The Conformational Stability of α-Helical Nonpolar Polypeptides in Solution*

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ABSTRACT: The effect of bulky nonpolar side chains on the stability of the α-helix has been studied in blocks of poly-L-leucine and poly-L-phenylalanine, and in mixed-batch copolypeptides incorporating one or the other of these residues. The helical stability of these preparations was examined under various conditions of solvent composition and temperature designed to alter the intensity of identifiable noncovalent interactions. In water, 8 M urea, 7.2 M guanidinium chloride, and quinoline, the block polypeptides retained complete helical stability, at room temperature and when heated to 95°.

These observations are consistent with the premise that the observed stability is an intrinsic property of the polypeptide backbone when it is disposed in the α-helix. The helical stability is dramatically reflected by the failure to detect any exchange of amide hydrogens in the blocks for deuterium in 24 hr under conditions in which the half-time of exchange for poly-L-glutamic acid is 70–80 min. L-Leucine residues are found to form more stable α-helical sequences than L-phenylalanine residues in two systems: in conformational titrations of the blocks in mixtures of chloroform and trifluoroacetic acid, and in aqueous solutions of mixed-batch copolypeptides. A thermodynamic treatment reveals that in chloroform, this difference in stability amounts to 1200 cal/mole of residues. The relative instability of L-phenylalanine residues is attributed to steric interference between the bulky side chain and the α-helical backbone. It is proposed that similar interference obtains with other aromatic amino acid residues. Bulky side chains, such as leucine, may enhance the stability of helical sequences in aqueous medium by the formation of hydrophobic interactions with neighboring side chains. These two effects, when present, are probably additive.

The structural and conformational properties of certain copolypeptides containing residues of DL-glutamate and L-leucine or L-phenylalanine are being examined in a series of two papers. In the previous paper (Auer and Doty, 1966) the synthesis and structure of the materials used here were considered; some spectroscopic observations were also reported. In this paper we present an investigation of the structural stability of the α-helices formed by the nonpolar components of the copolypeptides.

The conformations of synthetic polypeptides have long been studied as a means of elucidating the role played by the constituent amino acid residues in stabilizing the native conformations of proteins. In order to extend such studies to solutions of nonpolar polypeptides, which are normally insoluble in aqueous media, Gratzer and Doty (1963) introduced the technique of incorporating a block of poly-L-alanine between two flanking blocks of poly-DL-sodium glutamate. The block copolymer thus obtained was soluble in water. Observation of its optical rotatory dispersion (ORD)[1] could be employed to establish the α-helical conformation of the poly-L-alanine block, since only this portion of the copolymer is optically active. It was found that the α-helix remains stable in all the aqueous solvents examined, and is unfolded only in hydrazine or trifluoroacetic acid (TFA). Since side-chain interactions may not occur in α-helical poly-L-alanine and identifiable interactions of side chains with the main chain are too weak to explain the observations, it was concluded that the great conformational stability was an intrinsic property of the polypeptide backbone.[2] This may perhaps be attributed to such effects as electrostatic interactions between the dipole moments of the

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[1] Abbreviations used in this paper: DCA, dichloroacetic acid; ORD, optical rotatory dispersion; PBDLG, poly-γ-benzyl-DL-glutamate; PGA, poly-L-glutamic acid; TFA, trifluoroacetic acid; nmr, nuclear magnetic resonance.

[2] The stability of α-helical block poly-L-alanine has recently been ascribed solely to interamide hydrogen bonding and to a hydrophobic bond which, it is claimed, is formed between a methyl side chain and the α-carbon atom of the fourth residue down the helix (Bixon *et al.*, 1963). The authors conclude that this supposed hydrophobic bond "explains why the polymer . . . did not melt at 95°." In fact, however, their analysis shows only that this interaction, if it exists, may render an α-helix more stable; it does not demonstrate that other stabilizing factors are absent or unimportant.

amide groups (Arridge and Cannon, 1964; Brant and Flory, 1965) and the van der Waals repulsions between the groups comprising the main chain (Brant and Flory, 1965; De Santis *et al.*, 1963).

In the present work we begin with the premise, taken from the studies on poly-L-alanine, that the α-helical polypeptide backbone is intrinsically stable in solution. The amino acid residues studied here, L-leucine and L-phenylalanine, were chosen in order to examine the effects of larger nonpolar side chains (compared with the methyl side chain of L-alanine) on this assumed stability of the α-helical backbone. Our concern was mainly with these two facets: First, do bulky side chains enhance the stability of the helical main chain, *e.g.*, by further inhibition of solvation by polar solvents (Fasman, 1962), or do steric interactions between these side chains and the polypeptide backbone weaken the α-helical structure? And second, the side chains of both these amino acid residues are large enough to permit pairwise interactions with near neighbors in the α-helix, when Pauling–Corey models are examined. Are these important further sources of conformational stability?

The experimental approach to this problem was to investigate the conformational stability of block poly-L-leucine and block poly-L-phenylalanine, and of mixed-batch copolymers of L-leucine or L-phenylalanine and DL-glutamate, under a variety of conditions. In different solvents, the contributions of various noncovalent interactions, such as hydrogen bonding, dispersive interactions, and hydrophobic interactions, to the stability of the helical conformation may be expected to vary. An analysis of the relative importance of these contributions might shed some light on the problem posed here.

Materials and Methods

Reagents. Dichloroacetic acid (DCA) was dried over phosphoric anhydride prior to distillation at reduced pressure. Quinoline was twice distilled under dry nitrogen at reduced pressure after drying over anhydrous magnesium sulfate. Dioxane was purified according to the Fieser (1955) method. Water was glass distilled. Urea was twice recrystallized from a solution in 70% ethanol saturated at 50–60°. Guanidinium chloride was twice recrystallized from methanol by adding an equal volume of anhydrous ether (Greenstein and Jenrette, 1942). Deuterium oxide (D_2O) was 99.8% pure. Concentrated deuteriochloric acid was prepared by dropping D_2O into benzoyl chloride at elevated temperature and sweeping the deuterium chloride into a bubbler containing D_2O with dry nitrogen. The resulting solution was once distilled to remove traces of benzoic acid. All other materials were used as supplied.

Polypeptides. The synthesis and properties of the polypeptides used in these investigations are described in detail in Auer and Doty (1966). The block copolymers had the sequential structure poly-DL-glutamate–poly-L-(nonpolar amino acid)–poly-DL-glutamate. The weight-average degree of polymerization, DP_w, of the central block was about 50, out of a total value for DP_w of between 140 and 230. The mixed-batch copolymers contained about 30 mole % of nonpolar residues and had a DP_w of about 140. For studies in organic solvents the γ-benzyl ester forms of the copolymers were used, and in aqueous media the sodium salt derivatives were employed. It is shown in Auer and Doty (1966) that the block copolymerization products are indeed block copolypeptides, and that the natural conformation which residues of L-leucine and L-phenylalanine assume in favorable solvents is the α-helix.

Optical Rotation. Optical rotatory dispersions were obtained with a Rudolph Model 200S photoelectric spectropolarimeter. The light source was a Hanovia 910B-1 high-pressure xenon–mercury arc. The signal was detected with an RCA 7200 photomultiplier equipped with a separate, variable dc power supply whose maximum voltage was 800 v. When necessary to reduce the photomultiplier dark current, the photomultiplier tube was cooled to about −50° by the boil-off from liquid nitrogen. Thermostating of the sample at elevated temperatures was achieved by heating the entire sample trough with a circulating Haake bath. A mercury thermometer was inserted directly into the cell. About 1 hr was permitted for thermal equilibration. Cells were 1.00 and 2.00 cm supplied by Rudolph. They were equipped with removable quartz end windows specially selected for absence of birefringence, and were held in place by means of a Teflon gasket, 1.9-mm thick, and a gasket fashioned of steel wool, inserted in series into the window holder. This arrangement permitted routine cycles of heating the cell to about 100° and cooling, without pronounced change in the cell blanks.

Three or four determinations of α, the observed rotation, were made at each wavelength. These were usually reproducible to within ±0.001°, and always to within ±0.003°. Most readings were >0.100°, except with helical poly-L-leucine at high wavelengths.

A few ORD curves were obtained with a Cary Model 60 recording spectropolarimeter. The sample was contained in a 1-cm spectrophotometer cell held in a rigid mount in the light beam. Cell base lines were recorded after every experimental run.

Treatment of the ORD Data. The value of $[m']_\lambda$, the reduced residue rotation, was determined according to eq 1. Here, λ is the wavelength of the incident radiation, α_λ is the observed rotation, M is the residue weight, c is the concentration in g/dl, l is the optical path in decimeters, and n_λ is the index of refraction of the solvent at wavelength λ. All values of $[m']_\lambda$ are based on content of nonpolar amino acid residues in the copolymer. The dispersions of refractive index for all the organic solvents are taken either from the tables listed by Fasman (1963) or from the International Critical Tables (1930). For TFA, the value of n_D^{25} was applied throughout

$$[m']_\lambda = \frac{3M\alpha_\lambda}{(n_\lambda^2 + 2)cl} \quad (1)$$

the wavelength range studied. Values of n_λ for water are those given by Dorsey (1940). These values were also used for the disodium phosphate solutions. The values of n_λ for 8 M urea are given in Fasman's tabulation; those for 7.2 M guanidinium chloride were estimated by assuming that the ratio of $3/(n_\lambda^2 + 2)$ for 8 M urea to that for 7.2 guanidinium chloride measured at the sodium D line held across the entire wavelength range used. The values at the sodium D line were obtained with a Bausch and Lomb Abbé refractometer, Model 3L.

Concentrations of solutions were determined either by acid hydrolysis and subsequent amino acid analysis (Auer and Doty, 1966), or by determination of total amide N by micro-Kjeldahl analysis. They were corrected for expansion of the solutions on heating.

Determinations of fractional content of α-helix were based on the number of nonpolar L-amino acid residues in the copolymer. In the case of L-leucine, the Moffitt–

$$[m']_\lambda = \frac{a_0\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \qquad (2)$$

Yang (1956) equation (eq 2), in which a_0, b_0, and λ_0 are constants, was applied for this purpose. When λ_0 is set at 212 mμ, it has been shown in a careful analysis of the experimental application of this equation that $[m']_\lambda$, a_0, and b_0 may be used in a consistent fashion to determine f_h, the fractional helical content in polypeptides (Urnes and Doty, 1961). This is given in eq 3; the superscripts H, D, and obsd refer to helix, disordered, and observed, respectively. The right-hand equality assumes that b_0^D is 0. In the present case, only

$$f_h = \frac{[m']_\lambda - [m']_\lambda^D}{[m']_\lambda^H - [m']_\lambda^D} = \frac{a_0^{obsd} - a_0^D}{a_0^H - a_0^D} = \frac{b_0^{obsd}}{b_0^H} \qquad (3)$$

the b_0 criterion was used due to its relative independence of solvent effects, for the poly-L-leucines examined here could not be unfolded without drastic alteration in solvent conditions.

For the copolymers containing L-phenylalanine, eq 2 could not be used in a conventional fashion, due to the presence of the phenyl chromophore in the side chain (Auer and Doty, 1966). Furthermore, it was found that the ORD's for partially helical phenylalanine polymers exhibit an inflection point and an extremum within the wavelength range examined (see the curve for the random copolymer, Figure 2, Auer and Doty, 1966), so that the Moffitt–Yang equation would not be linear with any value of λ_0 chosen. Therefore, f_h was determined using the left-hand equality of eq 3.

Hydrogen–Deuterium Exchange. INFRARED SPECTROPHOTOMETRY. The exchange of amide hydrogens in the block copolypeptides for deuterium from a medium of heavy water was monitored by infrared spectrophotometry. The spectra were recorded on a Perkin-Elmer Model 21 double-beam spectrophotometer. The sample cell had a path length of 0.05 mm and the reference cell was a variable path cell adjusted to 0 absorption at a region where only solvent absorbs. The windows of the cells and the monochromator prism were all of calcium fluoride. The optical path was purged with dry nitrogen to eliminate the absorption of atmospheric water vapor.

The procedure adopted, a modification of the technique of Blout *et al.* (1961), records the rate of loss of the amide II band at about 1550 cm^{-1}, which is characteristic of the protonated α-helical peptide bond. Complete exchange of protons for deuterons in the poly-DL-sodium glutamate blocks at the outset of a run was effected as follows. The block copolymer was suspended in 1:1 D_2O–dioxane and the poly-DL-sodium glutamate blocks partially neutralized with concentrated deuteriochloric acid, until dissolution occurred. After a minute or two, the samples were further titrated to pD 3.5–4.0 (determined with narrow-range pH paper) thus protonating all the carboxylate groups. This is necessary in order to eliminate the intense carboxylate absorption at 1565 cm^{-1} which would otherwise obscure the remaining amide II intensity at 1545–1540 cm^{-1} due to the nonpolar blocks. At this low pD, the preparation is a gel. Scattering by the gel was minimal, amounting to only 0.02–0.03 optical density unit.

Extensive calibrations of the method were carried out on poly-L-glutamic acid (PGA) (Pilot Chemicals, Inc.) gelled at the same pD in this solvent. The rate of exchange for PGA obeyed first-order kinetics very well, with a first-order rate constant of about 9×10^{-3} min^{-1}. The rates of exchange of the block polypeptides were compared directly to that of gelled PGA, in order to offset the effects of gelation, if any.[3]

Conformational Stability

The conformational stability of the α-helical blocks of poly-L-leucine and poly-L-phenylalanine was investigated by seeking to induce helix–random coil transitions in these systems. By varying the conditions of solvent and temperature it was expected that the helix would be destabilized. Studies of the kinetics of hydrogen–deuterium exchange of the blocks and of the fraction of helical nonpolar residues in the mixed-batch copolymers were also undertaken as further criteria of helical stability.

Aqueous Media. The ORD of block poly-L-leucine (samples 4LG and A4LG; see Auer and Doty, 1966), and the optical rotation of block poly-L-phenylalanine (samples 2PG and 5PG, fraction 2) were determined as a function of temperature in three aqueous solvents, water, 8 M urea, and 7.2 M guanidinium chloride. In water (or 0.1 M Na_2HPO_4) the α-helices of these block polypeptides are likely to be stabilized by hydrophobic interactions between neighboring side chains, as well

[3] It was found that in 24 hr a gelled preparation of the block copolymer of L-alanine (Gratzer and Doty, 1963) exchanged about 10–20% fewer hydrogens than did a preparation allowed to remain dissolved in the D_2O–dioxane solvent during this time. Thus gelation does not critcally affect the rate of exchange in these systems.

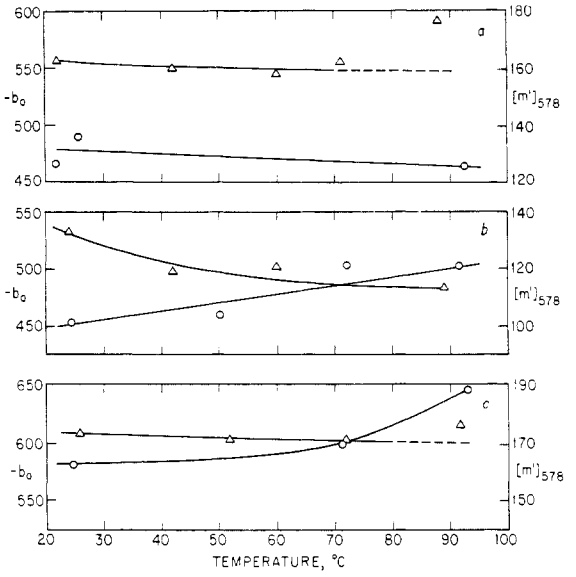

FIGURE 1: Temperature dependence of $-b_0$ of block poly-L-leucine (O) and of $[m']_{578}$ of block poly-L-phenylalanine (Δ) in aqueous solvents: (a) water; (b) 8.0 M urea; (c) 7.2 M guanidinium chloride.

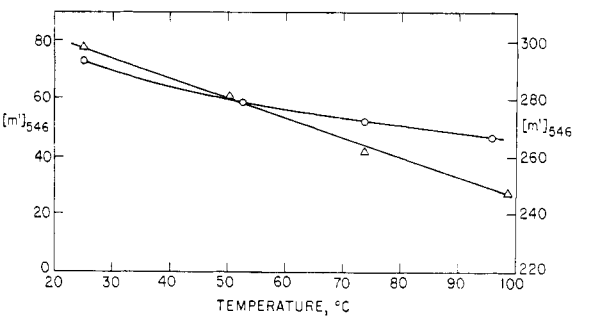

FIGURE 2: Temperature dependence of $[m']_{546}$ of block poly-L-leucine (O) and of block poly-L-phenylalanine (Δ) in quinoline. The scale for poly-L-phenylalanine is on the right; poly-L-leucine is on the left.

as by other types of noncovalent interactions. Concentrated urea or guanidinium chloride solutions should weaken many of these interactions, thus destabilizing the α-helical conformation.

Results of these determinations are shown in Figure 1, in which the values of $-b_0$ found for block poly-L-leucine and the values of $[m']_{578}$ for block poly-L-phenylalanine are plotted against temperature. It is seen that the rotatory power remains essentially constant up to temperatures exceeding 90° for all three solvents. The slight increase in optical rotatory power observed at the highest temperatures are probably ascribable to evaporation of water through the ground glass seals at the ends of the polarimeter cell. The somewhat low values of $[m']_{578}$ for block poly-L-phenylalanine in 8.0 M urea are probably due to a solvent effect on the rotatory power of the side chains. For if the decrease at room temperature had represented a loss of helical structure, there would have been further change with temperature toward values of $[m']_{578}$ representative of the disordered chain (Figure 3). This was not observed. From these experiments it may be concluded that even under relatively extreme conditions of temperature and solvent, the blocks of poly-L-leucine and poly-L-phenylalanine examined here remain helical in aqueous media.

Organic Solvents. The conformational stability of the block polypeptides was examined in two organic media. First, we consider the temperature dependence of the optical rotation in quinoline. These experiments were undertaken in order to eliminate any stabilization which hydrophobic bonding might have been contributing in aqueous media to the α-helices of these block polypeptides.

The results of these studies are shown in Figure 2. Quinoline is opaque below about 400 mμ, so only the monochromatic rotation was observed for both polymers. As in the aqueous case the changes are seen to be gradual and fairly small in magnitude, considering the extended temperature range examined. There is no obvious indication of a helix–random coil transition. The final values represent only about 10–15% disorder, if it is assumed that at room temperature the complete helix prevails, and that the values of $[m']_\lambda$ for the disordered states of the polymers are given approximately by the respective values of $[m']_\lambda$ for the polymers dissolved in TFA (see below).

An alternative interpretation of these small changes in $[m']_\lambda$ is that they are due not to a partitioning of residues between the helical state and the disordered state, but rather that thermal flexing of the rodlike helical segment with increased temperature decreases the intrinsic dissymmetry of the structure, thereby reducing the rotatory power. This interpretation is in the spirit of the expectation of Kauzmann and Eyring (1941) that the optical rotatory power of small molecules capable of unhindered rotation would decrease with increased temperature.

To decide which view of these results is to be preferred, let us consider three factors. First, there is no clear reason for attributing the rotatory power at room temperature to complete formation of the α-helix. The curves for both block polypeptides appear to continue indefinitely with the same slopes in both directions beyond the temperature range studied. Second, it may be shown on the basis of eq 7, below, that in the absence of TFA, no helix–random coil transitions in these polymers may be expected at accessible temperatures (Auer, 1965). Third, helical poly-L-glutamic acid in water and in 70% methanol shows a gradual decay of $-b_0$ with increasing temperature, in a similar fashion as observed here (Cassim and Taylor, 1965). From these considerations, therefore, it is likely that increased thermal fluctuations are the basis for the decrease in rotatory power at higher temperatures.

The relative conformational stability of the two block polypeptides was also investigated in binary mixtures

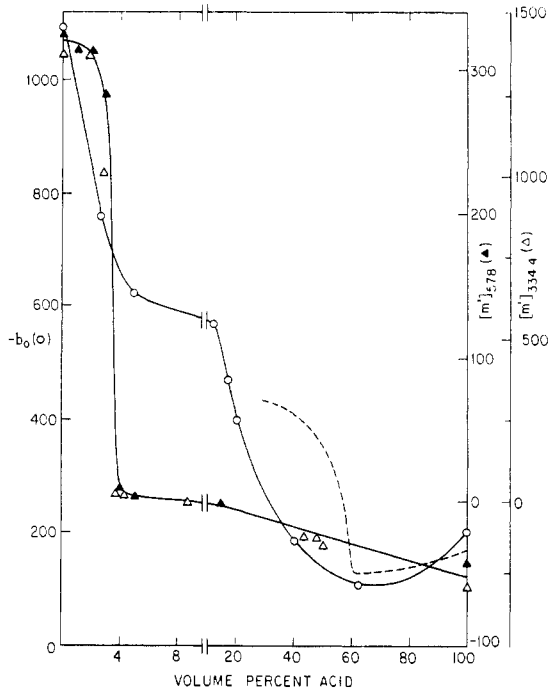

FIGURE 3: Dependence of optical rotatory power of the block polypeptides on volume per cent TFA in the solvent: (○), $-b_0$ of block poly-L-leucine in chloroform–TFA mixtures; (▲), $[m']_{578}$ of block poly-L-phenylalanine in chloroform–TFA mixture; (△) $[m']_{334.4}$ of block poly-L-phenylalanine in benzene–TFA mixtures; dashed curve, $-b_0$ of high molecular weight poly-L-leucine in chloroform–TFA mixtures (Fasman, 1962).

of chloroform (or benzene) and TFA by isothermal variation of the solvent composition. By using this solvent system, the block polymers could be placed on a scale of conformational stability already established for many high molecular weight polypeptides by Fasman (1962). The results are given in Figure 3 by plotting measures of the optical rotatory power of block poly-L-leucine and of block poly-L-phenylalanine *vs.* the volume per cent of TFA in the solvent. Fasman's (1962) curve for poly-L-leucine is also shown.

Considering the results from low contents of TFA to high, it is seen first that block poly-L-phenylalanine has a single apparent transition whereas block poly-L-leucine has two. However, in pure chloroform or pure benzene the values of $[m']_\lambda$ for the former and of $-b_0$ for the latter are anomalously high relative to their values in water. Although the causes for this are obscure, it may be due to some form of asymmetric ordering in chloroform and benzene involving the poly-γ-benzyl-DL-glutamate (PBDLG) blocks. Thus, *e.g.*, PBLG imposes its conformation on γ-benzyl-D-glutamate residues incorporated into it (Doty and Lundberg, 1957); the nonpolar blocks may have the same effect on the PBDLG blocks. Furthermore, superhelices of PBDG and of PBLG have been visualized in electron micrographs of fibers and films (Hall and Doty, 1958; Takashima, 1964; Ishikawa and Kurita, 1964), and films of PBLG have unusually high values of $-b_0$ (Elliott *et al.*, 1962). Such associations are presumably disrupted at very low concentrations of TFA.

The single transition for poly-L-phenylalanine, and probably the second transition for poly-L-leucine, then represent the true helix–random coil conformation transitions in this solvent system.[4] The midpoint of the former transition occurs at 3 vol % TFA, and that for the latter is at about 25% TFA. The transition for block poly-L-leucine proceeds at somewhat lower TFA contents than does that for poly-L-leucine itself (Fasman, 1962, see Figure 3); this is readily attributable to the considerably shorter length of the helical sequences in the L-leucine block polymer (Lifson and Roig, 1961, and references quoted therein).

The further changes in the optical rotatory properties of the two block polypeptides at high TFA concentrations, beyond the conformational transitions, are different for the two polymers. Block poly-L-phenylalanine exhibits a simple decay in $[m']_\lambda$ values to 100% TFA; this is indicative of a solvent effect. However, the increase observed in the values for $-b_0$ of block poly-L-leucine at the highest proportions of TFA is difficult to explain in this way. Rather, it may be that some order is in fact regenerated. For when the mixed-batch copolymer (sample 5LG) is dissolved in TFA, the value of b_0 obtained from the ORD curve is −63°, indicative of very little order remaining among the L-leucine residues. Since the only difference between these copolypeptides is in the sequential disposition of the residues, the relatively high value of $-b_0$ for the block copolymer suggests that some ordered structure is actually present.

The results of this solvent titration of conformation indicate that the conformational stability of α-helical poly-L-leucine is considerably greater than that of poly-L-phenylalanine in nonaqueous media.

Hydrogen–Deuterium Exchange. The rate of exchange of amide hydrogen for heavy isotopes from a doped medium in helical polypeptides reflects the extent of flexing or opening of the polypeptide backbone. Therefore, in order to examine the microscopic conformational stability of the α-helix in these block polypeptides, the kinetics of hydrogen–deuterium exchange was monitored by infrared spectrophotometry. The experiments were conducted on samples of the block polypeptides which were gelled in 1:1 D_2O–dioxane by the addition of DCl to a pD of about 4.0. They were carried out only after having shown that the gel did

[4] Hanlon and Klotz (1965) have recently published infrared evidence for protonation of polypeptides by DCA and TFA even at very low concentrations of acid, concluding thereby that the transitions observed by means of ORD are spurious. This allegation cannot be considered to be demonstrated until the hydrodynamic evidence for the transition (Doty and Yang, 1956; J. T. Yang and P. Doty, to be published; Perlmann and Katchalski, 1962; Doty, 1957) which parallels the changes in the ORD is found to be inadmissible.

not critically inhibit the exchange of protons (see Materials and Methods).

Both block poly-L-leucine and block poly-L-phenylalanine exhibited amide II bands at 1540–1543 cm^{-1}. Neither gave any indication of exchange during 24 hr, within an accuracy of about 10%. Under these conditions, however, it was found that gelled PGA exchanged with a half-life of about 70–80 min. This striking finding suggests that under these conditions, the α-helical structures formed in these blocks are dynamically rigid. No flexing or fluctuation of the helix occurs which is great enough or of sufficient duration for isotope exchange to occur.

Mixed-Batch Copolypeptides. The final step in these investigations was to determine the amount of net right-handed α-helix in mixed-batch copoly-L-leucine–DL-sodium glutamate and mixed-batch copoly-L-phenylalanine–DL-sodium glutamate, when dissolved in 0.1 M Na_2HPO_4. It was found that slightly less than 50% of the leucine residues coalesce into the α-helix, based on standard values for b_0 of $-500°$ for full helix (from the block polymer in water) and $-60°$ for complete disorder (from the mixed-batch copolymer in TFA). On the other hand, only about 10% of the phenylalanine residues are helical. This result comes from using $[m']_{578} = 163°$ for full helix (from the block polymer in water), and $-45°$ for complete disorder (from the block polymer in TFA, which gives a slightly more negative rotation than does the random copolymer in TFA; Auer, 1965).

This difference in apparent helix-forming ability for these two nonpolar residues when incorporated into mixed-batch copolymers is probably not due to significant differences in sequence distribution between the two copolymers. That is, it might be supposed that blocklike sequences of nonpolar residues are formed in the polymerization of the L-leucine copolymer but not in that of the L-phenylalanine copolymer. If this were so, a pseudo-first-order representation of the kinetics of copolymerization would deviate from linearity earlier in the case of L-leucine than in that of L-phenylalanine, for if either one of the components of the polymerization mixture containing L-leucine *N*-carboxyanhydride polymerized with the other much less readily than with itself, a preferential depletion of the fast-reacting component would result. The rate of copolymerization would then diminish as the slow-polymerizing component became predominant in solution. Concomitantly the polymer would be enriched in leucine residues at one end or the other. However, the pseudo-first-order kinetics plots for both of these mixed-batch copolymerizations have similar slopes, and both remain linear to about 90% of completion of reaction (Auer, 1965). This finding is consistent with a like, and probably small, degree of deviation from random incorporation in the two copolypeptides.

A further experiment was performed with these copolymers to ensure that sequence disparities were not responsible for the observations. The fraction of net right-handed helix was followed in distilled water as the charges on the DL-sodium glutamate residues were progressively neutralized. The results are shown in Figure 4. It is likely that in this medium, a random coil–helix transition tends to occur among the DL-glutamate residues in the range of $1 - \alpha$, the degree of protonation, from 0.5 to 0.8 (Wada, 1960). This results in a tendency to form equal extents of left- and right-handed helices (Tsuboi *et al.*, 1963) which is altered only by the presence of the L-nonpolar amino acid residues. Since at all values of $1 - \alpha$ studied the values of f_h for the leucine copolymer are higher than those for the phenylalanine copolymer, no plausible explanation involving differences of sequence distribution between the two preparations may be invoked to explain the observed behavior (Auer, 1965).

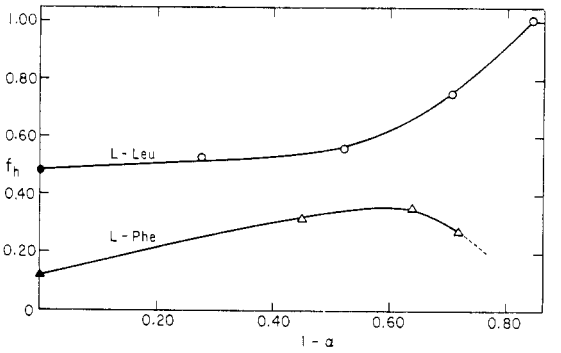

FIGURE 4: Fraction of net right-handed α-helix formation, f_h, based on the content of L-(nonpolar residues), of mixed-batch copoly-L-leucine–DL-sodium glutamate (circles), and of mixed-batch copoly-L-phenylalanine–DL-sodium glutamate (triangles) as a function of the degree of protonation, $1 - \alpha$, of the glutamate residues. Filled points represent 0.1 M Na_2HPO_4; open points, distilled water.

Lastly, a quantitative study of the sequence distribution of a number of mixed-batch copolypeptides was made by Salovey (1958) by simultaneous examination of the kinetics of the copolymerizations and of the dependence of copolymer composition on extent of completion of the polymerization reaction. For primary amino-initiated copolymerizations of γ-benzyl-L-glutamate *N*-carboxyanhydride and L-leucine *N*-carboxyanhydride (in the proportions 5:5 and 6:4) in chloroform, it was found that the two types of monomer add essentially at random to the growing polymer chain, resulting in very small and frequently unitary sequences of a given monomer species. In the random copolymers considered here, this situation is modified only by having about one-third D-amino acid *N*-carboxyanhydride in the reaction mixture. Any effect of the D-antipode should be primarily steric and, therefore, should be about equal for the two monomers, due to their similar large bulks.

From these considerations, it may be safely stated that disparities in the sequence distributions between the two copolypeptides cannot explain the greater extent of helix formation among L-leucine residues than

among L-phenylalanine residues when the glutamates are fully ionized. Consequently the result must be due to a greater inherent ability of the L-leucine residues to stabilize right-handed α-helical sequences.

Discussion

The Intrinsic Stability of the α-Helix. Heating curves for the block polypeptides dissolved in water, in aqueous denaturants, and in quinoline all revealed that the helical conformation remained the most stable structure throughout the entire temperature range examined, from 20 to about 100°. In aqueous media, there is the possibility that the side chains in the block polypeptides stabilize the α-helix by hydrophobic interactions. Such interactions, however, are not likely to exist in quinoline (Kauzmann, 1959; Némethy and Scheraga, 1962), or in concentrated urea or guanidinium chloride (Robinson and Jencks, 1963; Nozaki and Tanford, 1963; Wetlaufer *et al.*, 1964). [It may be that slight compensation favoring the maintenance of the helical conformation in quinoline arises from the formation of a slightly more stable intramolecular hydrogen bond in this solvent than in water (Klotz and Franzen, 1962).] The consistent stability of the helical conformation under a variety of conditions intended to disrupt known noncovalent interactions between portions of the helical polypeptide molecule suggests that such interactions are not essential for the helical stability. At the outset of this paper, we stated the premise that the α-helix composed of L-amino acids is inherently stable. The present observations are in accord with this view and offer it further support.

Helix–Random Coil Transitions in Chloroform. The conformational titration of α-helical polypeptides by strong acids (such as DCA or TFA) furnishes a means of comparing their conformational stabilities with one another (Fasman, 1962). In these systems, the apparent stability of the helical conformation is overcome by the protonation of the amide groups by the acid (Hanlon *et al.*, 1963; Klotz *et al.*, 1964) and contributions due to the side chains become observable.

The foregoing experiments clearly showed that in chloroform, the α-helix of block poly-L-leucine was more stable than that of block poly-L-phenylalanine of virtually the same length. This result can be placed on a semiquantitative basis with the aid of some thermodynamic considerations. The transition is formulated

$$\text{Helix} + n\text{RCOOH} \rightleftharpoons \text{Coil–(RCOOH)}_n \quad (4)$$

in eq 4, where RCOOH is either DCA or TFA in solvent mixtures containing these substances, and n is the number of peptide bonds in the polypeptide (equal to $DP_n - 1$), for only a single protonation is expected per peptide bond. The change in chemical potential, μ, for the reaction of eq 4 may be written

$$\mu_{C,i} - (\mu_{H,i} + n\mu_{S,i}) = \mu_{C,i}^\circ - (\mu_{H,i}^\circ + n\mu_S^\circ + RT[\ln a_{C,i} - (\ln a_{H,i} + n \ln a_{S,i})] \quad (i = L, P) \quad (5)$$

in which μ° is the standard chemical potential, R is the gas constant, T is the absolute temperature, and a is the thermodynamic activity. The subscripts L and P, indexed by i, refer respectively to block poly-L-leucine and block poly-L-phenylalanine, and the subscripts C, H, and S refer to the species Coil–$(RCOOH)_n$, helix, and RCOOH, respectively, of eq 4. RCOOH is TFA in this case. $a_{S,i}$ refers to the activity of TFA at the midpoint of the transition of species i.

Let us approximate thermodynamic activities by the corresponding concentrations c. Then at the midpoint of the transition, $c_{C,i}$ and $c_{H,i}$ are identical. Furthermore, for sharp transitions, the entire left-hand side of eq 5 vanishes at values of solvent concentration not far removed from $c_{S,i}$. Incorporating these developments, eq 5 becomes

$$\mu_{C,i}^\circ - (\mu_{H,i}^\circ + n\mu_S^\circ) = -RT \ln c_{S,i} \quad (i = L, P) \quad (6)$$

The pair of equations represented by eq 6 may then be subtracted from one another. Then, when the value of n in both cases is identical, we arrive at

$$\frac{1}{n}[\Delta\mu_{(H\rightarrow C),L}^\circ - \Delta\mu_{(H\rightarrow C),P}^\circ] = -RT \ln \frac{c_{S,L}}{c_{S,P}} \quad (7)$$

where

$$\Delta\mu_{(H\rightarrow C)}^\circ = \mu_C^\circ - \mu_H^\circ$$

The validity of this thermodynamic approach can be corroborated by the following considerations. In the statistical-thermodynamic treatment of helix–random coil transitions in polypeptides, the parameter s of Zimm and Bragg (1959) characterizes the statistical weight of a helical residue in the interior of a helical sequence. Furthermore, Applequist (1963) identified s with the equilibrium constant for the transfer of one residue from the nonhelical state to the end of a helical sequence, at constant number of helical sequences. For our purposes let us write explicitly that s is a function both of temperature T and of activity a_s of strong acid in the solvent, $s(T,a_s)$. Thus, according to Applequist

$$s(T,a_s) = \frac{a_C}{a_H} \quad (8)$$

and

$$-RT \ln s(T,a_s) = \Delta\mu_{(H\rightarrow C)}^+(T,a_s) \quad (9)$$

where $\mu_{(H\rightarrow C)}^+$ depends on a_s because s itself does. We may separate the dependence of $\mu_{(H\rightarrow C)}^+$ on a_s from its dependence on T

$$\Delta\mu_{(H\rightarrow C)}^+(T,a_s) = \Delta\mu_{(H\rightarrow C)}^*(T) - RT \ln a_s \quad (10)$$

This equation thus describes how s varies with both T and a_s.

However, at a given value of s the ratio a_C/a_H also depends on the value assigned to σ, the helix nucleation parameter (Zimm and Bragg, 1959); the transition is sharper as σ decreases in value toward 0. Thus eq 8–10 must also specify constant σ. There is one point at which s is independent of σ, namely when $s = 1$ (Zimm and Bragg, 1959). This is precisely the midpoint of the transition, which we selected for consideration above. At this point, from eq 9 and 10

$$\Delta\mu_{(H\rightarrow C)}^{+}(T,a_{s,i}) = 0 = \Delta\mu_{(H\rightarrow C)}^{*}(T) - RT \ln a_{s,i} \quad (11)$$

The various $\Delta\mu$ values in eq 9–11 refer to single residues, from the significance of s. Equation 11 is, therefore, identical with eq 6, save for the division by n in the latter. The two methods are, therefore, equivalent, after concentrations are substituted for activities in eq 11.

The left-hand side of eq 7 is seen to be an expression for the difference between the changes in chemical potential incurred by two polypeptides of identical length upon undergoing the helix–random coil transition, reduced to a per residue basis. Then, using the values given earlier for the midpoints of the transitions, the value of $-RT \ln (c_{S,L}/c_{S,P})$ in eq 7 is found to be about -1200 cal/mole. This result is independent both of the units of concentration chosen and of the choice of a standard state, because these factors cancel out when the ratio of activities is taken. If we now assume a common reference of chemical potential for the disordered (solvated) chains of the two blocks, it is seen from eq 7 that in nonpolar solvents, the chemical potential of the leucine residues in α-helical block poly-L-leucine is about 1200 cal/mole lower than that of the phenylalanine residues in α-helical block poly-L-phenylalanine.

The Instability of α-Helical Phenylalanine Residues. We have seen that under appropriate conditions the conformational stability due to the α-helical polypeptide backbone may be overcome. In such systems, slight differences in stability due to differences in side-chain interactions become discernible. It was shown in the present studies that L-phenylalanine residues form less stable α-helices than do L-leucine residues in two quite different circumstances: first, in random copolymers interacting with aqueous solvent, and second, in blocks interacting with nonpolar organic solvent. This suggests that the instability observed with these poly-L-phenylalanine preparations is intrinsic to the amino acid residue itself. Its source appears to be the sheer bulk of the benzyl side chain.

Examination of a space-filling Pauling–Corey model of right-handed α-helical poly-L-phenylalanine indicates that there is considerable restriction of the motion of the side chain. Due to unfavorable steric interaction with the polypeptide backbone, the rotation of the β carbon of the phenylalanine side chain about the bond joining it to the α carbon is severely restricted, and the rotation of the phenyl group in turn is also critically diminished. Steric repulsions between phenylalanine side chains in the α-helix appeared to be far less significant. Many of these interactions disappear if the main chain is not helical; in this case there is only slight interference of the motions of the side chain. Similar models with L-leucine residues, on the other hand, indicate much greater freedom of rotation of the groups comprising its side chain in the α-helix. Thus, many of the steric repulsions visualized in α-helical poly-L-phenylalanine are absent in α-helical poly-L-laucine.

This view is supported by the temperature dependence of the line narrowing in nmr spectral studies of pellets of these two polypeptides and a comparison of this with expectations derived from theoretical considerations (Kail *et al.*, 1962). It was concluded from this study that extensive rotation, and oscillations of large amplitude, occur at room temperature in the side chains of both poly-L-leucine and poly-L-phenylalanine. It may be assumed that these conclusions apply as well in solution. Accordingly, since models of the former polymer reveal less steric repulsions than do those of the latter, it would appear that a major factor contributing to the relative instability of α-helices comprising L-phenylalanine residues is the steric interaction entailed between the rotating side chain and the α-helical polypeptide backbone.

Bulky Side Chains and the α-Helical Backbone. In the introduction, the question of the interaction of bulky side chains with the polypeptide backbone of the α-helix was raised. We have suggested that the relative instability found with α-helical L-phenylalanine residues, compared with α-helical L-leucine residues, is an intrinsic property of these residues, which is primarily due to unfavorable steric interactions of the large side chain of L-phenylalanine and the helix backbone rather than to repulsions between side chains. Moreover, recent investigations of the helix–random coil transition of a mixed-batch copolymer of L-tyrosine and L-glutamic acid (5:95) have revealed that the tyrosine residues represent nuclei of relative instability in the α-helix (Doty and Gratzer, 1962; Urnes, 1963). This instability does not appear to be due to identifiable side-chain interactions in the copolymer. We may, therefore, presume that in analogy with the case of L-phenylalanine residues, the origin of the instability in the L-tyrosine residues also lies in steric interference between the side chain and the polypeptide backbone.

While there is no comparable study of the contributions of L-tryptophan or L-histidine to helix stability, their aromatic side chains can be expected to provide at least as much steric interference with the polypeptide backbone as do those of L-phenylalanine and L-tyrosine. Thus it would be reasonable to expect that all four aromatic amino acids are in some degree destabilizing to the α-helical conformation.

This conclusion suggests that a differentiation be added to the class of helix-forming polypeptides proposed by Blout (1962). This group of helix formers, it is suggested, should be further divided into the group of polypeptides with a single aliphatic substituent on the

β carbon, such as poly-L-alanine, which forms unhindered helices, and the group, comprising all the aromatic polypeptides, which forms helices destabilized somewhat by steric interactions between side chains and backbone.

Side-Chain Interactions in the α-Helix. The second question raised in the introduction concerned the possibility of interactions between bulky nonpolar side chains in the α-helix. Let us consider the relative helical stabilities of residues of L-alanine and L-leucine. In homopolypeptides in chloroform, poly-L-alanine is somewhat more stable to the disruptive effect of TFA than is poly-L-leucine (Fasman, 1962). (This may perhaps be due to the slight steric interaction of the side chains of the leucine residues with the helical main chain noted above; poly-L-alanine has no such interactions.) The situation is reversed in aqueous solutions of the mixed-batch copolymers of L-leucine or of L-alanine with DL-sodium glutamate. Here, in 0.1 M Na_2HPO_4, about 50% of the L-leucine residues are α-helical, while in an analogous preparation (in 0.2 M Na_2HPO_4), the L-alanine residues are completely disordered (Gratzer and Doty, 1963). Yet the sequence distribution in the alanine copolymer is favorable to the formation of helical sequences, for upon neutralization of most of the carboxylate groups, about 80% of the L-alanine residues become α-helical (Gratzer and Doty, 1963). In aqueous medium, then, L-leucine residues form more stable helical sequences than do L-alanine residues.

Molecular models suggest that in the α-helix, side-chain interactions between the isobutyl side chains of L-leucine residues may occur, whereas none are found between the methyl side chains of L-alanine residues. Such associations are an obvious basis for the stabilization by L-leucine residues in water. It has recently been suggested that hydrophobic interactions are important in stabilizing α-helical random copolymers of L-leucine and L-glutamic acid (Fasman *et al.*, 1964). Similarly, it is likely that the observed stabilization of the α-helical conformation in our mixed-batch copolymers is also due to this type of interaction. Hydrophobic interactions involving L-leucine residues in aqueous solution are thus capable of inverting the relative conformational stabilities of L-leucine and L-alanine found in chloroform. In view of this finding, we may respond to the question of side-chain interactions in the α-helix with the generalization that bulky nonpolar side chains may indeed participate in favorable noncovalent interactions which are capable of enhancing the over-all conformational stability of α-helical polypeptides.

In conclusion, it may be noted that the two effects found in this work are expected to be additive, to a first level of approximation. This requires that the steric interference of aromatic side chains with the polypeptide backbone be independent of noncovalent interactions between these side chains themselves. This is apparently the case. Block poly-L-phenylalanine in chloroform solution forms a less stable α-helix than does block poly-L-leucine, and a still less stable helix than poly-L-alanine. If the steric repulsion responsible for this were adversely to affect the formation of hydrophobic interactions in aqueous solutions of mixed-batch copoly-L-phenylalanine–DL-sodium glutamate, then it would be expected that virtually no helix formation occur in this system. In actual fact, however, there is a net generation of right-handed helix, involving about 10% of the L-phenylalanine residues. Since L-alanine residues were completely disordered in an analogous preparation, it may be seen that side-chain interactions, presumably hydrophobic bonds, are still operative in the L-phenylalanine system. This effect apparently overcomes the antagonistic steric factor in a linear fashion, and renders L-phenylalanine residues in this system not much less stable than L-leucine residues. (A straightforward thermodynamic calculation employing the values of f_h shows that the difference in free energies between L-phenylalanine residues and L-leucine residues in this aqueous system is on the order of 1 kcal, similar to the value found above for this difference in the block polypeptide–chloroform system.) Thus, having established the great ability of L-leucine residues to stabilize the α-helix in aqueous solution, the role of aromatic residues will be determined by the independent operation of steric repulsion and hydrophobic stabilization. They may be expected to contribute favorably toward the conformational stability of α-helical sequences in water, but, as in nonpolar solvents, this stabilization should be about 1 kcal less than that of L-leucine residues.

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