Nuclear Magnetic Resonance and Optical Rotatory Dispersion of L-Alanine Blocks Flanked by Benzyl L-Glutamate, Benzyl L-Aspartate, and N^{ϵ}-Carbobenzoxy-L-lysine

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ABSTRACT: Poly(L-alanine), solubilized by flanking blocks, is studied over the complete range of trifluoroacetic acid–chloroform mixtures. The L-alanine α-CH resonance is a single shifting peak throughout the helix–coil transition, observed at ~4.0 ppm in chloroform and at ~4.6 ppm in trifluoroacetic acid, values similar to those found for poly(γ-benzyl L-glutamate). Changes in b_0 over the transition parallel closely the shift of the α-CH peak for all the copolymers studied; it is concluded that b_0 is a reliable guide to the conformation of poly(L-alanine). Evidence is presented that small quantities of trifluoroacetic acid interact preferentially with the poly(L-alanine) blocks, rather than with the flanking blocks. A specific interaction of trifluoroacetic molecules with the helical form of poly(L-alanine) is proposed.

It is apparent from several publications[1–4] that the helix → coil transition of poly(L-alanine) (PLA) in organic solvent systems is not fully understood. This results largely from the insolubility of the polypeptide in nonpolar solvents such as chloroform, while in mixed solvents with sufficient organic acid to effect dissolution the optical parameters characteristic of the helical conformation are found to indicate a partially helical system. Further, in pure trifluoroacetic acid (TFA) the values of parameters such as b_0 are not those of the fully random-coil conformation, and it is not clear whether in these strong organic acid solutions PLA retains a small helical component. Attempts to resolve these problems have been made by the application of high-resolution nuclear magnetic resonance (nmr) spectroscopy to conformational studies of poly(L-alanine) and poly(D-alanine) (PDA) in $CDCl_3$–acid solvent systems.[1–4] On increasing the $CDCl_3$ proportion of a PLA or PDA solution it was found that the α-CH resonance peak retained its peak area for a large part of the coil → helix transition, as indicated by the optical rotatory dispersion (ORD) parameters, and also shifted upfield. The retention of peak area was attributed to rapid exchange of residues between the helix and coil conformations,[2–4] and the chemical shift behavior of the α-CH peak upfield, which closely followed by b_0 *vs.* solvent composition curve, was consistent with that expected for a partial coil → helix transition.[4] It was not possible in these studies to observe the fully helical state, as the polymer came out of solution at low acid contents. It was concluded[4] that both the α-CH chemical shift behavior and the parallel b_0 behavior were the result of a partial helix → coil transition and that there was no compelling reason to question the validity of the ORD data. This differed somewhat from the conclusions of Mandelkern and coworkers,[2,3] who suggested from their nmr data that no reliance could be placed on b_0 values as a measure of high helix content, and inferred that although the maximum b_0 in TFA–$CDCl_3$ solvent systems indicated a helix content of 54%, the helix content was, in fact, very much higher and that they were observing well-developed resonances for a highly helical polypeptide. More recently, Ferretti and Paolillo[1] have suggested from their nmr data that high molecular weight PLA is almost fully helical in 100% TFA, although the optical parameters of such solutions would indicate a largely random-coil form.

In view of these differences it was thought necessary to examine poly(L-alanine) in the complete range of chloroform–acid solutions and in particular to characterize—by ORD and nmr spectroscopy—the fully helical conformation of this polymer. Three block copolymers were synthesized of the form poly[A(*M*)-*co*-B(*N*)-*co*-A(*M'*)] in order to take poly(L-alanine) into nonpolar solvents. In each copolymer the central block B was of L-alanine (LA), while the flanking block A was one of the following: γ-benzyl L-glutamate (BLG), β-benzyl L-aspartate (BLA), or N^{ϵ}-carbobenzoxy-L-lysine (CLL).

Experimental Section

Nuclear Magnetic Resonance Spectroscopy. The nmr spectra were recorded on a Jeolco NM-4H 100-MHz spectrometer and on the Varian HR 220-MHz spectrometer belonging to the Science Research Council. Spectra were time averaged on a NS-544 Nuclear Instruments computer of average transients interfaced to the 100-MHz spectrometer.

All spectra within a single set were recorded under identical conditions. Chemical shift values were obtained from the reference peak of internal tetramethylsilane (TMS).

Optical Rotatory Dispersion. ORD curves were obtained for the same solutions as used for the nmr studies and were recorded over the range 210–400 mμ on a Bendix Polarmatic 62. Jacketed Opticell fused silica cells of path lengths, 0.1, 0.3, and 3 mm were used. The temperature of the solutions was controlled by circulating water from a Haake thermostated bath, Type F, through the outer jacket of the cell. The curves were analyzed by using the Moffitt and Yang equation to give the parameter b_0. A value of 212 mμ for λ_0 was found to give a linear plot in all cases.

Solvents for Spectroscopy. Trifluoroacetic acid (TFA) was purchased from B.D.H. and distilled three or four times before use. Deuteriochloroform was obtained from Prochem Ltd. and was used without further purification. TMS was obtained from CIBA.

Molecular Weights. For the copolymer [BLG(*M*)-*co*-LA(*N*)-*co*-BLG(*M'*)] the molecular weight of the first block of BLG was determined from the viscosity of a dichloroacetic acid (DCA) solution (using an Ostwald viscometer) by applying the molecular weight calibration of Doty, Bradbury, and Holtzer.[5] The average size of the L-alanine block was then obtained from the nmr spectrum in TFA of the BLG–LA block copolymer by comparing the inte-

(1) J. A. Ferretti and L. Paolillo, *Biopolymers*, **2**, 7 (1969).
(2) W. E. Stewart, L. Mandelkern, and R. E. Glick, *Biochemistry*, **6**, 143 (1967).
(3) R. E. Glick, L. Mandelkern, and W. E. Stewart, *Biochim. Biophys. Acta*, **120**, 302 (1966).
(4) E. M. Bradbury and H. W. E. Rattle, *Polymer*, **2**, 9 (1968).
(5) P. Doty, J. H. Bradbury, and A. M. Holtzer, *J. Amer. Chem. Soc.* **78**, 947 (1956).

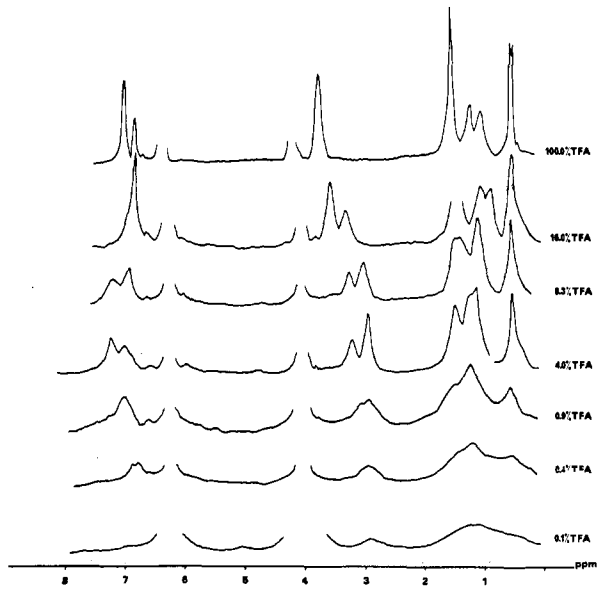

Figure 1. Spectra (220 MHz) in $CDCl_3$–TFA of block poly[benzyl L-glutamate(39)-*co*-L-alanine(46)-*co*-benzyl L-glutamate(33)].

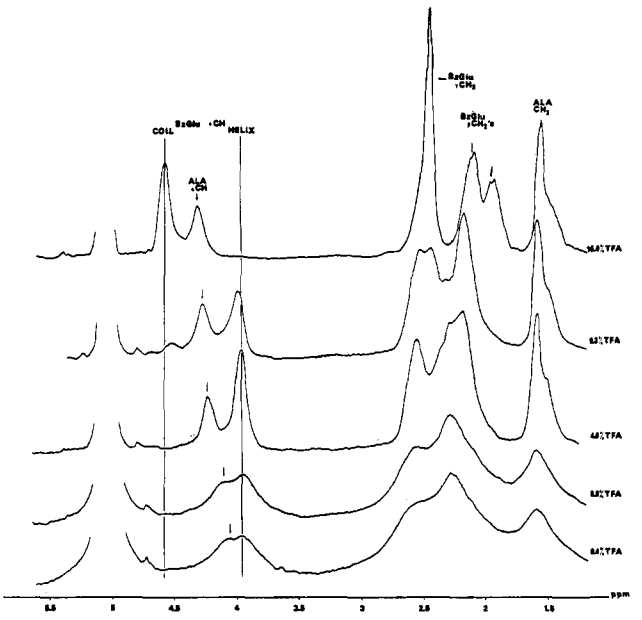

Figure 2. Expanded 220-MHz spectra in $CDCl_3$–TFA of block poly[benzyl L-glutamate(39)-*co*-L-alanine(46)-*co*-benzyl L-glutamate(33)].

grated area of the alanine CH_3 peak with that of the γ-CH_2 peak of BLG. The average size of the third BLG block was then found from the nmr spectrum of the complete copolymer by again comparing the integrated area of the γ-CH_2 peak of BLG to that of the CH_3 of alanine. For the block copolymers BLA(*M*)-*co*-LA(*N*)-*co*-BLA(*M'*), the same procedure was followed by assuming that the viscosity against molecular weight curve of PBLG in DCA could be used to obtain an estimate of the size of the first block of BLA. For the CLL(*M*)-*co*-LA(*N*)-*co*-CLL(*M'*) copolymers, the viscosity calibration was not considered accurate and thus only the relative sizes of the blocks were obtained, from the nmr spectra in TFA.

Preparation of Block Copolymers. (a) Poly[BLG(*M*)-*co*-LA(*N*)-*co*-BLG(*M'*)]. Polymerization of the first block of PBLG was achieved by initiation of the *N*-carboxyanhydride (NCA) in vacuum-distilled dimethylformamide (DMF) using *n*-hexylamine as the initiator.[6] The initiator to anhydride ratio was selected to give a M_w of 7000–10,000, although originally a M_w of 22,000 was intended. With the latter molecular weight, however, it was found that the resonance peaks in the nmr spectrum were broadened sufficiently to impair the spectral analysis. Two methods were used to follow the polymerization reaction, which was completed 4–7 days after the initiator was added. First, titration against Na–methanol using Bromothymol Blue as an indicator was tried but abandoned as being too inaccurate. Second, a weighing technique was applied together with the use of nmr spectroscopy. A small sample of the polymer was precipitated from the main batch and weighed after vacuum drying; as an extra check the polymer was dissolved in a known quantity of TFA and the peak height of the aromatic resonance under standard conditions was compared with the time of polymerization. This weighing method was found to be accurate and was employed on all first-block polymerizations. A small sample was also taken for molecular weight determination of the first block. On completion of the BLG polymerization, the solution was filtered and the L-alanine NCA added through the filter. The polymerization rate of the L-alanine depended considerably on the age of the NCA and the time to 80% completion varied from 1.5 to 24 hr. A total polymerization time of 4 days was therefore allowed for the alanine polymerization, achieving 95–100% completion. It was found that when the L-alanine block reached a length much in excess of the first BLG block, the copolymer precipitated from solution. Such material, comprising perhaps 10% of the total, was removed by centrifugation. The nmr spectrum in TFA was used to determine the rate of the polymerization of the L-alanine block by comparing the areas of the γ-CH_2 resonance peak of PBLG with that of the CH_3 resonance peak of PLA, the CH_3 reaching almost a constant value after 36 hr. When completion of the L-alanine block was achieved, BLG NCA was added and the completion of the third block was achieved in 4–7 days. All polymerizations were carried out at a concentration of 20 mg/ml at 20° in the dark. The polymer was precipitated by addition of absolute alcohol and washed three or four times with alcohol. It was found that vacuum drying did not remove DMF completely from the polymer and additional washing (three times) in distilled water was employed. The polymer was then dried under vacuum for 48 hr.

Two block copolymers were also synthesized by dissolving a homopoly(γ-benzyl L-glutamate) in dioxane and adding the NCA of L-alanine with diethylamine as initiator. This was left for 4 days to polymerize. When the L-alanine block became too large the copolymer precipitated from solution and was removed by filtration. The copolymer left in solution was precipitated with ethanol and dried.

(b) Poly[BLA(*M*)-*co*-LA(*N*)-*co*-BLA(*M'*)]. The techniques used in determining completion and purification of samples were identical with those of the previous case. It was found that DMF was not a suitable solvent owing to considerable precipitation of the copolymer at the second stage, and a 1:2 mixture of 1,2-dioxane and 1,2-dichloroethane was used. The dioxane was prepared by refluxing for 48 hr with sodium pellets in the solution and then distilling three times in the presence of fresh sodium wire in each case. 1,2-Dichloroethane was prepared by allowing Analar grade to stand over P_2O_5 for 48 hr and then distilled after decanting. All processes were the same as for the copolymers with γ-benzyl L-glutamate except that the block copolymer was washed five times in ethanol.

(c) Poly[CLL(*M*)-*co*-LA(*N*)-*co*-CLL(*M'*)]. These samples were prepared exactly the same as in the case of (a), and all products were dried under vacuum to give a pure white powder.

Results

Block Copolymers Poly[BLG(39)-*co*-LA(46)-*co*-BLG(33)] and Poly[BLG(100)-*co*-LA(70)-*co*-BLG(40)]. Both block copolymers were found to be soluble in $CDCl_3$, and the conformational transitions on addition of TFA were followed by using both ORD and nmr. Figure 1 gives the complete

(6) C. H. Bamford, A. Elliott, and W. E. Hanby, "Synthetic Polypeptides," Academic Press, New York, N. Y., 1956.

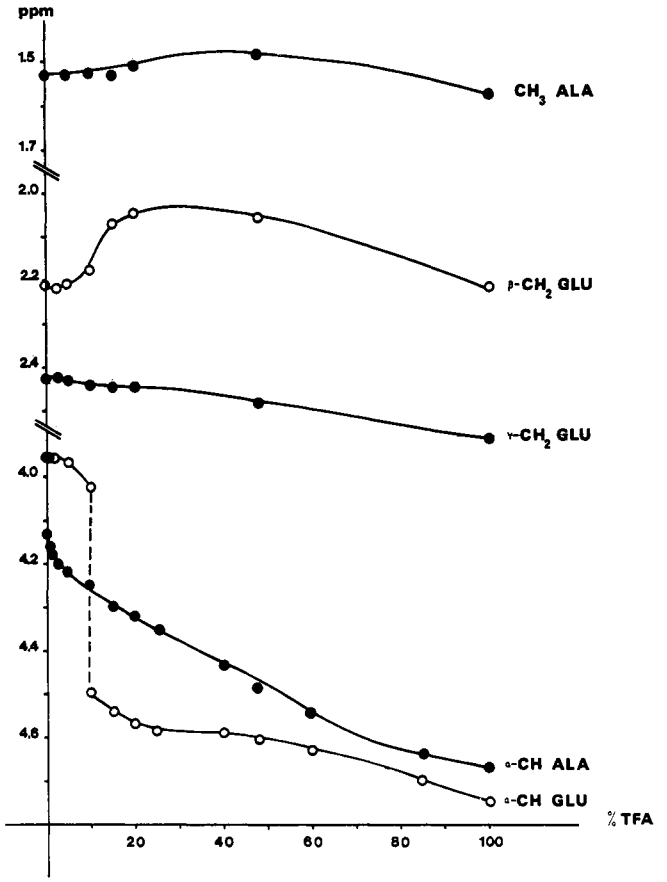

Figure 3. Nmr peak positions in $CDCl_3$–TFA of block poly[benzyl L-glutamate(39)-*co*-L-alanine(46)-*co*-benzyl L-glutamate(33)].

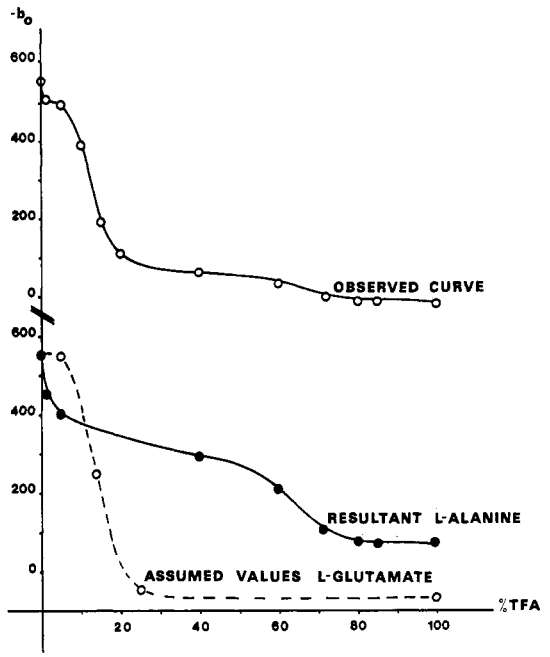

Figure 4. b_0 values in $CDCl_3$–TFA for block poly[benzyl L-glutamate(39)-*co*-L-alanine(46)-*co*-L-glutamate(33)].

spectral changes for the smaller of the two polymers, and the displacement of the α-CH peaks can be seen more closely in the expanded spectra of Figure 2. It can be seen that the α-CH peaks of the LA and BLG components overlap in $CDCl_3$, indicating very similar chemical shift values for the helical conformation in this solvent. On addition of small amounts of TFA, the composite α-CH peak becomes progressively more asymmetrical, and from the relative areas of the components it can be presumed that it is the α-CH peak of the alanine block which is principally affected by the interaction of small amounts of TFA. The α-CH peak of the benzyl L-glutamate blocks remains unchanged until 8–10% TFA is added in the same way as observed for PBLG homopolymers. In this TFA range two peaks are observed for the α-CH resonance of the benzyl L-glutamate blocks, one at 3.95 ppm, characteristic of the helical form of BLG, and the other at 4.55 ppm, the chemical shift of the random-coil form. By 12% TFA the transition is complete and a single α-CH peak is observed for the random-coil form of the benzyl L-glutamate. The helix → coil transition of the blocks can also be followed from the behavior of the NH resonance peak. In pure $CDCl_3$ and 0.1% TFA, the NH peaks from both blocks overlap to give a very broad peak centered at 8.2 ppm. On the addition of up to 1% TFA the acid OH peak obscures the NH peaks. At 1.3% TFA the NH peaks overlap to form a broad peak centered at 8.21 ppm with an upfield asymmetry at approximately 8.15 ppm. With further TFA the composite peak becomes increasingly asymmetrical, with the upfield component moving further upfield. At 5% TFA there are two distinct components, the glutamate NH peak at 8.21 ppm and the upfield peak at 8.03 ppm. The behavior of the upfield component parallels that of the alanine α-CH and is therefore probably the alanine amide NH. At 10% TFA the glutamate blocks have undergone a partial helix → coil transition and the NH peak for the random component is at 7.92 ppm. At 15% TFA the glutamate blocks are fully random with an NH at 7.87 ppm; the alanine block appears now to give an asymmetrical peak at about 7.97 ppm. The behavior of the NH peaks from the glutamate and alanine blocks is identical with that of the α-CH peaks. The helix → coil transition of the glutamate blocks can also be followed from the behavior of the β-CH_2 resonance peak, which shifts from 2.25 ppm at 10% TFA (helix) to two peaks centered at 2.05 ppm at 16% TFA (coil).

In Figures 1 and 2 the L-alanine CH_3 peak is seen to have an upfield asymmetry. Studies of random copolymers of L-alanine with BLG show that the position of the alanine CH_3 peak is sensitive to the presence of BLG residues; in copolymers of low alanine content the CH_3 is found upfield of the CH_3 peak of poly(L-alanine) under the same solvent conditions. The second component of the alanine CH_3 peak in the spectrum of the block copolymer is thought therefore to result from those alanine residues which overlap into the BLG flanking blocks. Similar behavior is found for the block copolymer of L-alanine with benzyl L-aspartate, and these observations are discussed in more detail in the next section.

The chemical shifts of the α-CH, β-CH_2, and the γ-CH_2 of BLG and the α-CH and CH_3 of L-alanine plotted against TFA content are given in Figure 3, while the b_0 values are given in Figure 4. The b_0 value for the copolymer in pure $CDCl_3$ is −550°, a somewhat lower value than for a fully helical polypeptide of high molecular weight, for which b_0 values are of the order of −630°. The lower value observed for the block copolymer is probably a result of the relatively small size of the blocks. On addition of TFA there is an initial sharp transition in the b_0 value to −500° at 2% TFA, which is parallel to the downfield shift seen for the α-CH and NH resonance peaks of the alanine block. At 10% TFA there is a second transition in the b_0 curve from −475 to −110°, which parallels helix → coil transition seen in the α-CH and

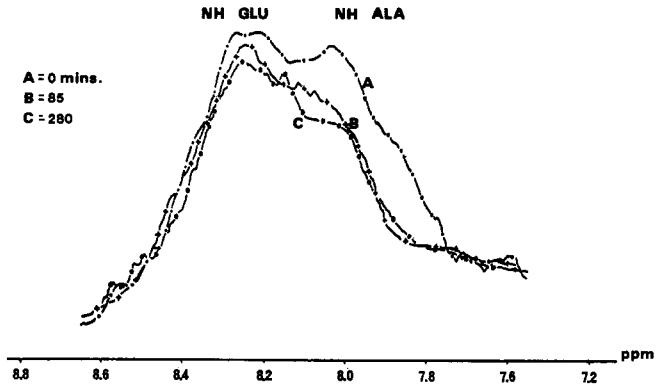

Figure 5. Deuteration in 5% TFA–95% $CDCl_3$ of block poly-[benzyl L-glutamate(39)-*co*-L-alanine(46)-*co*-benzyl L-glutamate(33)].

NH peaks of the benzyl L-glutamate blocks. At higher TFA contents, the b_0 curve is very similar to that found for poly(L-alanine) homopolymer. Also shown in Figure 4 as a broken line is the b_0 curve for PBLG of similar size to the flanking blocks. These values have been subtracted from the b_0 curve of the block copolymer to give an approximate transition for the L-alanine block. Comparison with Figure 3 shows this to be very similar to the curve obtained for the chemical shift of the L-alanine α-CH.

The behavior of the block copolymer poly[BLG(100)-*co*-LA(78)-*co*-BLG(60)] was found to be essentially identical with that of the smaller polymer. In pure $CDCl_3$ the b_0 was found to be $-700°$, and this higher value may result from the greater molecular weight of each block. On addition of small amounts of TFA there was a sharp transition in the b_0 value to $-500°$ in 2.5% TFA–$CDCl_3$. A similar sharp transition took place in the chemical shifts of the L-alanine NH and α-CH over the same range of TFA contents.

Both block copolymers show a sharp transition in b_0 values for small additions of TFA up to 5% which is accompanied by a parallel change in the chemical shifts of the α-CH and NH resonance peaks of the L-alanine residues only. The chemical shifts of the benzyl L-glutamate main-chain protons behave in a manner similar to those of the homopolymer and remain constant over this TFA range transition. It seems reasonable, therefore, to attribute the initial transition in the b_0 values to the interaction of TFA with the alanine blocks. The question then arises as to whether this interaction results in a destabilization of the L-alanine helix. An indication that this may be the case is obtained from the deuteration behavior of the block copolymer. It is known that in a stable α helix of poly(γ-benzyl L-glutamate) the amide NH is resistant to deuterium exchange.[7] As can be seen in Figure 1, the initial transitions are completed by 5% TFA and from the α-CH chemical shift the glutamate blocks are still fully helical. The changes in the NH resonance on exposure of the block copolymer to 5% O-deuterated TFA are shown in Figure 5. It can be seen that whereas the amide NH of benzyl L-glutamate is highly resistant to deuterium exchange, a large proportion of the alanine NH protons have exchanged over the same period. Clearly the interaction of 5% TFA-*d* much reduces the stability of the alanine block compared to the glutamate block.

Block Copolymers Poly[BLA(40)-*co*-LA(44)-*co*-BLA(36)] and [BLA(22%)-*co*-LA(35%)-*co*-BLA(43%)]. A major disadvantage in studying the behavior of L-alanine in blocks flanked by benzyl L-glutamate is that their NH and α-CH resonances overlap in both the helical and random-coil forms, and furthermore, when the glutamate of the flanking blocks undergoes a helix → coil transition the resonance peaks from its backbone protons cross those of the alanine block. Since it is known that the chemical shift of the α-CH of poly(β-benzyl L-aspartate) is about 0.5 ppm downfield from that of poly(L-alanine) for both the helical and random-coil forms,[8] the use of this residue as a flanking block would allow observation of the behavior of the alanine α-CH resonance peak over the complete range of solvents. Care has to be taken, however, in analyzing the results, because benzyl L-aspartate polymers form left-handed α helices of low stability.[7,9] Also, on account of the low stability of the aspartate helix the introduction of small amounts of L-alanine can cause a reversal of helix sense to the right-handed form.[9] The chemical shift of the aspartate amide NH and α-CH resonances have been correlated with helix sense; the α-CH resonance peak is at 4.40 ppm for the right-handed (RH) α helix and at 4.30 ppm for the left-handed (LH) form. The NH peak is at 8.3 ppm for the RH helix and at 8.70 ppm for the LH helix.[10] The LH poly(β-benzyl L-aspartate) has been found to give a b_0 of *ca.* $+630°$, while for the RH form as a copolymer b_0 is *ca.* $-630°$.[11]

Examination of the first block isolated as the pure poly(β-benzyl L-aspartate) showed that its b_0 was $+480°$, the value expected for a polymer of molecular weight corresponding to a DP of 40.[7] The chemical shifts observed were NH, 8.8; C_6H_5, 7.2; benzyl CH_2, a quartet centered at 5.08; α-CH, 4.28; and β-CH_2, a multiplet centered at 2.9 ppm. These values are characteristic of the LH form of the α helix of poly(L-aspartate esters).[10] The b_0 for the block copolymer BLA(40)-*co*-LA(44)-*co*-BLA(36) was found to be $-550°$ in $CDCl_3$. If the flanking blocks were fully left handed and the central alanine block were right handed, the expected b_0 value would be about $+200°$. The differences in these values must be attributed to the influence of the central alanine block in partially reversing the helix sense of the first aspartate block and of the overlap of L-alanine residues into the second flanking block, causing this to be in the fully right-handed α-helical conformation. This is supported by the chemical shifts of the aspartate main-chain protons in the spectrum of the block copolymer. The chemical shift values correspond to the RH α-helical form of benzyl L-aspartate together with indications, particularly for the amide NH resonance, of a component of benzyl L-aspartate in the LH conformation.

The changes in the 220-MHz spectra of the block copolymer are shown in Figure 6. In deuteriochloroform the peaks are broadened by the effects of aggregation. It can be seen that the NH resonances are more extended to low field than those of the α-CH proton, and it is thought that this is due to part of the first benzyl L-aspartate block being in the left-handed α-helical conformation, giving a broadened NH resonance at 8.70 ppm. On the addition of 0.3% TFA, the aggregation is largely broken down and the helical structure quite unaffected: the chemical shift of the aspartate α-CH is then at 4.40 ppm, characteristic of the RH helical form.

(7) E. M. Bradbury, A. R. Downie, A. Elliott, and W. E. Hanby, *Proc. Roy. Soc., Ser. A*, **259**, 110 (1960).

(8) E. M. Bradbury, B. G. Carpenter, C. Crane-Robinson, and H. W. E. Rattle, *Nature (London)*, **220**, 69 (1968).

(9) L. Paolillo, P. Temussi, E. Trivellone, E. M. Bradbury, and C. Crane-Robinson, *Biopolymers*, in press.

(10) E. M. Bradbury, B. G. Carpenter, C. Crane-Robinson, and H. Goldman, *Macromolecules*, in press.

(11) E. M. Bradbury, B. G. Carpenter, and H. Goldman, *Biopolymers*, **6**, 837 (1968).

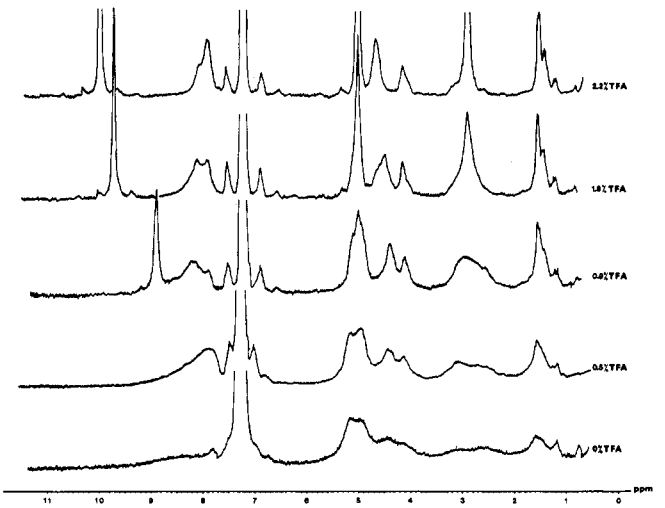

Figure 6. Spectra (220 MHz) of block poly[benzyl L-aspartate(40)-*co*-L-alanine(44)-*co*-benzyl L-aspartate(36)] in $CDCl_3$–TFA.

The presence of the alanine α-CH peak at 4.10 ppm partially obscures the upfield side of the aspartate α-CH peak, and it cannot be established with certainty whether or not there is a small peak at approximately 4.30 ppm which would confirm the presence of a LH helical component in the aspartate blocks. It is clear, however, that the aspartate blocks are largely in the RH α-helix form. The side-chain methylene groups, the β-CH_2 and the benzyl CH_2, both give multiplets thought to arise from some restriction of the side chain in the helical conformation.[10,12] On addition of more TFA the aspartate flanking blocks undergo a helix → coil transition which is shown by the behavior of the aspartate resonance peaks; the amide NH and α-CH peaks undergo transition from a chemical shift value characteristic of the RH α-helical form to that characteristic of the random-coil form, while the multiplets of the methylene group resonances undergo transition to single sharp peaks. The midpoint of the transition is close to 1.5% TFA, and the spectrum obtained at this TFA shows an α-CH peak with two components at 4.64 and 4.53 ppm. These are intermediate between the values for the RH helical and random-coil forms of 4.40 and 4.80 ppm, respectively.

Multicomponent α-CH resonances for partially helical polypeptides are now thought to be a manifestation of polydispersity of the sample[13] and have been discussed in detail for poly(γ-benzyl L-glutamate).[14] It is found that as the polydispersity is reduced the separation of the components of the α-CH peak at half-helicity is also reduced. For a monodisperse sample a single peak at an intermediate position between the values characteristic of the helix and random coil should be observed for a partially helical polymer. The small separation of the two α-CH components observed for the aspartate blocks could result from low polydispersity. It could also be due to the fact that the two flanking blocks are not identical in length and may have somewhat different transition midpoints.

The behavior of the L-alanine block is different from that of the flanking blocks in that there is not a complete transition to the random-coil form. Addition of small amounts of

(12) E. M. Bradbury, B. G. Carpenter, C. Crane-Robinson, and H. Goldman, *Nature (London)*, **225**, 64 (1970).
(13) R. Ullman, *Biopolymers*, **9**, 471 (1970).
(14) E. M. Bradbury, C. Crane-Robinson, and H. W. E. Rattle, *Polymer*, **11**, 277 (1970).

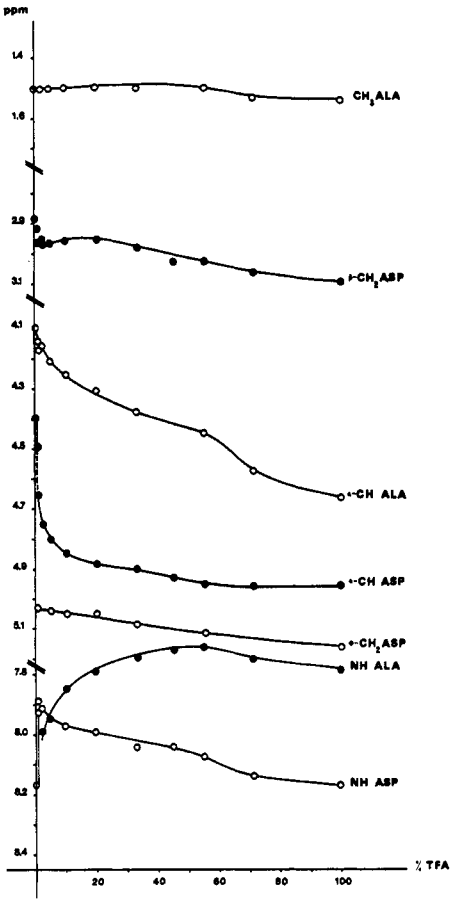

Figure 7. Nmr peak positions in $CDCl_3$–TFA of block poly[benzyl L-aspartate(40)-*co*-L-alanine(44)-*co*-benzyl L-aspartate(36)].

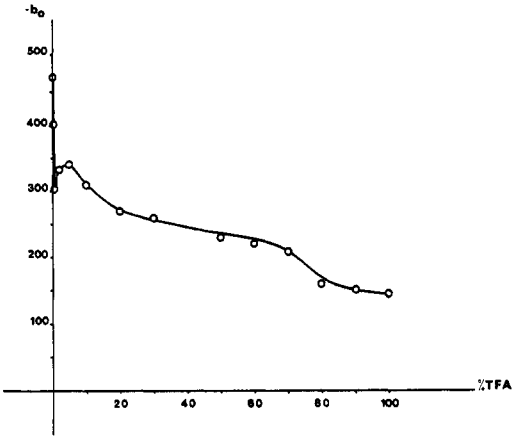

Figure 8. b_0 values in $CDCl_3$–TFA for block poly[benzyl L-aspartate(40)-*co*-L-alanine(44)-*co*-benzyl L-aspartate(36)].

TFA causes a marked shift in the position of the α-CH peak to lower fields and a shift upfield of the amide NH peak, though the displacement of the latter is somewhat obscured by the behavior of the aspartate NH peak. As the TFA content is increased the alanine CH_3 peak is seen to have two components, the main peak at 1.53 ppm and a secondary peak at 1.4 ppm. Similar behavior was found for the BLG–LA–BLG blocks, and this feature will be discussed later.

The dependence of the chemical shifts on TFA content for the resonance peaks of all the proton groups (except C_6H_5) is shown in Figure 7. The b_0 curve in Figure 8 shows a complex shape over the range 0–5% TFA: there is a fall in the

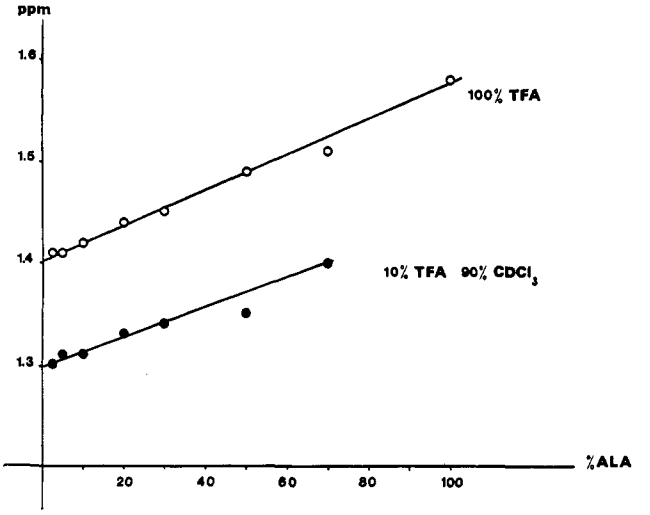

Figure 9. Alanine CH_3 peak positions in $CDCl_3$–TFA of random copolymers of benzyl L-aspartate and L-alanine.

b_0 from −500° in $CDCl_3$ to −300° in 1% TFA–$CDCl_3$ and then a gradual rise to −340° at 5% TFA. For higher TFA contents there is a gradual diminution in b_0 with a small transition from −220 to −160° between 60 and 80% TFA. The observation of a minimum in b_0 at about 1% TFA is unusual, and the shape of the curve cannot simply be a consequence of some of the polymer undergoing a RH helix → coil transition. It appears that a certain proportion of the L-aspartate undergoes transition to the LH form before becoming random coil in the 1–3% TFA region. The helix → coil transition of the L-aspartate blocks is also seen in the nmr spectrum. Since the conformational changes of the L-aspartate blocks are complex, no attempt has been made to separate the b_0 curve of Figure 8 into its contributions from the separate blocks as was possible with the L-glutamate copolymers in Figure 4.

The chemical shift of the alanine α-CH peak (Figure 7) is clearly observable over the complete range of solvent mixtures and shows an initial sharp downfield transition on the addition of up to 5% TFA. Although more difficult to estimate due to peak overlap, the alanine NH peak also undergoes a sharp upfield transition. From 10 to 55% TFA there are more gradual changes in the chemical shift values which follow the gradual change in b_0. From 55 to 80% TFA a small transition in b_0 is accompanied by transitions in the chemical shifts of the alanine backbone protons. This is significant, since above 10% TFA the aspartate blocks are fully random coil and changes in b_0 can be attributed to the alanine block with certainty.

The L-alanine CH_3 peak at 1.5 ppm shows a second component approximately 0.1 ppm to higher fields. This was originally thought to be an impurity. However, the compound resisted all attempts at purification, and it was eventually concluded that this peak resulted from the block copolymer and could be assigned to L-alanine CH_3 groups in a different environment from that of the CH_3 groups in the central alanine block. The method of synthesis of the block copolymer makes it possible for L-aspartate residues from the first block to overlap into the second L-alanine block and for L-alanine residues to be included in the second L-aspartate block. The observation that the L-aspartate blocks in $CDCl_3$ are largely in the RH and not the LH helical conformation confirms this as regards the second aspartate block. Some L-alanine residues will therefore be flanked by L-aspartates and be in a different environment than the bulk of the L-alanine residues in the central block. To confirm whether or not the chemical shift of the L-alanine CH_3 was dependent on its nearest neighbors, a series of random poly[L-alanine(x%)-*co*-benzyl L-aspartate(100 − x)%] was examined at 100 MHz, and the chemical shift values obtained are plotted in Figure 9. It is clear that when the L-alanine is largely random coil (100% TFA) and also when it is partly helical (10% TFA) incorporation of L-aspartate residues into poly(L-alanine) causes an upfield shift of the CH_3 resonance. It is concluded therefore that this upfield shift of the CH_3 peak is primarily a nearest-neighbor rather than a conformational effect. Thus the main CH_3 peak in the block copolymer spectrum at 1.5 ppm can be assigned to L-alanine residues in the central block alone, while the smaller upfield peak at 1.4 ppm is due to L-alanine residues adjacent to L-aspartate residues in either the central L-alanine block or the second L-aspartate block.

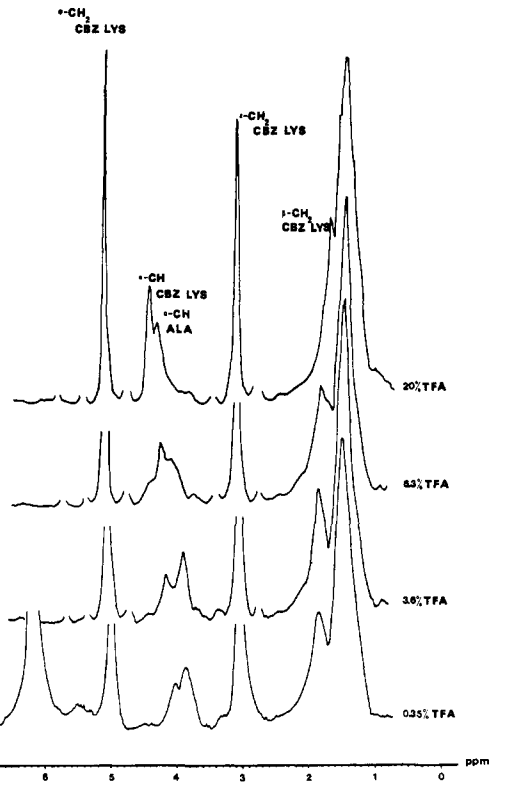

Figure 10. Spectra (220 MHz) of block poly[εCbz-L-lysine(32%) *co*-L-alanine(48%)-*co*-εCbz-L-lysine(20%)] in $CDCl_3$–TFA.

A second higher molecular weight block copolymer, poly-[benzyl L-aspartate(x)-*co*-L-alanine(1.6x)-*co*-benzyl L-aspartate(2.0x)], where $x \approx 200$, showed behavior similar to that observed for the smaller copolymer. On account of the higher molecular weight of the blocks, the transitions in chemical shifts and b_0 values occurred at slightly higher TFA contents.

Block Poly[CLL(32%)-*co*-LA(48%)-*co*-CLL(20%)]. In studies of the block copolymer described in the last section the complicated behavior of the benzyl L-aspartate end blocks prevented a comparison of the chemical shift behavior of the α-CH of the L-alanine block with the ORD parameters in solvent mixtures containing small proportions of TFA. To confirm the observation made in the first section, using the block copolymer of L-alanine flanked with benzyl L-glutamate, that at very low TFA contents a transition in the chemical shift values of the alanine α-CH is paralleled by a transition in b_0, a third block copolymer was prepared. This was block

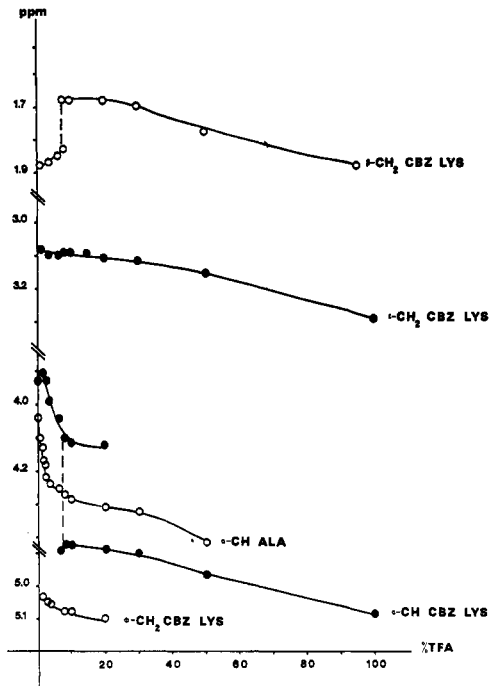

Figure 11. Nmr peak positions in $CDCl_3$–TFA of block poly[εCbz-L-lysine(32%)-*co*-L-alanine(48%)-*co*-εCbz-L-lysine(20%)].

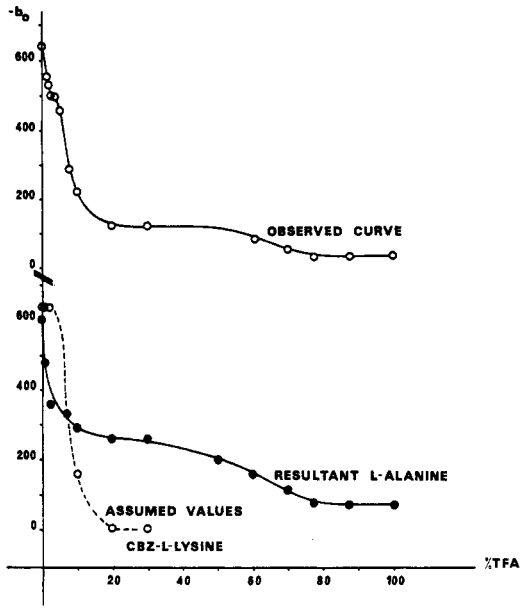

Figure 12. b_0 values in $CDCl_3$–TFA for block poly[εCbz-L-lysine(32%)-*co*-L-alanine(48%)-*co*-εCbz-L-lysine(20%)].

poly[N^{ϵ}-carbobenzoxy-L-lysine(32%)-*co*-L-alanine(48%)-*co*-N^{ϵ}-carbobenzoxy-L-lysine(20%)]. The α-CH chemical shift of helical ε-carbobenzoxy-L-lysine is slightly upfield of that of benzyl L-glutamate, which represents a slight advantage.

Since spectra of poly(N^{ϵ}-carbobenzoxy-L-lysine) have not been presented before, the spectral changes observed for the block copolymer are shown in Figure 10. The peak assignments are given in the figure and the chemical shift of the alanine α-CH is 4.08 ppm, in agreement with the results from the other copolymers. The α-CH resonance of the flanking ε-carbobenzoxy-L-lysine flanking blocks is at 3.90 ppm and is upfield of that of the alanine component. The transitions caused by the addition of TFA can be followed from the displacement of the backbone resonance peaks of both blocks as well as from the behavior of side-chain resonances, *e.g.*, the β-CH_2 resonance of the N^{ϵ}-carbobenzoxy-L-lysine. On addition of small amounts of TFA, the α-CH of the alanine block moves sharply downfield, while there is a less pronounced shift of the α-CH peak of the flanking blocks. Between 5 and 10% TFA the N^{ϵ}-carbobenzoxy-L-lysine flanking blocks undergo a helix → coil transition which can be seen first by a broadening of the α-CH and then by the emergence of a peak characteristic of the random-coil conformation to lowfield of the alanine α-CH at 4.4 ppm, which increases in peak intensity with additional TFA while the helix peak diminishes in intensity. The α-CH of the L-alanine is observed throughout the transition, and it appears to remain as a single peak, although small asymmetries would not be observed because of the proximity of the helix and coil α-CH peaks of the ε-carbobenzoxy-L-lysine. An interesting observation is that the β-CH_2 peak also shows evidence of double peak behavior on going through the helix → coil transition.

The chemical shifts of the L-alanine peaks and of several of the peaks of the flanking blocks are shown in Figure 11. In conjunction with this, Figure 12 gives the variation of b_0 with TFA content. The b_0 value of the block copolymer in $CDCl_3$ is −620°. Addition of up to 3.6% TFA causes a change in b_0 from −620 to −500°, and this is accompanied by a sharp transition in the chemical shift of the alanine α-CH from 4.08 to 4.2 ppm. Over this range of TFA content, the shift of the CLL α-CH is very much smaller. Between 5 and 20% TFA the flanking blocks undergo a helix → coil transition, as shown both by the behavior of the α-CH and β-CH_2 peaks of the CLL residues and by the transition of b_0 from −495 to −125°. Also included in this change of b_0 would be a contribution from the L-alanine block undergoing conformational changes in the 5–20% TFA range.

As it is known that poly(N^{ϵ}-carbobenzoxy-L-lysine) undergoes a normal helix → coil transition following a sigmoidal curve between b_0 values of −630 and 0°, it is possible by using data from the nmr studies to analyze the b_0 curve of the block copolymer into contributions from the L-alanine and N^{ϵ}-carbobenzoxy-L-lysine components. The nmr spectra show that the helix → coil transition of the N^{ϵ}-carbobenzoxy-L-lysine flanking blocks begins at 2.5% TFA, has its midpoint at 7.5% TFA, and is largely complete by 20% TFA. Knowing the composition of the polymer it is possible to make a rough estimate of the transition of the poly(N^{ϵ}-carbobenzoxy-L-lysine) flanking blocks, and by subtraction from the b_0 curve of the block copolymer obtain an approximation to the shape of the optical transition of the poly(L-alanine) central block. This is also shown in Figure 12. Bearing in mind that nmr data on the transition of the L-alanine block have not been utilized, it is striking that the shape of the b_0 curve obtained for the L-alanine block is identical with the chemical shift behavior of the L-alanine α-CH peak up to 50% TFA (beyond which it is overlapped by the α-CH of the CLL residues).

Discussion

The use of block copolymers with L-alanine as a central block flanked by (i) γ-benzyl L-glutamate, (ii) β-benzyl L-aspartate, and (iii) N^{ϵ}-carbobenzoxy-L-lysine has allowed a combined nmr and ORD study of poly(L-alanine) through the complete range of $CDCl_3$–TFA mixtures. The power of the nmr technique is that it allows each proton group in the block copolymer to report on its environment and hence its conformation. Thus it is possible to follow the conformational

transition of the different blocks in the copolymer and to obtain, for example, the starting point, midpoint, and end point of the transition of the flanking blocks. Since the behavior of homopolymers of residues such as γ-benzyl L-glutamate and N^{ϵ}-carbobenzoxy-L-lysine is well understood, these initial nmr data allow the contributions to the overall optical titration curve to be roughly estimated, and by subtraction the optical titration curve of the poly(L-alanine) block could be obtained over the complete range of $CDCl_3$–TFA mixtures. The transitions of the poly(L-alanine) blocks could also be followed from the chemical shift behavior of the α-CH peak on the addition of TFA: the curves obtained for the poly(L-alanine) block in the three copolymers are very similar, each showing a sharp downfield displacement of the α-CH on the addition of up to 2.5% TFA, followed by a more gradual change, a plateau, and lastly a falloff to the final chemical shift value. Moreover, the range of L-alanine α-CH shift values differs little from that found for other RH poly(L-amino acids) such as PBLG, *viz.*, helical α-CH at low TFA content, ~4.0 ppm; coil α-CH in pure TFA, ~4.6 ppm. In the case of the L-alanine blocks flanked by γ-benzyl L-glutamate and N^{ϵ}-carbobenzoxy-L-lysine, it is striking that the shift behavior of the L-alanine α-CH peaks is identical in all features with the estimated optical titration curve. The close similarity of these transition curves from the quite different techniques of nmr and ORD leads to the not unreasonable suggestion that both techniques are recording the same conformational changes and interactions of the poly(L-alanine) block. These observations, therefore, do not lend support to the proposals, based on nmr studies, that in the case of poly(L-alanine) the ORD parameter b_0 is a poor guide to the conformation of the polymer.[2]

The shapes of the ORD and nmr transition curves of poly(L-alanine) are very interesting and quite different from those of homopolymers such as poly(γ-benzyl L-glutamate), which exhibit the sigmoidal shape of a cooperative transition. The poly(L-alanine) curve shows discontinuities which probably indicate the presence of intermediate steps in the transition. Somewhat similar curves have been found for poly(L-α-amino-*N*-butyric acid),[15] poly(L-leucine), and poly(L-methionine).[16] For these polymers, however, the α-CH peak is usually multiple and not single as found for poly(L-alanine).[17–19] On the addition of *small* amounts of TFA to the block copolymers, it is clear that interactions occur specifically with the L-alanine block and not with the flanking blocks. The interactions cause a sharp transition in both the ORD parameter b_0 and the α-CH and NH chemical shifts of the L-alanine residues. The observation of a single resonance peak for both the α-CH peak and a single TFA OH peak throughout this initial transition shows that the TFA molecules are exchanging rapidly between their interacted and free states.

Two theories have been presented for the interaction of haloacetic acids with polypeptides leading to the helix → coil transition. The first is that strong hydrogen bonding occurs between TFA molecules and the polypeptide, and it is competition for the hydrogen-bonding sites on the peptide residues which causes a breakdown of the helix.[2,20–22] The second proposal put forward by Klotz, Hanlon, and coworkers,[23–25] is that in TFA–chlorinated hydrocarbon mixtures, protonation of amide groups in poly(γ-benzyl L-glutamate), poly(L-alanine), and poly(L-leucine) occurs at very low acid contents, the number of protonated groups increasing with increasing proportion of the acid component, until at high acid content the polymer is thought to be fully protonated. At acid contents lower than those required for the classical helix → coil transition, as monitored by ORD or hydrodynamic techniques, it is suggested that the density of charge is sufficiently high that helix disruption has already taken place and that the transition observed by the optical spectroscopic techniques "reflects the transformation of the protonated polypeptide chain to a form which is partially hydrogen bonded to the organic acid." Hanlon[25] has further studied poly(γ-benzyl L-glutamate), poly(L-alanine), and poly(L-methionine) in ethylene dichloride–dichloracetic acid (DCA) mixtures. Again it is proposed from near-infrared spectroscopic studies that all three polypeptides are protonated by the acid; poly(L-alanine), it is suggested, is largely protonated at only 6% DCA, poly(L-methionine) shows an increase in protonation throughout the range of DCA contents until it is largely protonated at high DCA concentration, and poly(γ-benzyl L-glutamate) is thought to show two stages of protonation, one at low acid contents and the other between 75 and 80% DCA. At acid contents lower than those required for the helix → coil transition of poly(γ-benzyl L-glutamate), as indicated by ORD and hydrodynamic studies, the polymer is thought to be 50% protonated. In all of these studies the random-coil form of the polypeptides is thought to be largely in the protonated form hydrogen bonded to the acid. There is considerable disagreement with this general thesis that haloacetic acids extensively protonate polypeptides in mixed solvent systems. Mandelkern and coworkers[2,3] have made nmr studies of poly(L- and DL-alanine), of *N*-methylacetamide, and of *N*,*N*-dimethylacetamide in TFA–$CDCl_3$ mixtures and conclude that whereas protonation of the model compounds occurs there is no protonation of the polypeptides. They favor the first mechanism given above, that competition of the acid for the intrachain peptide hydrogen bonds results in their rupture and solvation of the random-coil form. Quadrifoglio and Urry[26] found that in 10% TFA–90% chloroform and 30% DCA–70% chloroform the amplitude of the CD minimum at 222 mμ of the helical form of poly(γ-benzyl L-glutamate) was unchanged compared to the chloroform solution and concluded that protonation of the polymer did not occur at these acid concentrations. Steigman, *et al.*,[27] have studied a diamide, *N*-benzoylglycine-*n*-propylamide, in DCA–$CDCl_3$ and concluded that "neither amide was protonated in DCA solution, and that as a consequence polypeptides like poly(γ-benzyl-L-glutamate) exist in the coil conformation in DCA because of strong solvation rather than protonation." Preliminary infrared spectroscopic studies of poly(L-alanine)[4]

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in the fundamental region showed that in solutions of high acid content the polymer was not protonated, although *N*-methylacetamide under the same conditions was found to be fully protonated. At lower acid contents, with the polymer in the helical conformation, a new band was observed at 1616 cm^{-1} which was attributed to the interaction of the acid with poly(L-alanine) in the helical form. The same band was also observed on interacting acid vapor with oriented poly(L-alanine) molecules in sheared films, and although its position agreed with that of the antisymmetrical stretch of CO_2^{θ}, it was associated with the polymer rather than the acid part of the system, since it was observed to be dichroic. This, however, overlooked the possibility that steric reasons may exist for poly(L-alanine) which promote a strong interaction between the amide groups and acid molecules such that if this interaction involves a partial charge transfer then the resulting charged carboxyl group of the acid may also be oriented. Thus, although it is quite clear that in solvent mixtures of high acid content no protonation occurs in the random-coil form of poly(L-alanine), at low acid contents the helical form of poly(L-alanine) may present a particularly favorable binding site to the acid molecules, and the resulting interaction could involve a partial charge transfer. Volchek and Purkina[28] have likewise concluded on the basis of infrared spectra that protonation can occur in ordered conformations, while only hydrogen bonding takes place with random-coil forms. Haylock and Rydon[17] have supported this conclusion on the basis of certain nmr observations.

The nmr and ORD results presented in this paper show that a preferential interaction takes place in low acid solvent mixtures between the TFA molecules and the poly(L-alanine) block and that no similar interaction occurs with the flanking blocks of poly(γ-benzyl L-glutamate), poly(β-benzyl L-aspartate), or poly(N^{ϵ}-carbobenzoxy L-lysine). The compact side chain of L-alanine residues clearly results in a favorable situation for interaction with TFA which does not exist with the flanking blocks, all of which have long side chains with strongly interacting groups. It is probable, therefore, that different polypeptides have different modes of interaction with haloacetic acids. Either of the two proposals given above for the mechanism by which acid molecules interact with the polypeptide backbone may be invoked in order to explain the results of this paper. First, a regular interaction between TFA and the amide groups of helical poly(L-alanine) may take place through dipole–dipole interactions and hydrogen bonding. Such an interaction would change the chemical environment of the backbone protons and therefore their chemical shift. The parallel changes in the optical rotation parameter b_0 could be attributed to a weakening of the intramolecular interactions that give rise to the optically active transitions as a result of intermolecular interaction with the acid molecules. Secondly, the effect of a partial transfer of charge from the acid molecules to some of the amide groups could also affect both the chemical shifts of the backbone protons and the optical rotatory dispersion parameter b_0. Whatever the nature of the interaction between haloacetic acids and the poly(L-alanine) helix, the results of deuterium exchange on the block copolymer BLG–BLA–BLG show that the amide hydrogen of the L-alanine helix is more accessible to the acid proton than is the case for the benzyl L-glutamate helix.

In many low molecular weight samples of homopolypeptides, *e.g.*, poly(γ-benzyl L-glutamate), the helix → coil transition is often accompanied by the two-peak behavior seen in Figures 1, 6, and 10 for the α-CH peak of the flanking blocks of these copolymers. If this were regarded as resulting from slow exchange of residues between the helix and coil conformations through the helix → coil transition, then the lifetime of a residue in either conformation could be estimated as $t \geq 10^{-2}$–10^{-3} sec. Such a characteristic time is completely at variance with that obtained from relaxation experiments, which give estimates of the order of 10^{-7}–10^{-8} sec (see ref 13). Although several explanations have been advanced to account for this descrepancy, the most reasonable is that of Ullman[13] that the multicomponent α-CH peak is due to the molecular weight dependence of helicity present as a result of polydispersity. Experimental evidence has been given to support this proposal.[14] At high molecular weight there is little effect from polydispersity in poly(γ-benzyl L-glutamate), and a single shifting α-CH peak is observed. An exception to multicomponent peak behavior for the α-CH resonance (though claims to the contrary have been made[1,29]) has been that of poly(L- or D-alanine)[2–4,30] over a wide molecular weight range above DP $\sim$ 50, which, throughout the TFA-induced transition, remains as a single shifting peak. Although this had previously been observed only at TFA contents above 30%, the present measurements extend this observation of a single rather than a double peak down to pure chloroform as solvent. If the dynamics of the helix → coil transition are rapid, then the absence of multicomponent α-CH resonances implies that all molecules, irrespective of size, in a poly(L-alanine) sample have the same overall conformation in a given solvent mixture. Since it is the highly cooperative nature of the helix → coil transition in poly(γ-benzyl L-glutamate) that gives rise to the strong dependence of helicity on molecular weight, the poly(L-alanine) transition is probably of very low cooperativity and the helical lengths are short. It seems possible, however, that for very low molecular weight poly(L-alanine), in which the helical lengths become comparable with the chain length, a molecular weight dependence of helicity and multicomponent α-CH peaks could appear. In this case, the overall behavior of poly(L-alanine) would be similar to that of poly(γ-benzyl L-glutamate), with the double-peak α-CH spectrum occurring in a much lower molecular weight range. Some preliminary experiments suggest that this might be so.

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