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On the Crystal Structure of Poly(α-benzyl γ,DL-glutamate) of Microbial Origin

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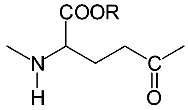
ABSTRACT: The conformation and crystal structure of the benzyl ester of microbial poly(γ,DL-glutamic acid) with a nearly racemic composition was investigated by X-ray diffraction, polarized infrared spectroscopy and molecular modeling simulations. Three different molecular arrangements were characterized. Form I was found to be a structure made of hydrogen-bonded sheets of chains in a nearly extended conformation with features similar to the well-known γ-form of nylons. Form III consisted of a hexagonal packing of 37/10 helices stabilized by intramolecular hydrogen bonds set between i and $i + 3$ amide groups. Form II was observed as a poorly oriented phase and identified with the 5/2 helical form previously described for optically pure poly(α-benzyl γ,L-glutamate). Energy calculations indicated that the helical conformations adopted by PAB(DL)G are incompatible with a polymer having a statistical distribution of D and L units and that a polymer made of D- and L-stereoblocks is the most appropriate to describe the crystal structure present in form I.

Introduction

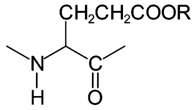
Poly(γ-glutamic acid) (PGGA) is a naturally occurring poly(γ-peptide) that is produced by several species of bacteria of the genus *Bacillus*. The biological occurrence of PGGA was accounted at the beginning of the past century and since then, the number of publications dealing with the biosynthesis, solution properties and chemical modifications of this fascinating biopolymer has been continuously increasing.[1] The biodegradable character and well-proven bioassimilation of PGGA and its derivatives justify the present interest for this substance as a potential material with applications in food packing and biomedicine.

PGGA is made of glutamic acid units linked between the γ-carboxylic and the α-amino functional groups. The biosynthesis of PGGA in the laboratory usually affords PGGA in satisfactory yields with high molecular weight and containing variable amounts of D- and L-enantiomeric units.[2] The stereochemical microstructure of biosynthetic PGGA with enantiomerically heterogeneous composition has remained uncertain for long time. Recently, strong evidences favoring the occurrence of a microstructure made of stereoblocks have been provided.[3] On the other hand, the ability of un-ionized PGGA to adopt regularly folded conformations in water solution was early revealed by spectroscopy measurements[4] and later supported by computational methods.[5]

Esterification appears to be the preferred procedure to modify the properties of microbial PGGA and to render non-water-soluble products. Thus, the preparation, characterization, and properties of a good number of poly(γ, DL-glutamate)s have been reported in the last 10 years.[6] Surprisingly, the structure adopted in the solid state by the PGGA esters, or even by PGGA itself, has been scarcely investigated. In fact, a communication describing some X-ray diffraction features of several poly(γ-glutamate)s[7] and a preliminary account of the crystal polymorphism observed for poly(α-ethyl γ,DL-glutamate)[3b] amount the only information available today on the crystal structure of biosynthetic poly(γ-glutamate)s. Some years ago we reported on optically pure poly(α-benzyl γ,L-glutamate) and poly(α-methyl γ,L-glutamate) obtained by chemical synthesis, and it was then discovered that these compounds can adopt helical conformations comparable to those characteristic of poly(α,L-glutamate)s.[8] This finding closely followed those obtained with poly(β,L-aspartate)s, a family of nylon 3 derivatives which distinguishes by its ability to adopt regular folded conformations of α-helix type.[9] Recent investigations carried out on oligo(β-amino acid)s and oligo(γ-amino acid)s[10] have shown that both types of peptides are able to adopt α-helix-like conformations confirming previous results reported for high molecular weight polymers.



poly(γ-glutamate)s



poly(α-glutamate)s

In the present paper, we wish to report on the conformation and crystal structure of racemic poly(α-benzyl γ,DL-glutamate) of microbial origin, hereafter abbreviated to PAB(DL)G. Previous work carried out on optically pure poly(α-benzyl γ,L-glutamate) prepared by chemical synthesis, hereafter abbreviated to PAB(L)G, will be used as the reference. It is known that PAB(L)G adopts two crystal forms, one made of hydrogen-bonded sheets stacked in a layered structure with chains in almost extended conformation (form I) and a second form proposed to consist of a hexagonal packing of intramolecularly hydrogen-bonded 5/2 helices (form II). It should be stressed that the polymer investigated in that case entirely consisted of L-enantiomeric units and had a molecular weight of about 30 000. In the present case, we are concerned with a polymer of much higher molecular weight and composed of approximately equal amounts of D and L units.

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Experimental Section

Materials and Synthesis. The sample of PAB(DL)G used in this work was a generous gift of Dr. Kubota from Meiji Co. The polymer was obtained by esterification of bacterially produced poly(γ-glutamic acid) and has an ester content higher than 98% as proved by ^{1}H NMR. The intrinsic viscosity of this sample measured in dichloroacetic acid was 2.43 dL g^{-1}, which corresponds to a molecular weight about 480 000 if the viscosity equation reported by Doty et al. for poly(γ-benzyl α,L-glutamate) is applied.[11] The enantiomeric composition of the polymer is D:L = 56:44 as determined by Marfey's reagent method reported elsewhere.[12]

Measurements. Densities were measured by the flotation method using water-KBr aqueous solution mixtures. Polymer films were obtained by casting at room temperature from the appropriate solvents and oriented by mechanical stretching under heating at the appropriate temperature. Dichroic infrared spectra (DIR) were recorded on a Perkin-Elmer 2000 instrument provided with an external gold polarizer. X-ray diffraction patterns were recorded on flat films in a modified Statton camera (W. Warhus, Wilmington, DE) using nickel-filtered copper radiation of wavelength 0.1542 nm and calibrated with molybdenum sulfide (d_{002} = 0.6147 nm).

Energy Calculations and Modeling Simulations. A preliminary search for the helical conformations compatible with experimental X-ray diffraction data was performed using the GEMOX (generation of polymer molecular models for X-rays) computer program.[13] This computational method is based on a corrected grid searching algorithm designed to generate all the molecular arrangements topologically feasible for a system with a given helical symmetry and a definite chain repeat length. Energy calculations were performed with the Amber 4.1[14] and PCSP[15] (prediction of crystal structure of polymers) computer programs. Force-field parameters were taken from the Amber set[16] with exception of the atomic charges, which were explicitly derived for the PBG(DL)G chain by fitting the quantum mechanics molecular electrostatic potential computed at the ab initio HF/6-31G(d) level to the Coulombic one.[17] Crystal modeling was made for the most favorable packing arrangements using the Cerius^2 program.[18] X-ray diffraction patterns were simulated for these models using sample textures similar to those used for the collection of experimental data. All the calculations were performed on SGI Indigo^2 workstations.

Results and Discussion

Three types of X-ray diffraction patterns were recorded for PAB(DL)G depending on the conditions used for the preparation of the sample. Two of them can be readily made to correspond to the two crystal forms (I and II) previously reported for optically pure PAB(L)G obtained by chemical synthesis.[9] The third one appeared to differ from the other two so much as to have to be attributed to a third new structure called henceforth form III.

Form I. This form was obtained by precipitation of the polymer from a solution in trifluoroacetic acid upon addition of methanol. The powder X-ray diffraction pattern produced by form I consists of a half a dozen of medium intensity rings including the characteristic inner one with a spacing of 1.05 nm. According to previous work on PAB(L)G,[8] this form may be interpreted as a layered structure of hydrogen-bonded sheets with chains arranged in almost extended conformation. Both polarized infrared spectroscopy and X-ray diffraction taken from oriented samples gave definite support to such interpretation. Oriented PAB(DL)G in form I was prepared by stretching at room temperature the isotropic film obtained by casting from $CHCl_3$−trifluoroethanol. The occurrence of an intermolecular network of hydrogen bonds running approximately normal to the polymer main chain, which is a distinctive feature of this structure, was evidenced by dichroism infrared. The DIR spectra showed perpendicular dichroism for both the amide A and amide I bands and parallel dichroism for the amide II band (Figure 1a). Such a dichroic

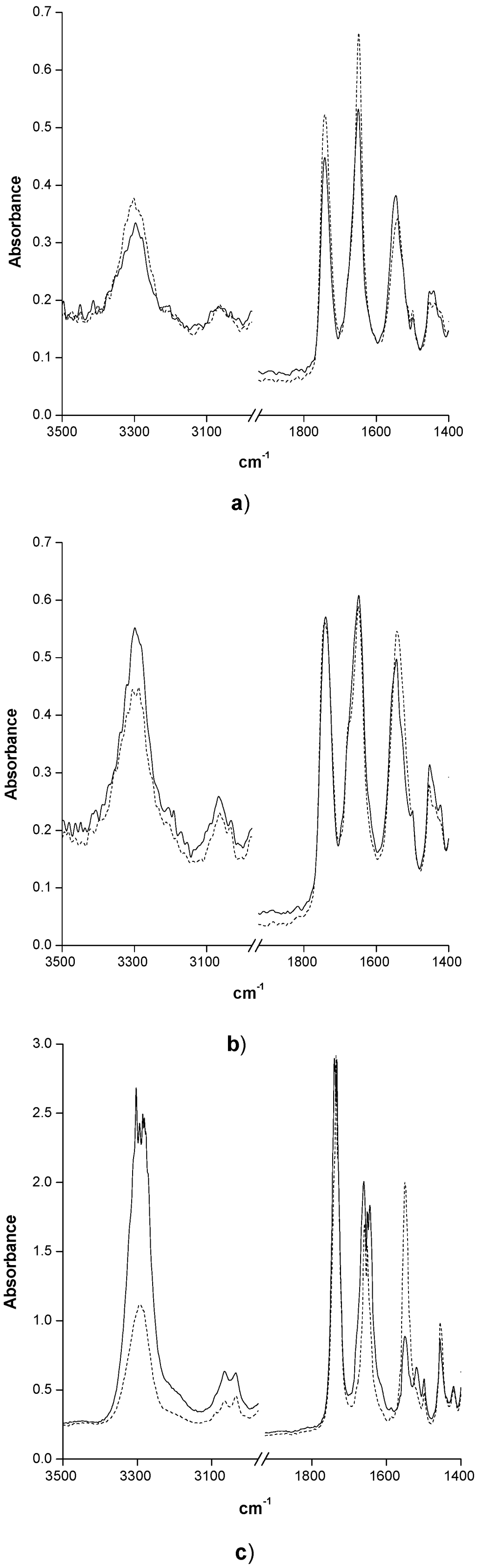


Figure 1. Polarized infrared spectra of PAB(DL)G in form I (a), in form II (b), and in form III (c). The solid and dotted lines correspond to the parallel and perpendicular orientation of the film axis to the polarized infrared vector, respectively.

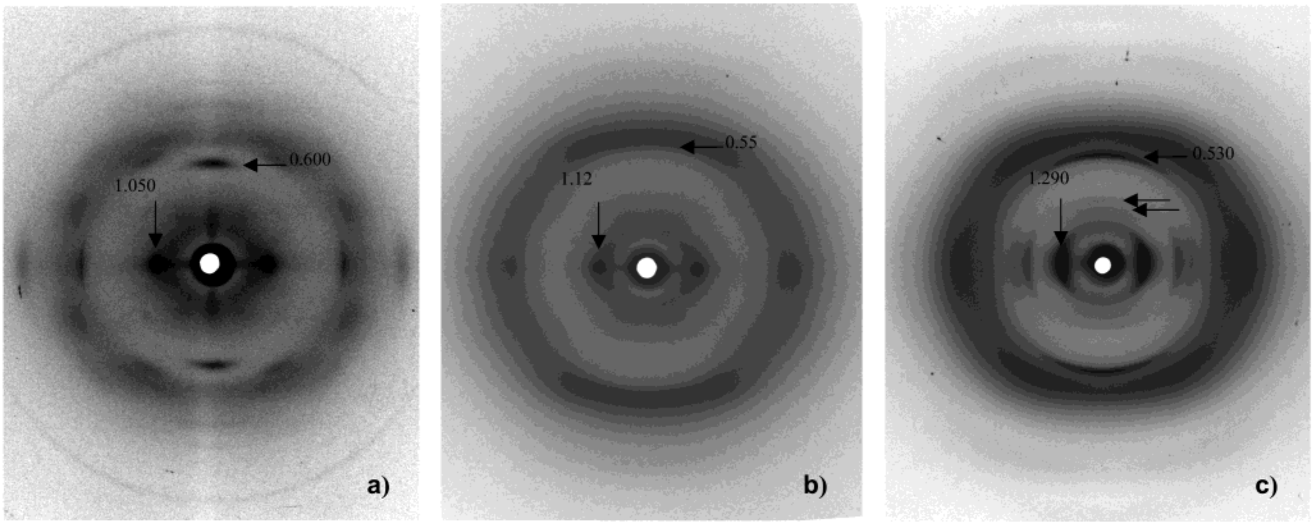


Figure 2. X-ray diffraction patterns of PAB(DL)G: (a) form I; (b) form II; (c) form III. Spacings in nanometers. The pair of arrows in part c indicate the fifth and sixth layer-lines of the 37/10 helical structure.

behavior is exactly what should be expected for a structure with the $C=O$ and $N-H$ bonds oriented approximately normal to the chain axis, the which runs parallel to the stretching direction.

The fiber pattern of PAB(DL)G in form I is shown in Figure 2a. The patterns displays on the equator two strong reflections with spacings at 1.05 and 0.47 nm as well as a prominent meridional reflection corresponding to an axial spacing of 0.60 nm. The 0.47 nm spacing can be made to correspond to the distance between adjacent polyamide chains within the H-bonded sheets provided that these are stacking in an orthogonal array with an intersheet distance of 1.05 nm. The spacing and intensity of reflections appearing along the first and second layer lines indicate that the *c* dimension of the structure, i.e., the axial repeating length, is 1.20 nm and that it must include two chemical residues. This implies that PAB(DL)G crystallizes in form I with the chain contracted about 0.03 nm per residue, which is similar to the contraction experimented by nylons in the γ-form with respect to the fully extended conformation that they adopt in the α-form.[29]

As seen in Table 1, the whole diffraction pattern can be satisfactorily indexed on the basis of an orthorhombic lattice with parameters $a_0 = 0.930$ nm, $b_0 = 1.045$ nm, and $c_0 = 1.200$ nm (chain axis) containing two chains in the unit cell. The density calculated for this structure is 1.26 g mL^{-1}, in excellent agreement with the experimental value of 1.25 g mL^{-1}.

Form II. This form was obtained by casting PAB(DL)G from a variety of solvents including dimethyl sulfoxide, hexafluoro-2-propanol, and $CHCl_3$−trifluoroethanol mixtures. The powder X-ray diffraction pattern produced by this form distinguishes by the presence of an inner strong ring at 1.12 nm and a very strong and broad ring covering the spacing range between 0.40 and 0.50 nm. Films in form II could be oriented while retaining the original structure by stretching at temperature below 90 °C. The polarized infrared spectra recorded from these oriented PAB(DL)G films displayed the bands arising from the amide group with dichroic ratios opposite to those observed for form I (Figure 1b), i.e., parallel dichroism for amide A and amide I bands and perpendicular dichroism for the amide II band. Such a dichroic behavior is characteristic of polypeptidic helices held by intramolecular hydrogen bonds where the $C=O$ and $N-H$ dipoles are aligned nearly parallel to the helical axis.

The diffraction pattern recorded from a PAB(DL)G film stretched at low temperature is shown in Figure 2b. The scattering is mainly concentrated on the equator and on the second layer-line as two rather diffuse strong reflections with associated spacings of 1.12 and 0.5−0.45 nm, respectively while the first layer-line at 1.0 nm is extremely weak and only discernible in the original photograph. Although with such paucity of X-ray data no structure can be determined with certainty, the characteristics of the pattern allow us to identify this form with the form II previously described for synthetic PAB(L)G.[8] The structure of this form should be accordingly described as a pseudohexagonal packing of 5/2 helices with an interchain distance of 1.30 nm and an axial periodicity of 1.0 nm. The density calculated for this structure is 1.25 g mL^{-1}, which compares satisfactorily with the value of 1.26 g mL^{-1} measured for a film of PAB(DL)G in form II.

Form III. The film obtained by evaporation of a solution of PAB(DL)G in $CHCl_3$ followed by annealing at 120 °C produced a well-defined diffraction pattern differing from the other two in both spacings and intensities. The distinctive feature of this pattern is the high spacing (1.29 nm) and intensity displayed by the innermost reflection. The density of the film was determined by flotation to be 1.29 g mL^{-1} and its polarized infrared spectrum showed the same dichroic properties as the film in form II (Figure 1c). When the film was stretched previous to be subjected to the annealing treatment, it produced the fiber diffraction pattern shown in Figure 2c. This pattern contains about 20 independent reflections and displays particularly strong scattering along the equator and along the layer line spacing ~5 nm. What is really significant in this pattern is the presence of several high-spacing layer-lines consistent with an axial periodicity of 5.45 nm. Moreover, the pattern recorded from the film tilted to the azimuth by 30° (not shown) clearly displayed a strong meridional reflection with a spacing of 0.150 nm revealing that it must be the height of the chemical repeating unit in the structure. On the basis of

Table 1. Observed and Calculated X-ray Diffraction Spacings (nm) of Crystalline Forms I, II, and III of PAB(DL)G

	form I			form II			form III		
layer line	d_{obsd}[a]	d_{calcd}[b]	$h\ k\ l$[b]	d_{obsd}[a]	d_{calcd}[c]	$h\ k\ l$[c]	d_{obsd}[a]	d_{calcd}[d]	$h\ k\ l$[d]
$l = 0$	1.050	1.045	0 1 0	1.12	1.12	1 10	1.290	1.282	1 0 0
						0 2 0			1 1 0
							0.730	0.740	2 0 0
									1 3 0
							0.640	0.641	0 4 0
									2 2 0
	0.470	0.465	2 0 0	0.46	0.45	1 5 0	0.485	0.485	2 4 0
									3 1 0
				0.41	0.40	3 2 0			
	0.340	0.348	2 2 0				0.390	0.390	3 4 0
		0.347	0 3 0						
	0.260	0.261	0 4 0				0.315	0.320	4 4 0
									0 8 0
$l = 1$	0.720	0.735	1 0 1	0.55	0.55	2 0 1	0.730	0.733	2 0 1
						1 3 1			1 3 1
	0.435	0.434	2 0 1				0.640	0.637	2 1 1
									2 2 1
							0.485	0.483	2 4 1
									3 1 1
	0.405	0.400	2 1 1				0.390	0.390	3 4 1
							0.315	0.320	4 4 1
									0 8 1
$l = 2$	0.600	0.600	0 0 2						
	0.500	0.504	1 0 2	0.50−0.40	0.50−0.40	$h\ k$ 2			
		0.519	0 1 2						
	0.450	0.454	1 1 2						
$l = 3$	0.390	0.374 0.400	0 1 3	0.32	0.32	0 2 3			
			0 0 3			1 1 3			
$l = 4$	0.280	0.285 0.288	1 0 4	0.25	0.25	$h\ k$ 4			
			0 1 4						
$l = 5$	0.234	0.233	0 1 5				1.000	0.997	0 1 5
$l = 6$							0.860	0.850	0 1 6
$l = 8$							0.660	0.654	0 1 8
$l = 10$							0.530	0.529	0 1 10
							0.465	0.457	0 3 10
							0.425	0.413	2 2 10
$l = 15$							0.325	0.332	0 3 15
$l = 20$							0.255	0.269	0 1 20
$I = 37$							0.150	0.150	0 0 37

[a] Spacings observed in X-ray diffraction patterns of oriented films. [b] Spacings calculated and indexed on the basis of an orthorhombic unit cell with parameters $a_0 = 0.930$ nm, $b_0 = 1.045$ nm, and $c_0 = 1.200$ nm. [c] Spacings calculated and indexed on the basis of an orthorhombic unit cell (pseudohexagonal) with parameters $a_0 = 1.30$ nm, $b_0 = 2.25$ nm, and $c_0 = 1.00$ nm. [d] Spacings calculated and indexed on the basis of an orthorhombic unit cell (pseudohexagonal) with parameters $a_0 = 1.478$ nm, $b_0 = 2.569$ nm, and $c_0 = 5.449$ nm (helix 37/10).

X-ray diffraction and density data, a hexagonal packing of 37/10 helices separated by 1.478 nm can be initially proposed for the crystal structure of form III. However, the presence of certain off-meridional reflections can be only explained by considering an array of chains arranged in antiparallel. The consequent result is an orthorhombic lattice with parameters $a_0 = 1.478$ nm, $b_0 = 2.564$ nm, and $c_0 = 5.449$ nm (chain axis) that contains two chains in the unit cell. The density calculated for this structure is 1.31 g mL^{-1} in good agreement with the density determined experimentally. A complete list of the observed and calculated reflections with the corresponding indexes for the finally proposed structure is given in Table 1.

Molecular Conformations. A preliminary search for the helical conformations compatible with experimental X-ray diffraction data was performed using the GEMOX program. X-ray diffraction data revealed that the chemical repeating unit of PAB(DL)G in form I is contracted about 0.03 nm per amide group with respect to the all-trans conformation. Such compression may be easily reached if some backbone torsional angles are allowed to deviate from 180°. By analogy with the γ-form of nylons,[19] the torsional angles most likely changed would be those adjacent to the amide groups, i.e., φ and ψ. Thus, a chain repeating length of 1.198 nm was attained for $\varphi = -\psi = 139.6°$, which entails a rotation of 35.5° between the amide plane and the plane containing the backbone carbon atoms in *zigzag* arrangement. Energy calculations carried out on an isolated chain of PAB(D)G revealed that this contracted conformation is only 0.3 kcal mol^{-1} residue^{-1} less stable than the fully extended one. Similar results would be attained using the PAB(L)G enantiomorph. The side and axial projections of the 2/1 helix made of D units are schematically depicted in Figure 3a.

Then the 5/2 and 37/10 helices occurring in forms II and III, respectively, were constructed and analyzed by energy calculations. The most stable arrangement for a PAB(D)G chain in 5/2 helical conformation was found

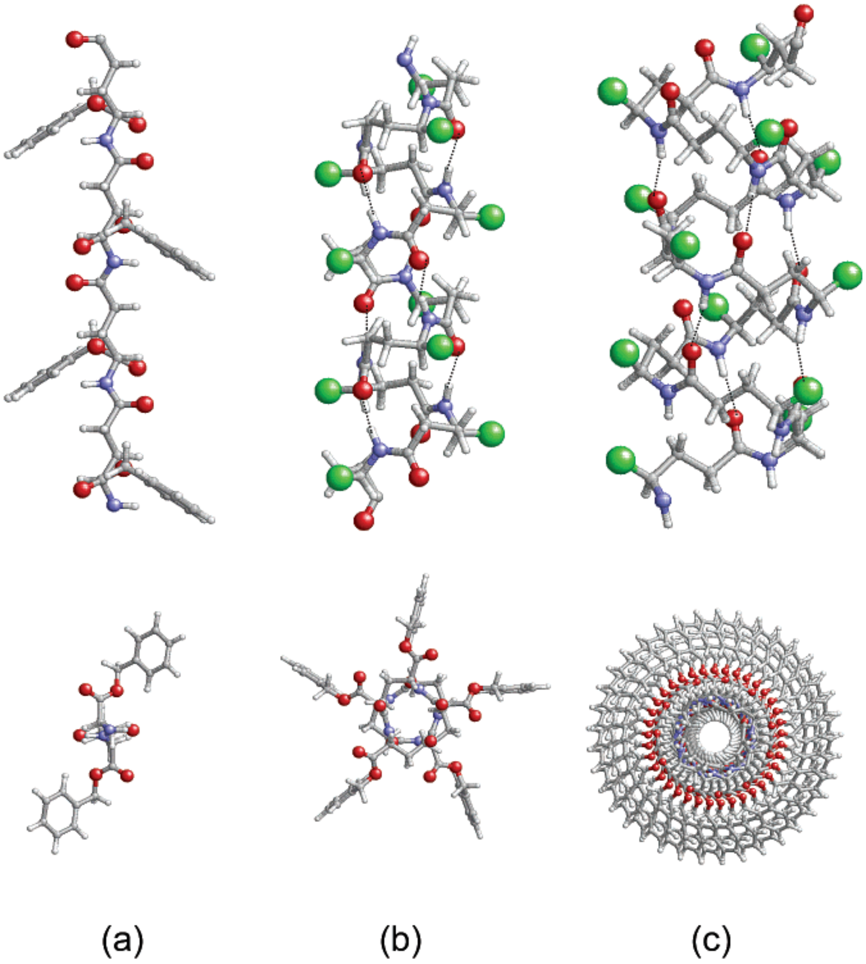


Figure 3. Schematic representation of PAB(D)G helices: (a) 2/1 helix present in form I; (b) right-handed 5/2 helix present in form II; (c) left-handed 37/10 helix present in form III. In the axial projections of parts b and c, the benzyloxy side groups have been replaced by green spheres for clarity.

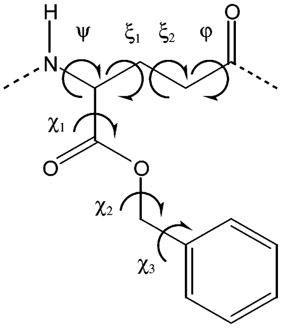
to correspond to a right-handed helix with intramolecular hydrogen bonds set between the C═O of the amide i and the NH of the amide $i + 2$. On the contrary, a left-handed conformation with an $i - i + 3$ scheme of hydrogen bonds appeared to be the most favored arrangement for the 37/10 helix. The equatorial and axial projections of both, the 5/2 and the 37/10 PAB(D)G helices, are schematically represented in Figure 3, parts b and c, and their respective conformational angles and hydrogen-bond parameters are given in Table 2. Similar values but with opposite sign for the angles would be obtained for these helices when constructed with a PAB(L)G chain.

Given the configurational heterogeneity of PAB(DL)G, the stability of the 5/2 and 37/10 helices was examined with regards to the enantiomeric composition and distribution along the polymer chain. This study was carried out using a chain segment containing 10 explicit residues and applying periodic boundary conditions along the helix axis. Energy results proved that both types of helices are incompatible with the occurrence of a racemic chain consisting of a statistical distribution of the enantiomeric units. These results are in agreement with recent observations reporting that poly(γ-glutamate)s derived from microbial PGGA are stereocopolymers composed of optically homogeneous poly-D and poly-L stereoblocks.

Crystal Modeling and Refinement. This study was limited to the analysis of form I due to technical reasons. The scarcity of X-diffraction data obtained for form II prevented to refine the structure with a minimum of confidence. On the other hand, the large number of atoms implied in the unit cell of form III largely exceeds our computer capacity to simulate the crystal lattice and to carry out the energy calculation of the structures.

The PAB(D)G 2/1 helix shown in Figure 3a was taken for building up the crystal structure of form I. The dimensions of the lattice were initially defined according to X-ray diffraction data. The space group $P2_122_1$ was specified with the cell containing two chains in antiparallel arrangement placed on the a-axis at $^1/_4a$ and $^3/_4a$ and displaced one to respect to the other by $^1/_2c$. This initial structure was refined with CERIUS by comparing the simulated diffraction pattern with the experimental one, which is that shown in Figure 2a. The resulting crystal structure is schematically represented in Figure 4a. Although this model was able to reproduce all the reflections observed in the experimental pattern, a significant number of additional noncorresponding spots were present. A much better concordance could be attained using a model made of equal amounts of PAB(D)G and PAB(L)G chains with an occupancy of 50% of each enantiomorph at every position of the lattice. Figure 4b shows the fiber pattern simulated for this structure, which satisfactorily compares with the pattern shown in Figure 2a.

Table 2. Conformational and H-Bond Parameters of Crystal Forms I, II, and III of PAB(DL)G



	helix		
	2/1	5/2	37/10
dihedral angles[a]			
φ	−139.6	−137.9	70.9
ξ_1	180.0	53.0	53.6
ξ_2	180.0	75.6	−172.8
ψ	−140.4	−143.8	164.0
rise per residue[b]	0.598	0.202	0.150
helix sense		right-handed	left-handed
H-bond type	intermolecular	intramolecular	intramolecular
H-bond scheme[c]	-	C=O(i)···H−N (i+2)	N−H(i)···O=C(i+3)
no. of atoms per H-bond	-	14	19
d(H···O)[b]	1.89	1.82	2.02
∠N−H···O[a]	175.8	164.8	165.1
ΔE[d]	4.5	2.3	0.0

[a] In deg. [b] In nm. [c] In parentheses is given the number of the amide groups linked by the hydrogen bond. [d] In kcal/mol·residue.

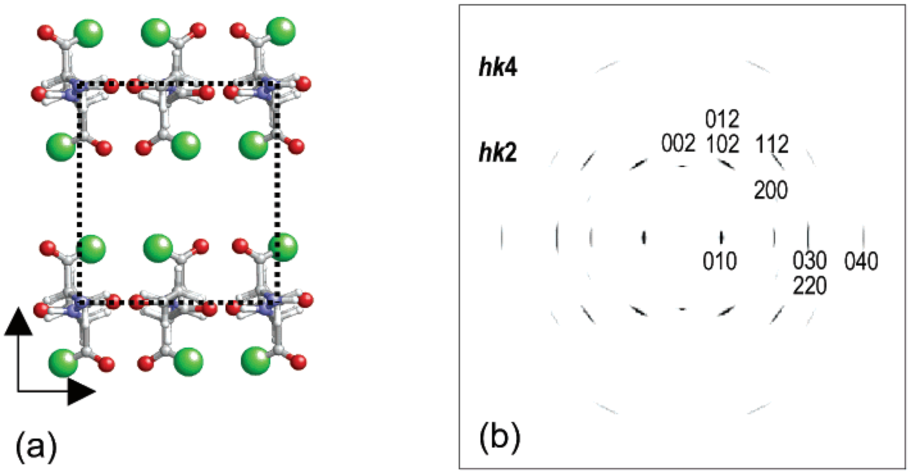


Figure 4. (a) Schematic representation of the unit cell of form I of PAB(D)G projected down the *c*-axis. The benzyloxy side group is represented by green spheres. (b) Simulated X-ray diffraction pattern produced by the statistically crystal structure modeled for PAB(DL)G in form I containing a mixture of PAB(D)G and PAB(L)G chains in equal amounts.

Concluding Remarks

Three crystal forms, I, II, and III, have been found for the benzyl ester of bacterially produced poly(γ-glutamic acid) with a nearly racemic composition. The crystal lattice parameters of these three forms are compared in Table 3. Form I is a layered structure with chains in nearly extended conformation reminiscent of the widely known γ-form of nylons. Form II is a structure made right-handed 5/2 helices stabilized by a $i - i + 2$ intramolecular scheme of hydrogen bonds. These two forms are essentially identical to those two described for the optically pure poly(α-benzyl γ,L-glutamate) prepared by chemical synthesis. Form III is a left-handed helical structure with 3.7 residues per turn and hydrogen bonds set between i and $i + 3$ amide groups, which is a conformation topologically close to the α-helix of poly(α-peptide)s. None of these structures are energetically acceptable for a racemic poly(γ-glutamate) having a statistical distribution of D and L

Table 3. Crystalline Forms of PAB(DL)G

	helix	crystal parameters (nm, deg)	density[a] (g mL^{-1})
form I	2/1	$a_0 = 0.930$; $b_0 = 1.045$; $c_0 = 1.200$ $\alpha = \beta = \gamma = 90°$	1.26 (1.25)
form II	5/2	a_0 1.30; $b_0 = 2.25$; $c_0 = 1.00$ $\alpha = \beta = \gamma = 90°$	1.25 (1.26)
form III	37/10	a_0 1.478; $b_0 = 2.564$; $c_0 = 5.449$ $\alpha = \beta = \gamma = 90°$	1.31 (1.29)

[a] Calculated and experimental (in parentheses) densities.

units. They will be not in disagreement however with a stereocopolymer made of homogeneous sequences of D and L units, as it is expected to be the case of PAB(DL)G obtained by esterification of bacterially produced PGGA. It should be remarked that in view of the limited amount of X-ray diffraction data available at present, the structures proposed in this work must be taken as the most probable ones, and they will be therefore susceptible to revision if new experimental data of significance were provided.

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