor rather than dielectric boundary conditions, allows a more robust implementation and leads to very similar results for polar solvents. Analytical energy first and second derivatives allow for geometry optimizations and harmonic frequency calculations in solution.[26]

The PCM has been adapted also to MM calculations and has already provided values of solvation energies in good qualitative agreement with the results obtained by accurate quantum mechanical calculations.[14]

Note that all the terms of the solvation energy can be dissected into contributions issuing from the different spheres forming the cavity. Since each sphere corresponds to a well-defined atom (or chemical group), this procedure allows a detailed analysis about the origin of differential solvation effects. However, the electrostatic contribution of each sphere originates from the electron density of the whole solute, and so this analysis should be considered only qualitative.

3. Results

Let us recall that the up puckering of the pyrrolidine ring is characterized by a negative value for the χ_1 torsion angle and a positive value for the χ_2 torsion angle, while the down conformation has a positive value for χ_1 and a negative value for χ_2 (see Figure 1).

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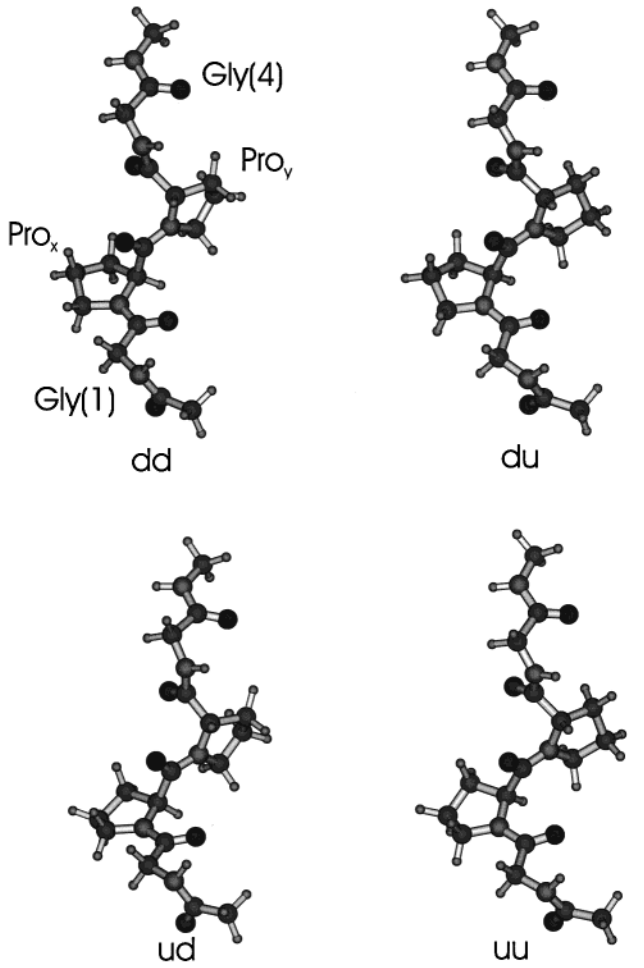

Figure 2. Minimum energy geometry (HF/6-31G(d) calculations) of the all-PII isomers of **dd du**, **ud**, and **uu**. ϕ and ψ dihedrals of the N-terminal glycine have been constrained to the average value of glycine residues in PPG_{10}.

3.1. Quantum Mechanical Study of GPPG. We have already verified that for peptides HF and correlated geometries are similar, and that the relative stability of up and down conformers predicted by correlated computations does not change if the geometry is optimized at the HF or correlated level.[11,12]

We started our analysis by optimizing at the HF/6-31G(d) level the geometry of the GPPG peptide, for all the different down−up combinations of proline puckering: Gly-Pro$_{down}$-Pro$_{down}$-Gly (hereafter **dd**), Gly-Pro$_{down}$-Pro$_{up}$-Gly (**du**), Gly-Pro$_{up}$-Pro$_{down}$-Gly (**ud**), and Gly-Pro$_{up}$-Pro$_{up}$-Gly (**uu**).

Since all the residues of collagen and collagen-like polypeptides adopt a PII conformation ($\phi \approx -70°$, $\psi \approx 160°$), we started our geometry optimizations from structures in which all the residues are in the PII conformation. The structures of the energy minima issuing from these optimizations are shown in Figure 2, and selected geometrical parameters are collected in Table 1. Except for Gly(1), which adopts a fully extended conformation (C5, $\phi \approx 180°$, $\psi \approx 180°$), the three remaining residues retain the PII conformation. This is a quite interesting result, since this conformation is not a gas-phase energy minimum for glycine and proline dipeptide analogues.[12] A statistical survey of protein X-ray structures shows indeed that the most probable conformation for a sequence of two prolines is the PII, and also residues adjacent to Pro-Pro sequences reveal a significant tendency toward that conformation.[27]

Table 1. Selected Geometrical Parameters of the GPPG Minima (Dihedral Angles in Degrees)[a]

		dd	du	ud	uu
		HF/6-31G(d) Calculations			
Gly(1	ϕ	−177.4	−177.5	−177.6	−178.3
	ψ	173.7	174.4	179.2	−179.6
	ω	−177.6	−177.7	−178.5	−178.2
Pro$_x$	ϕ	−71.7	−71.0	−62.5	−62.1
	ψ	153.4	141.2	149.0	137.0
	ω	176.0	177.8	177.6	175.4
	χ_1	32.1	29.6	−12.6	−17.0
Pro$_y$	ϕ	−72.6	−62.3	−73.2	−63.4
	ψ	155.4	151.8	154.6	150.1
	ω	172.6	168.0	174.2	170.9
	χ_1	31.2	−25.6	30.8	−27.0
Gly(4)	ϕ	−77.8	−77.8	−77.5	−78.6
	ψ	163.5	161.2	162.9	160.6
	ω	162.9	163.0	162.9	163.2
$\Delta E^{a,b}$		0.0	1.29	1.21	2.37
ΔE^{c}		0.0	1.03	0.35	0.89
ΔE^{d}		0.0	1.15	1.19	2.03
ΔE^{e}		0.0	0.86	0.92	1.43
		AMBER Calculations			
Gly(1)	ϕ	−178.0	−178.0	−179.2	−179.2
	ψ	179.4	179.4	−177.7	−177.5
	ω	−179.8	−179.8	−179.9	−179.9
Pro$_x$	ϕ	−72.2	−73.1	−56.1	−57.3
	ψ	161.7	166.2	155.0	160.2
	ω	177.2	176.8	174.6	174.3
	χ_1	30.9	31.7	−23.5	−22.9
Pro$_y$	ϕ	−72.7	−56.2	−73.2	−56.1
	ψ	166.1	157.3	165.4	155.4
	ω	172.6	168.6	173.6	169.2
	χ_1	31.5	−23.3	31.7	−23.4
Gly(4)	ϕ	−89.6	−81.9[f]	−90.3	−81.9[f]
	ψ	171.0	172.2	171.0	172.1
	ω	171.5	170.7	171.6	170.8
ΔE		0.0	1.22	1.13	2.46

[a] The differential energies (in kcal/mol) are relative to the **dd** conformer. [b] Gas phase. Energy(**dd**) = −1306.14431856 au. [c] Aqueous solution. Single-point CPCM/HF/631-G(d) calculations on the gas-phase optimized geometries. Energy (**dd**) = −1306.191784 au. [d] PBE0/6-31G(d) single-point calculations. Energy(**dd**) = −1312.55857598 au. [e] PBE0/6-31+G(d,p) single-point calculations. Energy(**dd**) = −1312.64363058 au. [f] Frozen in P−II conformation.

It is not surprising that the PII conformation is not a minimum for Gly(1): in collagen each Gly residue is sandwiched between two prolines, and the polypeptide chains are bound in a triple helix that, moreover, is strongly hydrated. All these effects are obviously lacking in GPPG, so that Gly(1) adopts an extended conformation which allows for a weak intraresidue hydrogen bond and is a minimum for the glycine dipeptide analogue in vacuo (GlyDA).[30] As a matter of fact, a CPCM/HF/6-31G(d) geometry optimization of the **du** structure predicts that in aqueous solution the PII structure is an energy minimum for Gly(1) already for GPPG (see Table 2).

From the structural point of view, our results confirm the interdependence between ring puckering and backbone dihedrals

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Table 2. Selected Geometrical Parameters of the All-PII GPPG Minima, Obtained at the HF/6-31G(d) Level (HF Calculations)[a]

		dd	du	ud	uu
Gly(1)	ϕ	−71.7	−71.7 (−76.8)	−71.7	−71.7
	ψ	175.9	175.9 (174.6)	175.9	175.9
	ω	159.9	160.5 (168.3)	159.8	160.4
Pro_x	ϕ	−73.1	−72.3 (−72.6)	−62.0	−60.7
	ψ	153.1	139.7 (145.2)	147.0	136.2
	ω	175.5	177.6 (−176.6)	172.2	173.9
	χ_1	31.3	28.5 (27.7)	−15.2	−19.1
Pro_y	ϕ	−72.9	−62.5 (−62.9)	−74.4	−63.6
	ψ	155.3	151.5 (152.9)	154.4	149.8
	ω	173.3	168.6 (171.9)	162.9	171.3
	χ_1	31.2	−26.1 (−24.5)	30.7	−27.3
Gly(4)	ϕ	−77.9	−78.2 (−75.6)	−77.6	−79.2
	ψ	162.5	160.4 (170.4)	161.8	159.5
	ω	162.9	163.3 (166.3)	162.9	163.3
ΔE^b		0.0	1.19	1.15	2.20
ΔE^c		0.0	1.14	1.57	1.99
ΔE^d		0.0	0.96	1.14	1.77
ΔE^e		0.0	0.69	0.86	1.31

[a] The results of the CPCM/HF/6-31G(d) geometry optimization of **du** in aqueous solution are given in parentheses. In gas−phase computations Gly(1) is constrained in the PII conformation as in $(PPG)_{10}$. The differential energies (in kcal/mol) are relative to **dd**. [b] Gas phase. Energy(**dd**) = −1306.141332 au. [c] Aqueous solutions. Single-point CPCM/HF/631-G(d) calculations on the gas-phase optimized geometries. Energy(**dd**) = −1306.190784 au. [d] PBE0/6-31G(d) single-point calculations. Energy(**dd**) = −1312.554054 au. [e] PBE0/6-31+G(d,p) single-point calculations. Energy(**dd**) = −1312.63861299 au.

Table 3. Selected Geometrical Parameters of the GPPG Minima, Obtained at the HF/6-31G(d) Level in the Gas Phase[a]

		dd	du	ud	uu
Pro_x	χ_1	32.8	33.1	−5.1	−7.7
Pro_y	χ_1	25.1	−22.8	27.0	−20.8
ΔE^b		0.0	0.44	1.35	1.79
ΔE^c		0.0	−0.09	1.15	1.06
ΔE^d		0.0	0.45	1.09	1.54
ΔE^e		0.0	0.19	1.06	1.32

[a] Backbone dihedrals are constrained to the corresponding experimental values of $(PPG)_{10}$. Gly: $\phi = -71.7°$, $\psi = 175.9°$, $\omega = 179.7°$. Pro_x: $\phi = -74.5°$, $\psi = 164.3°$, $\omega = 176.0°$. Pro_y: $\phi = -60.1°$, $\psi = 152.4°$, $\omega = 175.4°$. [b] Gas phase. Energy(DD) = −1306.133050 au. [c] Solvent (total). Energy (DD) = −1306.189420 au. [d] PBE0/6-31G(d). Energy(DD) = −1312.545951 au. [e] PBE0/6-31+G(d,p). Energy(DD) = −1312.631347 au.

that has been already highlighted by the analysis of X-ray structures and in our study on ProDA.[8,11]

In detail:

1. The value of the ϕ dihedral is $\approx -60°$ in up prolines and $\approx -70°$ in down prolines.

2. The average values of the ψ dihedrals are smaller for up puckerings than for down puckerings. This result is in agreement with previous experimental[29] and computational[11] results (vide infra).

3. The ψ dihedral of prolines is reduced, especially for Pro_x, when the adjacent residue adopts an up puckering. In contrast, the value of the ϕ dihedral does not depend significantly on the conformation of the adjacent residue.

4. The conformation of the pyrrolidine ring (χ_1 clustered around 25° and −30° in up and down puckering, respectively) is very similar to that found in previous calculations on ProDA.[11] However, it is noteworthy that the absolute value of χ_1 is much smaller for up prolines in the X position (vide infra).

Interestingly, the equilibrium geometry predicted in aqueous solution is more similar to that adopted in the PPG_{10} triple helix. As a matter of fact, the ω dihedrals have values closer to planarity, and all the backbone dihedrals of Gly(4) get closer to those found in collagen-like peptides (e.g., the value of the ψ dihedral goes from 160.4° to 170.4°, approaching the experimental value, 175.9°, found in PPG_{10}).

According to HF/6-31G(d) calculations, the **dd** conformer is ≈1 kcal/mol more stable than **du** and **ud** (exhibiting one up conformer), which are practically isoenergetic, and ≈2 kcal/mol more stable than **uu**. At the same computational level, the PII down conformer of ProDA is favored by 1.1 kcal/mol over its up counterpart. QM calculations thus strongly suggest that the presence of two adjacent prolines and the possible alternation of the up−down puckering in the chain do not affect the relative stability of different GPPG conformers. As already found for ProDA,[11,12] extension of the basis set and inclusion of correlation (by PBE0 computations) have only a negligible effect on the relative stabilities of the different structures, except for a slight reduction of the energy gap between down and up puckerings. Inclusion of solvent effects (by means of the CPCM) does not change the relative ordering of the different conformers, even if it decreases the energy gap between the **dd** conformer and the other three structures. This finding is also in line with the results obtained for the dipeptide analogue, whose down−up energy gap in aqueous solution (0.4 kcal/mol) is close to the corresponding energy difference between **dd** and **du** conformers (0.35 kcal/mol).

In the next step of our analysis, we checked whether the above picture depends on the Gly(1) conformation, by performing partial geometry optimizations in which this residue is forced to assume a PII conformation (see Table 2). ϕ and ψ dihedrals of that residue have thus been kept frozen at the values found in PPG_{10} (−71.7° and 175.9°, respectively).[8]

From an energetic point of view, the variation of the Gly(1) conformation has a negligible effect on the relative stability of the four conformers, except for a small stabilization of the **du** and **ud** conformers.

As it could be expected, only the **du** conformer exhibits the same trend of ϕ dihedrals ($Pro_x \approx -70°$, $Pro_y \approx -60°$) experimentally found in PPG_{10}.

We next performed partial geometry optimizations by constraining the backbone dihedral angles to the experimental values of the PPG_{10} triple helix. Gas-phase calculations (see Table 3) predict that **du** is more stable than **ud** and very close in energy to **dd**. At the PBE0/6-31+G(d,p) level, **dd** and **du** structures are nearly isoenergetic. When solvent effects are included by the CPCM, the **du** structure becomes slightly more stable than the **dd** one. Interestingly, **ud** and **uu** conformers are nearly isoenergetic and remarkably less stable than the **dd** and **du** ones.

When constrained to adopt backbone dihedrals ideal for a down proline, the puckering of up prolines in the X position (**ud** and **uu**) is severely distorted (χ_1 values close to 0°), confirming the interdependence between ring and backbone conformations.

3.2. AMBER Study of the (Pro-Pro-Gly)$_{10}$ Peptide. As a preliminary step, we performed some AMBER test calculations on the GPPG peptides, to check the reliability of an MM approach for the study of collagen-like peptides. AMBER full geometry optimizations predict, in agreement with QM results, that Gly(1) always adopts an extended conformation (see Table 1), whereas the PII conformation is an energy minimum for

Table 4. Selected Geometrical Parameters of the PPG_{10} Minima, Obtained at the AMBER Level in the Gas Phase

		DD	DU	UD	UU
		Single Chain			
Pro_x	ϕ	-72.3 ± 0.2	-73.2 ± 0.2	-56.2 ± 0.2	-57.3 ± 0.2
	ψ	161.4 ± 0.2	165.8 ± 0.2	154.3 ± 0.3	159.4 ± 0.5
	ω	176.2 ± 0.03	175.8 ± 0.05	173.3 ± 0.05	173.1 ± 0.1
	χ_1	30.9 ± 0.06	31.7 ± 0.05	-23.5 ± 0.03	-23.0 ± 0.09
	χ_2	-35.5 ± 0.00	-35.9 ± 0.03	33.8 ± 0.03	33.6 ± 0.07
Pro_y	ϕ	-72.9 ± 0.06	-56.2 ± 0.2	-73.3 ± 0.2	-56.2 ± 0.3
	ψ	166.7 ± 0.2	158.0 ± 0.8	166.1 ± 0.6	156.9 ± 1.3
	ω	172.4 ± 0.1	168.5 ± 0.2	173.6 ± 0.1	169.1 ± 0.2
	χ_1	31.7 ± 0.06	-23.4 ± 0.2	31.8 ± 0.2	-23.4 ± 0.2
	χ_2	-35.8 ± 0.03	33.8 ± 0.09	-35.8 ± 0.06	33.8 ± 0.1
Gly[a]	ϕ	-83.9 ± 0.1	-87.0 ± 0.2	-86.5 ± 0.1	-89.9 ± 2.1
	ψ	172.4 ± 0.07	172.6 ± 0.05	176.5 ± 0.03	177.0 ± 0.3
	ω	170.7 ± 0.05	171.1 ± 0.05	170.4 ± 0.05	171.0 ± 0.3
		Triple Helix			
Pro_x	ϕ	-71.6 ± 1.4	-73.3 ± 1.4	-57.4 ± 1.6	-57.8 ± 1.5
	ψ	163.3 ± 1.5	165.4 ± 2.4	154.7 ± 2.8	154.1 ± 4.1
	ω	179.2 ± 1.1	178.5 ± 1.5	176.6 ± 0.9	175.6 ± 1.4
	χ_1	29.2 ± 1.1	30.9 ± 0.7	-23.5 ± 1.0	-23.6 ± 0.6
	χ_2	-34.0 ± 1.0	-35.2 ± 0.7	33.2 ± 0.6	33.5 ± 0.4
Pro_y	ϕ	-63.1 ± 2.8	-51.3 ± 1.1	-64.0 ± 2.1	-51.3 ± 1.3
	ψ	153.9 ± 6.3	149.6 ± 6.2	149.5 ± 6.0	146.7 ± 8.3
	ω	179.6 ± 2.1	174.2 ± 2.7	172.4 ± 1.8	166.4 ± 2.1
	χ_1	24.7 ± 2.0	-26.5 ± 1.1	27.7 ± 1.1	-25.5 ± 0.8
	χ_2	-33.9 ± 0.8	34.4 ± 0.7	-34.2 ± 0.6	34.8 ± 0.4
Gly[a]	ϕ	-73.2 ± 2.4	-73.7 ± 0.7	-75.5 ± 1.4	-74.6 ± 1.8
	ψ	178.0 ± 1.9	-179.2 ± 7.8	179.3 ± 1.3	-178.1 ± 1.5
	ω	-178.2 ± 3.2	179.6 ± 2.2	-170.8 ± 1.6	-174.5 ± 1.8

[a] Excluding C-terminal glycine.

both proline residues. Gly(4) retains a PII conformation in the energy minima of **dd** and **ud** peptides, whereas it prefers an extended conformation for **du** and **uu**. When the geometry of the C-terminal glycine is constrained in a PII conformation also for **du** and **uu**, the relative stability of the four isomers is remarkably close to that obtained at the QM level. The equilibrium geometries are also similar, except for larger ψ values at the MM level that, already for the tetrapeptide, are similar to those found in collagen-like chains. These results thus support the reliability of the conclusions inferred from the analysis of AMBER results for longer Pro-Pro-Gly chains.

3.2.1. PPG_{10} Monomer. Table 4 collects selected geometric parameters of the four regular up−down conformers of PPG_{10} (capped with an acetyl group at the N-terminus and an *N*-methylamino group at the C-terminus), optimized in vacuo at the AMBER level. The PII conformation is an energy minimum, and the stability trend (Table 5) is in agreement with the results of QM calculations on GPPG. The conformer exhibiting all the prolines in the down conformation (DD) is more stable than the pair of nearly isoenergetic isomers containing half the prolines with up puckering (DU and UD) by ≈10 kcal/mol, and more stable than the all-up conformer (UU) by ≈20 kcal/mol. Since AMBER calculations predict that for ProDA the down puckering is ≈1 kcal/mol more stable than the up one, these results confirm the substantial independence of the proline puckerings' stability on its position in the chain and on the conformation of the adjacent residues.

From a structural point of view, the main conclusions drawn from the analysis of the QM optimizations are confirmed. Both ϕ and ψ dihedrals strongly depend on the pyrrolidine puckering, whereas only ψ depends also (albeit to a much lower extent) on the puckering of the adjacent residues (see Table 4). The structures are regular, except for the geometry of the C-terminal Gly residue that, in three of the four chains, assumes an extended conformation (ϕ and $\psi \approx 180°$) in order to form a weak intraresidue hydrogen bond.

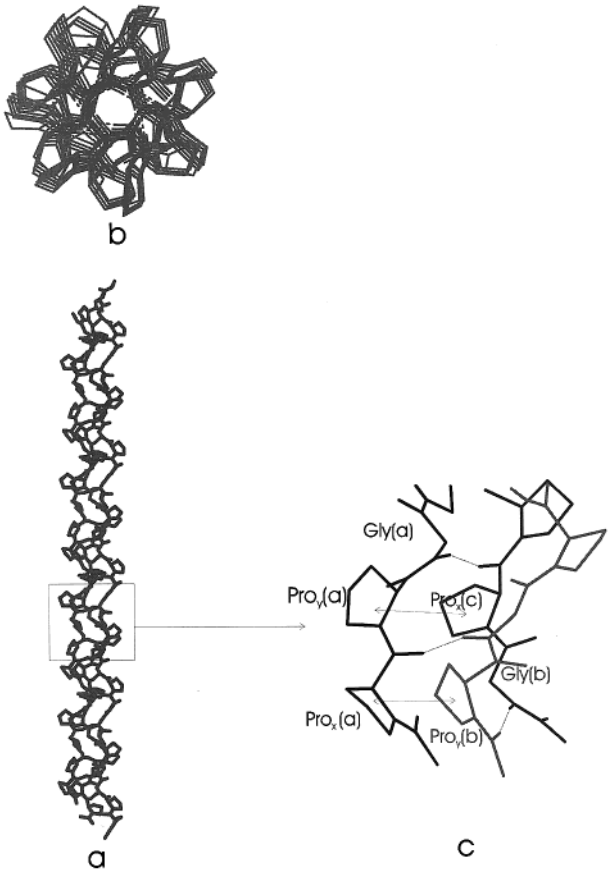

Figure 3. Minimum energy geometry (AMBER calculations) of 3DU: (a) side view, (b) top view. (c) Schematic drawing of the most important interactions stabilizing the triple helix.

From a general point of view, the structure predicted by AMBER calculations is in good agreement with the experimental determination on collagen-like polypeptides.[6−8]

3.2.2. PPG_{10} Triple Helix. In the next step of our analysis, we optimized at the AMBER level the triple helix formed by three PPG_{10} peptides for each of the four regular down−up alternations (hereafter 3DD, 3DU, 3UD, and 3UU, respectively). All the sequences of puckerings are compatible with the formation of the triple helix, and all the residues adopt a PII conformation (see Figure 3), except for the C-terminal glycine of 3UU, which prefers an extended conformation. This conformation allows the formation of an interchain hydrogen bond with the C-terminal carbonyl of an adjacent chain.

In agreement with experimental results, 3DU is the preferred conformer for a triple helix of the PPG_{10} trimer. It is more stable than 3DD and 3UU by ≈21 and ≈35 kcal/mol, respectively. Interestingly, the 3UD compound is predicted to be the least stable (≈43 kcal/mol less than 3DU). Table 4 collects the average values of the backbone and ring dihedral angles: it is gratifying that they are quite similar to the corresponding experimental values, especially for the 3DU species, thus supporting the reliability of a structural analysis based on AMBER calculations.

The backbone conformation changes to some extent upon formation of the triple helix, approaching the experimental one.

Table 5. Energy Contributions[a] (in kcal/mol) to the AMBER Energy for the Optimized Structures of the Single Chain and the Trimer of PPG_{10}

	DU	UD	UU
	Single Chain		
bond stretching	0.35	0.35	0.82
angle bending	0.06	0.91	0.71
dihedral torsion	7.28	5.33	13.86
1−4 electrostatic	2.98	3.45	5.66
1−4 van der Waals	−2.39	−2.87	−5.66
nonbonded electrostic	−2.85	−3.07	−3.13
nonbonded van der Waals	4.69	6.19	11.20
ΔE_{tot}	10.11	10.35	23.46
	Triple Helix		
bond stretching	0.13 (−0.92)	0.42 (−0.63)	0.88 (−1.58)
angle bending	7.21 (7.03)	8.38 (5.65)	15.44 (13.31)
dihedral torsion	−6.51 (−28.35)	31.11 (15.12)	42.82 (1.24)
1−4 electrostic	0.57 (−8.37)	7.64 (−2.71)	11.25 (−5.73)
1−4 van der Waals	−6.21 (0.96)	−4.30 (4.31)	−12.91 (4.07)
nonbonded electrostic	−10.58 (−2.03)	−35.32 (−26.11)	−51.28 (−41.89)
nonbonded van der Waals	−5.95 (−20.02)	14.12 (−4.45)	9.02 (−24.58)
ΔE_{tot}	−20.7 (−51.03)	22.1 (−8.23)	14.4 (−55.98)
ΔE in solution[d]	−15.8	16.4	13.1
	Triple Helix[b]		
bond stretching	−0.78	1.86	1.33
angle bending	4.21	26.20	22.02
dihedral torsion	20.56	17.93	36.71
1−4 electrostatic	2.74	0.72	0.99
1−4 van der Waals	−9.35	3.15	−15.40
nonbonded electrostatic	−16.41	−25.02	−22.02
nonbonded van der Waals	−10.88	11.81	−0.24
ΔE_{tot}[a]	−9.87 (−40.2)	37.31 (6.26)	23.36 (−47.2)
	Triple Helix[c]		
bond stretching	−0.70	2.89	0.74
angle bending	−1.08	5.31	5.12
dihedral torsion	−26.61	35.60	16.86
1−4 electrostatic	−2.27	Z	−1.02
1−4 van van der Waals	−1.27	3.20	1.11
nonbonded electrostatic	3.71	−13.11	−15.17
nonbonded van der Waals	−6.13	25.25	19.59
ΔE_{tot}[a]	−34.35 (−14.18)[e]	60.10 (1.59)[e]	27.23 (−13.10)[e]

[a] Energy terms defined according to ref 20. All the results are relative to those of 3DD. Interchain interactions (obtained by subtracting 3 times the single helix contributions) are given in parentheses. [b] Backbone dihedrals constrained to the values assumed in the corresponding optimized single chain. [c] Backbone dihedrals constrained to the values assumed in 3DU. [d] After adding solvation free energies calculated at the AMBER PCM level.

The most remarkable differences between the single helix and the triple helix concern the ψ dihedral of Pro_y and the ϕ dihedrals of Gly. Their absolute values decrease by ≈10°, likely to improve the hydrogen bond packing.

The comparison of the geometry of the four bundles reveals that all the species tend to assume a backbone geometry as similar as possible to that of 3DU, the most evident features being the following:

1. The prolines in the Y position of the 3DD bundle exhibit an average ϕ value of −63°, typical of an up puckering. It is interesting that also in the 3UU conformer the absolute value of the ϕ dihedral in Pro_x is larger than that in Pro_y.

2. The ψ dihedrals of Pro_x are consistently larger than those of Pro_y. This feature, even if less evident, is present also in 3UD, which has an opposite puckering alternation.

Although an exhaustive analysis of the main determinants of the triple helix assembly is outside the scope of the present study, our results seem to confirm the importance of proline/proline steric interactions in stabilizing the bundle (see Table 5).[31] As a matter of fact, van der Waals interactions are responsible for ≈60% of the interchain interaction energy, whereas electrostatic and H bond interactions account for the remaining ≈40%. For example, when the 3DU triple helix is formed, the attractive nonbonded van der Waals interactions increase by 311.2 kcal/mol and the nonbonded electrostatic interactions by 212.8 kcal/mol.

As expected, the most important interactions for the stability of the triple helix are the van der Waals interactions between proline rings belonging to adjacent chains and the hydrogen bond (electrostatic) interaction between the carbonyl groups of prolines in X positions and amino groups of glycines. Even if it is not easy to discriminate between purely electrostatic and hydrogen bond interactions, residue/residue and atom−atom decompositions of the AMBER results show that ≈20% of the electrostatic stabilization is due to the interaction between the oxygen of Pro_x and the amidic hydrogen of glycines (for 3DU it is ≈ −13 kcal/mol for each of the 30 hydrogen-bonded pairs, while the total nonbonded electrostatic energy is −1916.1 kcal/mol).

Van der Waals interactions always involve one proline in the X position and one in the Y position (see Figure 3c). So,

(31) Bhatnagar, R. S.; Pattabiraman, N.; Sorensen, K. R.; Langridge, R.; MacElroy, R. D.; Renugopalakrishnan, V. J. *Biomol. Struct. Dyn.* **1988**, *6*, 223.

Table 6. Solvent-Exposed Surface of the Different Groups (Hydrogen Atoms Included in the Heavy Atom to Which They Are Bonded) of 3DU, 3DD, 3UD, and 3UU Calculated According to the GePol Method

		N	C	O	Cα	Cβ	Cγ	Cδ
Pro$_x$	DD	0.45 ± 0.04	0.0 ± 0.0	0.3 ± 1.4	8.2 ± 1.0	33.8 ± 0.6	27.3 ± 4.0	9.4 ± 7.6
Pro$_x$	DU	0.4 ± 0.1	0.0 ± 0.	0.0 ± 0.	6.8 ± 0.8	34.9 ± 0.6	25.5 ± 4.3	9.0 ± 7.5
Pro$_x$	UD	0.4 ± 0.0	0.0 ± 0.0	0.4 ± 2.4	8.6 ± 0.6	19.8 ± 1.7	46.6 ± 2.3	3.4 ± 7.8
Pro$_x$	UU	0.3 ± 0.1	0.0 ± 0.0	0.4 ± 2.2	7.6 ± 0.6	20.7 ± 1.4	43.2 ± 2.9	3.3 ± 8.0
Pro$_y$	DD	0.0 ± 0.0	0.0 ± 0.0	10.3 ± 1.3	0.0 ± 0.0	11.6 ± 3.5	47.6 ± 3.3	25.6 ± 0.6
Pro$_y$	DU	0.0 ± 0.0	0.0 ± 0.0	9.9 ± 1.2	0.0 ± 0.0	16.0 ± 4.3	41.6 ± 1.5	27.4 ± 0.3
Pro$_y$	UD	0.0 ± 0.0	0.0 ± 0.0	11.1 ± 1.3	0.0 ± 0.0	12.1 ± 4.3	47.3 ± 3.0	25.3 ± 1.0
Pro$_y$	UU	0.0 ± 0.0	0.0 ± 0.0	11.0 ± 1.8	0.0 ± 0.0	16.6 ± 5.5	39.6 ± 2.8	27.3 ± 0.7
Gly	DD	0.1 ± 1.1	0.8 ± 0.5	6.3 ± 4.2	0.1 ± 0.6			
Gly	DU	0.3 ± 1.8	0.9 ± 0.7	6.6 ± 4.4	0.0 ± 0.0			
Gly	UD	0.0 ± 0.0	1.0 ± 0.6	6.0 ± 4.1	0.2 ± 0.8			
Gly	UU	0.1 ± 0.3	0.9 ± 0.3	6.1 ± 5.6	0.4 ± 2.8			

depending on the system, they can involve either prolines with parallel puckerings (as in 3DD or in 3UU) or prolines with antiparallel puckerings (as in 3UD and 3DU). Although this could affect the stability of triple helix bundles, an analysis of the AMBER results shows that this is not the case, since the largest difference in interchain Pro$_x$-Pro$_y$ van der Waals interactions occurs between 3DD and 3UU, which are both characterized by parallel puckerings.

3.2.3. AMBER Calculations in Aqueous Solution. To ascertain whether solvent effects can play some role in determining the relative stability of the different down−up conformers, we performed single-point AMBER/PCM calculations on the gas-phase minima of 3DD, 3UD, 3DU, and 3UU (see Table 5 and Figure 4). The differential solvation energy is quite similar for the four compounds examined (within ≈10 kcal/mol), and thus it does not change the stability trend predicted in vacuo.

The 3DU conformer is the most compact, and thus it is the most favored by nonelectrostatic contributions. Electrostatic contributions favor, instead, 3DD and 3UD conformers (≈15 and ≈13 kcal/mol more than 3DU, respectively). However, a decomposition of the total solvation energy shows that this effect is mainly due to the contribution of the acetyl N-terminal capping groups, that is, −14.5 kcal/mol in 3DD, −8.6 kcal/mol in 3DU, −13.2 kcal/mol in 3UD, and −13.4 kcal/mol in 3UU. As mentioned above, the N-terminal part of 3DU is the most closely packed, while in the remaining three conformers it is slightly unwound. As a matter of fact, test calculations performed on the analogues of the four trimers where a hydrogen atom substitutes the acetyl as the capping group predict that the electrostatic contributions of all four conformers are in a range of just ≈5 kcal/mol.

Table 6 collects the solvent-accessible surfaces calculated by our modified GePol procedure[22] for all the isomers of PPG$_{10}$, obtained by using explicit hydrogen atoms and standard van der Waals radii for the peptide and an effective radius of 1.4 Å for the water molecule. The regularity of the triple helix is confirmed by our results, since the solvent-exposed surface of each group exhibits small deviations from the corresponding average value. The only exceptions are the oxygen atoms of glycine residues and the Cδ group of Pro$_x$. In both cases, however, the large standard deviation is due exclusively to the terminal residues that are much more exposed to the solvent. If the contribution of those residues is discarded, the standard deviation strongly decreases.

CPCM calculations show that solvent accesibility should play a minor role in determining the relative stability of the four

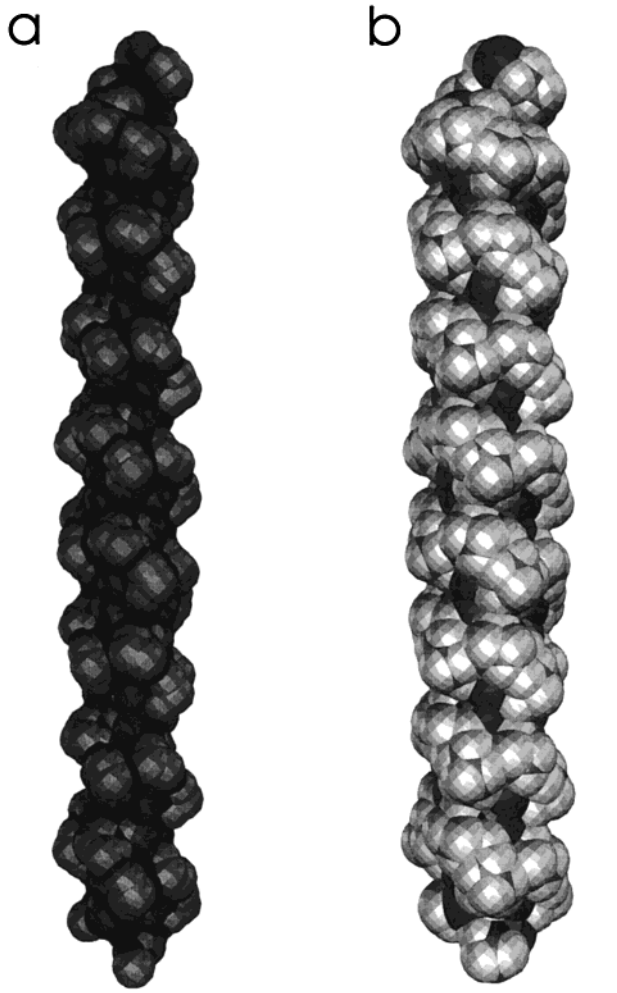

Figure 4. Solvent-contact (a) and solvent-accessible (b) surface of 3DU, calculated by the modified GePol procedure (see text for details).

isomers. The hydrophilic exposed surface is indeed very similar in the compounds examined, as is the total hydrophobic exposed surface. The four isomers differ only in the relative contributions of the carbon atom of the pyrrolidine ring to the total exposed surface. The most relevant feature concerns CγH_2 and CβH_2 groups in Pro$_x$: the former is more exposed to the solvent in up puckerings, the latter in down puckerings. However, this effect could be potentially relevant only in the presence of hydrophilic ring substituents. An analysis of the contribution of different groups to the total electrostatic solvation energy confirms the considerations based on the solvent-exposed

Table 7. Average H Bond Geometry (Bond Distances in Angstroms, Bond Angles in Degrees) for 3DD, 3DU, 3UD, and 3UU

	3DD	3UD	3DU	3UU
		Free Optimization		
no. of H bonds	28	29	29	29
O−H distance	1.969 ± 0.044	1.992 ± 0.059	1.893 ± 0.031	1.907 ± 0.039
H−N−O angle	16.71 ± 1.75	17.73 ± 3.24	14.40 ± 1.31	15.11 ± 2.32
		Single Chain-like[a]		
no. of H bonds	21	28	28	29
O−H distance	1.936 ± 0.166	2.061 ± 0.069	2.015 ± 0.093	2.110 ± 0.098
H−N−O angle	14.24 ± 9.35	24.00 ± 3.88	22.34 ± 3.47	27.29 ± 4.16
		3DU-like[b]		
no. of H bonds	29	29	29	29
O−H distance	1.998 ± 0.079	1.992 ± 0.059	1.946 ± 0.056	1.940 ± 0.045
H−N−O angle	16.98 ± 2.23	17.73 ± 3.22	16.02 ± 2.21	16.64 ± 2.86

[a] Backbone dihedrals constrained to the values assumed in the corresponding optimized single chain. [b] Backbone dihedrals constrained to the values assumed in 3DU.

surface. For example, the contribution of the spheres associated with carbonyl oxygens is similar in the four compounds examined.

Finally, it is noteworthy that the description of the exposed surface we obtained for 3DU is in good agreement with the surface analysis performed starting from the experimental geometry in the solid state.[8a]

4. Puckering Alternation and Triple Helix Stability

In the preceding paragraphs we have shown that AMBER calculations predict that the triple helix formed by the $(Pro_{down}$-Pro_{up}-$Gly)_{10}$ peptide is the most stable one. This result could be, in principle, due to two different effects: (i) the backbone dihedrals of the DU peptides are the closest to those allowing the best packing of the triple helix, and (ii) the stability order is determined by the different ring−ring interactions of the four isomers. For example, the steric repulsions between two prolines with opposite puckering, as is the case in 3DU (see above) and in 3UD, could be smaller than those between two prolines with equal puckering.

The results of the AMBER geometry optimizations support the first hypothesis, since the stabilization due to the formation of the triple helix of the UU isomer is slightly larger than that of DU, and, thus, 3UU is less stable than 3DU just because the PII single helix is remarkably less stable for UU than for DU. The next task to tackle is then to understand why the PPG_{10} triple helix tends to adopt backbone dihedrals typical of a down−up alternation. To gain some insights on this question, we performed some AMBER geometry optimizations constraining all the backbone dihedrals of 3DD, 3DU, 3UD, and 3UU to their equilibrium values in the single helix (see Table 4). Interestingly, the 3DD bundle is now prevented from forming a regular triple helix, since one of the three chains is quite distant from the remaining two. This feature is evident when looking at Table 7, where the most relevant parameters of the H bond network are collected. The number of hydrogen bonds is remarkably smaller than in a normal triple helix, since all the hydrogen bonds involving the "distant" chain are actually lacking. The backbone dihedrals of the DU chain seem to be the most favorable to the maximization of the interchain interactions, especially the electrostatic ones. As a matter of fact, when constrained to the single helix geometry, 3DU recovers more than 92% (−196.1 kcal/mol of the total −212.8 kcal/mol) of the electrostatic interchain stabilization obtained by a full geometry optimization. On the other hand, the percentages for the remaining three isomers are smaller, going from 80% (−200.9 kcal/mol of the total −252.7 kcal/mol for 3UU) to 89% (−188.3 kcal/mol of the total −210.8 kcal/mol for 3DD). Van der Waals interactions seem instead less dependent on the backbone dihedrals, since all the isomers recover more than 90% of the final van der Waals energetic stabilization when frozen at the insulated helix geometry. Not surprisingly, the 3DD chain, which does not exhibit a perfect triple helix, is the system which is less stabilized by van der Waals interactions (−268.5 kcal/mol of the total −291.2 kcal/mol, i.e., ≈92%).

The above considerations are confirmed by AMBER calculations where all the systems have been forced to adopt the backbone dihedrals of the 3DU triple helix. As a matter of fact, all the systems exhibit a hydrogen bond geometry similar to that reached after full geometry optimizations. Analogously, the energetic stabilization coming from the electrostatic interactions is very similar to that of the optimized bundle (for 3DD it is even larger).

Interestingly, for 3DU the stabilization coming from interchain interactions is similar to that experienced by the three remaining systems (within ≈10 kcal/mol, see last row of Table 5), especially if one thinks that the backbone geometry used in the computations should be ideal for maximizing the packing of 3DU. This result rules out the possibility that the down−up alternation is intrinsically favored by the interchain interactions.

5. Discussion and Conclusions

In this paper we have presented a QM/MM study of $(Pro\text{-}Pro\text{-}Gly)_n$ polypeptides, aimed to shed light on the influence of the proline puckering on the stability of the collagen triple helix.

Accurate QM calculations of the Gly-Pro-Pro-Gly peptide analogue show that, both in vacuo and in aqueous solution, the inclusion of interresidue interactions does not lead to any significant stabilization of the X-down/Y-up conformation. In agreement with the slight intrinsic preference of Pro for a down puckering, the **dd** isomer is always more stable than the **du** and **ud** ones, which are nearly isoenergetic.

The experimental finding that X-down/Y-up alternation is present in collagen and in collagen-like peptides cannot thus be due to an intrinsic preference of the Pro-Pro-Gly sequence. However, the study of the models including this sequences is relevant for understanding the interplay between backbone and ring parameters. It is confirmed that ring puckering and backbone dihedrals are strongly correlated. Not only, as it could

be expected, do down prolines adopt ϕ dihedrals different from those of up prolines ($-72°$ and $-62°$, respectively), but also the ψ dihedrals have a different behavior for the two puckerings. In agreement with the results obtained at the dipeptide analogue level, ψ dihedrals have a larger conformational freedom in the down puckerings, and they can adopt more easily large values (up to $\approx$165°). It is important to highlight that this result is confirmed also by a statistical survey of X-ray protein structures, showing that the ψ dihedrals of most up prolines fall in the range [120°,150°], whereas the ψ dihedrals of down prolines span quite uniformly the interval [120°,180°].[28,29]

The above considerations are confirmed by the reversed stability trend obtained when the chain is forced to assume the backbone dihedrals of PPG_{10}: the **du** conformer becomes the most stable, and the **uu** conformer is relatively stabilized over the **dd** one. The **ud** peptide, which exhibits a puckering alternation opposed to that experimentally found, is the less stable conformer. But why do collagen-like peptides adopt an X-down/Y-up conformation?

Since both QM and MM calculations rule out the possibility that this could be due to intrachain effects, we studied the four possible triple helices of PPG_{10} by means of AMBER calculations. In vacuo geometry optimizations show that the triple helix formed by down−up chains is the most stable. This result is essentially due to the fact that the backbone dihedrals typical of the down−up alternation are those allowing the best packing of the triple helix and the maximization of the interchain hydrogen bond strength. The triple helix formed by a regular alternation of down and up puckerings suffers the smallest internal strain, as confirmed by the fact that it is the less unwound at the termini.

On the other hand, the other three regular peptides tend to adopt a backbone conformation as close as possible to that of the 3DU chain, but this causes severe intrachain strain, even if the interchain interactions are comparable (and, for 3UU stronger) with those of 3DU. The reliability of this analysis is supported by test calculations performed on GPPG showing that MM and QM computations are in good qualitative agreement. Furthermore, the optimized geometry of 3DU is close to that obtained by X-ray diffraction studies of PPG_{10}.[6−8]

Finally, CPCM calculations show that solvent effects should play a minor role in determining the preference between the different kinds of puckering. However, it is noteworthy that 3DU is the more compact bundle, confirming the conclusion issuing from the calculations performed in the gas phase.

In conclusion, collagen is certainly a much more complex system than a Pro-Pro-Gly model peptide, and a number of effects can influence its stability.[32,33] Furthermore, we have not considered the role possibly played by dynamic effects. However, our study gives a convincing explanation of the role of hydroxyproline. The stability of the triple helix increases if the iminoacid in the X position adopts a down puckering and the one in the Y position an up puckering. Down puckerings are slightly more stable for proline, and this is the residue present in vivo in the X position. As we have shown in the first paper of this series,[11] an increase in the electronegativity of the 4(*R*) substituent stabilizes the up puckering. In vivo, prolines in the Y position are indeed hydroxylated.

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