TABLE III
CHARACTERIZATION OF THREE LOWEST ENERGY MINIMUM CONFORMATIONS FOR R_H = 1.275 Å

	C′	D′	E′
Pro_4	II	II	II
Pro_5	II	II	II
φ_1	66.27	47.10	84.37
ψ_1	104.09	127.42	63.37
φ_2	100.07	113.63	127.43
ψ_2	221.33	162.11	151.55
φ_3	340.17	13.15	77.75
ψ_3	55.83	79.77	45.89
ψ_4	124.96	123.36	123.88
ψ_5	99.28	104.30	105.90
CO_1	↓	←	↓
CO_2	↑	←	←
E_{tot}	11.42	10.36	9.29
E_{tor}	0.16	−0.12	−0.21
E_{nb}	4.76	2.07	−0.57
E_{es}	6.51	8.41	9.88

freedom to the statistical weights will also be discussed.

The complete energy surface could be calculated for the pentapeptide considered here because it is cyclic and it contains only eight variable dihedral angles in the backbone. For more complex molecules, it is impossible to calculate the complete energy surface, and other methods than those used here must be employed to find local minima within a reasonable energy range of the global minimum. The complete energy surface obtained in the present calculation can now be used to test the efficiency of such methods.[29a]

(29a) NOTE ADDED IN PROOF. A reviewer has raised the point that the introduction of the possibility for flexibility of bond angles and bond lengths would greatly expand the domain of the ψ_1, ψ_5 space within which ring closure could be effected. This is correct, even though we have no quantitative information as to how the domain would be expanded. As was discussed elsewhere,[3] the conformation of a flexible molecule can be treated by a two-step procedure, the first step being the minimization of the conformational energy of the rigid molecule having fixed standard bond lengths and bond angles, with the second one being the energy minimization in a larger space corresponding to the flexibility of bond lengths and bond angles *starting* from the conformation obtained by the first step. In this paper, we are concerned with the first step.

Conformational Studies on Polypeptides. Circular Dichroism Properties of Random Copolymers of Lysine and Phenylalanine in Aqueous Solutions at Various pH Values

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ABSTRACT: Circular dichroism (CD) measurements have been performed on random copolymers of lysine and phenylalanine in 0.1 *M* KCl at various pH values. On increasing the pH, copolymers containing 3:1 and 1:1 molar ratios of lysine to phenylalanine undergo a conformational transition from a random coil to an ordered structure. The shape of the CD patterns, which are affected by the presence of side-chain aromatic chromophores, suggest that the ordered forms do correspond to β structures. In the limits of the examined copolymer compositions, the higher is the phenylalanine content of the copolymers, the higher is the stability of the β form in alkaline aqueous solutions.

It is well known that the overall stability of any conformation of natural and synthetic polypeptides is due to contributions from different forces like inter- or intramolecular hydrogen bonds, hydrophobic bonds, van der Waals interactions, dipole–dipole interactions, and so on.[1]

The relative importance of such forces are strongly dependent on the nature of the solvent. Thus, in aqueous solutions, hydrophobic bonds among nonpolar side chains are mostly responsible for the tendency of peptides and proteins to assume definite conformations.[2]

In organic solvents like 1,2-dichloroethane, chloroform, etc., hydrogen bonds are suggested to play an important role in stabilizing ordered conformations. Strong interacting organic solvents like dichloroacetic or trifluoroacetic acids compete successfully with amide groups of peptides in the formation of hydrogen bonds, and then in such media occurrence of ordered structures is generally prevented.

In a previous investigation[3] we studied the conformational properties of random copolymers of N^{ϵ}-carbobenzoxy-L-lysine (Z-Lys) and L-phenylalanine (Phe) in tetrahydrofuran as the solvent; it was found that increasing proportions of aromatic residues apparently do not perturb the α-helical conformation of poly-N^{ϵ}-carbobenzoxy-L-lysine (PCBL). However the presence of aromatic residues in the copolymer weakened the conformational stability of the α-helical form, owing to steric interference between side chains and peptide

(1) For a comprehensive review on this topic, see "Structure and Stability of Biological Macromolecules," G. N. Timasheff and G. D. Fasman, Ed., Marcel Dekker, Inc., New York, N. Y., 1969.

(2) H. A. Scheraga in "The Proteins," Vol. 1, 2nd ed, H. Neurath, Ed., Academic Press, New York, N. Y., 1963.

(3) E. Peggion, A. S. Verdini, A. Cosani, and E. Scoffone, *Macromolecules*, 2, 170 (1969).

backbone.[3,4] In aqueous solutions one could expect that bulky nonpolar side chains may participate in favorable hydrophobic interactions which are capable of enhancing the stability of the helical form.

In the present paper we report the conformational properties of random copolymers of lysine and phenylalanine in aqueous solutions, by CD measurements of solutions at different pH values.

Experimental Section

Solvent and Materials. Dimethylformamide (DMF) and tetrahydrofuran (THF) were both of reagent grade and were purified as previously described.[5] Ethyl ether (Carlo Erba RP) was dried overnight over sodium metal and then fractionally distilled.

Random Copolymers of Lysine and Phenylalanine. Random copolymers of lysine and phenylalanine have been prepared by decarbobenzoxylation of the parent Z-Lys and phenylalanine copolymers. The blocked copolymers containing different proportions of phenylalanine have been prepared by copolymerization of the corresponding N-carboxyanhydrides (NCA's), as described in our previous work.[3]

Copolymers 1, 2, and 3 of ref 3, containing, respectively, 3:1, 1:1, and 1:3 molar ratios of Z-Lys to phenylalanine, were decarbobenzoxylated according to the method of Fasman, *et al.*[6] The detailed procedure for copolymer 1 was the following. To a solution of the protected copolymer in anhydrous, freshly distilled dioxane (1.3 g in 130 ml), chloroform (320 ml) was added. Dry gaseous HCl was bubbled for 0.5 hr through the solution which remained perfectly clear. Then dry gaseous HBr was bubbled through for 2 hr, and the deblocked copolymer precipitated as a white powder. The mixture was left standing for 2 hr. The copolymer was then recovered by filtration and exhaustively washed with dry acetone (Merck puriss). The dry white powder so obtained was dissolved in dilute HCl and dialyzed for 4 days against 0.01 *N* HCl. Dialysis was performed with a 4465-A2 dialyzing tubing (A. Thomas Co., Philadelphia, Pa.) which retains materials with molecular weight 12,000 and higher. Finally the copolymer hydrochloride was recovered by lyophilization and dried under vacuum over phosphorus pentoxide.

For copolymers 2 and 3 the same above procedure was followed. The only difference was in copolymer 3 which does not dissolve in aqueous HCl; in this case we dialyzed the heterogeneous copolymer solution against 0.01 *N* HCl.

The composition of each deblocked copolymer was checked by amino acid analysis, using a Carlo Erba Model 3A27 automatic amino acid analyzer. The results of this determination are collected in Table I. On the average, all

TABLE I

Copolymer	Lys/Phe ratio	
	Theoretical	Found
1	3:1	3:1.05
2	1:1	1:1.05
3	1:3	1:3.01

samples contained about 10% moisture. The exact title of each copolymer was determined either by elemental analysis or by micro-Kjeldhal nitrogen determinations on standard solutions of the various copolymers, and by amino acid analysis.

As discussed in the previous paper,[3] in all copolymers there is a statistical distribution of lysine and phenylalanine residues along the copolymer chain.

Apparatus and Measurements. CD measurements have been carried out on a Roussel-Jouan 185 Model II dichrograph, which records directly the so-called dichroic optical density $A_L - A_R$, namely, the difference in absorbance between left-handed and right-handed circularly polarized radiation. From this quantity the molar circular dichroism, $\Delta\epsilon$, and the molar ellipticity, $[\theta]$, can be calculated according to the well-known relations[7]

$$A_L - A_R = (\Delta\epsilon)cd$$

$$[\Theta] = 3300(\Delta\epsilon)$$

where c is the molar concentration and d the optical path length.

In this paper we report the CD curves directly recorded by the instrument.

The pH of each copolymer solution was determined using a Metrohm Model E 388 precision potentiometer with a Metrohm UX combined glass electrode. CD measurements on copolymer solutions at various pH values were carried out in the following way. The pH of each solution (~20 ml) was adjusted to the desired value by adding to the copolymers solution in 0.1 *M* KCl–0.8 *N* NaOH with a Methrom precision microburet. Then 2.5 ml of solution was quickly transferred into the CD fused quartz optical cell and the CD spectrum was immediately run.

All successive measurements were carried out on aliquots of the mother solution brought to the desired pH value.

It was not possible to carry on measurements on copolymer 3 because the sample did not dissolve in aqueous HCl. CD measurements on copolymers 1 and 2 at pH higher than 10.50 were prevented by copolymer precipitation.

Results

The CD spectra of copolymer 1 (Lys:Phe 3:1) in 0.1 *M* KCl solution at various pH values are reported in Figures 1 and 2. At pH 8.68 the CD pattern is typical for polypeptides in a random coil conformation.[8] A positive dichroic band ($\Delta\epsilon$ = 1.1) is located at 218 mμ, followed by a strong negative band centered below 200 mμ. The intensity of the positive band at 218 mμ is quite high with respect to that observed for randomly coiled polypeptides. This band, however, is quite sensitive to the solvent[9,10] and to the nature of amino acid residues in the peptide chain.[11] In this particular case also contributions from the side chain aromatic chromophores are probably involved.

At pH higher than 9.28 the CD spectrum of the copolymer drastically changes (Figure 2), and a strong negative band centered at about 218 mμ becomes more and more evident. The change of the CD pattern corresponds to a conformational transition which is strongly cooperative in character. This is even more evident in Figure 3 where the molar dichroic absorption

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(6) G. D. Fasman, M. Idelson, and E. R. Blout, *J. Amer. Chem. Soc.*, **83**, 709 (1961).
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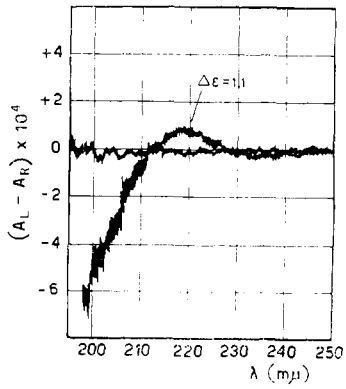

Figure 1. CD spectrum of copolymer 1 at pH 8.68. The copolymer concentration was 0.2760 g/l. in a 0.05 cm path length cell.

at 218 mμ is reported as a function of the pH. The conformational transition occurs within less than one pH unit; at pH 10.05 the transition is essentially complete. The complete CD spectrum at pH 10.05 recorded in the range 250–190 mμ is shown in Figure 4.

In addition to the negative band at 218 mμ there is a strong positive band ($\Delta\epsilon$ = 6.9) centered at 195 mμ. This pattern closely resembles that of poly-L-lysine (PLL) in the β conformation,[12-14] the negative band being due to the n–π* transition and the positive one arising from excitation resonance interactions of the NV1 transition.[15,16]

The intensity of the negative band at 218 mμ is about 50% lower than that found in poly-L-lysine in the β

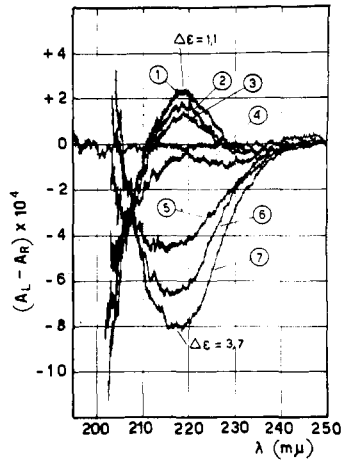

Figure 2. CD spectra of copolymer 1 in 0.1 *M* KCl at various pH values: curve 1, pH 8.68; curve 2, pH 9.01; curve 3, pH 9.28; curve 4, pH 9.48; curve 5, pH 9.67; curve 6, pH 9.85; curve 7, pH 10.05. The copolymers concentration was 0.6822 g/l. in a 0.05 cm path length cell.

(12) B. Davidson, N. Tooney, and G. D. Fasman, *Biochem. Biophys. Res. Commun.*, **23**, 156 (1966).
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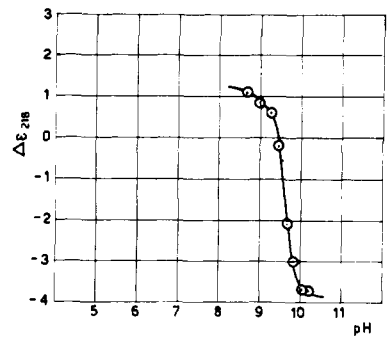

Figure 3. pH induced coil–β form transition of copolymer 1. Δε values at 218 mμ plotted *vs.* pH.

form. However, as pointed out by Quadrifoglio and Urry[17] in their investigation on poly-L-serine, the β-type CD patterns are variable as to position and intensity of bands when comparing different polypeptides. Moreover, in the specific case of lysine–phenylalanine random copolymers possible contributions to the optical activity from the side chain aromatic chromophores could distort the β form CD pattern. From all above considerations we interpret the results of Figure 3 as a coil-to-β-form transition.

CD measurements on copolymer 2 (Lys–Phe 1:1) in 0.1 *M* KCl at various pH values are shown in Figure 5 and Figure 7. At pH 4.38 (Figure 5) the CD pattern is quite unusual; there is a negative band ($\Delta\epsilon$ = −0.32), located at 232 mμ, followed by a very weak positive band centered at about 220 mμ. Below 220 mμ the CD pattern is quite indefinite because of the unfavorable $\Delta\epsilon/\epsilon$ ratio. There is in fact a surprisingly weak dichroism in this region.

The spectrum is not consistent with that of PLL and PLP in the random coil conformation. In fact both homopolymers show typical CD spectra with a positive band at 220 mμ (which is quite strong in PLP) and a strong negative band below 200 mμ.[11] On the contrary, Figure 5 shows that copolymer 2 exhibits very weak dichroism below 220 mμ. The complete CD pattern in the range 250–190 mμ seems consistent with the simultaneous presence of random coil and β conformations; this hypothesis could explain the weak dichroism around 200 mμ, which should arise from overlapping of coiled form and β structure CD bands, which are opposite in sign. The minimum at 232 mμ could be

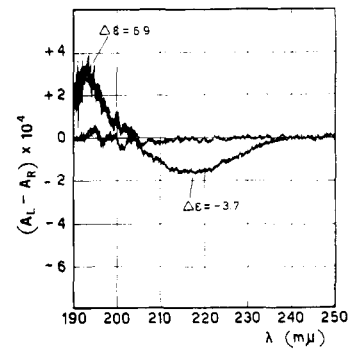

Figure 4. Complete CD spectrum of copolymer 1 at pH 10.05. The copolymer concentration was 0.6822 g/l. in a 0.01 cm path length cell.

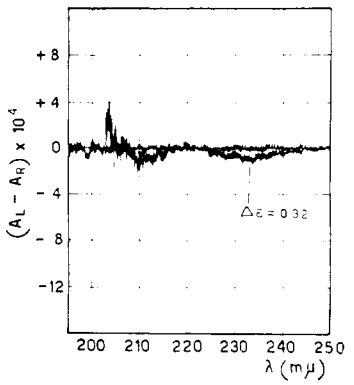

Figure 5. CD spectrum of copolymer 2 in 0.01 *M* KCl at pH 4.36. The copolymer concentration was 0.3032 g/l. in a 0.1 cm path length cell.

explained with overlapping of a positive band at 220 mμ with a negative one centered at about 215 mμ.

Therefore we tentatively conclude that the CD spectrum of Figure 5 corresponds to a partly ordered structure (mixture of coil and β form) which is stable in 0.1 *M* KCl at pH 4.38. It is important to note that this conformation is stable only in the presence of KCl. In fact in pure water, at the same pH conditions, the CD spectrum of copolymer 2 is totally consistent with the spectrum of a random coil conformation (Figure 6), the high intensity of the positive band at 220 mμ being due to the presence of aromatic residues in the chain.[11]

On increasing the pH, the CD pattern of copolymer 2 in 0.1 *M* KCl drastically changes (Figure 7). A strong negative band becomes more and more evident which is initially located at 220 mμ, and progressively shifts to the blue, the final position being at 215 mμ ($\Delta\epsilon$ −2.64). In Figure 8 we reported the $\Delta\epsilon$ values at 220 mμ as a function of the pH; from the graph the pH-induced conformational transition is clearly evident. We note first that the transition is not as sharp as in the case of copolymer 1; it occurs in fact within 2–2.5 pH units. In addition, the pH at which the conformational change takes place is about 0.5 units lower than that of copolymer 1. Also in this case, the interpretation of the CD spectrum in terms of conformation is not univocal, probably because of contributions to the optical activity from the aromatic chromophores. The CD spectrum at pH higher than 10 is different

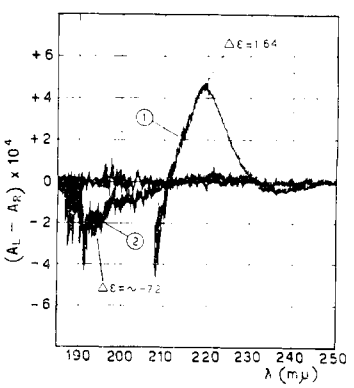

Figure 6. CD spectrum of copolymer 2 in water at pH 4.36. Curve 1 has been recorded using a 0.1 cm path length cell, and curve 2 has been recorded using a 0.01 cm path length cell. The copolymer concentration was 0.4306 g/l.

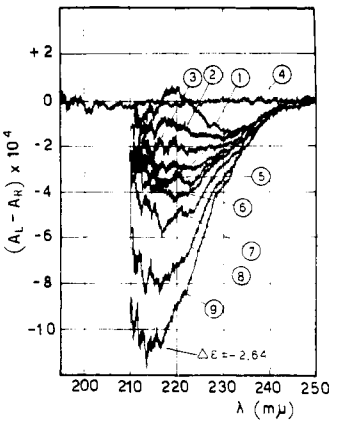

Figure 7. CD spectra of copolymer 2 in 0.1 *M* KCl at different pH values: curve 1, pH 4.36; curve 2, pH 8.18; curve 3, pH 8.51; curve 4, pH 8.67; curve 5, pH 8.83; curve 6, pH 8.93; curve 7, pH 9.16; curve 8, pH 9.52; curve 9, pH 9.78. The copolymer concentration was 0.6192 g/l. in a 0.1 cm path length cell.

either from that of copolymer 1 recorded under same conditions, or from that of a 1:1 Z-Lys-Phe random copolymer in THF, for which the right-handed α-helical conformation has been found.[3] Certainly the α-helical form for copolymer 2 is unlikely, since we should have observed a CD spectrum very similar in shape to that found in THF with two negative bands at 222 and 210 mμ, whose positions and intensities are affected by the presence of the phenyl groups (see Figure 3 of ref 3).

The CD spectrum observed in alkaline aqueous solutions is more similar to that of a β structure, except for position and intensity of the negative band. However, as previously pointed out, both position and intensity of such a band are quite different for different polypeptides even when there are no optically active chromophores in the side chains. On this basis, it seems reasonable to assume that the CD spectrum of copolymer 2 in alkaline aqueous solutions is that of a β structure, the distortions of the spectrum arising from the presence of a substantial amount of aromatic chromophores in the side chains.

Then we conclude that the plot of Figure 8 corresponds to a conformational change from a partly ordered structure to a completely ordered β form.

Discussion and Conclusions

The results presented in this paper show that, on the basis of CD measurements, random copolymers of

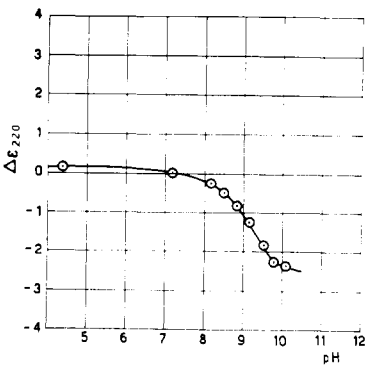

Figure 8. pH-induced conformational transition of copolymer 2. $\Delta\epsilon$ values at 220 mμ plotted *vs.* pH.

lysine and phenylalanine in 0.1 *M* KCl at alkaline pH's assume the β conformation. This result is quite surprising if one considers that neither PLL nor PLP (dissolved in water using the sandwich technique of Gratzer and Doty[18]) in alkaline aqueous solutions are in the β form. In both cases the α-helix is the preferred conformation.

Furthermore we stress the point that random copolymers having the same amino acid composition as copolymer 1 and copolymer 2, but with N^{ϵ}-Z-lysine in place of lysine, in THF assume the conformation of a right-handed α-helix.[3]

From all these facts it seems quite reasonable to conclude that the occurrency of the β conformation for both the examined copolymers has to be related both with the simultaneous presence of phenylalanine and lysine side chains, and with the solvent medium as well.

As pointed out in the beginning of this present paper, the importance of the solvent medium lies on the fact that it may change the relative importance of the different forces which contribute to the overall conformational stability of natural and synthetic polypeptides. In our case the equilibrium between these different forces has been changed in favor of the β structure which becomes stable in alkaline aqueous solution of KCl. It is sufficient to change the salt in order to alter this equilibrium and to have a completely different situation.[19] Furthermore one should remark that while copolymer 1 undergoes the conformational transition at pH >9.5, copolymer 2 apparently is not completely in the randomly coiled form even at acid pH's, and undergoes the transition to a completely ordered β structure at pH >9.0. It seems, therefore, that, in the limits of the examined copolymer compositions, the higher the phenylalanine content of the copolymers, the higher the conformational stability of the β form. Two factors can be invocated in order to explain these facts. First in copolymer 2 two lysine residues are statistically separated by one phenylalanine residue. Therefore destabilizing electrostatic repulsions among charged side chain amino groups are weaker than in the case of copolymer 1. Consequently the β form of copolymer 2 is stable at higher degrees of protonation than the β form of copolymer 1. Second, the aromatic side chains might participate in favorable hydrophobic interactions with the aliphatic portions of lysine side chains, then enhancing the conformational stability of copolymers having high phenylalanine contents.

In light of our data it seems quite unlikely to evaluate or to predict the conformation of synthetic polypeptides and of proteins as well in terms of helix forming or helix destabilizing amino acid residues. We show in fact that in spite of the high conformational stability of the α-helical form of both PLL and PLP, random copolymers containing different proportions of lysine and phenylalanine assume the β conformation.

The data presented in this work are not sufficient to explain in terms of the various contribution interactions why the β form and not the α-helix is the preferred conformation. One main difficulty for a tentative explanation of the findings reported in this paper is that we are dealing with statistically random copolymers prepared *via* the usual method of NCA's copolymerization. In such a way copolymers are obtained which do not possess a rigorous structural order along the chain in the sense that the two amino acids residues are not regularly located along the chain. It could be that this structural disorder is partly responsible for the destabilization of the α-helix in favor of the β form in alkaline aqueous solutions. In this connection it is worth remembering that the β conformation has been found also in other lysine containing copolymers.[20] For this reason conformational studies on sequential copolymers of lysine and phenylalanine are needed.

In addition more details have to be known on the salt effects on peptide conformation. We show in fact that KCl is responsible of a partly ordered structure of copolymer 2 at acid pH values.

Results of further investigations on this area are underway and will be reported elsewhere.

Acknowledgments. The authors thank Professor Mario Mammi, Professor F. Quadrifoglio for the useful discussions and criticism, and Dr. Bruno Filippi for the technical assistance. The financial support from the Institute of Macromolecular Chemistry of C.N.R. (Milan) is gratefully acknowledged.

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