Synthesis and Conformational Study of Alternating Poly(γ-benzyl D,L-glutamates)

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ABSTRACT: Alternating poly(γ-benzyl D,L-glutamates) have been prepared using the active-ester method. As some racemization seems to occur during polycondensation the composition of the polymer is about 53% D and 47% L residues. It has a helical conformation, mainly left-handed, as shown by optical rotatory dispersion studies.

The existence of helical structures in polypeptide chains containing D and L residues is still the subject of experimental and theoretical studies. In such structures, there are the same destabilizing interactions which exist in left-handed helices of poly(L-peptides). These interactions appear in particular between the C_β atoms of the side chains and the oxygen of the carbonyl group.[1]

In meso polymers, examination of molecular models shows that other interactions may take place. For example, in a right handed α helix, the β-carbon atom of the side chain of the nth L residue is close to the same atom of the side chain of the $(n + 3)$ residue if this one is a D residue. The number of side chains involved in these interactions depends on the primary structure of the polypeptide. If the distribution of residues in a meso polypeptide is random, these interactions affect 50% of the β-carbon atoms; in the case of alternating poly(D,L-peptides) all the β-carbon atoms interact and none if the primary structure is $(LLD)_n$. The lower stability of the structures of meso polypeptides dissolved in organic solvents is attributed to these interactions, which on the contrary could give rise to hydrophobic forces in aqueous medium.[2]

One of the most studied meso polypeptides is poly(γ-benzyl D,L-glutamate). (We denote as PBD,LG a meso copolymer in which the sequence is unknown and as PBD-LG the alternating copolymers. For other abbreviations, see the Experimental Section.) Downie, *et al.*,[3] studied the variation of the molar rotation $[m']_D$ with the ratio L/(D + L) in different solvents and concluded that PBD-LG may exist in a helical conformation. Moreover, molecules of samples containing less than 30% of D residues have a right helical sense. The same conclusion has been reached by Doty and Lundberg,[4] who have also calculated by linear extrapolation the rotatory dispersion of a right-handed α helix of PBD,LG.

The existence of two forms of PBD,LG has been demonstrated by studies in *N*,*N*-dimethylformamide (DMF) as solvent.[5,6] Forms A and B were obtained by polymerization of the *N*-carboxy anhydride using aprotic initiators. Form A was flexible in DMF, whereas form B was rigid in this solvent and had a hydrodynamic behavior comparable to that of PBLG. Both forms have different primary structures, and it has been established by stability measurements that form B is not a mixture of PBDG and PBLG. These results have been confirmed by dielectric absorption studies and Kerr-effect measurements in ethylene dichloride solution.[7] Form A has some helical structure in chloroform, as shown by a transition in viscosity in mixed solvents.[6]

Wada[8] has studied preparations of PBD,LG with different D/L ratios by dielectric dispersion in ethylene dichloride. He concluded that PBD-LG molecules are mainly helical and proposed for the polymerization a kinetic model which is based mainly on the following assumptions. (1) At the beginning of the polymerization, the composition of the growing chain in D and L residues is random and, because of this, there is no helical conformation. (2) The α structure appears only after a sequence of more than four residues of the same configuration has been formed. Helical structures are then formed in which only one-fifth of the D (or L) residues are incorporated.

Similar results have been found by Tsuboi, *et al.*,[9] who studied the infrared spectra of the polymers in the solid state and who proposed an analogous model for the polymerization of the D,L-*N*-carboxy anhydride.

In order to study the conformation of a copolypeptide with a known primary structure we have prepared the alternating poly(D,L-peptides), PBD-LG, in which steric interactions are less favorable for a helical structure. A preliminary report has already appeared.[10]

Synthesis and Characterization of the Polymer

The samples of PBD-LG were prepared as shown in the Scheme I.

The ONp activated ester of the dipeptide was polycondensed in concentrated solution in DMF (samples DL 1 and DL 2) or in DMSO (sample DL 3). Other results from this laboratory[11] have shown that higher molecular weight can be obtained using the OPcp ester polycondensed in benzene. This has been done for samples DL 4, DL 5, and DL 7. The infrared spectra in KBr pellets of the crude samples obtained by precipitation as described later show bands mainly at 1660 and 1554 cm^{-1}. These values are also found for *meso*-PBD,LG.[12] The infrared spectra also reveal in all samples, and especially in sample DL 3, the presence of diketopiperazines (1680 cm^{-1}). A band characteristic of β structures (1620 cm^{-1}) occurs in all samples obtained through the ONp

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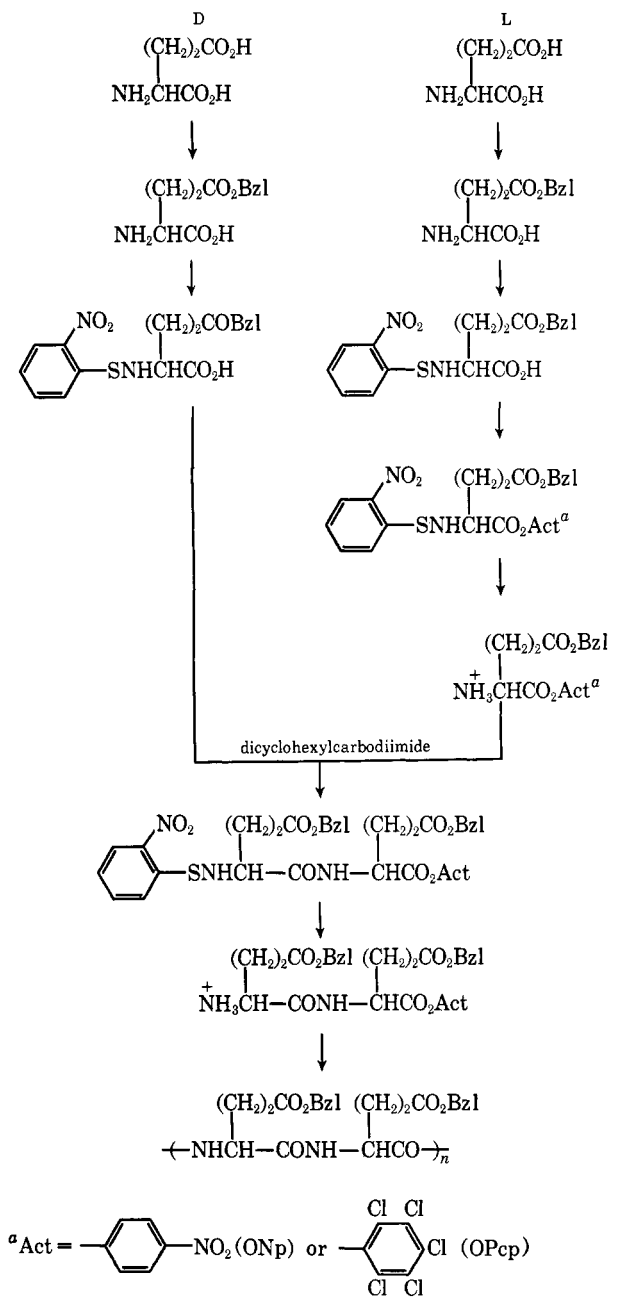
TABLE I

Samples	DL 1	DL 2	DL 3	DL 4	DL 5	DL 7
Activated ester	ONp	ONp	ONp	OPcp	OPcp	OPcp
Solvent of polycondensation	DMF	DMF	DMSO	Benzene	Benzene	Benzene
Mol wt[a]	15,000	12,000		23,000	21,000	16,000
$[\alpha]_D^{DCA}$, deg	+0.3	+0.4	+0.5	+1.2	+0.8	+1.4
Ratio D/(L + D)	50.9	51.2	51.8	53.6	52.5	54
Polymer yield after dialysis, %	48	40	10	50	45	45

[a] Estimated by viscosity measurements in DCA.

SCHEME I



ester and to a lesser extent in samples obtained through the OPcp ester. The cyclic products are eliminated by dialysis in DMF. However, the β fraction, which is partly soluble in this solvent, cannot be removed completely, either by dialysis or by extraction with formic acid. Therefore, most of our results refer to samples DL 4 and DL 5, which are free of β structures. On sample DL 7, β structures were eliminated by filtration of a solution in ethylene dichloride.

The molecular weights have been estimated by viscosity measurements of solutions in dichloroacetic acid (DCA) and by using the relation established between molecular weight and viscosity for PBLG in DCA (Table I)[13] These values may be underestimated in view of the conclusion of Miller, *et al.*,[14] who predicted that in a racemic D,L-polypeptide the chain dimensions are strongly dependent on the distribution of D and L residues. As the sequence distribution is changed to give successively pure stereopolymer, block copolymer, random copolymer, and finally alternating D and L copolymer, the chain dimensions are predicted to fall continuously. Indeed, molecular weight measurements of sample DL 7 in DCA by light scattering give a value of 21,000.

In contrast to the optical activity $[\alpha]_D$ of the A and B forms of PBD,LG in DCA, that of alternating PBD–LG is not zero in this solvent. This discrepancy could have its origin in the optical purity of the starting amino acids ($[\alpha]_D$ $-30.9°$ for D-glutamic acid and $[\alpha]_D$ $+30.3°$ for L-glutamic acid). A more probable explanation is that some racemization occurs during the polycondensation, affecting the L residue of the peptide. The optical purity of the polymer seems to be related to the ester used (Table I) and is retained to a lesser degree with the OPcp ester.

In DCA no conformational effects should influence the optical activity. Therefore, we have estimated the ratio D/(L + D) (Table I) in the different samples by measuring the ratio of $[\alpha]_\lambda^{DCA}$ of alternating PBD–LG over $[\alpha]_\lambda^{DCA}$ of PBLG. This ratio is constant for all wavelengths. The same measurements were made in trifluoroacetic acid and give a higher value for the ratio D/(L + D); for example, in sample DL 7 we find 0.57 instead of 0.54 in DCA. This calculation indicates that about 15% of C-terminal L residues are affected by a racemization reaction during the polymerization of the OPcp ester. This means that there is, in molecules of DP 100 (which is approximately the value found for sample DL 4), an average of 46.4 L residues and 53.6 D residues. The primary structure is mainly the repetition of a D–L sequence, but with some D–D defects which are probably randomly distributed.

To summarize, polycondensation of the OPcp ester in benzene leads to high-molecular-weight samples, free of β structures and with better yield than the ONp ester, but with apparently higher racemization.

Conformational Studies in Solutions

In contrast to the A and B forms of random PBD,LG, which in DMF solutions give a value of $[\alpha]_D$ near zero, the values of $[\alpha]_D$ obtained for the PBD–LG samples are high and negative in DMF and in solvents promoting helical structures (chloroform, *m*-cresol, and dioxane). The comparison of

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TABLE II

Sample	Mol wt	b_0 DMF	b_0 $CHCl_3$	b_0 *m*-Cresol	b_0 HF2P[a]
DL 1	15,000	150			
DL 2	12,000			290	
DL 4	23,000			320	
DL 5	21,000	310	360	315	
DL 7	16,000	365			330

[a] Hexafluoro-2-propanol.

the value of

$$[m']_D = \frac{3}{n^2 + 2}\frac{M}{100}[\alpha]_D$$

(n is the refractive index of the solvent and M the molecular weight of a benzylglutamyl residue) with that obtained by linear extrapolation by Elliott, *et al.*,[3] in DMF and *m*-cresol suggests in PBD–LG a high proportion of the left-handed helical conformation.[10]

It is also interesting to compare (Figure 1) the rotatory dispersion in dioxane of sample DL 7 with the curve calculated by Doty and Lundberg[4] for a left-handed α helix of PBD,LG. The two curves show a striking similarity between 360 and 600 nm.

We therefore favor the existence of an α-helical structure for PBD–LG. More evidence is obtained from the circular dichroism spectrum of a solution in chloroform, which shows a positive maximum at 225 nm, also indicating the presence of left-handed helices. In hexafluoro-2-propanol, between 195 and 250 nm the spectrum is also in agreement with a left-handed α-helical structure ($\Delta\epsilon$ +4 at 222 and 209 nm).

Another point is that the solid-state X-ray diffraction patterns of sample DL 7 reveal a hexagonal lattice and a meridional reflection at 1.49 Å which are characteristic of an α-helical structure.

The values of the parameter b_0, calculated using the Moffitt equation, are reported in Table II (λ_0 2120 Å). These positive values suggest that the polymer is at least partially helical and mostly left-handed. A similar conclusion was reached by Marlborough and Rydon,[15] who studied poly(*tert*-butyl D,L-glutamates). Taking a b_0 value of zero as indicative of the absence of any helical structure and a value of +600 for a fully left-handed α-helical structure, and assuming the absence of any other ordered structure, a value of +300 would indicate that we are dealing with a mixture of 50% left-handed helices plus 50% random coil or, in the case of a mixture of fully helical molecules, 75% left-handed and the rest right-handed. This point is examined in the following papers.[16,17]

Stability of the Helical Structure of PBD–LG

In Figure 2 we show the variations with temperature of the coefficient b_0 for sample DL 5 in chloroform and *m*-cresol. On the same figure, we have similarly shown the behavior of PBLG (mol wt = 20,000) and of PBLA in *m*-cresol.

These experiments show that the stability of PBD–LG with respect to temperature is comparable to that of PBLG and that these two polymers do not show a helix–coil transition as observed with PBLA. The same behavior is observed in DMF.

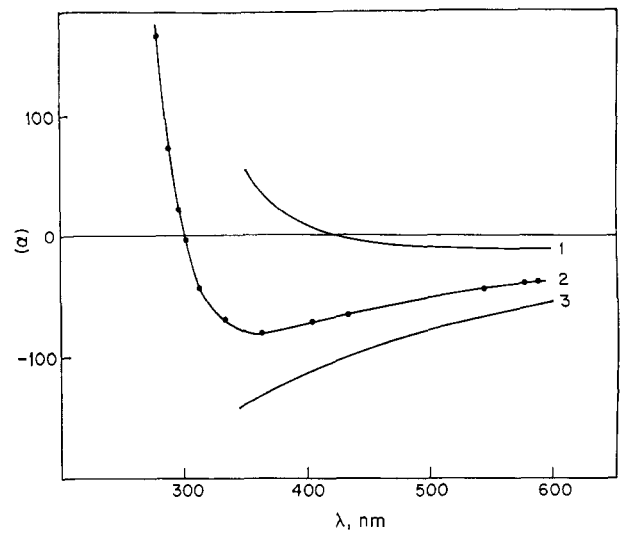

Figure 1. ORD spectra of γ-benzyl glutamate polymers in dioxane: (1) PBDG; (2) PBD–LG (sample DL 7; see Table II), a_0 = −445, b_0 = +427; (3) PBD,LG, a_0 = −680, b_0 = +500, calculated by extrapolation for a meso left-handed α helix.[4]

Addition of DCA to chloroform or *m*-cresol solutions of sample DL 5 generates a transition at room temperature when 17% DCA is added to the chloroform solution of PBD–LG or about 25% to the *m*-cresol solution. Heating a solution of mixed solvents to the transition region produces in PBD–LG, as for PBLG, an increase of b_0. Above 70° degradation of the polymer occurs, as shown by a slow decrease of b_0 and the irreversibility of the phenomenon.

Taking the percentage of DCA in chloroform as a measure of the stability of the conformation, the polymer stabilities can be compared as follows (polymer, % DCA): PBLA, 6% < PBD,LG (A), 14% ≤ PBD–LG, 17% < PBD,LG (B), 30% < PBLG, 75%.

Attempt to Modify the Configuration of Residues in PBD–LG

We have seen that residues having an L configuration can become engaged in a left-handed helix. It was then interesting to use this skeleton as an asymmetrical matrix to promote the inversion of the configuration of the L residues and increase the ratio D/(L + D). The easiest method was the reversible removal of the α proton. In order to do this, a solution in

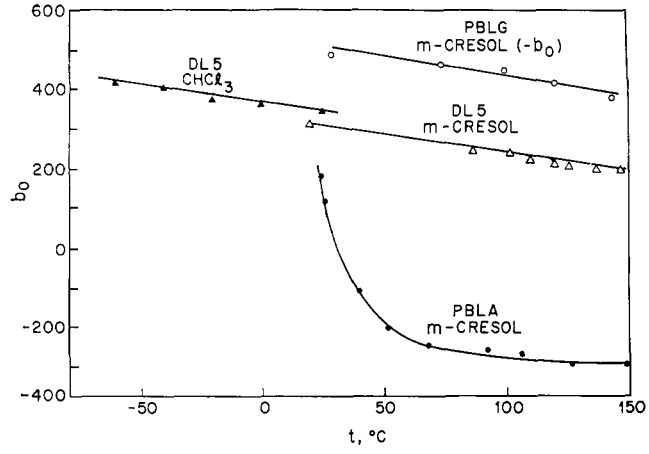

Figure 2. Comparison of the stability of different polypeptides with respect to temperature.

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DMF of sample DL 5 was subjected to reaction at 65° with 3 equiv of triethylamine (TEA) for 2 days. The optical rotatory dispersion was monitored but did not show the expected trend and approached a zero value. That the polypeptide was degraded, with formation of diketopiperazines, was shown by the infrared spectrum.

A PBLG sample subjected to the same conditions underwent no modification.

Discussion

We have obtained samples of PBD–LG of DP about 100 containing 47 L and 53 D residues, the excess of D residues being presumably randomly distributed.

In solvents favoring the α-helical form of polypeptides, an ordered structure does exist for PBD–LG. We have presented arguments indicating that this structure is α helical, with the possibility of being slightly distorted. A small excess of one enantiomer is sufficient to favor one helix sense over the other. This helical structure is stable to temperatures below 150° and has an intermediate stability toward DCA addition.

Our results demonstrate that, even in the most unfavorable case, where all the β-carbon atoms of the side chains are close together, it is possible to have a helical structure, the exact amount of which cannot, however, be determined from our measurements. This conclusion indicates that a previous hypothesis used by Wada and Tsuboi[9] for the kinetic scheme of polymerization of D,L-*N*-carboxy anhydride may be oversimplified. Alternating or random copoly(benzyl D,L-glutamate) can exist in the helical conformation, and a long sequence of residues of the same configuration is not needed to promote a helical structure. It remains to determine the minimum molecular weight required to induce a helix. It would be also interesting to know the influence of the composition of the helical end of the last turn on the kinetics of incorporation of D and L residues in a growing chain.

Experimental Section[18]

***N*-(*o*-Nitrophenylsulfenyl)-γ-benzyl-D-glutamyl-γ-benzyl-L-glutamate *p*-Nitrophenyl Ester. Nps-D-Glu(OBzl)-L-Glu(OBzl)-ONp.** Nps-D-Glu(OBzl)-OH,DCHA[19] (6.2 g, 0.011 mol) was dissolved in 150 ml of chloroform. To this solution, cooled to 0°, 2.4 g (0.011 mol) of dicyclohexylcarbodiimide and 4.4 g (0.011 mol) of HCl,H-L-Glu(OBzl)-ONp[20] were added.

The mixture was stirred for 1 hr at 0° and then overnight at room temperature. The solvent was removed under vacuum and the resulting product taken up in EtOAc. Dicyclohexylurea was filtered off and the filtrate evaporated to dryness under vacuum. The resulting oil crystallized by triturating under ether. The crude product was recrystallized in absolute ethanol giving 7.2 g (93%) of protected dipeptide, mp 84–86°; $[\alpha]_D$ +29.6° (5% in EtOAc).

γ-Benzyl-D-glutamyl-γ-benzyl-L-glutamate *p*-Nitrophenyl Ester Hydrochloride. HCl,H-D-Glu(OBzl)-L-Glu(OBzl)-ONp. To a solution of Nps-D-Glu(OBzl)-L-Glu(OBzl)-ONp (4.8 g, 0.0066 mol) in chloroform (45 ml) a solution of HCl in ether (0.014 mol in 750 ml) was added. The resulting oil was crystallized in 2-propanol, giving 2.6 g (66%) of activated peptide hydrochloride: mp 117–119°; $[\alpha]_D$ −30.8° (5% in DMF), −33.2° (5% in $CHCl_3$).

Poly(γ-benzyl-D-glutamyl-γ-benzyl-L-glutamyl). [D-Glu(OBzl)-L-Glu(OBzl)]$_n$ (Residue Weight 438). HCl,H-D-Glu(OBzl)-L-Glu-(OBzl)-ONp (2.45 g, 0.0039 mol) was dissolved in a mixture of DMF–TEA (1.36 ml, 2 vol of DMF or DMSO for 1.4 vol of TEA). The mixture was left for 2 days at room temperature. The crude polymer (1.76 g) was obtained by precipitation with water. The impurities were removed either by dialysis in DMF or by extraction with formic acid.

***N*-(*o*-Nitrophenylsulfenyl)-γ-benzyl-D-glutamyl-γ-benzyl-L-glutamate Pentachlorophenyl Ester. Nps-D-Glu(OBzl)-L-Glu(OBzl)-OPcp.** Nps-D-Glu(OBzl)-OH,DCHA (6.0 g, 0.01 mol) and HCl,H-L-Glu-(OBzl)OPcp[21] (5.2 g, 0.01 mol) were worked up as for the ONp ester to obtain 5.2 g (72%) of protected dipeptide, mp 106–107°; $[\alpha]_D$ +12.5° (1% in EtOAc).

γ-Benzyl-D-glutamyl-γ-benzyl-L-glutamate Pentachlorophenyl Ester Hydrochloride. HCl,H-D-Glu(OBzl)-L-Glu(OBzl)-OPcp. To a solution of Nps-D-Glu(OBzl)-L-Glu(OBzl)-OPcp (6.0 g, 0.0069 mol) in EtOAc (150 ml), a solution of HCl in ether (0.014 mol in 1000 ml) was added. The precipitate was purified with ethanol and ether, giving 3.2 g (70%) of activated peptide: mp ≃140° dec; $[\alpha]_D$ −27.5° (1% in DMF), −26.6° (1% in DCA).

Poly(γ-benzyl-D-glutamyl-γ-benzyl-L-glutamyl). HCl,H-D-Glu-(OBzl)-L-Glu(OBzl)-OPcp (3 g, 0.00405 mol) was dissolved in a mixture of benzene (1 vol)–TEA (1.4 vol, 8.1×10^{-3} mol). After 10 min, 1 ml of benzene was added, then the mixture was left for 2 days at room temperature. The crude polymer was obtained by evaporating the solvent, then dissolving in DMF and precipitating with water. Purification was done by dialysis in DMF.

Optical Rotatory Measurements. The optical rotatory measurements were made at room temperature at 365, 436, 546, 578, and 589 mμ with a Perkin-Elmer Model 141 polarimeter.

The optical rotatory dispersion data were analyzed using the Moffitt equation (λ_0 2120 Å), and the calculated b_0 values are reported in Table II.

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(18) Abbreviations used: Glu, Glutamic acid; PBLG, poly(γ-benzyl L-glutamate); PBDG, poly(γ-benzyl D-glutamate); PBLA, poly(γ-benzyl L-aspartate); DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DCA, dichloroacetic acid; TEA, triethylamine; DCHA, dicyclohexylamine; DCCI, dicyclohexylcarbodiimide; Nps, *o*-nitrophenylsulfenyl; Bzl, benzyl; ONp, *p*-nitrophenyl; OPcp, pentachlorophenyl; DP, degree of polymerization; EtOAc, ethyl acetate.

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