Nuclear Magnetic Resonance Investigation of the Helix to Random Coil Transformation in Poly(α-amino acids).
II. Poly(γ-benzyl L-glutamate)

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ABSTRACT: High-resolution nuclear magnetic resonance spectra at 100 and 200 MHz have been obtained on three samples of poly(γ-benzyl L-glutamate) of molecular weights 9200, 35,000 and 65,000. Separate helix and random coil peaks are observed for the α-CH and peptide NH backbone proton resonances. In the region of the transformation the behavior of the helix peaks is found to be strongly dependent on chain length. Lifetimes associated with the helix and random coil peaks have lower limits of *ca.* 10^{-1} sec.

Poly(γ-benzyl L-glutamate) (PBLG) is known to undergo a transformation from an ordered and presumably helical structure to a disordered (random coil) structure.[2a] The transformation to the random coil is induced by the addition of organic acids such as trifluoroacetic acid (TFA) to chloroform solutions of the polypeptide.[2b] The transformation can also be elicited by changing the temperature of the solution at an appropriate acid concentration.[3] This paper is primarily concerned with using high-resolution nuclear magnetic resonance (nmr) spectroscopy to study the behavior of PBLG in the region of transformation. A systematic nmr investigation will yield much information about the mechanism involved in the process.

A number of nmr studies on PBLG have been reported.[4-7] These studies have been performed on a wide variation of molecular weight samples (degree of polymerization, DP $\simeq$ 12–700). Despite this wealth of data, there is still considerable controversy in the literature concerning the nature and interpretation of the data. The purpose of this paper is to present new and anomalous data at 220 MHz on PBLG of DP $\simeq$ 250. It will be shown that these data, together with data on lower molecular weight samples (DP $\leqslant$ 200), are understandable in terms of an exactly solvable kinetic model of the transformation.[8]

High resolution nmr at 100 and 220 MHz has been shown to be a very useful probe for studying conformational changes such as the helix–random coil transformation for a number of polypeptides.[9-11] In these examples, separate peaks were observed for the α-CH and peptide NH protons in helical and random coil environments. On the basis of this observation, minimum limits of lifetimes of *ca.* 10^{-1} sec for protons in the two environments were obtained.[11] However, it has been postulated that the observation of separate helix and random coil peaks is not a general phenomenon, but rather that it depends on such factors as the size and nature of the side chains, the molecular weight of the polypeptide, and also on the nature of the solvent system employed.[6] Further questions concerning the effect of a molecular weight distribution on the separate peak observation have also been raised.[12] The nmr observation is clearly of major importance, since it is closely related to the question of backbone mobility and the mechanism and nature of the transformation. However, in order to understand fully the nmr data, it is necessary to examine also the kinetics of the process. Dielectric relaxation studies on PBLG of DP $\simeq$ 1750 have yielded a time scale presumably associated with the kinetics of the transformation of the order of 10^{-6} sec.[13] These data would appear to be in gross contradiction with the nmr lifetimes by at least five orders of magnitude. However, a consequence of the nmr data on PBLG of different molecular weights together with a collective model of the transformation is that the kinetic times are associated with propagation rate constants while the nmr lifetimes are related to nucleation rate constants.[8] The purpose of this paper and the succeeding paper which describes the kinetic model is to demonstrate the resolution of any presumed conflict between the two techniques.

The 100- and 220-MHz spectra show that separate "average helix" and "random coil" peaks exist for

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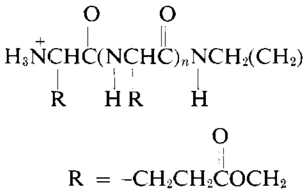
both low and high molecular weight PBLG samples. However, the nature of the "average helix" peak is dependent on the degree of polymerization.

Experimental Section

Polypeptide Characterization. The PBLG samples used in these experiments were obtained from Miles-Yeda Ltd. (Elkhart, Ind.). According to the manufacturer, the low molecular weight PBLG had a weight average molecular weight of 9200 ($DP_w = 40$). Since the polymer was synthesized using *n*-hexylanine as an initiator, the secondary structure in solutions containing TFA may be written as[7]



$$\mathrm{H_3\overset{+}{N}\underset{R}{\underset{|}{C}}H\overset{O}{\overset{|}{C}}(\underset{H}{\underset{|}{N}}\underset{R}{\underset{|}{C}}H\overset{O}{\overset{\|}{C}})_{n}\underset{H}{\underset{|}{N}}CH_2(CH_2)_4CH_3}$$

$$\mathrm{R = -CH_2CH_2\overset{O}{\overset{\|}{C}}OCH_2}$$

Since the *n*-hexylamino end group is present, the number-average molecular weight of the low molecular weight PBLG may be determined by measuring the ratio of the areas of a backbone to an end group resonance (see Figure 1 and *cf.* Results and Peak Assignments). The peaks centered around 0.8, 1.3, and 3.1 ppm correspond to the end group CH_3, $(CH_2)_4$, and CH_2N resonances, respectively. Although the NH_3^+ end group should occur around 7.3 ppm, its presence is masked by the phenyl group. The α-CH proton adjacent to the NH_3^+ end group has a resonance frequency of *ca.* 4.2 ppm (see Figure 1 and *cf.* Results and Discussion). The observation of this end group α-CH proton peak is consistent with the observations of Bradbury, *et al.*,[6] on a PBLG sample of DP ≃ 13. Measurements of the ratio of the areas of the β and γ side chain proton and the $(CH_2)_4$ end group proton resonances yielded a number-average molecular weight of 8000 ($DP_n \simeq 37$). From the DP_n, it is readily apparent that $DP_w/DP_n \simeq 1.1$. A ratio of $M_w/M_n \simeq 1.1$ indicates a relatively narrow molecular weight distribution. This observation of a narrow molecular weight distribution for the low molecular weight sample of PBLG has also been substantiated by gpc chromatography.

According to Miles-Yeda Ltd. the other two samples of PBLG had weight average molecular weights of 35,000 and 65,000, corresponding to DP_w's of 160 and 300, respectively. These two samples will be referred to as intermediate molecular weight and high molecular weight, respectively. Grubisic, *et al.*[14] have reported for PBLG synthesized by various methods including the one used by Miles-Yeda Ltd., that above molecular weights of 20,000, the distribution is quite sharp ($M_w/M_n \simeq 1.3$). For the intermediate and high molecular weight samples, it was not possible to determine DP_n from nmr spectra due to the low relative concentration of the end groups.

Nmr Spectroscopy. All 100-MHz spectra were taken on a Varian HA-100 nmr spectrometer which was equipped with a variable-temperature probe. The ambient temperature near the receiver coils of the probe was *ca.* 31°. The 220-MHz spectra of the high molecular weight PBLG were run on a Varian HR-220 superconducting magnet spectrometer system (Varian Associates, Palo Alto, Calif.). Small amounts of tetramethylsilane (TMS) were added to the solutions to give an internal reference, and also to serve as a sharp signal for the HA-100 field-frequency servo loop.

Solutions. Solutions of *ca.* 5% by weight of polypeptide were found to produce adequate signal-to-noise ratios. All solutions were clear and appeared to be true solutions. Furthermore, all spectra were run immediately after solubilization of the polypeptide. The TFA was introduced into $CDCl_3$ solutions with a microsyringe to adjust the TFA–$CDCl_3$ concentration ratio. The $CDCl_3$ was a spectrograde product (NMR Specialties) and the TFA was freshly distilled and stored in a drybox.

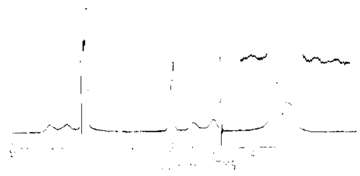


Figure 1. 100-MHz spectrum of PBLG of $DP_w = 40$ in a solution of 10% TFA in $CDCl_3$ (inset run with gain increased a factor of 10).

Results and Peak Assignments

The 100-MHz spectrum of PBLG of $DP_w = 40$ in solution of 10% TFA in $CDCl_3$ (v/v) at 31° is shown in Figure 1. The β- and γ-methylene protons occur in the range from 1.8 to 2.8 ppm, the α-CH proton resonance falls in the region from 3.9 to 4.8 ppm depending on the concentration of TFA, and the benzyl CH_2 proton resonance is observed at *ca.* 5.1 ppm. The phenyl ring protons occur at *ca.* 7.2–7.3 ppm, and the peptide NH proton resonance is seen in the region from 7.5 to 8.2 ppm. Although the α-CH and peptide NH proton resonances are largely dependent on the TFA concentration, the precise chemical shift of all of the protons depends on the particular TFA–$CDCl_3$ concentration ratio of the solution being investigated.

Attention will be focused primarily on the backbone α-CH and peptide NH proton resonances, since these proton frequencies have been shown to be most sensitive to changes in the conformation of the polypeptide. The 100-MHz spectra of the α-CH and peptide NH proton resonances of low molecular weight ($DP_w = 40$) PBLG at various concentrations of TFA in $CDCl_3$ are presented in Figure 2. These spectra show that two separate peaks are resolved for both the α-CH and peptide NH proton resonances over essentially the entire range of values of the TFA concentration. Furthermore, the ratio of the areas of these separate peaks varies as a function of TFA concentration. For the α-CH proton resonance, the lower field peak grows at the expense of the upper field peak as the TFA concentration is increased, whereas for the peptide NH proton resonance, the upper field peak grows at the expense of the lower field peak with increasing TFA concentration. Since at 31° below *ca.* 3% TFA in $CDCl_3$, PBLG is known from ORD and viscosity studies to be in the helical form, and above 25% TFA it is known to exist as a random coil, the higher field peak of the α-CH proton resonance and the low-field peak of the peptide NH proton resonance must correspond to the helical form of the polypeptide. Therefore, the lower field peak of the α-CH proton resonance and the higher field peak of the peptide NH proton resonance are those of the random coil form of the polypeptide. There is also a small peak at *ca.* 4.2 ppm which does not change in intensity or frequency as the TFA concentration is changed. This peak is assigned to the resonance of the α-CH proton

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[14] Z. Grubisic, L. Reibel, and G. Spach, *C. R. Acad. Sci., Paris*, **264**, 1690 (1967).

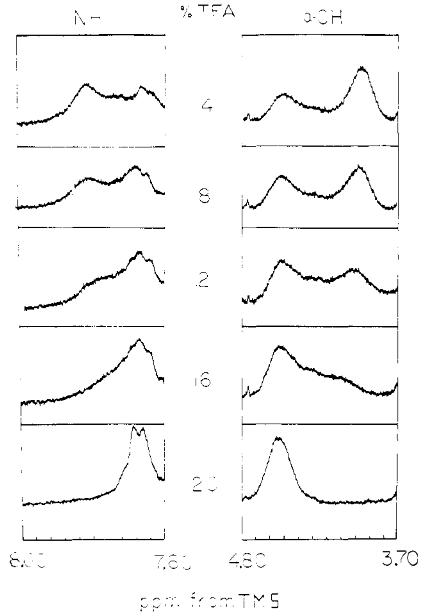

Figure 2. 100-MHz spectra of the α-CH and peptide NH proton resonances of PBLG of DP_w = 40 at four different TFA–$CDCl_3$ solvent compositions.

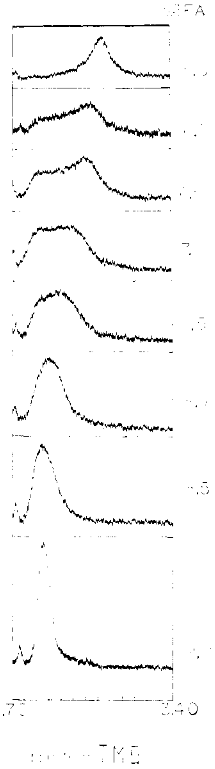

Figure 3. 220-MHz spectra of the α-CH proton resonances of PBLG of DP_w = 300 at eight different TFA–$CDCl_3$ solvent compositions.

adjacent to NH_3^+ amino group in agreement with Bradbury, *et al.* Essentially identical separate peak behavior on a PBLG sample of DP 92 was also observed by Bradbury, *et al.*,[6] with the exception that the end group peak is no longer present.

In addition to the observed intensity changes in both the α-CH and peptide NH proton resonances in going through the helix–random coil transformation, the chemical shifts of the separate peaks are observed to change only slightly. These changes in chemical shifts are small and of the same order of magnitude as those observed in other polypeptide systems. These changes may be associated with a solvent shift due to the difference in susceptibilities of $CDCl_3$ and TFA.

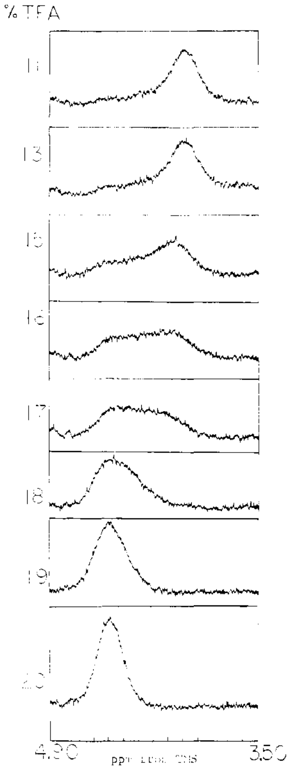

Figure 4. 100-MHz spectra of the α-CH proton resonances of PBLG of DP_w = 160 at eight different TFA–$CDCl_3$ solvent compositions.

The 220-MHz spectra of the α-CH proton resonance of high molecular weight PBLG (DP_w = 300) at various TFA concentrations in $CDCl_3$ are shown in Figure 3. As in the low molecular weight PBLG case, two separate peaks are observed in the region of the transformation. Furthermore, the high-field peak decreases in intensity and the low-field peak increases in intensity as one goes from helix to random coil. However, in sharp contrast to the behavior of the low molecular weight material, the high-field (helix) peak in the DP_w = 300 sample shifts toward the low-field (random coil) peak and eventually (when the helix is completely degraded) becomes indistinguishable from the low-field (random coil) peak. The chemical shifts of the random coil peaks of both the low and high molecular weight samples are essentially identical for all concentrations of TFA.

The 100-MHz spectra of the α-CH proton resonance of intermediate molecular weight (DP_w = 160) PBLG at various TFA concentrations in $DDCl_3$ are shown in Figure 4. As for both low and high molecular weight PBLG, separate peak behavior is observed in the region of the transformation. However, the magnitude of the shift of the high-field (helix) peak is intermediate between the corresponding shift magnitudes of the low and high molecular weight samples. Furthermore, in contrast to the behavior of the low and high molecular weight samples, the high-field (helix) peak of DP_w = 160 PBLG appears to broaden in the region of the transformation.

Discussion

Helix and Random Coil Lifetimes. For both low and high molecular weight PBLG two and only two well-defined peaks are observed for a given proton resonance in the region of the helix–random coil transformation. On the basis of the theory developed in the succeeding paper,[8] the equation for the transformation may be written as

$$\mathrm{H} \underset{k_{\mathrm{B}}}{\overset{k_{\mathrm{F}}}{\rightleftarrows}} \mathrm{C} \qquad (1)$$

where H and C represent (respectively) "average helix" and "random coil," and k_{F} and k_{B} are the related rate constants. The observation of separate "average helix" and "random coil" peaks permits the determination of minimum limits for the lifetimes, τ_1 ($\tau_1 = k^{-1}$), of the protons in the two environments,[14] since

$$\tau_1 \geqslant \frac{1}{\omega_\alpha - \omega_\beta} \qquad (2)$$

In eq 2, $\omega_\alpha - \omega_\beta$ is the chemical shift difference in hertz between protons in the two environments (represented by α and β). Furthermore, the above observations on PBLG show that, over essentially the entire region of the transformation, $\tau_1 \geqslant 10^{-1}$ sec.

The differences in the behavior of the "average helix" peak for low and high molecular weight PBLG imply that the dynamics of the transformation differ for the two cases. The fact that the "average helix" peak shifts and eventually becomes indistinguishable from the "random coil" peak only for the high molecular weight case indicates that the environment associated with the peak is changing continuously throughout the transformation. Therefore, this peak is best characterized as "average helix" in nature. This type of behavior is in agreement with the theory developed in the succeeding paper.

In the intermediate molecular weight case the helix peak appears to shift although not as dramatically as in the case of high molecular weight PBLG. However, in precisely this molecular weight range, the effect of any polydispersity in molecular weight should be very important. The broadening of the "average helix" peak therefore results from higher molecular weight fractions shifting to a larger extent than the lower molecular weight fractions. However, this observation that the "average helix" peak is still well defined is in agreement with Grubisic, *et al.*, *i.e.*, that the polypeptide is reasonably monodisperse.

Ullman[12] has attempted to attribute the observation of separate peak behavior to polydispersity in the molecular weight rather than to a fundamental property of the transformation. He assumes that the process is very fast and shows that certain molecular weight distributions give rise to two or more peaks in the region of the transformation. However, his theory predicts that, even for low molecular weights, the "average helix" peak shifts from the helix frequency to the random coil frequency in proceeding through the transformation. This prediction is not borne out by the experiments on the low molecular weight PBLG, since the frequency of the "average helix" peak remains essentially constant over the entire region of the transformation. Therefore, the observed separate peak behavior cannot be explained on the basis of polydispersity.

Chemical Shift Dependencies. The chemical shift behavior of the "average helix" peak may yield some information about the nature of the TFA–polypeptide interaction. The precise effect of the TFA is still the subject of considerable controversy. Hanlon and Klotz[15] have concluded, on the basis of infrared studies, that TFA protonates the amide moiety. On the other hand, Stewart, *et al.*,[16] have suggested that TFA hydrogen bonds to the peptide group of the helix. As a result of detailed circular dichroism studies on a number of polypeptides in $\mathrm{CDCl_3/TFA}$, Quadrifoglio and Urry[17] conclude there is neither protonation nor hydrogen bonding of TFA to the helix. A similar conclusion has been obtained by Balasubramian based on our results.[18a] Furthermore, Bovey[18b] has indicated, after a detailed circular dichroism and nmr study of both polypeptides and rigid small molecules, that protonation of amide groups by TFA is highly unlikely. However, to the extent that the concepts of hydrogen bonding and protonation have clear significance in this case, none of the above studies can eliminate the presence of a small quantity of either protonated or hydrogen bonded helical polypeptide, perhaps as an intermediate or activated complex.

The present nmr results, coupled with the results of Quadrifoglio and Urry[17] and of Bovey,[18b] indicate that protonation is not responsible for the transformation, and that TFA does not strongly interact with the helical peptide $>\mathrm{H{-}H\cdots O{=}C}<$ hydrogen bonds. This conclusion follows, in part, from the fact that neither the helix peptide NH peak chemical shift nor its line width changes appreciably as a function of TFA concentration, whereas the coil NH peak does show a marked solvent dependency. These results seem to suggest that hydrogen-bond competition is responsible for the transformation

$$\underset{\text{Helix}}{>\mathrm{C{=}O\cdots H{-}N}<} + 2\mathrm{TFA} \rightleftarrows \underset{\text{Random Coil}}{\mathrm{TFA\cdots HN}\!<\,>\!\mathrm{C{=}O\cdots TFA}}$$

The TFA monomer is supplied by the equilibrium

$$(\mathrm{TFA})_2 \rightleftarrows 2\mathrm{TFA}$$

The helix–random coil transformation is controlled by such factors as the nonbonded interaction energies. The concentration of TFA and solvation of the polypeptide by the acid will also influence the stability of the helix and must be considered in any more rigorous treatment of the phenomenon.

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