Polymer Journal, Vol. 13, No. 9, pp 837—844 (1981)

Helix–Coil Transition Behavior of Terminal Group of Poly(γ-benzyl L-glutamate) Studied by Depolarization of Fluorescence

Hideharu USHIKI and Itaru MITA

Institute of Space and Aeronautical Sciences, University of Tokyo, Komaba, Meguro-ku, Tokyo 153, Japan.

(Received November 18, 1980)

ABSTRACT: The emission anisotropy r of rhodamine B chemically bound to the terminal amino group of poly(γ-benzyl L-glutamate) (PBLG) was measured in a series of chloroform–dichloroacetic acid mixtures at room temperature. The value of r for the chromophore bound to PBLG differs appreciably from that for a free chromophore in chloroform a helix forming solvent for PBLG, but the values are similar to each other in dichloroacetic acid, a helix breaking solvent. The change in the rotational relaxation in the neighborhood of terminal group of PBLG with solvent composition does not parallel the content of a helix measured by ORD but shows two sharp increases at 0—10% and 60—75% dichloroacetic acid. The implication of these results is briefly discussed.

KEY WORDS Rhodamine 6G / 5-Isothiocyanatorhodamine B / Emission Polarization / Emission Anisotropy / Neighborhood of Terminal Group / Chloroform–Dicholoroacetic Acid Mixtures / Helix–Coil Transition / Helix–Breaking Ratio / Poly(γ-benzyl L-glutamate) /

The fluorescence probe method in the study of biomacromolecules has recently been reported by many authors. A fluorescent probe is defined as a fluorescent molecule or residue whose spectral response (quantum yield, excitation, emission) varies as a function of the physical and chemical environment with which the probe is associated.[1] The fluorescence polarization method, first studied by Perrin,[2] is also very useful for analyzing the rotational Brownian motion of a fluorescent molecule or group in a certain environment, and this method has been used for the investigation of polyacrylamide, polystyrene gels, etc.[3-5]

The mechanism of helix–coil transition in polypeptides has become an interesting subject. Reviews on the conformation-dependent properties of synthetic polypeptides in the helix–coil transition region have been reported by Teramoto and Fujita.[6,7] According to Doty[8] who has studied the specific rotation and intrinsic viscosity of high molecular weight sample of poly(γ-benzyl L-glutamate) (PBLG) in isothermal mixtures of chloroform (CF) and dichloroacetic acid (DCA) over the entire range of composition, the polypeptide maintains a helical conformation up to a DCA composition of about 70% and then undergoes a transition to random-coil within a very narrow range of DCA composition. On the other hand, the intrinsic viscosity continues to decrease, first sharply, then gradually, and again sharply, as the content of DCA increases.

As mentioned above, the helix-coil transition behavior of polypeptides as a whole has already been measured by well-known methods (ORD, CD, etc.).[6,7] Furthermore, a fluorescence polarization study on the behavior of polypeptides side chains in helix–coil transition has been reported.[18]

On the other hand, research on the behavior of terminal group of polypeptide in helix–coil transition has not been reported very much. The behavior of the chain end group of PBLG has recently been studied by the spin labeling technique.[16] Therefore, it may be assumed that the technique of fluorescence polarization is excellent for the study of the helix–coil transition behavior in the neighborhood of the terminal group of the polypeptide, though this technique has not yet been applied in

the field of helix–coil transition at the end group.

In the present work, rhodamine B was chemically bound to the terminal group of PBLG and the emission anisotropy r of PBLG-RhB was measured in a series of CF–DCA mixtures at room temperature in order to study the differences in their behavior as a whole and in the neighborhood of the terminal group in the helix–coil transition of PBLG.

EXPERIMENTAL

Preparation of Rhodamine B Terminated PBLG

PBLG was prepared in a way similar to that used by many authors.[9] γ-Benzyl L-glutamate prepared from L-glutamic acid and benzyl alcohol (yield 54%) was converted to γ-benzyl L-glutamate-*N*-carboxyanhydride (BLG-NCA) with trichloromethyl chloroformate (TCF) in tetrahydrofuran (yield 41%). The BLG-NCA was polymerized in tetrahydrofuran with a trace of water for a long time at low temperature. The PBLG thus obtained was precipitated in petroleum ether, filtered, and dried. The total yield of PBLG was 98%.

The rhodamine B group was introduced into the chain end of PBLG by a reaction between the amino group of PBLG and 5-isothiocyanatorhodamine B (RhB-NCS). A mixture of PBLG (1.8 g) and RhB-NCS (0.08 g) in tetrahydrofuran (50 ml) was stirred for 23 hours at room temperature. PBLG-RhB was precipitated in a petroleum ether–methanol (1 : 1) mixture, filtered, and then dried repeatedly in order to remove any free RhB-NCS from PBLG-RhB.

Other Materials

Dried tetrahydrofuran, hexane, and ethyl acetate, distilled with metallic sodium or calcium hydride, were used as the solvents in the above-mentioned experiments. RhB-NCS, rhodamine 6G, and acridine orange (AO) were purchased from Eastman Kodak Co. and Tokyo Kasei Co., respectively, and used as received.

Characterization of Polymer

The average degree of polymerization DP of PBLG was calculated from its intrinsic viscosity in *N,N*-dimethylformamide (DMF) at 25°C.[10]

$$[\eta]_{25°C}=2.9\times10^{-7}M_w^{1.70} \quad (1)$$

The content of rhodamine B group at the chain end was determined from its fluorescence spectra.

Table I. Characterization of polymers

Polymer	M_v[a]	M_n[b]	DP	RhB group at the chain end
				%
Polystyrene	—	4.5×10^4	430	—
PBLG260-RhB	5.6×10^4	—	260	20
PBLG480-RhB	1.1×10^5	—	480	22

[a] M_v, average molecular weight calculated from eq 1.
[b] M_n, number average molecular weight.

Pertinent data are shown in Table I.

Fluorescence Measurement

A JASCO FP-550 type spectofluorometer was used for measuring fluorescence spectra (the maximum wavelength λ_{em} and intensity I_F) as well as fluorescence depolarizations in the stationary state with the aid of polarizing filters. Dotite–Luminasol chloroform (CF) and DMF were used as solvents for fluorescence depolarization measurements.

The emission polarization p and emission anisotropy r can be calculated from eq 2 and 3, respectively,[11]

$$p=\frac{I_{\parallel}-I_{\perp}}{I_{\parallel}+I_{\perp}}=\frac{I_{vv}-GI_{vh}}{I_{vv}+GI_{vh}} \quad (2)$$

$$r=\frac{I_{\parallel}-I_{\perp}}{I_{\parallel}+2I_{\perp}}=\frac{I_{vv}-GI_{vh}}{I_{vv}+2GI_{vh}} \quad (3)$$

where the intensities of emitted light polarized parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the exciting light are related to the observed intensities I_{vv}, I_{vh}, I_{hv}, I_{hh} and a correction factor $G=I_{hv}/I_{hh}$, and the subscripts v and h denote vertically and horizontally polarized lights, the first letter in the subscript refers to the exciting light and the second refers to the emitted light. The G factor in eq 2 and 3 is necessary for to correcting the depolarization characteristics of the apparatus.

The fluorescence depolarization of PBLG-RhB was measured within a polymer concentration ranging from 0.28 to 0.55 g dl^{-1}. The fluorescent quenching of RhB bound to PBLG by the polymer itself (amide group) is not important in this concentration range because we confirmed that the fluorescent intensity I_F of free Rh6G is hardly affected by the addition of PBLG: $I_F=363$

([PBLG]=0 g dl^{-1}), I_F=344 ([PBLG]=0.98 g dl^{-1}), I_f=325 ([PBLG]=17.8 g dl^{-1}).

RESULTS AND DISCUSSION

(A) Fluorescence Depolarization of Chromophores and PBLG-RhB

Table II shows the emission polarization p and the emission anisotropy r for rhodamine 6G (Rh6G), RhB-NCS, and PBLG-RhB in several solvents calculated by eq 2 and 3. Data for acridine orange (AO) are also listed as reference. The emission anisotropy r is related to rotational relaxation time ρ by,

$$\frac{r_0}{r}=1+\frac{3\tau}{\rho} \tag{4}$$

where τ is the excited singlet lifetime of the chromophore and r_0 is the emission anisotropy in rigid solution. It should be noted that the measurement of fluorescence polarization of small molecular chromophores is possible only for compounds having sufficiently short excited singlet lifetimes since the rotational relaxation time of small molecular chromophores is also very short. The excited singlet lifetimes τ of the chromophores used are 2.0—5.5 ns (AO in ethanol) and 4.8—5.8 ns (Rh6G in water).[12] But even with these lifetimes, the difference between $I_{\parallel}$ and $I_{\perp}$ for these chromophores in DMF are not sufficiently large. On the contrary, the values of r for these compounds in DCA are large because the viscosity of the DCA is larger than that of DMF ($\eta_{25°C}$: 4.0 cp (DCA), 0.85 cp (DMF)).

Even when a chromophore is incorporated into the chain end of a polymer, r_0 and τ may not change appreciably. r may actually be a relative measure of the mobility of the same chromophore bound to the polymer or in free state. As Table II shows, the values of r of the Rh6G chromophore are nearly the same for RhB-NCS and PBLG-RhB in DCA, showing that in the random coil solvent for PBLG, the movement of RhB group as the terminal group of PBLG is in fairly good agreement with that of small molecules. On the other hand, they differ from each other appreciably in CF, a helix solvent for PBLG. There is even observed a molecular weight effect on PBLG. This shows clearly that the terminal RhB group chromophore is rather tightly bound to the PBLG helix.

To make sure that the presence of polymer does not cause any anomalous behavior in emission anisotropy r due to specific adsorption of the chromophores on the polymers, the effect of the addition of polymer on the emission anisotropy r of chromophores in DMF or DCA was examined and the results are shown in Figure 1. As shown in Figure 1, in the random coil solvent (DCA) as well as in the helix solvent (DMF), the addition of PBLG to Rh6G and AO does not affect their emission anisotropy appreciably, showing that there is no special interaction between the chromophores and the polymers. It is interesting to note that the addition of polystyrene (DP=430) up to *ca.* 20% in CF to a solution of helical PBLG-RhB does not affect very much the emission anisotropy r.

(B) Helix–Coil Transition Behavior in the Neighborhood of the Terminal Group of PBLG-RhB in a Series of CF–DCA Mixtures

Fluorescence spectra of RhB-NCS and PBLG-RhB in CF and DCA with a light source of 557 nm

Table II. Fluorescence depolarization of chromophores and PBLG-RhB

Chromophores	Solvent	Concentration	λ_{ex}	λ_{em}	I_F	I_{vv}	I_{vh}	$I_{vh}G$	G	p	r
		M	nm	nm							
AO	DMF	1.5×10^{-5}	498	533	41	4.8	5.5	4.6	0.83	0.027	0.018
AO	DCA	1.5×10^{-5}	498	575	10	1.5	1.3	1.0	0.78	0.19	0.13
Rh6G	DMF	5.0×10^{-6}	540	564	363	36	43	34	0.79	0.029	0.020
RhB-NCS	CF	5.0×10^{-5}	557	579	350	33	40	31	0.78	0.024	0.016
RhB-NCS	DCA	5.0×10^{-5}	557	593	171	19	19	14	0.76	0.14	0.10
PBLG260-RhB	CF	1.0×10^{-5}	557	573	70	8.0	8.6	6.8	0.79	0.086	0.059
PBLG260-RhB	DCA	1.0×10^{-5}	557	588	35	4.3	4.0	3.1	0.78	0.15	0.11
PBLG480-RhB	CF	1.1×10^{-5}	557	573	76	9.5	8.7	6.9	0.79	0.16	0.12
PBLG480-RhB	DCA	1.1×10^{-5}	557	586	29	3.8	3.2	2.5	0.77	0.20	0.14

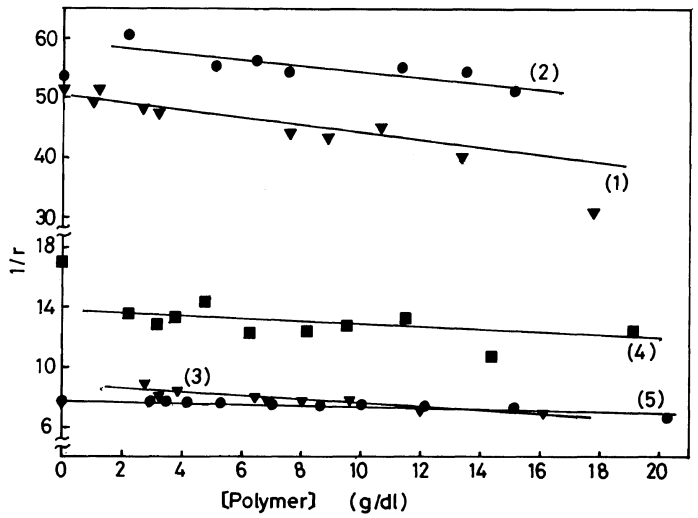

Figure 1. Dependence of emission anisotropy r of chromophores or PBLG-RhB on the concentration of polymer: (1) Rh6G in DMF (polymer; PBLG260); (2) AO in DMF (polymer; PBLG260); (3) AO in DCA (polymer; PBLG260); (4) PBLG260-RhB in CF (polymer; PS430); (5) PBLG480-RhB in CF (polymer; PS430).

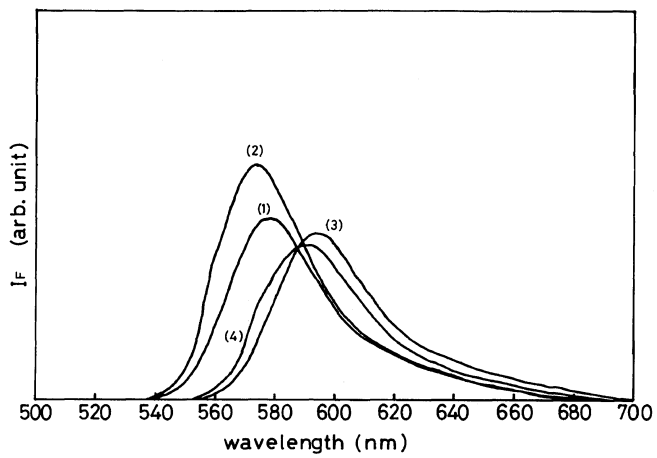

Figure 2. Fluorescence spectra of RhB-NCS and PBLG-RhB in CF and DCA: λ_{em}, 557nm: (1) RhB-NCS in CF (×1.0); (2) PBLG260-RhB in CF (×4.7); (3) RhB-NCS in DCA (×1.0); (4) BPLG260-RhB in DCA (×4.7).

as the excitation wavelength are shown in Figure 2. The fluorescence intensities I_F, emission depolarization p, and emission anisotropy r of RhB-NCS and PBLG-RhB in a series of CF–DCA mixtures are shown in Table III. The fluorescence intensity relative to that in CF (I_F/I_F^{CF}) of RhB-NCS and PBLG-RhB decreases with increasing content of DCA, and the fluorescent maximum wavelengths λ_{em} shift slightly with the content of DCA (Figure 3). The change in these fluorescence intensities may be attributed to the excited singlet lifetime of these chromophores. If the rate constant of fluorescence k_f is independent of the solvent composition, the ratio of I_f/I_f^{CF} is proportional to τ/τ^{CF} by the relationship of $I_F \propto k_f\tau$.[13] Figure 3 shows that variations in I_F/I_F^{CF} with DCA, content for PBLG-

Table III. Fluorescence depolarization of PhB-NCS and PBLG-RhB in a series of CF–DCA mixtures

CF/DCA	λ_{em}	PhB-NCS			λ_{em}	PBLG260-RhB			λ_{em}	PBLG480-RhB		
	nm	I_F	p	r	nm	I_F	p	r	nm	I_F	p	r
100/0	579	350	0.024	0.016	573	70	0.086	0.059	573	76	0.16	0.12
89/11	587	398	0.027	0.018	580	81	0.052	0.035	580	86	0.11	0.074
80/20	589	387			583	78	0.061	0.041	583	80	0.11	0.979
73/27	590	371	0.034	0.023	584	77	0.060	0.041	584	76	0.11	0.077
61/39	591	345	0.045	0.030	584	74	0.065	0.045	584	71	0.11	0.083
53/47	592	325	0.048	0.032	585	71	0.066	0.045	585	67	0.12	0.086
50/50	592	316	0.048	0.032								
47/53	592	311	0.053	0.036	585	68	0.077	0.053	585	63	0.12	0.086
39/61	593	290	0.059	0.040	585	63	0.081	0.055	585	57	0.13	0.092
27/73	593	258	0.083	0.059	586	57	0.095	0.065	586	50	0.14	0.099
20/80	593	235			586	53	0.11	0.078	586	44	0.16	0.11
11/89	593	205	0.11	0.077	588	44	0.12	0.083	586	36	0.18	0.12
0/100	593	171	0.14	0.10	588	35	0.15	0.11	586	29	0.20	0.14

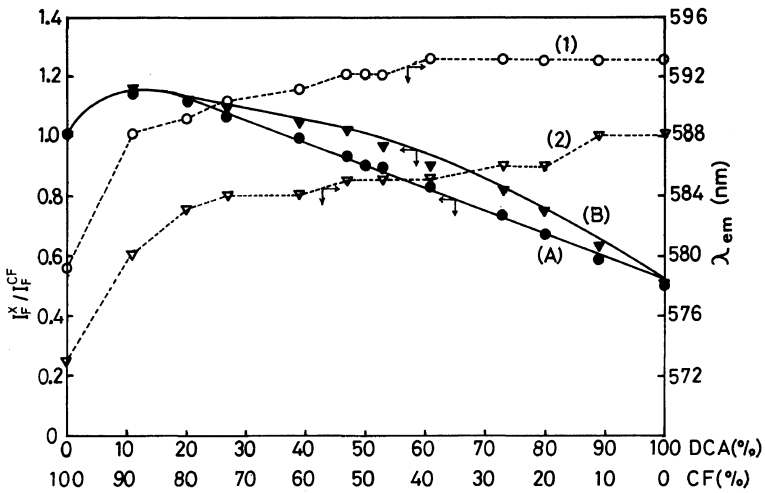

Figure 3. The change of I_F^x/I_F^{CF} and fluorescent maximum wavelength λ_{em} for RhB-NCS and PBLG-RhBin a series of CF–DCA mixtures: λ_{em}, (1) RhB-NCS; (2) PBLG260-RhB; I^x_F/I_F^{CF}, (A) RhB-NCS; (B) PBLG260-RhB.

RhB and RhB-NCS agree fairly well. This indicates that the lifetimes of excited singlet states of free and bonded RhB groups are practically the same in each solvent mixture. Variation in λ_{em} with solvent composition is also nearly the some for both chromophores, showing that no special interaction with solvent is operative for either one of the two chromophores.

The dependence on the composition of CF–DCA mixtures of the reciprocals of emission anisotropy r^{-1} of RhB-NCS and PBLG-RhB is shown in Figure 4. The behavior of r^{-1} for RhB-NCS may be explained by the increase in a solvent viscosity and the decrease in a excited singlet lifetime with increasing DCA content. As already mentioned, changes of in r_0 and τ with solvent composition are practically the same for both RhB-NCS and BPLG-RhB. Consequently, the effect of these two factors is best eliminated for PBLG-RhB by using the relative value of $(r^p/r^s)^{-1}$ instead of $1/r^p$ in the discussing on

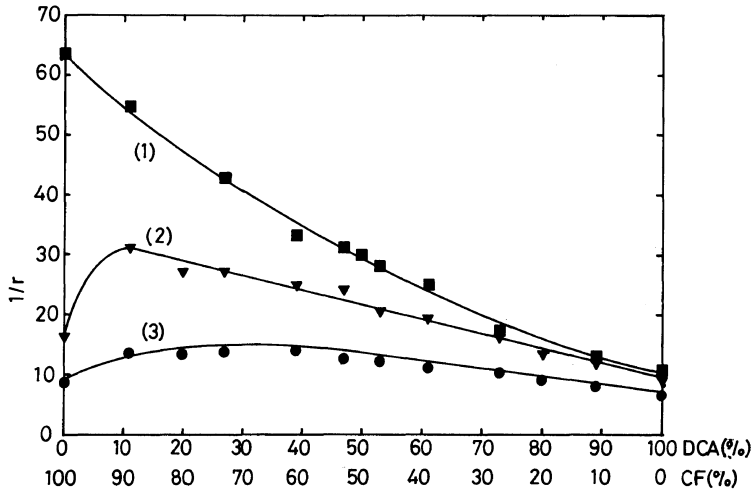

Figure 4. Dependence of reciprocal of emission anisotropy on various CF–DCA mixtures: (1) RhB-NCS; (2) PBLG260-RhB; (3) PBLG480-RhB.

the mobility of the terminal RhB group where the superscripts p and s denote the polymer and the small molecules (RhB-NCS). The ratio r^s/r^p at each CF–DCA mixture can be expressed by eq 5,

$$\frac{r^s}{r^p}=\frac{r_0^p(1+3\tau^p/\delta^p)}{r_0^s(1+3\tau^s/\delta^s)}=\frac{r_0^p\delta^s(\rho^p+3\tau^p)}{r_0^s\rho^p(\rho^s+3\tau^s)} \quad (5)$$

Equation 5 can be rewritten as follows by assuming $r_0^p \approx r_0^s$, $\tau^p \approx \tau^s$, and $3\tau^s \gg \rho^s$ at each CF–DCA mixture,

$$\frac{r^s}{r^p}\approx\frac{\rho^s(\rho^p+3\tau)}{3\tau\rho^p}\approx\frac{\rho^s}{3\tau}+\frac{\rho^s}{\rho^p}\approx\frac{\rho^s}{\rho^p} \quad (6)$$

The final assumption $3\tau^s \gg \rho^s$ is not unreasonable since the value of ρ^s for RhB-NCS is calculated by the equation $1/\rho=kT/3V\eta$, to be about 0.8 ns for $V=2\times10^{-21}\,\mathrm{cm}^3$, $\eta=0.54$ cP (CF), and $T=298$ K. The dependence on the composition of CF–DCA mixtures of the ratio r^s/r^p for PBLG260-RhB and PBLG480-RhB is shown in Figure 5.

In order to understand more easily helix–coil transition behavior in the neighborhood of the terminal group of PBLG-RhB in CF–DCA mixtures, the apparent helix-breaking ratio in the neighborhood of the terminal group b_x at a certain composition of CF–DCA mixture was calculated by,

$$b_x=\frac{a_x-a_{CF}}{a_{DCA}-a_{CF}}\times 100\ (\%) \quad (7)$$

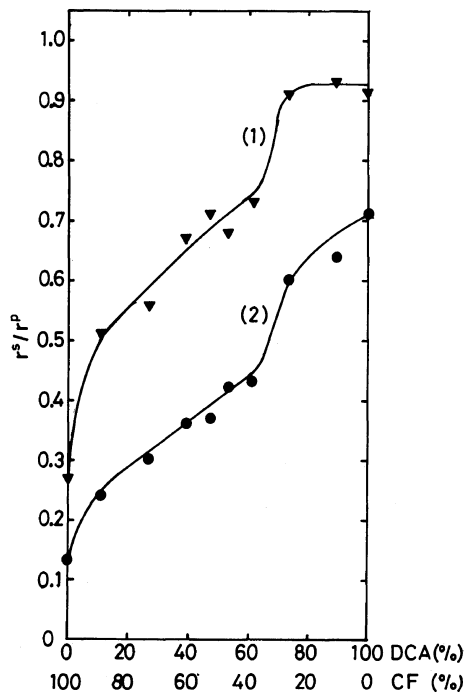

Figure 5. Dependence of r^s/r^p on various CF–DCA mixtures: (1) PBLG260-RhB; (2) PBLG480-RhB.

where a_x is the ratio of $r_x^s/_x^p$, and the subscripts x, DCA, and CF were used when necessary for denoting a certain compositon of CF–DCA mixture, in DCA, respectively. The plots of b_x for the apparent

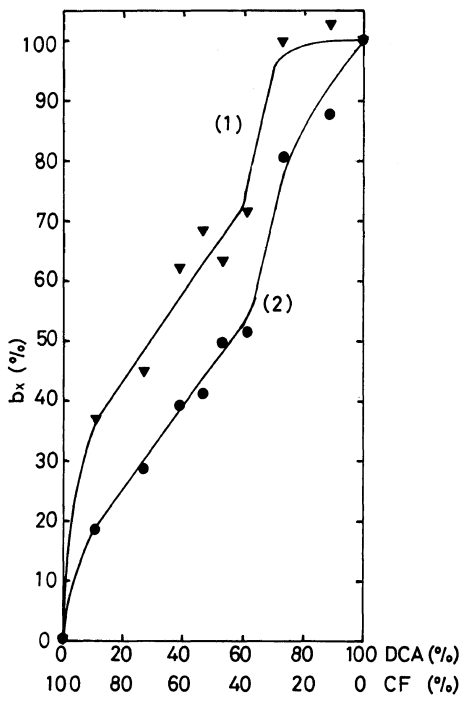

Figure 6. Graph of helix-breaking ratio in the neighborhood of the terminal group b_x against content of DCA: (1) PBLG260-RhB; (2) PBLG480-RhB.

helix-breaking ratio in the neighborhood of terminal group of PBLG against the composition of CF–DCA mixtures are shown in Figure 6.

According to the two curves in Figure 6, the apparent helix-breaking ratio b_x in the neighborhood of the terminal group of PBLG increases rather sharply in two regions, *i.e.*, at 0—10% and 60—75% DCA. The important thing is that the apparent helix–breaking ratio b_x increases considerably during the increase in the DCA content from 10—60%. This behavior strongly contrasts with a sharp and almost exclusive increase (from 0% to 100%) in the content of helix at *ca.* 60% DCA observed by several other methods such as ORD,[8] intrinsic viscosity,[8] and laser light scattering.[14] The behavior observed in the present experiment, however, corresponds to the fact that the rate of H–D exchange in the neighborhood of the terminal group of the polypeptides is faster than that in the center of polypeptides.[15] It seems that form of the helix in the neighborhood of the terminal group is broken up in proportion to the content of DCA in the range of 10—60% DCA. It is reasonable that this effect should be more important for PBLG having a smaller molecular weight (DP=260 for curve (1) and 480 for curve (2) in Figure 6). The somewhat sharp increase in b_x at 0—10% DCA may be ascribed to the dissociation of the head-to-tail type aggregation of PBLG often observed in helicogenic solvents.[17]

In conclusion, measurement of the emission anisotropy r of rhodamine B chemically bound to the terminal amino group of PBLG shows that the values of r for PBLG-RhB and RhB-NCS in DCA (helix-breaking solvent) are nearly the same, but differ from each other appreciably in CF (helix-forming solvent), and that the apparent helix-breaking ratio b_x in the neighborhood of the terminal group of PBLG increases gradually before leaping to 100% at 60—75% DCA indicating gradual increase in the freedom of motion of the terminal group.

In closing, we should like to maintain that for a detailed analysis of the mechanism of helix–coil transition behavior of polypeptides, it is desirable to examine the behavior of a particular part of the polypeptide chain too. For this purpose, the fluorescence and triplet probe methods are of importance.

Acknowledgement. The authors express their gratitude to Dr. T. Kōmoto of the Tokyo Institute of Technology for his helpful comments on the synthesis of BLG-NCA and PBLG.

REFERENCES

1. R. F. Steiner and H. Edelhoch, *Chem. Rev.*, **62**, 457 (1962).
2. F. Perrin, *Ann. Phys.*, **12**, 169 (1929).
3. Y. Nishijima, *J. Polym. Sci.*, **C31**, 353 (1970).
4. J. P. Jarry and L. Monnerie, *J. Polym. Sci.*, **16**, 443 (1978).
5. K. Horie, I. Mita, J. Kawabata, S. Nakahama, A. Hirao, and N. Yamazaki, *Polym. J.*, **12**, 319 (1980).
6. A. Teramoto and H. Fujita, *Adv. Polym. Sci.*, **18**, 65 (1975).
7. A. Teramoto and H. Fijita, *J. Macromol. Sci., Rev. Macromol. Chem.*, **C15**, 165 (1976).
8. P. Doty, Collection, *Czech. Chem. Commun.*, **22**, 5, 11 (1957).
9. T. Kōmoto, private communication.
10. P. Doty, *J. Am. Chem. Soc.*, **78**, 947 (1956).
11. A. M. North and I. Soutar, *J. Chem. Soc. Faraday Trans. 1*, **68**, 1101 (1972).
12. R. F. Chen, G. G. Vurek, and N. Alexander, *Science*,

156, 949 (1967).
13. Y. Kawasaki, K. Mihashi, H. Tanaka, and H. Ohnuma, *Biochem. Biophys. Acta*, **446**, 166 (1976).
14. J. N. C. Ford, W. Lee, and F. E. Karasz, *J. Chem. Phys.*, **50**, 3098 (1969).
15. M. Nakanishi and M. Tsuboi, *J. Mol. Biol.*, **64**, 363 (1972).
16. E. L. Wee and W. G. Miller, *J. Phys. Chem.*, **77**, 182 (1973).
17. H. Kihara, *Polym. J.*, **9**, 443 (1977).
18. T. Iio, Y. Iwashita, and H. Watanabe, *Bull. Chem. Soc. Jpn.*, **45**, 2206 (1972).