

## Conformational Studies on Poly-L-phenylalanine. Circular Dichroism Properties of Copolymers of L-Phenylalanine and N<sup>ε</sup>-Carbobenzoxy-L-lysine

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**ABSTRACT:** Circular dichroism (CD) measurements have been carried out on pure poly- $\epsilon$ -carbobenzoxy-L-lysine (PCBL) and on copolymers of L-phenylalanine and N<sup>ε</sup>-carbobenzoxy-L-lysine in tetrahydrofuran as the solvent. The CD spectrum of pure PCBL does correspond to that of a polypeptide having the right-handed  $\alpha$ -helical conformation. When increasing amounts of L-phenylalanine residues were introduced into the peptide chain, a gradual change in the CD spectra was observed. From these data it was concluded that an increasing proportion of L-phenylalanine residues does not change the  $\alpha$ -helical conformation of PCBL. Furthermore, by optical titration experiments it was found that the  $\alpha$ -helical structure of copolymers containing increasing amounts of the aromatic residues is less stable than that of pure PCBL.

Conformational properties of poly( $\alpha$ -amino acids) in solution have been extensively studied by optical rotatory dispersion (ORD) and circular dichroism (CD).<sup>1</sup> Different ORD and CD spectra are observed when the polymers in solution exist in the  $\alpha$ -helical form, in the  $\beta$  form or in the coiled form. These differences in the optical rotatory properties arise from different modes of coupling of the transition moments of the peptide chromophores and permit the determination of the conformation of polypeptides in solution when only peptide chromophores are present in the polymer. The problem becomes much more complicated when optically active side-chain chromophores are present, whose contribution to the optical activity may overlap those from the peptide group. A further complication arises from the fact that electronic interactions among side-chain chromophores and between side-chain and main-chain chromophores are possible, so that all the different contributions to the optical activity may not be simply additive. In most cases a theoretical analysis of all contribution interactions to the optical activity is not yet available, and CD or ORD spectra are not sufficient to establish safely the conformation in solution. This is the case of polypeptides containing aromatic amino acids like L-tyrosine, L-tryptophan, L-histidine, and L-phenylalanine. The interpretation of CD and ORD spectra of these polymers in terms of conformation is always difficult and ambiguous. In such cases useful, if not completely unequivocal, information on the conformation in solution can be obtained by studying the optical rotatory properties of copolymers of the aromatic amino acids with a simple amino acid whose homopolymer has a known conformation in solution. If the CD or ORD spectra of the copolymers change gradually when the molar composition is correspondingly varied, one can assume that no conformational transition occurs on varying the copolymers composition. In other words the conformation of the aromatic poly(amino acid) is assumed to be identical with the conformation of the

known homopolymer. This approach has been already used in conformational studies on poly-L-tryptophan (PLTR).<sup>2</sup> Following the same method, in the present paper we tried to obtain information on the conformation in solution of poly-L-phenylalanine (PLP). Previous investigations carried out in water by ORD and CD measurements on the pure L-phenylalanine sequence (dissolved in water by using the sandwich technique of Gratzer and Doty)<sup>3</sup> and on random copolymers of L-glutamic acid and L-phenylalanine do not permit an unambiguous establishment of the conformation of PLP.<sup>3-6</sup> The CD spectrum of the L-phenylalanine sequence in water shows a negative band at 227 m $\mu$  whose position and intensity are not properly those of a polypeptide in the  $\alpha$ -helical conformation. These results have also been recently confirmed in nonaqueous solvents.<sup>7</sup>

It is well known that monosubstituted benzene rings exhibit three major transitions—a weak band in the 255–275-m $\mu$  region, corresponding to a symmetry-forbidden  $\pi \rightarrow \pi^*$  transition, and two stronger bands near 210 and 185 m $\mu$ .<sup>8</sup> Very recent work carried out on optically active compounds containing the phenyl chromophore<sup>9</sup> present definitive evidence that, in addition to the transition at 255 m $\mu$ , the transitions at 210 and at 185 m $\mu$  also substantially contribute to the optical activity.<sup>10-12</sup> In view of this fact it appears that the actual CD spectrum of PLP in the 180–220-m $\mu$

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region arises from contributions to the optical activity both from the side-chain chromophores and from the peptide chromophores. In the present work we have measured the CD spectra of copolymers of L-phenylalanine with N<sup>ε</sup>-carbobenzoxy-L-lysine in tetrahydrofuran as the solvent. Moreover, by optical titration experiments we studied the relative conformational stability of copolymers containing various amounts of aromatic residues.

## Experimental Section

**Solvents and Initiators.** Dimethylformamide (DMF) and tetrahydrofuran (THF) were both of reagent grade and were purified as previously described.<sup>13</sup> Dichloroethane (DCE, Fluka Puriss) was fractionally distilled. Ethyl ether (Carlo Erba RP) was dried over sodium metal and then distilled. Dichloroacetic acid (DCA) (Carlo Erba RP) was dried over P<sub>2</sub>O<sub>5</sub> and then fractionally distilled under reduced pressure. Ethyldiisopropylamine (Fluka Puriss) was dried over potassium metal and then fractionally distilled under reduced pressure.

**Monomers.** L-Phenylalanine N-carboxy anhydride (L-Phe-NCA) and N<sup>ε</sup>-carbobenzoxy-L-lysine N-carboxy anhydride (N<sup>ε</sup>-CBZ-L-Lys-NCA) were prepared from L-phenylalanine and from N<sup>ε</sup>-carbobenzoxy-L-lysine (N<sup>ε</sup>-CBZ-L-Lys), respectively, according to the literature.<sup>14,15</sup> L-Phe-NCA was recrystallized several times from ethyl acetate and *n*-hexane, mp 90°. N<sup>ε</sup>-CBZ-L-Lys-NCA was recrystallized several times from ethyl acetate–*n*-hexane. The final product has a mp 98°. N<sup>ε</sup>-CBZ-L-Lys was prepared from the corresponding copper complex<sup>16</sup> according to the following procedure. To a hot solution of 50 g of triethylenetetrammonium disulfate (TETD) in 1700 ml of 2 *N* NaOH, 50 g of N<sup>ε</sup>-CBZ-L-Lys copper complex was added under vigorous stirring. In less than 1 min all the material dissolved completely, forming a deep blue solution. The solution was rapidly filtered, cooled to 0° and neutralized with 210 ml of glacial acetic acid. A white precipitate formed, which was recovered by filtration, washed with cold water and with alcohol–water mixture, and finally recrystallized three times from water: mp 274–275°;  $[\alpha]_{589}^{25} +14.5$  (*c* 1.6, 2 equiv of HCl). *Anal.* Calcd for C<sub>14</sub>H<sub>20</sub>N<sub>2</sub>O<sub>4</sub>: C, 60; N, 10; H, 7.15. Found: C, 59.55; N, 9.97; H, 7.14.

**Polymers and Copolymers.** Poly- $\epsilon$ -carbobenzoxy-L-lysine (PCBL) was obtained by polymerization of the corresponding NCA in DMF, using ethyldiisopropylamine as the initiator. The initial monomer concentration was 0.1 *M* and the monomer to initiator molar ratio  $[M]_0/[I]_0$  was 95. The reaction was followed by ir (disappearance of the monomer band at 1860 cm<sup>-1</sup>). When the polymerization was complete the solution was poured into ethyl ether and PCBL precipitated as a white powder. It was recovered by filtration, washed and dried under vacuum overnight at 50°. The final polymer has an intrinsic viscosity  $[\eta]$  in DCA of 0.624 dl/g. Copolymers of N<sup>ε</sup>-CBZ-L-Lys and L-Phe were prepared in the following way. Various solutions were made up containing the desired proportions of the two monomers in DMF. The total monomer concentration of each solution was 0.1 *M*. The various solutions were poly-

merized at room temperature using ethyldiisopropylamine as the initiator. At the end of the polymerization (checked by ir) each solution was poured into a dilute solution of HCl. Each copolymer was recovered by filtration, washed abundantly with water, and dried under vacuum at 50°.

**Determination of the Relative Reactivity of the Two Monomers.** In order to determine the relative reactivity of L-Phe-NCA and N<sup>ε</sup>-CBZ-L-Lys-NCA toward polymerization, copolymerization experiments have been carried out and the copolymers composition was determined as a function of the initial composition of the monomer mixture. The reactions were initiated by diisopropylamine in DMF. When the polymerization reached about 20% conversion (checked by ir) the unreacted monomers were deactivated by adding a large excess of isopropylamine. The reaction mixture was then poured into 3 *N* HCl, in which only the copolymers, and not the reaction product of the primary amine with the monomers, are insoluble. The copolymers were then recovered by filtration, washed with water and finally dried under vacuum over P<sub>2</sub>O<sub>5</sub>. Known amounts of copolymers were dissolved in THF and the N<sup>ε</sup>-CBZ-L-Lys content was determined by measuring the absorption of the ester band at 1740 cm<sup>-1</sup>. The calibration was made using the copolymers of Table I, which have a known composition.

**CD measurements** have been carried out on a Roussel-Jouan 185 Model II dichrograph. The instrument records directly the *dichroic optical density*  $A_L - A_R$ .<sup>17</sup> From this quantity, the molar circular dichroism,  $\Delta\epsilon$ , can be calculated according to the equation

$$A_L - A_R = \frac{(\Delta\epsilon)cd}{M}$$

where *c* is the concentration in grams per liter, *d* is the cell path in centimeters, and *M* is the molecular weight per residue.

## Results and Discussion

**Copolymer Composition.** Table I shows the composition of various copolymers of L-Phe and N<sup>ε</sup>-CBZ-L-Lys. With the exception of copolymer sample 4, all samples are soluble in THF. TFE dissolves only samples 1 and 2. Copolymer 4 was dissolved in THF containing about 8% (by volume) concentrated sulfuric acid. Since all the copolymerization experiments have been carried out to 100% conversion, the actual composition of the copolymers corresponds to the initial composition of the monomer mixture. In this connection one must ask whether we are dealing with truly random copolymers in which the two different monomers units are statistically distributed along the polymer chains. According to the literature on the copolymerization of L-Phe and N<sup>ε</sup>-CBZ-L-Lys NCA's in DMF,<sup>18</sup> we can assume that in the bimolecular propagation reaction it is the monomer which determines the copolymerization rate rather than the nature of the active end of the growing chains. In other words the two active ends display the same preference for one monomer over the other. Over a narrow range of conversion the copolymer composition is uniquely determined by the relative reactivity of the

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TABLE I  
COPOLYMERS OF L-PHE AND N<sup>ε</sup>-CBZ-L-LYS

Sample no.	<i>A</i> / <i>I</i> <sup>a</sup>	Mol % L-Phe in the initial monomer mixture	N <sup>ε</sup> -CBZ-L-Lys/L-Phe molar ratio in the copolymers	Average molecular weight per residue
1	95	25.0	3:1	233
2	95	50.0	1:1	205
3	95	75.0	1:3	176
4	95	85.7	1:6	163

<sup>a</sup> Total monomer to initiator molar ratio. The initial concentration of the two monomers in the polymerization solution was 0.1 M.

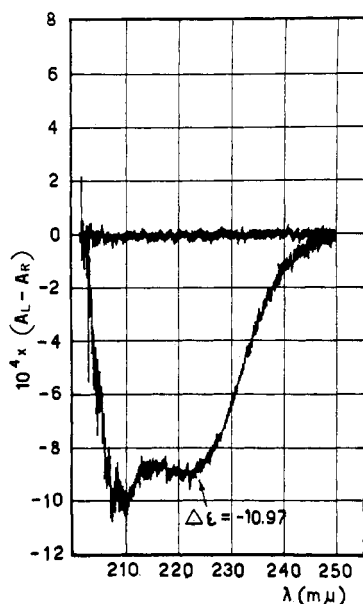


Figure 1. CD spectrum of PCBL in THF. Dichroic optical density ( $A_L - A_R$ ) is reported on the ordinate scale. The  $\Delta\epsilon$  value (calculated as described in the Experimental Section) at the wavelength of maximum dichroic absorption is also shown in the figure. The polymer concentration was 2.15 g/l. in a 0.1-mm cell.

two monomers and by their concentrations in the feeding monomer mixture. Since the L-Phe monomer reacts with the growing peptide chains three times more rapidly than the N<sup>ε</sup>-CBZ-L-Lys monomer, the free N<sup>ε</sup>-CBZ-L-Lys monomer will increase during the copolymerization, and the composition of the copolymers will change during the reaction. This result was also confirmed by us (Table II). It follows that in the copolymers formed at the beginning of the reaction the molar fraction of L-Phe will be higher than that of the initial monomer mixture. On the contrary in the copolymers formed toward the end of the reaction the molar fraction of L-Phe will be smaller than that of the initial monomer mixture. Of course the over-all composition of the copolymers will be identical with the composition of the initial monomer mixture if the reaction is carried out to 100% conversion.

**CD Studies.** CD spectra of PCBL and of copolymers of N<sup>ε</sup>-CBZ-L-Lys and L-Phe in THF are shown in

TABLE II<sup>a</sup>  
COPOLYMERIZATION OF L-PHE-NCA AND N<sup>ε</sup>-CBZ-L-LYS-NCA IN DMF. ANALYSIS OF THE COPOLYMER COMPOSITION AT 20% CONVERSION

% conversion	Molar fraction of L-Phe in the initial monomer mixture	Molar fraction of L-Phe in the copolymer
20.0	0.25	0.50
23.0	0.75	0.79

<sup>a</sup> The same monomer to initiator ratio and the same total monomer concentration as in Table I were used.

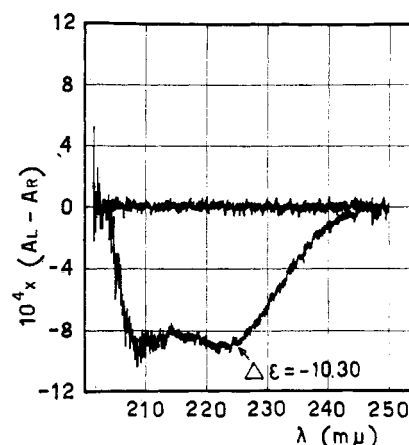


Figure 2. CD spectrum of copolymer sample 1 (N<sup>ε</sup>-CBZ-L-Lys/L-Phe 3:1) in THF. The copolymer concentration was 2.035 g/l. in a 0.1-mm cell.

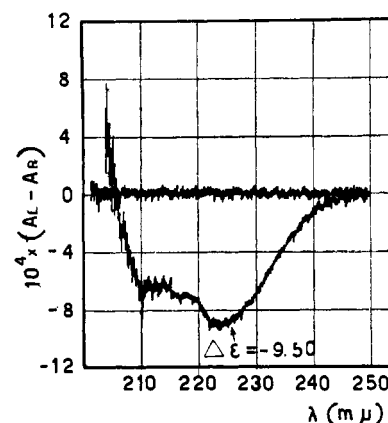


Figure 3. CD spectrum of copolymer sample 2 (N<sup>ε</sup>-CBZ-L-Lys/L-Phe 1:1) in THF. The copolymer concentration was 1.943 g/l. in a 0.1-mm cell.

Figures 1–5. The CD spectrum of pure PCBL is typical of a right-handed  $\alpha$  helix, with negative bands at 222 and 208 m $\mu$ , whose positions and intensities are in agreement with the literature data on helical polymers.<sup>19</sup> It can be observed from Figure 1 that the relative intensities of the two negative dichroic bands are not the same as in other helical polypeptides.

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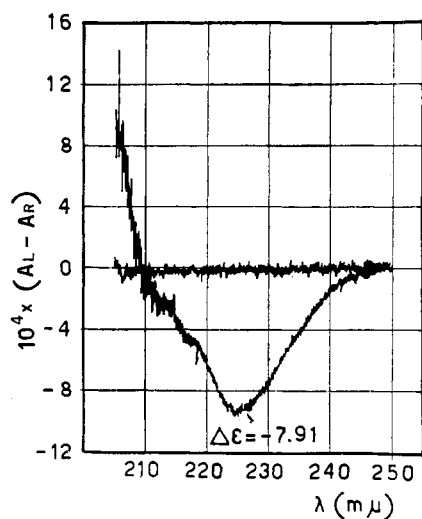


Figure 4. CD spectrum of copolymer sample 3 (N-CBZ-L-Lys/L-Phe 1:3) in THF. The copolymer concentration was 2.048 g/l. in a 0.1-mm cell.

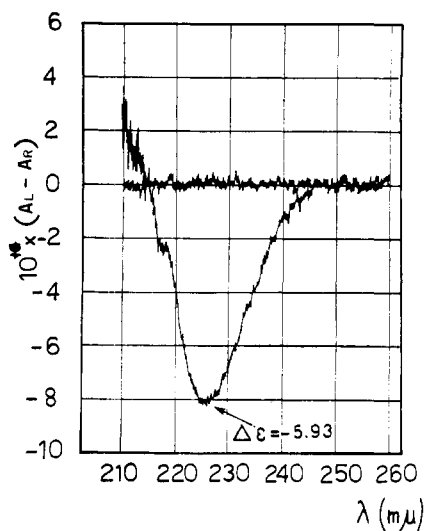


Figure 5. CD spectrum of copolymer sample 4 (N-CBZ-L-Lys/L-Phe 1:6) in THF containing ~8% concentrated sulfuric acid. The copolymer concentration was 2.200 g/l. in a 0.1-mm cell.

The explanation probably rests on the fact that the band at 208 mμ, which arises from exciton splitting of the  $\pi \rightarrow \pi^*$  transition of the amide chromophore, is quite sensitive to the nature of the solvent.<sup>7,20</sup> Then it is possible that different  $\alpha$ -helical polypeptides in different solvents exhibit CD spectra in which the two characteristic negative bands do not have the same relative intensities.

Proceeding from pure PCBL to copolymer 4 which contains 85.7 mol % L-Phe residues, a gradual change in the CD spectra is observed. The intensity of the negative band at 208 mμ continually decreases and ultimately disappears. At the same time the band at 222 mμ also decreases and slowly shifts to the red: in copolymer 4 it is located at 226 mμ (Figure 5). The

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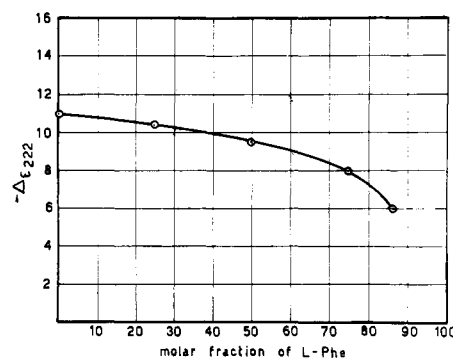


Figure 6.  $\Delta\epsilon$  values at 222 mμ plotted against the copolymer composition.

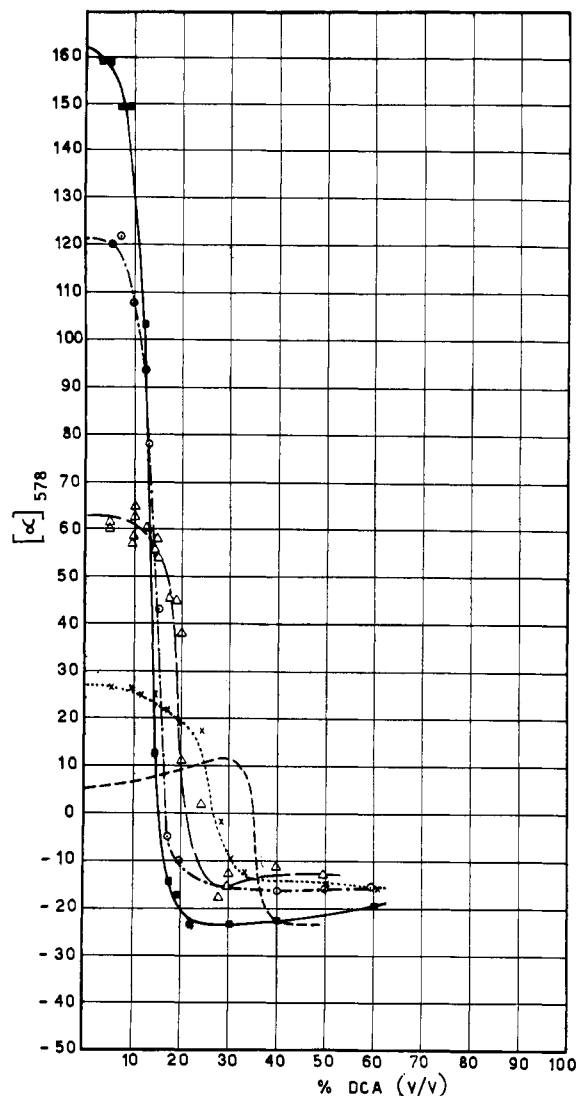


Figure 7. Optical titration experiments in DCE-DCA mixtures:  $\times \cdots \times \cdots \times \cdots \times \cdots$ , copolymer 1;  $\triangle$  — — —  $\triangle$ , copolymer 2;  $\circ$  — — —  $\circ$ , copolymer 3;  $\blacksquare$  — — —  $\blacksquare$ , copolymer 4; — — — — —, pure PCBL [this curve is taken from the paper of G. D. Fasman ("Poly- $\alpha$ -Aminoacids, Polypeptides and Proteins," M. A. Stahmann, Ed., University of Wisconsin Press, Madison, Wis., 1963, p 221) and refers to the  $\text{CHCl}_3$ -DCA solvent mixture. If DCE is used as the cosolvent instead of  $\text{CHCl}_3$  the transition occurs about at the same volume per cent of DCA].

CD spectrum of this copolymer, which is the richest in L-Phe, almost corresponds to that reported for PLP sequence in water.<sup>4</sup> In our case, however, the intensity of the negative band at 226 m $\mu$  is higher than that on the pure PLP. Unfortunately it was not possible to carry out CD measurements of the pure PLP, since this polymer is not soluble in organic solvents which are transparent in the far-uv region.

Figure 6 shows  $\Delta\epsilon$  ellipticity values at 222 m $\mu$ , plotted against the copolymer composition. In the range of composition which has been examined the graph does not show any evidence of a sharp conformational transition on increasing the L-Phe content. These results are quite similar to those found for random copolymers of  $\gamma$ -ethyl-L-glutamate and L-tryptophan.<sup>2</sup> As in that case, it is not clear whether such behavior is a consequence of a progressive change of the polypeptide structure because of the increasing amounts of the aromatic residues, or whether it merely reflects the fact that amino acid residues with chromophores in the side chains have been progressively introduced into the copolymer chain without any change of the conformation. The answer to this problem cannot be completely unambiguous. According to what has already been discussed in the first part of the present paper, we assume that no conformational transition takes place on increasing the L-Phe content of the copolymers, and therefore the right-handed  $\alpha$ -helical conformation of pure PCBL is maintained through the entire range of copolymer composition which has been examined. Furthermore, since the CD spectrum of pure PLP is almost identical with that of copolymer 4, we assume that this polymer also has the right-handed  $\alpha$ -helical conformation. For the nonlinear behavior of the graph of Figure 6 we propose the same explanation as in the previously mentioned case of tryptophan-containing copolymers:<sup>2</sup> that is, the progressive introduction of increasing amounts of the aromatic amino acid containing the optically active phenyl chromophore in the side chain makes possible electronic interactions among the phenyl groups and between phenyl groups

and the peptide chromophores, so that the sum total of all contributions to the measured optical activity are not simply additive. Of course this conclusion is a tentative one, since nothing is actually known at the present time about these interactions and about their possible effects on the total optical activity of helical polypeptides. Decisive evidence of the right-handed  $\alpha$ -helical structure of PLP can arise from the study by ORD and CD of polycyclohexylalanine (polyhexahydrophenylalanine), which does not contain chromophores in the side chains. Preliminary results obtained on this polymer indicate unambiguously that it exists in the  $\alpha$ -helical conformation in organic solvents. These data, which will be fully reported elsewhere, strongly support our hypothesis about the conformation of PLP in solution.

**Helix-Coil Transitions.** According to Auer and Doty,<sup>6</sup> the aromatic amino acids to some degree tend to destabilize the  $\alpha$ -helical conformation. This behavior arises from a steric interference between the side chain and the peptide backbone. From our CD measurements we concluded that an increasing proportion of L-Phe residues does not change the  $\alpha$ -helical conformation of PCBL. However, according to Auer and Doty, the presence of aromatic residues in the peptide chain must induce some instability of the  $\alpha$ -helical conformation. This prediction has been confirmed by optical titration experiments carried out in DCA-DCE mixtures on copolymers of L-Phe and N<sup>ε</sup>-CBZ-L-Lys having different compositions (Figure 7). We found that increasing amounts of L-Phe residues in the copolymers induce a progressive instability of the helical structure. Pure PCBL requires about 35% (by volume) DCA in order to undergo the helix-coil transition while copolymer 4 requires only 13% DCA to bring about the same transition. It must be pointed out that this situation occurs in organic solvents only. In aqueous solutions, bulky nonpolar side chains may participate in favorable noncovalent interactions like hydrophobic bonds which are capable of enhancing the stability of the  $\alpha$ -helical form.<sup>5,6</sup>