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Correlation between internal motion and emission kinetics of tryptophan residues in proteins

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Time-resolved fluorescence anisotropy measurements of tryptophan residues were carried out for 44 proteins. Internal rotational motion with a sub-nanosecond correlation time (0.9 ± 0.6 ns at 10°C) was seen in a large number of proteins, though its amplitude varied from protein to protein. It was found that tryptophan residues which were almost fixed within a protein had either a long (> 4 ns) or short (< 2 ns) fluorescence lifetime, whereas a residue undergoing a large internal motion had an intermediate lifetime (1.5–3 ns). It is suggested that the emission kinetics of a tryptophan residue is coupled with its internal motion. In particular, an immobile tryptophan residue emitting at long wavelength was characterized by a long lifetime (> 4 ns). It appears that a tryptophan residue fixed in a polar region has little chance of being quenched by neighboring groups.

Fluorescence depolarization is caused by rotational Brownian motion of a fluorophore during the lifetime of the excited singlet state. Recent advances in the instrumentation used for fluorometry has made it possible to measure time-resolved fluorescence depolarization and thus to get information on the dynamic structure of macromolecules [1–7].

Fluorometry with extrinsic probes has been utilized for the structural characterization of a specified region of a protein. However, the procedure of specific labeling is sometimes too tedious. If intrinsic chromophores, e.g. tryptophan and tyrosine residues, can be used as optical probes, it will become much easier to carry out structural analyses of proteins. Furthermore, internal motion of intrinsic chromophores may be coupled to important biological processes.

For fluorescence depolarization measurements of tryptophan residues, several technical problems remain to be solved. One is in data analyses of fluorescence anisotropy decay curves of proteins containing many tryptophan residues. The anisotropy decay does not necessarily reflect a simple average of the rotational motions, unless all tryptophan residues undergo homogeneous motions or have the same fluorescence lifetime. Munro et al. [4] have successfully studied protein dynamics by investigating proteins containing a single tryptophan. The appearance of more than two tryptophan residues is more common, however. It is important to develop an adequate method by which the fluorescence signal from several tryptophan residues in a protein can be distinguished. The aim of the present work was to demonstrate the usefulness and limitations of fluorescence depolarization measurements of tryptophan residues for the determination of the static and dynamic structure of a protein.

MATERIALS AND METHODS

Chymotrypsinogen, carboxylesterase, concanavalin A, egg albumin, elastase, lactate dehydrogenase, pyruvate kinase and wheat-germ agglutinin were purchased from Boehringer Co. Ltd and bovine serum albumin from Seikagaku Co. Ltd. Myosin subfragment 1 and actin were prepared from rabbit skeletal muscle (gifts from Dr H. Yoshimura). Bacterioopsin was obtained by bleaching purple membrane (from *Halobacterium halobium* JW3) with hydroxylamine. Other proteins were from Sigma Co. Ltd. Tryptophan was from Nakari Co. Ltd. G-actin was suspended in 0.2 mM ATP, 0.1 mM $CaCl_2$, 1 mM NaN_3 and 1 mM 2-mercaptoethanol, and polymerized in 0.1 M KCl. Apoferritin was suspended in 0.1% sodium dodecyl sulfonic acid; its native form did not fluoresce. Other proteins were suspended in the following buffers: 10–50 mM phosphate at pH 7, 10–50 mM acetate at pH < 5 or 25 mM Tris/HCl at pH > 8.

Fluorescence emission spectra (corrected) were measured with a Hitachi 650-60 fluorometer.

Fluorescence decay kinetics were measured with a single-photon-counting apparatus [8]. Light pulses, with a duration of less than 0.8 ns and at a frequency of around 10 kHz, were provided by a free-running discharge in H_2 gas at 11.1 MPa (11 atm). Light at 300 nm was selected by combination of a monochromator, an interference filter (bandwidth of 12 nm) and a Ni/Cu solution filter. The excitation beam was polarized vertically by a Glan prism, and focused onto the sample placed in a thermostatted cell holder. Two beams of fluorescence emitted at 90° to the excitation beam were separately collected and, in this T-type optical arrangement, vertically and horizontally polarized component of fluorescence, $I_{\parallel}(t)$ and $I_{\perp}(t)$, were measured simultaneously. Each emission was passed through a series of optical filters (Fuji-Color SC37 and Hoya UV340) which transmitted light in the range 370–400 nm, an analyzer (Polaroid HNP'B) and then a depolarizer, and detected by a photomultiplier tube (Hamamatsu R943-02). The direction of the polarization plane of the analyzer was changed alternatively, and thereby slight differences in the detection sensitivity and in the temporal response for the two polarized components were nullified. Experimental decay curves of total $S(t)$ and difference $D(t)$ intensity were computed as follows:

$$S(t) = I_{\parallel}(t) + 2I_{\perp}(t)$$
$$D(t) = I_{\parallel}(t) - I_{\perp}(t) .$$

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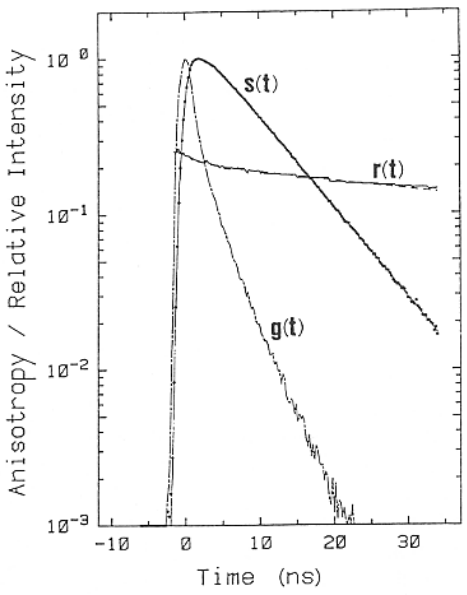

Fig. 1. *An example of the fluorescence intensity and anisotropy decay curves of tryptophan residues in protein.* Sample: lactate dehydrogenase in 50 mM phosphate buffer at pH 7 and 10 °C. In this example, the total intensity decay (dots) was described with two lifetimes (solid line): $\tau_1 = 7.30$ ns, $\alpha_2 = 0.63$, $\tau_2 = 2.46$ and $\alpha_2 = 0.37$. The anisotropy decay (zigzag curve) was described with two correlation times (broken line): $\phi_f = 1.24$ ns, $\phi_s = 69.6$ ns, $r_o = 0.259$ and $r_{os} = 0.205$. The chain line represents the response function of the apparatus

The experimental curves $S(t)$ and $D(t)$ correspond to the convolution products:

$$S(t) = \int_0^t g(T)s(t-T)\mathrm{d}T$$

$$D(t) = \int_0^t g(T)d(t-T)\mathrm{d}T.$$

Here $s(t)$ and $d(t)$ are the fluorescence intensities that would be obtained with an infinitely short excitation pulse. The apparatus response function, $g(t)$, was measured at 300 nm with a dilute Ludox (Du Pont) suspension in place of the sample.

The fluorescence decay curves were analyzed by an iterative least-square method as previously described [9]. The curve fitting was performed in the time region between −2 ns and 35 ns with reference to the peak position of $g(t)$. In the analysis of the total intensity decay, $s(t)$ was assumed to be a sum of exponential: $s(t) = \Sigma_i \alpha_i \cdot \exp(-t/\tau_i)$. The number of exponential components was increased until the following condition was satisfied: the quantity $|S(t_j) - S_c(t_j)|/\sqrt{S(t_j)}$ did not exceed five at any time region where $S(t_j)$ and $S_c(t_j)$ are the experimental and the calculated fluorescence intensity (count number) respectively, at the jth channel. The average lifetime was calculated using the equation $\langle\tau\rangle = \Sigma_i \alpha_i \tau_i$. Although the triple-exponential analysis usually provided a much better fitting than the double-exponential analysis did, the two gave similar results with respect to the average lifetime; the difference was less than 20%. It seemed unlikely that a large systematic error was involved in the calculation of the average lifetime. The experimental curves $D(t)$ were analyzed using the equation:

$$d(t) = r(t)s_c(t)$$

where $s_c(t)$ is the calculated total intensity decay and $r(t)$ is the anisotropy decay. The anisotropy decay was assumed to be a single- or double-exponential function: $r(t) = r_0 \exp(-t/\phi_s)$, or $r(t) = (r_o - r_{os})\exp(-t/\phi_f) + r_{os}\exp(-t/\phi_s)$. When the analysis with two relaxation times was unsuccessful, the curve fitting was performed in a restrictred time region so that only an interesting decay parameter (e.g. r_0) was determined.

RESULTS AND DISCUSSION

Internal motion and emission kinetics of tryptophan residues

Fig. 1 shows a typical example of the fluorescence decay curves of tryptophan residues. The intensity decay curves observed for several proteins were well described with two lifetimes. For other proteins, much better fittings were obtained by triple-exponential analyses. None of the proteins investigated exhibited a single-exponential decay. The decay parameters obtained are summarized in the middle columns of Table 1. The longest lifetime was 9.13 ns; this value is close to the lifetime (9.58 ns) of a free tryptophan with deprotonated amino group (Table 2).

Lifetime values listed in Table 1 tend to cluster around three lifetime regions: 6−8 ns, 2−3 ns, and 0.3−0.8 ns. This does not seem to be an artifact of the fitting procedure, because our result is consistent with previous results in the literature; for instance, the lifetime reported for bovine serum albumin [10] are 7.1 ns (7.41 ns) and 2.7 ns (2.43 ns) (our data in parenthesis); for nuclease [10], 5.7 ns (6.2 ns) and 2.0 ns (2.03 ns); for myosin S1 [11], 8.8 ns (9.13 ns), 4.5 ns (4.60 ns) and 0.72 ns (0.63 ns); for phospholipase A_2 [12], 6.93 ns (6.83 ns), 2.49 ns (2.81 ns) and 0.55 ns (0.52 ns). The lifetimes of tryptophan at neutral pH and at 20 °C (Table 2) are also consistent with previously reported values [6]: 3.0−3.2 ns (3.18 ns) and 0.53−0.85 ns (0.67 ns).

The anisotropy decay curves observed for most proteins were well described with a single- or double-exponential function. More complicated anisotropy decay curves were observed for a few proteins. In the anisotropy decay curves that were well described with a double-exponential function, the following common feature was found; the time constant ϕ_f of the fast component ranged over a narrow time region (0.9 ± 0.6 ns) and the time constant of the slow component reflected the rotation of the whole protein.

Even when tryptophan residues are immobile, an apparent decay of fluorescence anisotropy could be caused by energy migration among tryptophan residues. In the present study, tryptophan residues were excited at 300 nm so that energy migration via Förster's mechanism was minimized (Weber's red-edge effect [13]). Thus this type of energy migration was expected not to affect the anisotropy decays. Experimentally, the amplitude of the sub-nanosecond component, $r_o - r_{os}$, scarcely correlates with the density of tryptophan residues, although a low limiting anisotropy ($r_o < 0.2$) was occasionally seen for the proteins containing a very high density of tryptophan residues. Except for such proteins, the sub-nanosecond decay components were suggested to come from the internal motion of tryptophan residues. This assignment is consistent with the previous observations of sub-nanosecond motion in single-tryptophan-containing proteins; $\phi_f = 0.51$ ns at 8 °C in azurine [4]; $\phi_f = 0.69$ ns at 10 °C in adrenocorticotropin [14]; $\phi_f = 0.42$ ns at 19 °C in glucagon [15].

A noticeable correlation was found between the anisotropy decay kinetics and the fluorescence lifetime (Fig. 2). When the amplitude r_{os} of the slow component in the ani-

Table 1. *Parameters of emission kinetics of tryptophan residues in proteins at 10°C*
The fluorescence intensity decays $s(t)$ were described with double- or triple-exponential functions; $s(t) = \Sigma_i \alpha_i \exp(-t/\tau_i)$. $\langle\tau\rangle$ is the average lifetime $\Sigma_i \alpha_i \tau_i$. The anisotropy decays $r(t)$ were analyzed with single- or double-exponential functions: $r(t) = r_o \exp(-t/\phi_s)$, or $r(t) = (r_o - r_{os})\exp(-t/\phi_f) + r_{os}\exp(-t/\phi_s)$. Other symbols: λ_{max}, the wavelength of emission peak; na, not available; (*), not well analyzed with a double-exponential function; e.g. in the case of F-actin, $r(t)$ first decreased to 0.11 and then increased to 0.188: *S. griseus, Streptomyces griseus; S. aureus, Staphylococcus aureus; M. lysodeikticus, Micrococcus lysodeikticus; A. niger, Aspergillus niger*

Protein class	Sample (source)	Mol. mass	No. of sub-units	No. of Trp/ subunit	pH	λ_{max}	$\langle\tau\rangle$	τ_1	τ_2	τ_3	r_o	r_{os}	ϕ_f	ϕ_s
		kDa				nm	ns						ns	
Albumin	(bovine serum)	66	1	2	7.0	341	6.38	7.41	2.43		0.249	0.249	–	50.1
	(chicken egg)	45	1	3	7.0	335	5.00	7.28	1.31		0.243	0.243	–	32.0
	(human serum)	66	1	1	7.0	339	3.86	7.51	0.98		0.234	0.234	–	44.0
Protease	α-chymotrypsin (bovine)	25	1	8	5.0	328	1.41	(5.48)	2.05	0.30	0.197	0.138	–	17.4
	chymotrypsinogen (bovine)	27	1	8	1.3	326	1.37	(6.45)	2.24	0.57	0.189	0.138	0.96	16.9
	elastase (pig)	27	1	7	5.0	328	1.82	4.18	2.06	0.28	0.194	0.146	0.67	16.1
	carboxylesterase (pig)	83	2	na	7.0	332	2.98	4.18	2.06	0.28	0.210	0.185	0.80	79.3
	kallikrein (pig)	25	1	6	5.0	332	2.11	6.16	2.49	0.40	0.241	0.216	0.51	15.1
	pepsin (pig)	35	1	5	5.0	342	4.99	7.36	0.79		0.277	0.229	0.50	27.4
	pronase E (*S. griseus*)	na	1	na	5.0	327	3.33	6.24	2.54	0.35	0.194	0.194	–	22.6
	protease K (pilzen)	19	1	na	5.0	328	4.76	5.65	1.88		0.198	0.198	–	16.3
	thermolysin	36	1	3	3.8	335	3.36	6.90	4.06	1.01	0.178	*	*	20.5
	trypsin (bovine)	25	1	4	4.5	327	1.84	4.71	2.33	0.49	0.232	0.163	0.45	12.1
Nuclease	nuclease (*S. aureus*)	17	1	1	7.0	333	4.48	6.20	2.03		0.251	0.232	0.65	15.6
	DNase I (bovine)	31	1	2	7.0	334	2.68	8.29	3.78	0.98	0.207	0.181	0.66	38.4
	DNase II (bovine)	45	1	na	7.0	333	2.15	7.20	2.80	0.26	0.205	0.139	0.57	30.7
Lectin	Concanavalin A (jack bean)	26	4	4	7.0	336	3.07	6.61	3.01	0.33	0.204	0.159	1.49	53.5
	wheat germ agglutinin	18	2	1–5	7.0	346	4.93	6.93	2.71		0.224	0.162	0.78	21.8
Dehydro-genase	alcohol dehydrogenase (horse)	40	2	2	7.0	324	4.16	7.46	3.84	0.35	0.248	0.234	0.72	60.0
	lactate-dehydrogenase (pig)	37	4	5	7.0	340	5.51	7.30	2.46		0.259	0.205	1.24	69.6
Reductase	glutathione reductase (yeast)	51	2	3	7.0	329	2.17	5.97	2.67	0.58	0.248	0.209	1.39	19.7
	ferredoxin-$NADP^+$ reductase (spinach)	35	1/2	na	7.0	335	2.08	7.20	2.63	0.48	0.221	0.170	0.87	31.1
Inhibitor	trypsin-inhibitor (soybean)	22	1	2	7.0	340	5.06	6.88	4.13		0.223	0.223	–	16.7
Muscle protein	G-actin (rabbit)	43	1	3	8.3	325	3.01	6.22	2.49	0.42	0.204	0.088	0.31	18.0
	F-actin (rabbit)	43	∞	3	7.0	323	2.93	5.11	1.78		*	0.188	*	∞
	fructose-*P*-aldolase (rabbit)	40	4	3	8.1	320	0.95	(5.36)	1.90	0.29	0.222	0.222	–	51.9
	myosin S1 (rabbit)	115	1	5	7.0	336	4.98	9.13	4.60	1.04	0.236	0.198	0.58	103.1
	triose-*P*-isomerase (rabbit)	27	2	5	7.0	326	1.25	(4.65)	1.82	0.47	0.239	0.239	–	28.2
	phosphorylase b (rabbit)	97	2	12	7.0	340	4.89	6.29	2.55		0.231	0.207	0.92	80.4
	pyruvate kinase (rabbit)	60	4	3	7.0	331	3.73	6.82	3.39	0.97	0.250	0.168	0.67	*
Membrane protein	bacterioopsin (*H. halobium*)	27	∞	8	7.0	330	1.66	(7.26)	2.74	0.47	0.280	0.098	0.36	∞
Miscella-neous	alkaline phosphatase (bovine)	69	2	na	7.0	336	2.93	6.99	3.42	0.77	0.206	0.111	0.83	111.6
	apoferritin (horse) in SDS	18	1	1	7.0	333	1.75	5.70	2.50	0.31	0.288	0.186	0.15	*
	carbonic anhydrase (bovine)	30	1	4	7.0	338	4.46	6.97	3.67	0.17	0.220	0.104	0.99	21.0
	citrate synthase (pig)	50	2	9	8.5	334	3.33	5.93	2.90	0.42	0.221	0.204	0.78	49.5
	DNA polymerase (*M. lysodeikticus*)	na	na	na	7.0	334	4.48	8.28	4.30	1.30	0.251	0.185	1.28	62.2
	glucose oxidase (*A. niger*)	52	2	na	7.0	336	2.02	(7.99)	2.70	0.64	0.232	0.155	0.34	40.8
	hexokinase (yeast)	51	2	3	7.0	325	3.21	6.60	3.52	0.58	0.238	0.209	0.29	35.2
	lactogloblin A (bovine)	18	2	na	7.0	328	1.74	(6.89)	2.02	0.57	0.280	0.280	–	22.3
	lysozyme (egg white)	14	1	6	7.0	336	1.98	(5.68)	2.53	0.49	0.227	0.204	0.23	7.2
	mannosidase (jack bean)	na	na	na	7.0	328	3.09	6.03	2.93	0.62	0.221	0.158	*	*
	phospholipase A_2 (pig)	14	1	1	7.0	342	1.91	(6.83)	2.81	0.52	0.208	0.157	0.43	8.4
	rhodanase (bovine)	32	1	7	8.3	333	3.46	6.37	1.66		0.193	0.193	–	22.0
	superoxide dismutase (human)	17	2	1	7.0	331	1.33	(6.12)	2.08	0.38	0.246	0.218	0.70	13.3
	urease (jack bean)	96	6	na	7.0	332	3.66	6.67	1.88		0.287	0.200	0.38	56.6

Table 2. *Parameters of fluorescence decay kinetics of tryptophan in water*

Temperature	pH	λ_{max}	$\langle\tau\rangle$	α_1	τ_1	τ_2
°C		nm	ns		ns	
10	2	347	1.58	0.39	0.67	2.60
	7	350	3.00	0.33	0.45	4.23
	10	358	7.91	0.19	0.81	9.58
20	7	350	2.45	0.29	0.67	3.18

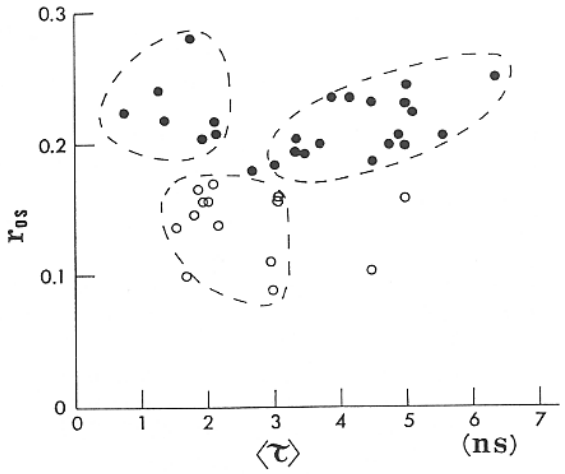

Fig. 2. *The amplitude of the slow anisotropy decay component* r_{os} *vs the fluorescence lifetime* $\langle\tau\rangle$. (●) and (○), data for proteins exhibiting high (>0.18) and low (<0.18) r_{os} values, respectively

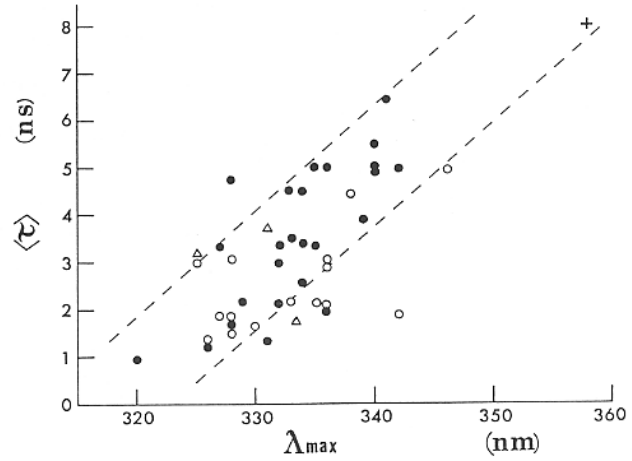

Fig. 3. *The wavelength of the emission maximum* λ_{max} *vs the fluorescence lifetime* $\langle\tau\rangle$. (●) and (○), data for proteins exhibiting high (>0.18) and low (<0.18) r_{os} values, respectively; (△), data for proteins exhibiting complicated anisotropy decays; (+), the value for free tryptophan in an alkaline solution (pH 10)

sotropy decay had a high value (>0.18), the average lifetime was found to be either long (>4 ns) or short (<2 ns). When the r_{os} value was low, the average lifetime $\langle\tau\rangle$ fell in a narrow time range of 1.5 – 3 ns with a few exceptions. A similar correlation was seen between the lifetime $\langle\tau\rangle$ and the amplitude of the fast-decay components $r_o - r_{os}$, but the correlation was somewhat obscure due to a larger experimental error in the r_o value. Since a lower value of r_{os} is expected in the presence of a larger internal motion, the correlation observed suggests

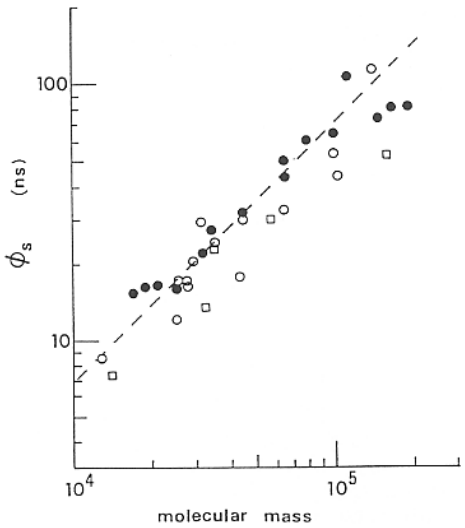

Fig. 4. *The rotational correlation time,* ϕ_s*, of the slow anisotropy decay component vs the molecular mass of protein.* Proteins exhibiting low r_{os} values (<0.18) are represented by (○) and proteins exhibiting high r_{os} values are represented by (●) (long lifetime) or by (□) (short lifetime). The dashed line shows the rotational correlation time that would be calculated for a spherical protein with 75% hydration [3]: i.e. $\phi_s = M(v + h)\eta/(RT)$, where M is the molecular mass of protein (dalton), v is the partial specific volume (0.72 $cm^3 \cdot g^{-1}$), h is the degree of hydration (0.75 v), η is the viscosity of the medium (0.013 P), R is the gas constant and T is the absolute temperature (283 K)

that the internal motion of a tryptophan residue has a great influence on its decay kinetics.

Fluorescence lifetime vs Stokes' shift

The polarity of the environment is another factor affecting the emission kinetics. In Fig. 3, the fluorescence lifetime $\langle\tau\rangle$ is plotted against the wavelength at emission peak λ_{max}. The closed circles in Fig. 3 represent the proteins exhibiting high r_{os} values, whereas the open circles represent the proteins exhibiting low r_{os} values. It can be seen that the closed circles appear in a narrow band, in which the protein exhibiting a longer mean lifetime tends to emit at longer wavelength. Since the polarity of its einvironment is a main factor determining the magnitude of the Stokes' shift, the distribution of the closed circles in Fig. 3 suggests that the tryptophan residue in an environment of higher polarity tends to have a longer fluorescence lifetime.

The above result is consistent with the previous observations concerning the wavelength-dependent lifetime of tryptophan residues. From the investigation of 17 proteins, Grinvald and Steinberg [10] noticed that proteins showing red-shifted emission spectra contained decay components characterized by long lifetimes (>7 ns). Time-resolved emission spectra of several proteins have been measured and all of them have the common feature that the component with a longer decay time has an emission maximum at longer wavelength [11, 16 – 18]. In order to explain the wavelength-dependent lifetime of tryptophan residues, Grinvald and Steinberg [10] pointed out the possibility that some photoreaction, e.g. electron ejection from the excided indole ring, is more active in a non-polar einvironment than in a polar environment. It has, however, been reported that the single tryptophan residue in apoazurine has an emission maximum at 315 nm and a considerably long lifetime (4.8 ns) [19, 20].

To discuss quantitatively the excited-state dynamics of tryptophan residue, we need more information of the tertiary structures of proteins. More investigation of the influence of the internal motion is also required.

Molecular mass vs rotational correlation time

In Fig. 4, the time constant ϕ_s of the slow component in anisotropy decay is plotted against the molecular mass of protein. When tryptophan residues in a protein exhibit heterogeneous internal motions and heterogeneous lifetimes, the time constant ϕ_s is not necessarily expected to reflect the rotational correlation time of the whole protein. Nonetheless, the closed circles in Fig. 4, which represent the data for the proteins exhibiting high r_{os} values, appear around a linear line in the log/log plot. When the r_{os} value is high, the emitting residues are expected to have little freedom of internal motion. In such a case, the fluorescence anisotropy decay can be interpreted in a straightforward manner, irrespective of the multiplicity of lifetime; i.e. the time constant ϕ_s can reflect the rotational motion of the whole protein. The rotational correlation time thus obtained was, on the average, about twice as long as the value that would be expected for an unhydrated spherical protein.

Here we propose a convenient method for estimating the molecular size of protein from the fluorescence anisotropy decay of tryptophan residue. The first point to be taken into account is to excite at the red edge of the absorption band so that the effect of energy transfer via Förster's mechanism can be minimized. The second point is to collect tryptophan emission at a long wavelength so that the signals from tryptophan residues exhibiting high r_{os} values and long lifetimes are emphasized. The third point is to carry out the fluorescence anisotropy measurements at a low temperature so as to restrict internal motion of tryptophan residues; a significant reduction in the r_{os} value was occasionally seen when the temperature was increased.

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