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The Behaviour of the Copolymer of L-Proline and *O*-Benzyl-L-Tyrosine in a *n*-Propanol and Water System

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A copolymer consisting 95.8% of L-proline and 4.2% of *O*-benzyl-L-tyrosine was transformed in conformation from Form I to Form II as the propanol content changed from 100% to 74%. In Form I, the copolymer took a rod-like structure like that of poly-L-proline Form I, and the *O*-benzyl -L-tyrosyl residue moved independently of the molecular motion of the copolymer. On the other hand, the copolymer became flexible in Form II and the components associated with each other, forming an amorphous aggregation, when the propanol content decreased further, below the transition point. The hydrophobic interaction between the *O*-benzyl-L-tyrosyl side chain and the prolyl residue was the main factor which made the components of the copolymer associate with each other and form an amorphous aggregation. This was verified by a fluorescence study of the *O*-benzyl-L-tyrosyl residue.

Poly-L-proline exists in two forms, Form I and Form II, the structure of which have been elucidated by X-ray crystallography.[1–3] Form I forms, in the solid state, a right-handed helix with a translation of 1.90 Å and a rotation around the helix axis of 108° per proline residue, which the relationship between two successive C_α atoms around an amide bond is *cis*.[3] It has been shown by various investigators that Form I in solution maintains this structure and that its molecular shape is rod-like as a whole.[4] Form II possesses, in the solid state, a left-handed helical conformation with a translation of 3.12 Å and a rotation around the helix axis of 120° per proline residue, and the relationship between two successive C_α atoms around an amide bond is *trans*.[1,2] In solution, however, it has been shown that the polymer in Form II possesses a certain degree of flexibility.[4,5] The interconversion from Form I to Form II is induced by changing the solvent composition from unsubstituted aliphatic alcohol to aliphatic acid or water; this transformation is reversible.[5] The two conformations are very stable even when the temperature is changed.[5] On the other hand, *O*-benzyl-L-tyrosine is an amino acid forming α-helical polypeptide.[6]

We were interested in obtaining information about the states of fluorescent amino - acid side chains in polypeptides and proteins, and also about their secondary or higher structure, by a fluorescence study. One of the available methods was the method of fluorescence polarization measurements which was developed by Weber[7] and which has been applied to many proteins and polypeptides. For instance, Gill *et al.*[8] studied the helix-coil transition of polypeptide using this method. We planned to apply this method to the copolymer containing L-proline and *O*-benzyl-L-tyrosine. To avoid energy transfer between an excited chromophore and a non-excited one, which would complicate the analysis of fluorescence polarization, we used a sample with only a small amount of *O*-benzyl-L-tyrosine (4.2%). Since L-proline and *O*-benzyl-L-tyrosine were amino acids forming different polypeptide conformations, we first investigated the overall shape of the copolymer. In the course of our investigation we found a new fact, that the

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components of the copolymer in Form II associated with each other and formed an amorphous aggregation. The hydrophobic interaction between the *O*-benzyl-L-tyrosyl side chain and the L-prolyl residue was the main factor which made the copolymer components associate with each other. This was verified by a fluorescence study of the *O*-benzyl-L-tyrosyl residue of the copolymer.

Experimental

The random copolymer of L-proline and *O*-benzyl-L-tyrosine was obtained by copolymerizing L-prolyl-*N*-carboxyanhydride (L-prolyl-NCA)[9] and *O*-benzyl-L-tyrosyl-NCA[9] in pyridine,[10] the molar ratio of L-prolyl-NCA to *O*-benzyl-L-tyrosyl-NCA being 21 : 1. The insoluble parts which appeared in the course of the polymerization were removed, and from the soluble parts the desired polymer was obtained by precipitation with ether. The precipitant was dissolved in acetic acid, and any insoluble parts which contained the *O*-benzyl-L-tyrosine rich polymer were removed. Then the polymer was reprecipitated with ether and dried under a vacuum. The molar ratio of the L-prolyl residue to the *O*-benzyl-L-tyrosyl residue of the copolymer was 22.6 : 1 (the molar content of *O*-benzyl-L-tyrosine was 4.2%). The average degree of polymerization, n, was determined as $n=72$ by the titration of the free carboxyl group with sodium methoxide in *n*-propanol, using a thymol blue indicator.

The vacuum-dried polymer was dissolved in *n*-propanol. The solution was then left overnight, and a portion was diluted by *n*-propanol and water to give a solution of the required solvent ratio. The final concentration was adjusted to 0.395 g/100 cc for solutions of various solvent ratios. These solutions were then used for measurements of the optical rotatory dispersion (ORD), the viscosity, the ultraviolet (UV) absorption, and the electric birefringence. The concentration of the solutions for the fluorescence measurements was 0.0152 g/100 cc.

The ORD was measured with a JASCO ORD/UV 5 apparatus made by the Japan Spectroscopic Co., Ltd., using a 1-cm cell; the UV absorption spectra with a Carl Zeiss spectrophotometer PMQ II, using a 1-cm cell, and the fluorescence spectra, with a Hitachi Fluorescence Spectrophotometer MPF-2A. The values of polarization,[11] p, were calculated at the 305-mμ emission peak of the *O*-benzyl-L-tyrosine fluorescence spectra with an excitation light of 282 mμ. The relative viscosities were measured in an Ostwald-type viscometer. The flowtime of the water at 25°C was 60 sec. The electric birefringence was measured using the optical method of O'Konski and Zimm[12] according to the usual procedure[13] at 20—23°C.

Results

Two weeks were necessary to obtain a constant value of specific rotation at 300 mμ and at room temperature. The equilibrium values thus obtained are shown in Fig. 1(a). After the equilibrium was attained, the specific rotation did not change even when the temperature was raised to 56°C. As may be seen in Fig. 1(a), a rapid change in the specific rotation from −615 to −1952 was observed in the range of the propanol content from 100 to 74%, and the rotation was almost unchanged below 74%. This behaviour shows that the copolymer is transformed in conformation from one to another as the solvent composition changes from 100% propanol to 74%. We here call the conformation in 100% propanol "Form I", and that in 74% or less propanol content, "Form II", following the studies of poly-L-proline.[4]

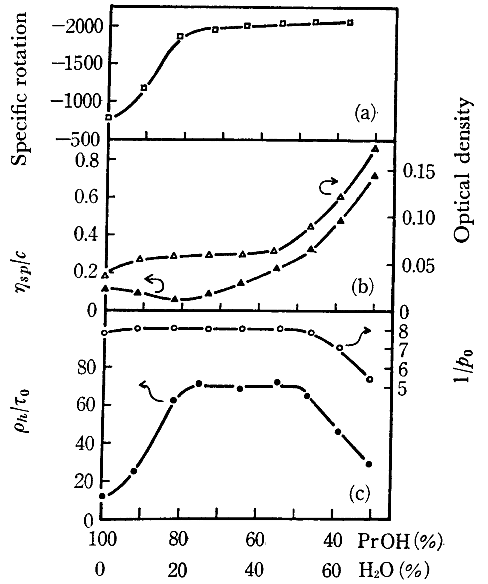

Fig. 1. Variations of specific rotation at 300 mμ (□), optical density at 500 mμ (△), η_{sp}/c (▲), $1/p_0$ (○) and ρ_h/τ_0 for $T/\eta_0=30000$ (●) with solvent composition.

A positive electric birefringence was observed in the range from 100 to 83% of propanol, but it disappeared below 74%. In 100% propanol, a rotational relaxation time of 0.20 μsec was obtained at 20°C. It was suggested from the decay of the birefringence that the distribution of the molecular size was fairly narrow (see Fig. 2).[14]

On the other hand, the reduced viscosity, η_{sp}/c

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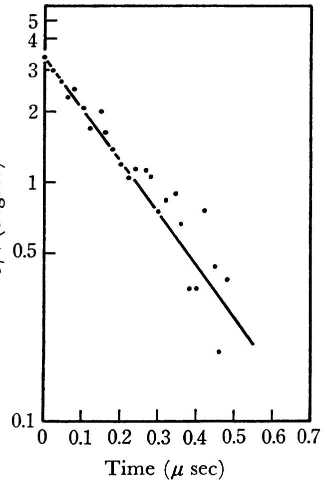

Fig. 2. Decay of electric birefringence of the copolymer in 100% propanol at 20°C.

(c=0.395 g/100 cc), in the same solvent system had its minimum at 83% propanol and rapidly increased below 50% propanol. The optical density at 500 mμ rapidly increased when the propanol content became less than 55% (Fig. 1(b)). Although we observed a small amount of precipitation in 30% propanol, we did not observe any precipitation above that.

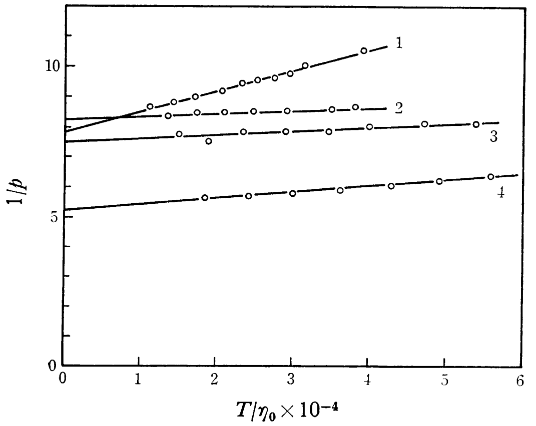

Fig. 3. Plots of reciprocal of polarization, $1/p$, against T/η_0 (degrees Kelvin per centipoise) for the copolymer in 100% propanol (1), 74% propanol (2), 38% propanol (3) and 30% propanol (4).

As may be seen in Fig. 3, linear plots of the reciprocal polarization, $1/p$, against T/η_0 were obtained, where p is the degree of the polarization of light emitted at a right angle to the direction of the excitation and where η_0 is the viscosity of the solvent. Therefore, we can calculate the value of ρ_h/τ_0 according to the Perrin equation:[15]

$$\left(\frac{1}{p}-\frac{1}{3}\right)=\left(\frac{1}{p_0}-\frac{1}{3}\right)\left(1+\frac{3\tau_0}{\rho_h}\right), \tag{1}$$

where τ_0 is the life time of the excited state of O-benzyl-L-tyrosine and where ρ_h is the harmonic mean of the two principal relaxation times of the rotation of the ellipsoidal molecule. The value of ρ_h/τ_0 increased at first as the propanol content decreased from 100% to 83%, stayed constant until 50%, and then decreased below a 50% propanol content (see Fig. 1(c)). The $1/p_0$ values stayed constant in the range from 100 to 50% of propanol, and then decreased below 50%.

Discussion

Assuming that the copolymer takes a rod-like structure in 100% propanol, we can obtain information about the molecular dimensions. We calculated the axial ratio, P, from the viscosity data alone, using the equation of Simha:[16]

$$[\eta]=\frac{\bar{v}\nu}{100} \tag{2}$$

where $\bar{v}$ is the partial specific volume and where ν is the well-known shape factor. We adopted the value of $\bar{v}=0.758$ cm^3/g which was found for poly-L-proline.[17] An axial ratio of 8.5 was found for $[\eta]=0.0868$. The expected value of P, as calculated for the rod-like particle possessing the Traub and Shmueli configuration,[3] is 7.6 for $n=72$. The inconsistency of these two figures is reasonable, because the former assumes an ellipsoidal shape, and the latter, a rod-like one; therefore the value calculated by Eq. (2) may be expected to be larger than the latter.

On the other hand, we can calculate P from the rotational relaxation time, τ_{rot}, if the polymer length is known, by using the equation of Broersma:[18]

$$\frac{1}{6\tau_{\text{rot}}}=\frac{3kT}{8\pi\eta_0 a^3}\left[\ln 2P-1.57+7\left(\frac{1}{\ln 2P}-0.28\right)^2\right], \tag{3}$$

where η_0 is the viscosity of the solvent and where $2a$ is the polymer length. If we assume, based on $n=72$, a rod-like molecule with a length of 137 Å, Eq. (3) leads to $P=7.8$, which agrees well with the expected value of $P=7.6$. Therefore, it is confirmed that the copolymer in 100% propanol takes the same rod-like shape as poly-L-proline in Form I. This fact shows that the incorporation of a small amount of O-benzyl-L-tyrosine (4.2%) into poly-L-proline does not destroy the structure of Form I.

On the other hand, when the copolymer was transformed to Form II the electric birefringence disappeared. If the copolymer took a rod-like

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shape possessing the Cowan-McGavin configuration[1] (Form II), the electric birefringence should be observed beyond the transition point between Form I and II. Therefore, judging from the absence of the electric birefringence below 74% of propanol, the copolymer should be very flexible in this range. The reduced viscosity had a minimum value at 83% propanol, and increased rapidly below 50%. Even if the copolymer took the structure, Form II, of poly-L-proline, we could not expect a value of reduced viscosity larger than 0.24, judging from Simha's equation[16] and assuming a value of $P=14.4$. The value of the reduced viscosity should actually be smaller than this, since the copolymer chain is flexible rather than stiff and rodlike. Nevertheless, the observed values were larger than this value in the range of low propanol content. The optical density at 500 mμ, where the copolymer should not show any optical absorption, increased below 55%. All these observations suggest that, when the copolymer was transformed from Form I to Form II, the rod-like structure collapsed and the polymer chain became flexible, and that when the propanol content decreased further beyond the transition point between Form I and Form II, the flexible components of the polymer associated with each other, forming an amorphous aggregation which did not contribute to the electric birefringence.

Although poly-L-proline in Form II had a certain degree of flexibility,[4,19] its components have not been reported to associate with each other or to form an aggregation in water at room temperature. In higher temperatures (above 55°C), poly-L-proline in water takes a crystalline phase,[20] but in this discussion we can ignore this phase separation because all our experiments except for the fluorescence study were performed at room temperature, while the fluorescence study was performed using dilute solutions. Therefore, it is very interesting that the copolymer with only a small amount of *O*-benzyl-L-tyrosine (4.2%) became flexible and formed an amorphous aggregation which made solutions very viscous without causing any precipitation when transformed from Form I to Form II in these solvent systems.

It has been shown that the hydrophobic bond is one of the main factors which maintain the secondary or higher structure of polypeptides and proteins.[21] A hydrophobic interaction should exist between the benzyl or tyrosyl group and the prolyl residue. Although this interaction may be absent in 100% propanol, it may be a main factor in making the copolymer components associate with each other and form an amorphous aggregation in the range of low propanol content. This inference was verified by the following fluorescence study of the *O*-benzyl-L-tyrosyl residue.

From the value of ρ_h/τ_0, we can study the motion of the *O*-benzyl-L-tyrosyl residue.[22] For this purpose, it is necessary to know the value of τ_0, which is proportional to the quantum yield of the fluorescent molecule.[23] Since there were insufficient data about the lifetime of the *O*-benzyl-L-tyrosine, we adopted L-tyrosine as a reference. Therefore, it should be noted that the following calculation gives merely a rough estimation of the lifetime of the *O*-benzyl-L-tyrosyl residue in the copolymer. The ratio of the relative quantum yield of the *O*-benzyl-L-tyrosine of the copolymer in 100% propanol to that of L-tyrosine in water (pH 7.0) was obtained as 0.05. The lifetime of the excited state of L-tyrosine in water (pH 7.0) was 2.6 nsec.[24] From these values, $\tau_0=1.3\times10^{-10}$ sec was obtained for the *O*-benzyl-L-tyrosyl residue of the copolymer in 100% propanol, giving a value of $\rho_h=3.0\times10^{-9}$ sec at 20°C. On the other hand, the mean harmonic of the principal relaxation times of the ellipsoidal copolymer, whose volume and axial ratio were 3.48×10^{-20} cm^3 and 8.5 respectively, was 1.3×10^{-7} sec at 20°C in 100% propanol. This value is larger than ρ_h, so it may be concluded that the *O*-benzyl-L-tyrosyl residue moves independently of the molecular motion of the copolymer in Form I.

This fact showed that the hydrophobic interaction between *O*-benzyl-L-tyrosyl residue and prolyl residue did not exist in 100% propanol. It has been reported that alcohols break the hydrophobic bond.[25] In this case also, *n*-propanol might break the hydrophobic interaction between the *O*-benzyl-L-tyrosine side chain and the prolyl residue by covering the side chain with *n*-propanol; the aliphatic groups of *n*-propanol came in contact with the *O*-benzyl-L-tyrosine side chain, and the OH groups were arranged outside. Therefore, it was understandable why the intermolecular association was not observed and the *O*-benzyl-L-tyrosyl residue moved independently of the molecular motion of the copolymer in 100% propanol.

When the propanol content changed from 100% to 80%, the copolymer was transformed in conformation from Form I to Form II, and the ρ_h/τ_0 value increased according to the conformational change. The value was then almost unchanged

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over the range from 80% to 50% propanol. We can calculate the ρ_h value by the same procedure as has been described above. In 80% propanol, $\tau_0=0.98\times10^{-10}$ sec and $\rho_h=1.4\times10^{-8}$ sec at 20°C. On the other hand, we have no equation for determining the rotational relaxation time, ρ, of a flexible polymer, so we here calculate the ρ value for a sphere with the same volume as the rod-like poly-L-proline Form II (Cowan-McGavin's configuration), with $n=72$, by means of this equation:

$$\rho=\frac{3\eta_0 v}{kT}$$

where v is the volume of the sphere. We obtain $\rho=8.4\times10^{-8}$ sec for the sphere in 80% propanol at 20°C.

This value is not very different from the ρ_h value. Therefore, it is sure that the hydrophobic interaction between the *O*-benzyl-L-tyrosine side chain and the prolyl residue begins to occur when the propanol content decreases beyond the transition point between Form I and Form II, and that then the motion of the *O*-benzyl-L-tyrosyl residue is suppressed. Although this hydrophobic interaction may exist between intramolecular residues, it may also exist between intermolecular residues. Judging from the behaviour of the reduced viscosity and the value of ρ_h/τ_0 beyond the transition point from Form I to Form II, it is reasonable to conclude that intermolecular hydrophobic interaction between the *O*-benzyl-L-tyrosine side chain and the prolyl residue is a main factor in making the components of the copolymer associate with each other and form an amorphous aggregation.

The suppression of the motion of the *O*-benzyl-L-tyrosine side chain by the hydrophobic interaction was observed more clearly in 100% water. The value of p in 100% water was much larger than that in the mixed solvents, and it increased as the temperature was raised to 45°C, as may be seen in Fig. 4. This increment can be explained in terms of the general nature of the hydrophobic interaction.[21] Because of the positive ΔH value and the negative ΔF value, the hydrophobic interaction is more strengthened as the temperature is raised. The value of p decreased slowly above 45°C. This decrease may reveal a thermal rotational Brownian motion by a part of the polymer chain in which the side chain of the *O*-benzyl-L-tyrosine is fixed by the hydrophobic interaction.

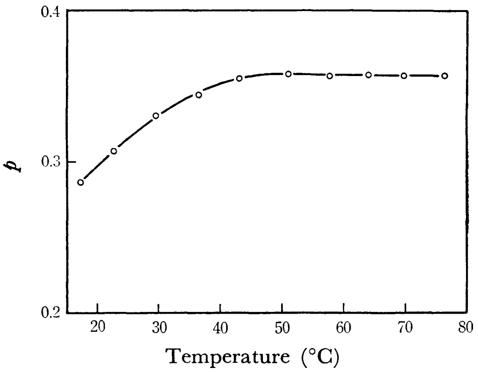

Fig. 4. Plot of polarization, p, against temperature for the copolymer in 100% water.

In the propanol-content range from 50% to 30%, where the intermolecular associations became dominant and an amorphous aggregation is formed, the apparent decrease in ρ_h/τ_0 and $1/p_0$ was obvious. One possible interpretation was that the distance between the *O*-benzyl-L-tyrosyl residues was decreased because of the large amount of intermolecular associations and because energy transfer between the chromophores began to occur; this energy transfer might obviously decrease the value of ρ_h/τ_0.

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