Steady-State and Time-Resolved Fluorescence Studies on the Ligand-Induced Conformational Change in an Active Lysozyme Derivative, Kyn62-Lysozyme

Shoji Yamashita,*,‡ Etsuko Nishimoto,‡ Arthur G. Szabo,§ and Nobuyuki Yamasaki||

Institute of Biophysics and Laboratory of Biochemistry, Faculty of Agriculture, Kyushu University, Hakozaki, Higashi-ku, Fukuoka 812, Japan, and Department of Chemistry and Biochemistry, University of Windsor, Windsor Ontario N9B 3P4, Canada

Received February 3, 1995; Revised Manuscript Received November 6, 1995⊗

ABSTRACT: The ligand-induced conformational change of an active lysozyme derivative, Kyn62-lysozyme, in which Trp62 of hen egg-white lysozyme (EC 3.2.1.17) was selectively modified to kynurenine, was investigated by steady-state and time-resolved fluorescence spectroscopy. Kyn62 formed an intramolecular energy transfer donor−acceptor pair with a tryptophan residue as a donor. The energy transfer was related to the conformation of the active site. The spectral overlap integral (J) of the kynurenine−tryptophan pair is large as it was determined to be 4.92×10^{-15} M^{-1} cm^3. Time-resolved fluorescence properties of Kyn62-lysozyme and its complex with a trimer of *N*-acetyl-D-glucosamine [$(GlcNAc)_3$] show that the energy donor is Trp28 or Trp111 in the hydrophobic matrix box of the free Kyn62-lysozyme. In the complex, it appears that the kynurenine residue drastically changed its orientation or approached closer to Trp108 to accept more efficiently the excitation energy from Trp108 on the binding of Kyn62-lysozyme with $(GlcNAc)_3$.

It has been generally accepted that proteins exhibit conformational flexibility and that this flexibility would play an essential role in allowing bioactive proteins to fulfill their biological functions (Brooks *et al*., 1988). The catalytic reactions of enzymes are initiated through initial complex formation with their appropriate substrate. Occasionally their function is regulated through the interaction with a specific activator or suppressor. In enzyme systems, therefore, the conformational flexibility would be comparable to that in many other biological systems such as the immune system and large-scale molecular assemblies. Hen egg-white lysozyme (HEWL)[1] is a model enzyme whose biochemical properties are well understood, and its crystallographic structure has been established. According to the X-ray analysis, HEWL is considered to have a relatively rigid structure (Blake *et al.*, 1965). However, evidence that the lysozyme segments in the vicinity of the active site are altered on binding of the oligomer of *N*-acetyl-D-glucosamine is provided by nuclear magnetic resonance and fluorescence spectroscopic studies (Lehrer, 1971). This contradiction demonstrates that additional studies are required on the ligand- or substrate-induced conformational changes in order to improve our understanding of the specific enzymatic reactivity of HEWL.

Steady-state and time-resolved fluorescence spectroscopies are important methods in studies of protein conformation because of the high sensitivity and the ability to uncover subtle changes in protein structure. Such studies have provided information on the protein conformational changes occurring in bioactive proteins in their complexes with ligands (Yamasaki *et al.*, 1989), the conformational heterogeneity demonstrated in proteins and peptides (Willis & Szabo, 1992), and the motional freedom found in some proteins (Rholam *et al.*, 1984). Fluorescence studies of proteins usually take advantage of the spectroscopy of the tryptophan residue. This fluorescent amino acid is found in limited quantities in proteins, and its photophysical properties make it a useful probe for monitoring protein structure, conformation, and dynamics.

HEWL contains six tryptophan residues; three of them are located in the substrate binding site, two are in the hydrophobic matrix box, and one is separate from the others. Trp62 and Trp108 are the most dominant fluorophores (Imoto *et al.*, 1972), both being located in the substrate binding site. Therefore, fluorescence changes created by the interaction with substrates can be largely attributed to the conformational change in the binding site, but would be an average of the fluorescence of Trp62 and Trp108. In those cases of a protein consisting of a single tryptophan residue, the local conformation and interactions with the surroundings can be localized as being in the segment containing the tryptophan residue (Harris & Hudson, 1990; Hutnik *et al.*, 1989). However, when a protein has more than one tryptophan residue in the same domain, like HEWL, it would be more informative if another fluorophore could be substituted for one of the tryptophan residues. Hogue *et al.* biosynthetically incorporated 5-hydroxytryptophan, which has an extended red-shifted absorption spectrum, into an oncomodulin mutant using site-directed mutagenesis (Hogue *et al.*, 1992). This has advantages in studies of protein−protein interactions where one protein contains a tryptophan analogue and the other contains normal tryptophan. Chemical modification of tryptophan may also be effective, if it is possible to

* To whom correspondence should be addressed.
‡ Institute of Biophysics, Kyushu University.
§ University of Windsor.
|| Laboratory of Biochemistry, Kyushu University.
⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1995.
[1] Abbreviations: Kyn, kynurenine; Kyn62-lysozyme, an active derivative of hen egg-white lysozyme in which Trp62 is replaced with kynurenine; HEWL, hen egg-white lysozyme; $(GlcNAc)_n$, $\beta(1\rightarrow4)$-linked *n*-mer of *N*-acetyl-D-glucosamine.

0006-2960/96/0435-531$12.00/0

selectively change one amino acid residue to another chromophore without affecting the protein structure and activity. Trp62 of HEWL can be preferentially converted to kynurenine, maintaining its enzymatic activity afterward (Yamasaki *et al.*, 1979). Kyn62-lysozyme, in which Trp62 is modified to kynurenine, permits the detection of the conformational change in the active site. Conveniently, the absorption band of kynurenine extends from 300 to 400 nm, and it overlaps the fluorescence band of tryptophan residues (Charchich, 1972). This spectroscopic property of kynurenine allows us to examine the probability of intramolecular energy transfer from a tryptophan residue to kynurenine in Kyn62-lysozyme. The donor−acceptor distance can be uniquely obtained using the Förster energy transfer formalism and is a useful criterion for conformational changes in proteins.

In the present work, we have analyzed the steady-state and time-resolved fluorescence properties of Kyn62-lysozyme in detail and are able to describe conformational changes induced by oligomers of *N*-acetyl-D-glucosamine.

MATERIALS AND METHODS

Materials and General. Hen egg-white lysozyme (HEWL) was purchased from Seikagaku Kogyo Co. Kyn62-lysozyme was prepared by the ozonization of HEWL following the method established by Sakiyama and Natsuki (1976). Chitin was hydrolyzed to obtain tri-, tetra-, and penta-*N*-acetyl-D-glucosamine [$(GlcNAc)_3$, $(GlcNAc)_4$, and $(GlcNAc)_5$] based on the method described by Rupley (1964). Their purity was checked by high-performance liquid chromatography.

Solutions of Kyn62-lysozyme, HEWL, and $(GlcNAc)_n$ were prepared in 0.1 M acetate buffer (pH 5.5). The concentrations of the lysozymes were determined spectrophotometrically using molar extinction efficients $\epsilon = 4.7 \times 10^3$ M^{-1} cm^{-1} at 360 nm and $\epsilon = 3.9 \times 10^4$ M^{-1} cm^{-1} at 280 nm for Kyn62-lysozyme and HEWL, respectively. The absorption spectra were measured with a Varian DMS 200 UV Spectrophotometer.

Solutions of Kyn62-lysozyme−$(GlcNAc)_n$ complexes were prepared by adding $(GlcNAc)_n$ stock solutions to the enzyme solution. Temperature was controlled with Neslab Endocal thermo-regulation bath.

Time-Resolved Fluorescence. Time-resolved fluorescence measurements were performed using the technique of time-correlated single photon counting. The excitation pulse was obtained from a cavity-dumped dye laser synchronously pumped by a mode-locked argon ion laser (Spectra Physics) operating at 825 kHz with a pulse width of 15 ps. Fluorescence emission, following vertically polarized excitation, was detected on a Hamamatsu 1564U-01 microchannel plate photomultiplier after passing through a Jovin-Yvon H-10 monochromator with a 4 nm band-pass. The channel width of the multichannel analyzer was set to 21.2 ps, and data were collected in 1024 channels. The instrument response function was determined by measuring the scattered light from a suspension of glycogen in sodium cacodylate buffer. The ratio of laser pulse to single photon events was greater than 100:1. Data were analyzed with a nonlinear least-squares iterative convolution method based on the Marquart algorithm (McKinnon *et al.*, 1977). Adequacy of the fluorescence decay was judged by inspection of the plots of weighted residuals and by the statistical parameters such as reduced chi-square (χ^2) and the serial variance ratio (SVR). The detailed instrumentation and procedures for the time-resolved fluorescence measurements have been described elsewhere (Willis & Szabo, 1989).

For measurements of the time-resolved fluorescence anisotropy, a Glan−Taylor polarizer was placed between the sample holder and the emission monochromator. It was set at 0° or 90° relative to the vertical excitation polarization direction, and the decay of parallel (F_{VV}) and perpendicular (F_{VH}) components was collected. The equations $F_{VV}(t) = (1/3)F(t)\{1 + 2r(t)\}$ and $F_{VH} = (1/3)F(t)\{1 - r(t)\}$, derived from the definition of the fluorescence anisotropy, $F_{VV}(t)$ and $F_{VH}(t)$, were fit simultaneously to obtain the parameters for the fluorescence anisotropy decay kinetics. $F(t)$ and $r(t)$ are the fluorescence emission intensity and anisotropy decay functions, respectively. $F(t)$ and $r(t)$ were described by sums of exponentials as follows: $F(t) = \Sigma\alpha_i e^{-t/\tau_i}$, and $r(t) = \Sigma\beta_i e^{-t/\phi_i}$, where τ_i and ϕ_i are the fluorescence lifetime and the rotational correlation time of the ith component, respectively, and α_i and β_i are their corresponding amplitudes. The G-factor or sensitivity scaler was determined by measuring $F_{VV}(t)$ and $F_{VH}(t)$ of a solution of azanaphthene or 9-cyanonaphthalene in ethanol.

Steady-State Fluorescence. Steady-state fluorescence measurements were performed with an SLM 8000C spectrofluorometer. The fluorescence spectra were taken with excitation and emission band-passes of 4 nm. Correction was made for the contribution from the blank and the wavelength dependence of the instrument response.

The fluorescence quantum yields of kynurenine in Kyn62-lysozyme were calculated relative to the standard quinine sulfate ($\Phi = 0.56$) fleshly prepared in 0.05 M sulfuric acid (Meech & Phillips, 1983).

The steady-state fluorescence anisotropy of the kynurenine of Kyn62-lysozyme was measured with T-format detection configuration using Glan−Taylor polarizers. The emission wavelength was set at 450 nm, and Kyn62-lysozyme was excited at 285 or 360 nm. Band-passes for excitation and emission were 4 nm.

Evaluation of Energy Transfer Rate and Distance between Energy Donor/Acceptor Pair. The distance between the energy donor and acceptor (R) can be related to the energy transfer efficiency (E) according to the equation:

$$(R_0/r)^6/\tau_d^0 = k_e \qquad (1)$$

R_0 is the critical distance which gives 50% efficiency of energy transfer and is expressed by eq 2

$$R_0^6 = (8.785 \times 10^{-25})K^2\Phi_0 n^{-4} J \text{ (cm)} \qquad (2)$$

$$J = \int \epsilon_A(\lambda)F_0(\lambda)\lambda^4 \, d\lambda / \int F_0(\lambda) \, d\lambda \qquad (3)$$

where J is the spectral overlap integral, n is the refractive index of the medium, K^2 is an orientation factor which is determined by the mutual orientation of donor and acceptor molecules, and Φ_0 is the fluorescence quantum yield of the donor. ϵ_A is the absorption coefficient of the donor. J was determined by integrating the overlap of the fluorescence spectrum of the tryptophan residues and the absorption spectrum of kynurenine.

The random distribution approximation was applied to K^2 for the estimation of R_0. Because this approximate value,

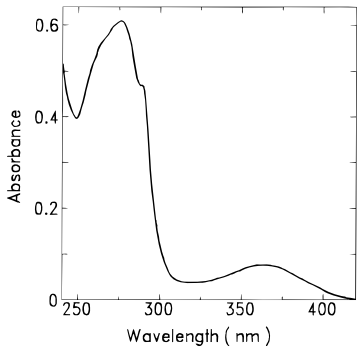

FIGURE 1: Absorption spectrum of Kyn62-lysozyme. The spectrum was recorded at 20 °C with a concentration of 1.7×10^{-5} M for Kyn62-lysozyme in sodium acetate buffer (pH 5.5, 0.1 M).

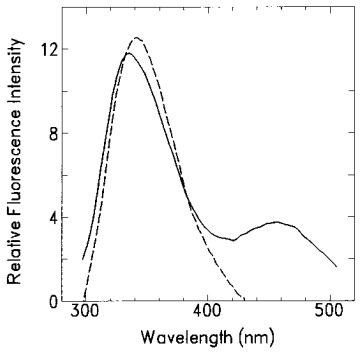

FIGURE 2: Fluorescence spectra of native lysozyme (- - -) and Kyn62-lysozyme (—). The concentrations of native lysozyme and Kyn62-lysozyme were 10^{-6} M. Excitation wavelength, 285 nm.

$K^2 = {}^2/_3$, is adequate only when donor/acceptor pairs have isotropic rotations on the time scale of the fluorescence lifetime, the upper ($\langle K^2\rangle^{max}$) and lower ($\langle K^2\rangle^{min}$) limits were calculated for the orientation factor based on Dale (Dale *et al.*, 1979). The polarization factor, $\langle d^x\rangle$, is related to the anisotropy (r) by $\langle d^x\rangle^2 = r/r_0$, where r_0 is the anisotropy at $t = 0$ in the anisotropy decay. $\langle K^2\rangle^{max}$ and $\langle K^2\rangle^{min}$ are then given by

$$\langle K^2\rangle^{max} = (2/3)(1 + \langle d_D{}^x\rangle + \langle d_A{}^x\rangle + 3\langle d_D{}^x\rangle\langle d_A{}^x\rangle)$$

$$\langle K^2\rangle^{min} = (2/3)[1 - (\langle d_D{}^x\rangle + \langle d_A{}^x\rangle)/2]$$

RESULTS

The absorption spectrum (Figure 1) of Kyn62-lysozyme consisted of two large absorption bands attributed to the aromatic amino acids, tryptophan and kynurenine. The high-energy band shows two maxima at 286 and 280 nm, similar to that of native HEWL.

Figure 2 shows the fluorescence spectra of the native and modified lysozyme excited at 285 nm. In addition to the emission from tryptophan residues, the fluorescence of Kyn62-lysozyme shows another maximum at 450 nm, which is assigned to the fluorescence of kynurenine 62. The fluorescence peak of native HEWL is centered at 342 nm, while the tryptophyl fluorescence of Kyn62-lysozyme has a spectral maximum at 335 nm, a 7 nm blue shift. The full absorption band-shape of the kynurenine residue in Kyn62-lysozyme was obtained by extrapolating the absorption line from 420 to 330 nm to the shorter wavelength side. It showed a maximum at 350 nm. This spectrum is shown in Figure 3 together with the normalized fluorescence spectra

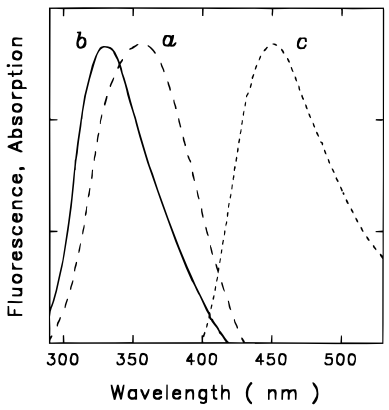

FIGURE 3: Spectral overlap of the kynurenyl absorption band (curve a) with the tryptophyl fluorescence band (curve b). Curve c, fluorescence band of the kynurenine residue in Kyn62-lysozyme. Curve a was obtained by extrapolating the lowest absorption band of Kyn62-lysozyme to the shorter wavelength side.

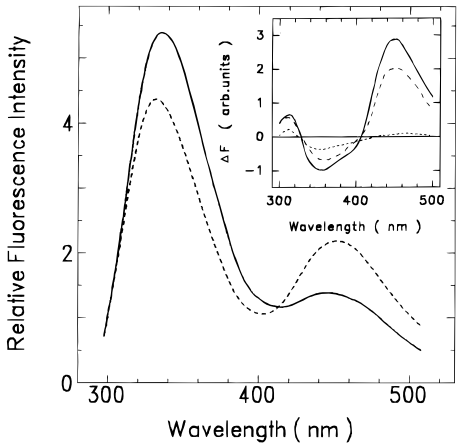

FIGURE 4: Effect of $(GlcNAc)_3$ on the fluorescence spectra of Kyn62-lysozyme. The concentration of Kyn62-lysozyme was 2.5×10^{-6} M. (—) Kyn62-lysozyme; (- - -) in the presence of 250×10^{-6} M $(GlcNAc)_3$. Inset: the fluorescence difference spectra between Kyn62-lysozyme in the absence and in the presence of $(GlcNAc)_3$. The reference was Kyn62-lysozyme. (—) Kyn62-lysozyme + 250×10^{-6} M $(GlcNAc)_3$; (- - -) Kyn62-lysozyme + 50×10^{-6} M $(GlcNAc)_3$; (•••) Kyn62-lysozyme + 5×10^{-6} M $(GlcNAc)_3$.

of tryptophan and kynurenine residues. The overlap of the absorption spectrum of kynurenine with tryptophan fluorescence was significant, and the spectral overlap integral (J) was estimated to be 4.92×10^{-15} M^{-1} cm^3.

In the presence of $(GlcNAc)_3$, the fluorescence intensity of tryptophan was reduced above 340 nm, and that of kynurenine was enhanced, when Kyn62-lysozyme was excited at 285 nm. The difference spectra between the free Kyn62-lysozyme and the Kyn62-lysozyme−$(GlcNAc)_3$ complex are shown in the inset in Figure 4. This difference spectrum shows a large negative band in the tryptophan fluorescence spectral region and small but clear positive maxima at 460 nm and at 320 nm, respectively.

The fluorescence quantum yield of kynurenine in Kyn62-lysozyme was estimated to be 0.03 when kynurenine was excited at 360 nm. Although the absorption of kynurenine is considered to be negligible at 285 nm (Teshima *et al.*, 1980), the fluorescence emission of kynurenine was clearly observed on excitation at 285 nm, and its quantum yield was estimated to be 0.003. The ratios of the fluorescence quantum yield (ϕ_{285}/ϕ_{360}), where ϕ_{285} and ϕ_{360} are the

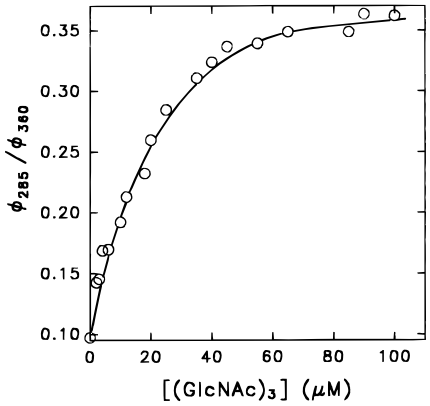

FIGURE 5: Effect of $(GlcNAc)_3$ on the ratio of the fluorescence quantum yield of kynurenine in Kyn62-lysozyme excited at 285 nm (ϕ_{285}) to one excited at 360 nm (ϕ_{360}). Emission was measured at 450 nm. The concentration of Kyn62-lysozyme was 10^{-6} M.

Table 1: Fluorescence Decay Parameters of the Kynurenine Residue in Kyn62-Lysozyme[a]

	α_1	α_2	α_3	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)
Kyn62-lysozyme (ex = 320 nm)	0.06	0.31	0.63	6.31	1.95	0.36
Kyn62-lysozyme + $(GlcNAc)_3$ (ex = 285 nm)	0.36	2.49	−1.58	4.24	1.71	0.15

[a] The emission wavelength is 450 nm.

fluorescence yields of kynurenine excited at 285 and 360 nm, respectively, were plotted against the concentrations of $(GlcNAc)_3$. It increased with increasing concentration of $(GlcNAc)_3$, reaching a constant value $\phi_{285}/\phi_{360} = 0.352$ at a ratio of $(GlcNAc)_3$/enzyme = 80:1 (Figure 5).

The fluorescence decay parameters of Kyn62-lysozyme and its complex with $(GlcNAc)_3$ measured at the emission maxima of kynurenine are summarized in Table 1. When this sample was excited at 320 nm, the fluorescence of kynurenine (450 nm) gave a good fit to three exponential decay components. Excitation at 285 nm, in the absorption band of the tryptophan residues, resulted in a component at 450 nm with a negative preexponential factor. This is evidence for the energy transfer from tryptophan to kynurenine in the fluorescence of the Kyn62-lysozyme−$(GlcNAc)_3$ complex. The decay times measured at 450 nm which were determined with 285 nm excitation are significantly different from those measured with 320 nm excitation. This is a consequence of complex decay kinetics resulting from energy transfer from tryptophan to kynurenine when 285 nm excitation was used. As the temperature increased, the decay time corresponding to the negative amplitude decreased.

The fluorescence decay of the tryptophan residues (W_{em} = 340 nm) of Kyn62-lysozyme was more complicated and best described by four exponential decay components (Table 2). This is obviously the consequence of a combination of the fluorescence of each of the other tryptophan residues remaining in the protein. These decay times represent

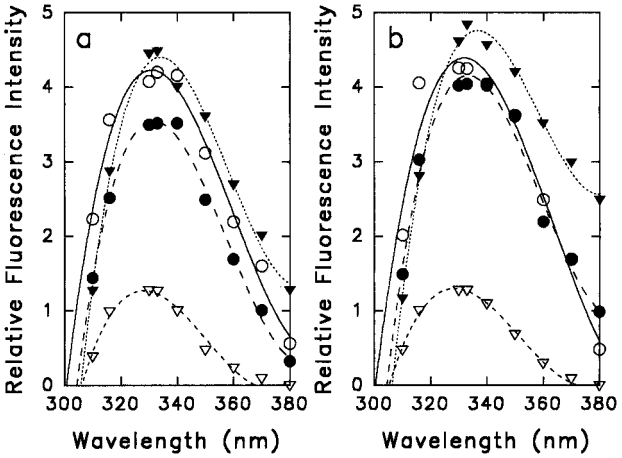

FIGURE 6: Fluorescence decay-associated spectra of Kyn62-lysozyme (b) and its complex with $(GlcNAc)_3$ (a). (●) Component with longest lifetime (τ_1); (▼) component with second longest lifetime (τ_2); (○) component with second shortest lifetime (τ_3); (▽) component with shortest lifetime (τ_4).

average values of the decay components of the individual tryptophans.

When the fluorescence decay times, τ_i, are constant across the spectral band, then the individual data sets can be combined into a global analysis and decay associated spectra (DAS) of each decay component can be constructed (Knutson *et al.*, 1982). The DAS of the tryptophan residues are shown in Figure 6. If the decay components τ_i are discussed in order of increasing magnitude, where τ_1 is the longest decay time and τ_4 is the shortest, then the τ_3 and τ_4 components exhibit maxima at 330 nm and the spectral maxima of τ_1 and τ_2 components were observed at 340 nm. The binding of $(GlcNAc)_3$ decreased the relative emission of the τ_1, τ_2, and τ_3 components at wavelengths >350 nm. On the other hand, the intensity of each component was greater at the short wavelength side of the spectra. The DAS of each decay component in the kynurenine fluorescence band (400−520 nm) all had a similar shape. Hence, the preexponential term at any wavelength can represent the relative proportions of the three decay components.

When the average fluorescence lifetime of tryptophan residues of Kyn62-lysozyme, which is defined as $\tau_{av} = \Sigma\alpha_i\tau_i$, was plotted against the emission wavelength, it increased linearly with wavelength from 310 to 360 nm to reach a constant value, τ_{av} = 0.50 ns (Figure 7). This τ_{av}−wavelength curve was different in the $(GlcNAc)_3$ complex (Figure 7, solid circles) with the τ_{av} of the tryptophan residues of Kyn62-lysozyme in the complex being lower than that for the uncomplexed enzyme at wavelengths >340 nm.

The steady-state fluorescence anisotropy of kynurenine was observed to be 0.1 when Kyn62-lysozyme was excited at 360 nm, and increased to 0.2 in the complex of Kyn62-lysozyme with $(GlcNAc)_3$. Time-resolved anisotropy measurements showed that the dynamics of the enzyme and

Table 2: Fluorescence Decay Parameters of Tryptophan Residues in Hen Egg-White Lysozyme and Kyn62-Lysozyme[a]

	SVR	σ	α_1	α_2	α_3	α_4	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_4 (ns)	τ_{av} (ns)
HEW-lysozyme	2.07	1.15	0.14	0.43	0.42		3.16	1.31	0.38		1.16
Kyn62-lysozyme	2.00	1.12	0.03	0.09	0.19	0.69	3.25	1.26	0.37	0.13	0.37
Kyn62-lysozyme + $(GlcNAc)_3$	2.00	1.14	0.03	0.12	0.27	0.57	2.92	1.09	0.33	0.16	0.37

[a] Excitation wavelength, 295 nm; emission wavelength, 340 nm.

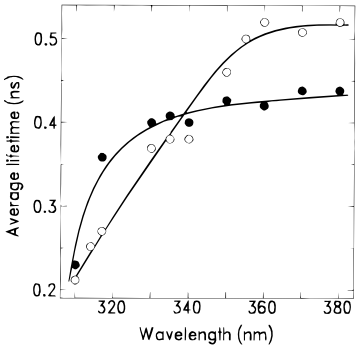

FIGURE 7: Emission wavelength dependence of the average lifetime of Kyn62-lysozyme (○) and its complex with $(GlcNAc)_3$ (●). The average lifetime was defined as $\tau_{av} = \sum\alpha_i\tau_i$, where α_i and τ_i are the normalized preexponential factor and the fluorescence lifetime of the ith component, respectively.

Table 3: Dynamic Parameters of Segmental Motions of Kynurenine and Tryptophan Residues in Kyn62-Lysozyme

	β_1	β_2	β_3	ϕ_1 (ps)	ϕ_2 (ns)	ϕ_3 (ns)	r_0
kynurenine (free)[a]	0.16	0.13	0.03	150	1.40	4.20	0.32
kynurenine (complex)[a]	0.13		0.18	170		4.50	0.31
tryptophan (free)[b]	0.23		0.06	<20		4.44	0.29
tryptophan (complex)[b]	0.24		0.08	<20		4.56	0.32

[a] Excitation wavelength, 320 nm; emission wavelength, 450 nm. [b] Excitation wavelength, 295 nm; emission wavelength, 340 nm.

enzyme−ligand complex reflected both the overall rotational motion of the enzyme and the local segmental motion of the kynurenine residue (Table 3). The 4.20 and 4.50 ns correlation times may be assigned to the overall rotational tumbling of the enzyme in solution. The two shorter correlation times in the cases of Kyn62-lysozyme represent the segmental motion of the kynurenine residue. In the complex, the 1.40 ns component disappears, but the 4.50 ns component becomes more dominant ($\beta = 0.18$). The steady-state fluorescence anisotropy of tryptophan residues measured at 340 nm (W_{ex} = 295 nm) gave a value of 0.1, in the presence or absence of ligand. The internal motion of tryptophan residues appeared to be very rapid with a value of the rotational correlation time of <20 ps being observed.

DISCUSSION

The fluorescence emission band of tryptophan has a high degree of overlap with the absorption band of kynurenine, satisfying one of the essential conditions for intramolecular energy transfer in Kyn62-lysozyme. The fluorescence of kynurenine was readily observed even when the excitation was in the absorption band of the tryptophan residue. The fluorescence intensities of tryptophan and kynurenine residues complementarily decreased and increased on binding of $(GlcNAc)_n$. This suggests that the excitation energy of the tryptophan residue was transferred to kynurenine and the efficiency of this transfer was enhanced in the Kyn62-lysozyme−ligand complex. Previous reports on the fluorescence properties of Kyn62-lysozyme and HEWL showed that the fluorescence intensity of kynurenine increased on formation of the Kyn62-lysozyme−$(GlcNAc)_3$ complex even when kynurenine was directly excited at 360 nm (Teshima *et al.*, 1980). Furthermore, the tryptophyl fluorescence of HEWL which contained no energy acceptor was quenched by the presence of $(GlcNAc)_3$ at pH 5.0 (Lehrer *et al.*, 1971).

Table 4: Energy Transfer Parameters for Kyn62-Lysozyme

	Kyn62-lysozyme	Kyn62-lysozyme−$(GlcNAc)_3$ complex
ϕ_{285}/ϕ_{360}	0.099	0.352
τ_d (ns)	0.37[a]	0.15[b]
k_e (10^8/s)	2.60	23.46
τ_d^0 (ns)	0.41	0.23
$\langle K^2\rangle^{RDA}$	0.67	0.67
$\langle K^2\rangle^{max}$	1.91	2.51
$\langle K^2\rangle^{min}$	0.31	0.21
R_0^{RDA} (Å)	16.5	16.5
R_0^{max} (Å)	19.8	20.7
R_0^{min} (Å)	14.6	13.7
R^{RDA} (Å)	23.8	18.3
R^{max} (Å)	28.8	22.9
R^{min} (Å)	21.2	15.2

[a] The average fluorescence lifetime of tryptophan residues measured at 340 nm. [b] The fluorescence decay time of kynurenine with the negative preexponential factor.

At first sight, these seem to be inconsistent with our conclusion. However, the concomitant changes in the tryptophyl and kynurenyl fluorescence intensities are doubtlessly results from the promotion of the intramolecular energy transfer efficiency. The normalized fluorescence quantum yield of kynurenine excited at 285 nm to one excited at 360 nm (ϕ_{285}/ϕ_{360}) was increased 3.5-fold on formation of the Kyn62-lysozyme−$(GlcNAc)_3$ complex. If the energy transfer was not enhanced, ϕ_{285}/ϕ_{360} would decrease.

The ratio ϕ_{285}/ϕ_{360}, which substantially corresponds to the energy transfer efficiency (E) between tryptophan and kynurenine, is related to the energy transfer rate, k_e, through the rates of the relaxation processes of Kyn62-lysozyme from the excited singlet state as in eq 4c:

$$\phi_{360} = k_f^K/(k_f^K + k_i^K) \quad (4a)$$

$$\phi_{285} = [k_e/(k_f^T + \sum k_i^T + k_e)][k_f^K/(k_f^K + \sum k_i^K)] \quad (4b)$$

$$\phi_{285}/\phi_{360} = k_e\tau_d \quad (4c)$$

where k_f and k_i are the rates of the radiational and radiationless transition, respectively, superscripts K and T correspond to kynurenine and tryptophan, respectively, and τ_d is the lifetime of tryptophan working as an energy donor.

According to the Förster formalism (Förster, 1965), the energy donor−acceptor distance (R) and other parameters related to energy transfer in Kyn62-lysozyme are evaluated by using eqs 1−3 and 4c. The parameters decided experimentally are summarized in Table 4. The fluorescence lifetime of the energy donor in the absence of the energy acceptor (τ_d^0) was calculated with k_e and τ_d. Two types of fluorescence lifetime of the energy donor (τ_d) were employed for the evaluation of the energy transfer rates. One is the average lifetime of the tryptophan residues in Kyn62-lysozyme, and the other is the decay time with the negative preexponential factor in the decay kinetics of kynurenine. According to the well-known energy transfer kinetics, the fluorescence lifetime of the energy donor corresponds to the decay time of the component with a negative preexponential factor in the fluorescence decay kinetics of the energy acceptor (Schiller, 1975). Theoretically, the latter must be valid for the lifetime of the energy donor. Because the former includes the contributions from the fluorescence of

tryptophan residues which do not take part in the energy transfer, it must be estimated to be longer than the real lifetime of the tryptophan residue working as the energy donor. But, when the energy transfer rate is smaller than the fluorescence decay rate of the tryptophan residue (in the absence of ligand), adopting the average lifetime is not the source of the serious errors.

When the random distribution approximation ($K^2 = {}^2/_3$) is used, the critical distance (R_0) is found to be 16.5 Å, and the corresponding energy donor−acceptor distances of the free and bound states of the enzyme were estimated to be 23.8 and 18.3 Å, respectively. Although the upper and lower limits of the orientation factor resulted in a fairly large uncertainty in the estimation of R_0, the possible ranges of the donor−acceptor distances were within 28.8−21.2 and 22.9−15.2 Å in the free and ligand binding Kyn62-lysozyme, respectively. These results suggest that the energy donor (acceptor) is about 6 Å closer to the acceptor (donor) if the orientation factor is not extremely changed by the binding of $(GlcNAc)_3$. However, it should be noted that the lower limit of the donor−acceptor distance in the free Kyn62-lysozyme is shorter than the upper limit in the complex with $(GlcNAc)_3$. This fact demonstrates the possibility that the donor−acceptor pair in Kyn62-lysozyme might exclusively change the mutual orientation on the interaction with $(GlcNAc)_3$ to increase K^2 without reducing its distance.

To support a substantial conformational change based on these results, the energy donor should be designated. We can reasonably exclude two of five tryptophan residues, Trp63 and Trp123, from consideration because it is confirmed that their excited singlet states are almost completely quenched by the Dexter-type electron exchange interactions with the adjacent disulfide linkages (Cys76−Cys96, Cys6−Cys127) (CowGill, 1967). The fluorescence properties of Kyn62-lysozyme and the complex with $(GlcNAc)_3$ consistently demonstrate that the energy donor in free Kyn62-lysozyme is Trp28 or Trp111 and it switches to Trp108 on the formation of Kyn62-lysozyme with $(GlcNAc)_n$. The fluorescence of the tryptophan residues was reduced at the longer wavelengths and was enhanced at the shorter wavelength side in the fluorescence and decay-associated spectra in the presence of $(GlcNAc)_3$. This trend was more clearly seen in the average lifetime−wavelength curves. All of these results suggest that the fluorescence of the tryptophan residues located in the hydrophobic region is enhanced by being free from the energy donor and, contrarily, one of tryptophan residues surrounded by the polar circumstances is quenched by coming closer to kynurenine to participate in the energy transfer. Trp28 and Trp111 are buried in the hydrophobic matrix box, and their fluorescence maxima are seen around 320 nm. As shown in a specific Raman line, Trp108 is surrounded in a polar atmosphere (Miura *et al.*, 1988), and, therefore, its fluorescence deflects to the longer wavelength side. According to the X-ray crystallographic study, the distances between Trp62 and the other five tryptophan residues in HEWL, Trp28, Trp63, Trp108, Trp111, and Trp123, which were evaluated with the distance between 8th carbons in the indole ring of tryptophans, were 19.32, 5.73, 13.09, 18.63 and 27.13 Å, respectively (Diamond, 1974). The donor−acceptor distance in free Kyn62-lysozyme evaluated under the random distribution approximation (23.8 Å) is longer, but its lower limit distance almost corresponds to the distance between Trp62 and Trp28 or Trp111. One or both of these tryptophan residues probably transfers the excitation energy to kynurenine.

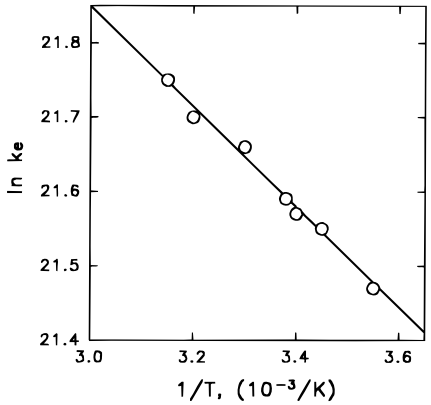

FIGURE 8: Arrhenius plots of the energy transfer rate (k_e) in the Kyn62-lysozyme−$(GlcNAc)_3$ complex. k_e was evaluated with the fluorescence lifetime of the energy donor and ϕ_{285}/ϕ_{360} at various temperatures using eq 4c (see text).

Unfortunately, our results cannot definitely confirm the kynurenine-Trp108 distance in the free Kyn62-lysozyme as far as considering Trp28 or Trp111 as the energy donor. But our data suggest that kynurenine occupies a position apart from Trp108 by more than 21.2−28.8 Å in the free Kyn62-lysozyme. Although this anticipated distance does not correspond to one between Trp62 and Trp108 in the crystallographic structure of HEWL, it may be reasonable to consider that the kynurenine ring is kept apart from the peptide chain of Kyn62-lysozyme. Because kynurenine is created by cleavage of the indole ring of Trp62, therefore it may be connected to the peptide chain with the longer arm.

The active site of HEWL is composed of six subsites (subsites A, B, C, D, E, and F). Trp62 is a constituent residue of subsite C, and it binds directly with $(GluNAc)_n$ through a hydrogen bond between the N-atom of the indole ring and the O-atom of *N*-acetyl-D-glucosamine. Trp108 is located at the opposite side of this binding cleft. In the case of Kyn62-lysozyme, such an arrangement of kynurenine and Trp108 is ordinarily retained, and Kyn62 is also linked to $(GlcNAc)_3$. On the basis of the crystallographic structure of HEWL and our experimental results, we can draw a picture for the ligand-induced conformational change in Kyn62-lysozyme such that the kynurenine may flip toward Trp108 to play the role as subsite C. Then, kynurenine would come closer to Trp108 more than 6 Å or change the orientation such that the overlap of its molecular plane with one of Trp108 might be larger. Proton magnetic resonance studies provide a possibility that Trp108 may be pulled to the side toward kynurenine by the binding of other subsites with $(GlcNAc)_3$ or the binding of itself with other amino acid residues such as Leu56 (Perkins *et al.*, 1981). We have no direct evidence for objecting to their assumption. But the small activation energy for the conformational change, which was estimated to be 930 cm^{-1} (1.73 kcal/mol) in the Arrhenius plot for the energy transfer rate (Figure 8), suggests the fragment volume involved in the conformational change may be relatively small. The movement of Trp108, which is surrounded with polar residues, might demand a larger activation energy.

Although fluorescence spectroscopic methods provide practical information on protein conformations, their results and interpretations should be examined with other physico-

chemical studies. The ligand-induced conformational change in Kyn62-lysozyme shown in the present work was mostly consistent with the conformation of HEWL studied with NMR spectroscopy. But a loosely packed conformation was drawn for Kyn62-lysozyme and the complex with oligosaccharide. Such a conformation is rather interesting because the potential flexibility of the protein conformation must be more vividly exhibited in physiological conditions.

REFERENCES

Blake, C. C. F., Koenig, D. F., Mair, G. A., North, A. C., Phillips, D. C., & Sarma, V. R. (1965) *Nature 206*, 757−761.

Brook, C. L., III, Karplus, M., & Pettitt, B. M. (1988) *Adv. Chem. Phys. 71*, 7−21.

Charchich, E. J. (1972) *Biochim. Biophys. Acta 285*, 91−98.

CowGill, R. W. (1967) *Biochim. Biophys. Acta 140*, 37−44.

Dale, R. E., Eisinger, J., & Blumberg, W. E. (1979) *Biophys. J. 26*, 161−193.

Diamond, R. (1974) *J. Mol. Biol. 82*, 371−390.

Förster, T. (1965) *Istanbul Lecture* (Sinanoglu, O., Ed.) Part III, pp 93−137, Academic Press, New York.

Harris, D. L., & Hudson, B. S. (1990) *Biochemistry 29*, 6276−6285.

Hogue, W. V. C., Rasquinha, I., Szabo, A. G., & MacManus, J. P. (1992) *FEBS Lett. 310*, 269−272.

Hutnic, C. M., & Szabo, A. G. (1989) *Biochemistry 28*, 3935−3939.

Imoto, T., Foster, L. S., Ruoley, J. A., & Tanaka, F. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1151−1155.

Knutson, J. R., Walbridge, D. G., & Brand, L. (1982) *Biochemistry 21*, 4671−4679.

Lehrer, S. S. (1971) *Biochemistry 10*, 3254−3263.

McKinnon, A. E., Szabo, A. G., & Miller, D. (1977) *J. Phys. Chem. 81*, 1564−1570.

Meech, S. R., & Phillips, D. (1983) *J. Photochem. 23*, 193−217.

Miura, T., Takeuchi, H., & Harada, I. (1988) *Biochemistry 27*, 88−94.

Perkins, S. J., Johnson, L. N., Phillips, D. C., & Dwek, R. A. (1981) *Biochem. J. 193*, 553−572.

Rholam, B. P., Scarlata, S., & Weber, G. (1984) *Biochemistry 23*, 6793−6796.

Rupley, J. A. (1964) *Biochim. Biophys. Acta 83*, 245−255.

Sakiyama, F., & Natsuki, R. (1976) *J. Biochem. (Tokyo) 79*, 225−228.

Schiller, P. W. (1975) *Biochemical Fluorescence: Concepts* (Chen, R. F., & Edelhoch, H., Eds.) pp 295−303, Marcel Dekker, Inc., New York.

Teshima, K., Kuramitsu, S., Hamaguchi, K., Sakiyama, F., Mizuno, K., & Yamasaki, N. (1980) *J. Biochem. (Tokyo) 87*, 1015−1027.

Willis, K. J., & Szabo, A. G. (1989) *Biochemistry 28*, 4902−4908.

Willis, K. J., & Szabo, A. G. (1992) *Biochemistry 31*, 8924−8931.

Yamasaki, N., Tsugita, T., Eto, T., Masuda, S., Mizuno, K., & Sakiyama, F. (1979) *J. Biochem. (Tokyo) 86*, 1291−1300.

Yamasaki, N., Absar, N., & Funatsu, G. (1989) *J. Mol. Recognit. 1*, 153−157.

Zuker, M., Szabo, A. G., Bramall, L., & Krajcarski, D. T. (1985) *Rev. Sci. Instrum. 56*, 14−22.

BI9502553