

Steady State and Time-Resolved Fluorescence Studies on the Structure and Dynamics of Lysozyme

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on the Structure and Dynamics of Lysozyme

YOSUKO NISHIMOTO

1983

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ABBREVIATIONS:

HEWL,	hen egg-white lysozyme
W62Y-lysozyme,	hen egg-white lysozyme of which Trp62 is replaced with tyrosine
W108Y-lysozyme,	hen egg-white lysozyme of which Trp108 is replaced with tyrosine
Kyn62-lysozyme,	hen egg-white lysozyme of which Trp62 is replaced with kynurenine
(NAG) ₃ ,	(1→4) linked trimer of <i>N</i> -acetyl-D-glucosamine
TCSPC,	time-correlated single photon counting
MCA,	a multichannel analyzer
TAC,	a time-to-amplitude converter
ADC,	an analogue-to-digital converter
MCP,	a microchannel plate type photomultiplier
FWHM,	full width of half maximum
SVR,	serial variance ratio
WSSR,	weighted sum of squares of differences or residuals
K _{sv} ,	Stern-Volmer constant
k _q ,	collisional fluorescence quenching constant
τ _i ,	fluorescence decay constant of i-th component when the fluorescence decay is described with linear combination of exponentials
α _i ,	amplitude of i-th component corresponding to τ _i
φ _i ,	rotational correlation time of i-th component when the fluorescence anisotropy decay is described with linear combination of exponentials
β _i ,	amplitude of i-th component corresponding to φ _i
S ² ,	order parameter of the orientation of tryptophan residue
r _s ,	steady state fluorescence anisotropy

CHAPTER 1

General Introduction

Fluorescence spectroscopy has been deeply concerning with biochemistry. It is a commencement that Becchari found a luminescence correspond to the fluorescence in protein-enriched tissue in eighteenth century. Since then, fluorescence methods enable to detect specific chemical reaction and interaction of excited molecule have given constantly fundamental information on biochemistry. Recently, validity and reliability on fluorescence spectroscopy were revolutionally improved because of development of sensitive detection apparatus, high performed excitation source such as picosecond laser, and applications of computer technology. Fluorescence is an electronic transition process with radiation between the singlet states. The molecules excited to so-called Fank-Condon state by light absorption immediately lose their excess energy to reach the thermal equilibrium in the excited state. In condensed matter like biological system, the excited molecule experiences vibrational relaxation, solvent re-orientation and other processes during this energy relaxation process. The fluorescence emitted from this equilibrium state competes with internal conversion to the ground state, intersystem crossing to the triplet state, energy transfer process from and/or to the other molecules, and other chemical reactions according to the circumstances surrounding the excited molecule. In the case of tryptophan molecules, which is the most important intrinsic fluorophore in proteins, the emission state is 1L_a or 1L_b created by the photon absorption at 275 nm or 286 nm (Fig. 1.1). The tryptophan residue excited to 1L_a state reorients the surrounding polar groups or molecules under the strong dipole-dipole interactions to relax to the equilibrium state. The most important process competing with the tryptophyl fluorescence is the proton transfer, or electron exchange processes from and with the surroundings. The tryptophan residue in protein is quenched by the protonated amino or carboxylic groups of lysine or glutamate, respectively. The rotational or translational movement of tryptophan residue is not, itself, the quenching source for the fluorescence but it closely relates with the depolarization property of tryptophan residue. The nature and behavior exhibited by the excited molecule are characterized by following experimental parameters.¹⁾

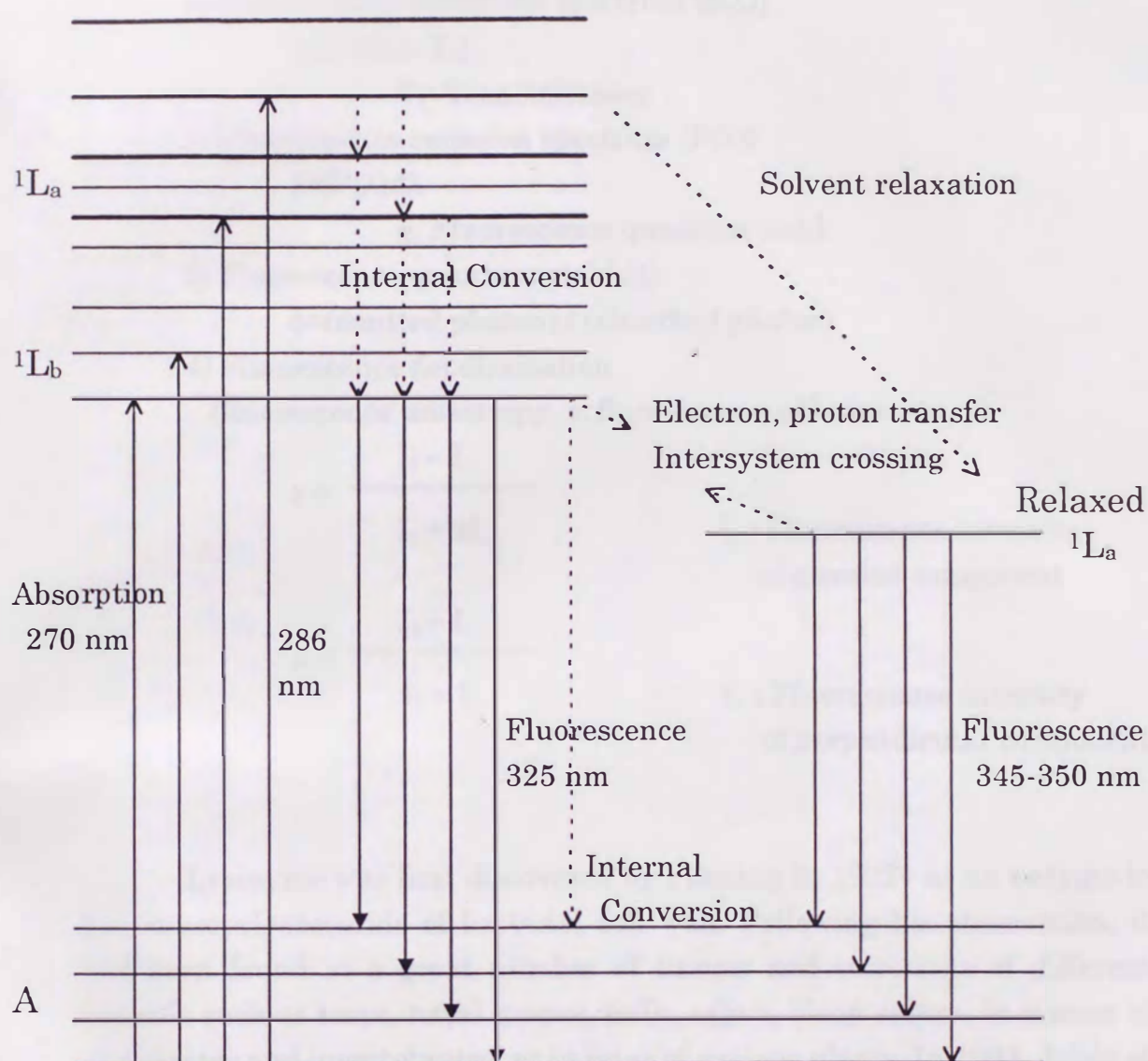


Fig. 1.1 Energy level diagram of electronic state of L-Tryptophan.

- 1) Fluorescence excitation spectrum ($\phi(\lambda)$)

$$\phi(\lambda) = K(1 - T_\lambda)$$

T_λ ; Transmittance

- 2) Fluorescence emission spectrum ($F(\lambda)$)

$$\phi = \int F(\lambda) d\lambda$$

ϕ ; Fluorescence quantum yield

- 3) Fluorescence quantum yield (ϕ)

$$\phi = (\text{emitted photon}) / (\text{absorbed photon})$$

- 4) Fluorescence depolarization

(fluorescence anisotropy, r ; fluorescence polarization, p)

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

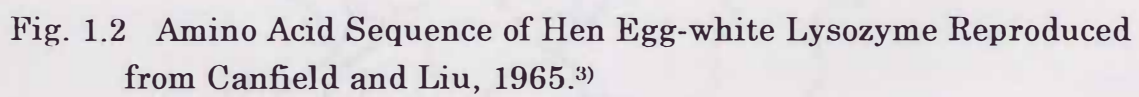
$I_{\parallel}$; Fluorescence intensity
of parallel component

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

$I_{\perp}$; Fluorescence intensity
of perpendicular component

Lysozyme was first discovered by Fleming in 1922²⁾ as an enzyme to lyse mucopolysaccharide of bacterial cell wall. Following his observation, it had been found in a great number of tissues and secretions of different animals such as tears, nasal mucus, milk, saliva, blood serum, in tissues of vertebrates and invertebrates, or in latex of various plants. In 1963, Jollès et al. and Canfield determined primary structure of lysozyme after the establishment of the procedure to isolate a great amount of this enzyme from egg-white indicating that it was constituted of 129 amino acid residues in a single chain as shown in Fig. 1.2.³⁻⁵⁾ In 1962, X-ray analysis of lysozyme crystalline was carried out with 6 Å resolution by Blake et al.⁶⁾, and more closer analysis with 2 Å resolution was succeeded so that three-dimensional structure of lysozyme was confirmed.⁷⁾ Fig. 1.3 shows the structure of hen egg-white lysozyme (HEWL).

Lysozyme catalyzes the hydrolysis of a polysaccharide that is the major constituent of the cell wall of certain bacteria. Lysozyme has a well-defined deep cleft, running down one side of the ellipsoidal molecule, for binding the substrate. The cleft is divided into six sites, A, B, C, D, E and F. In



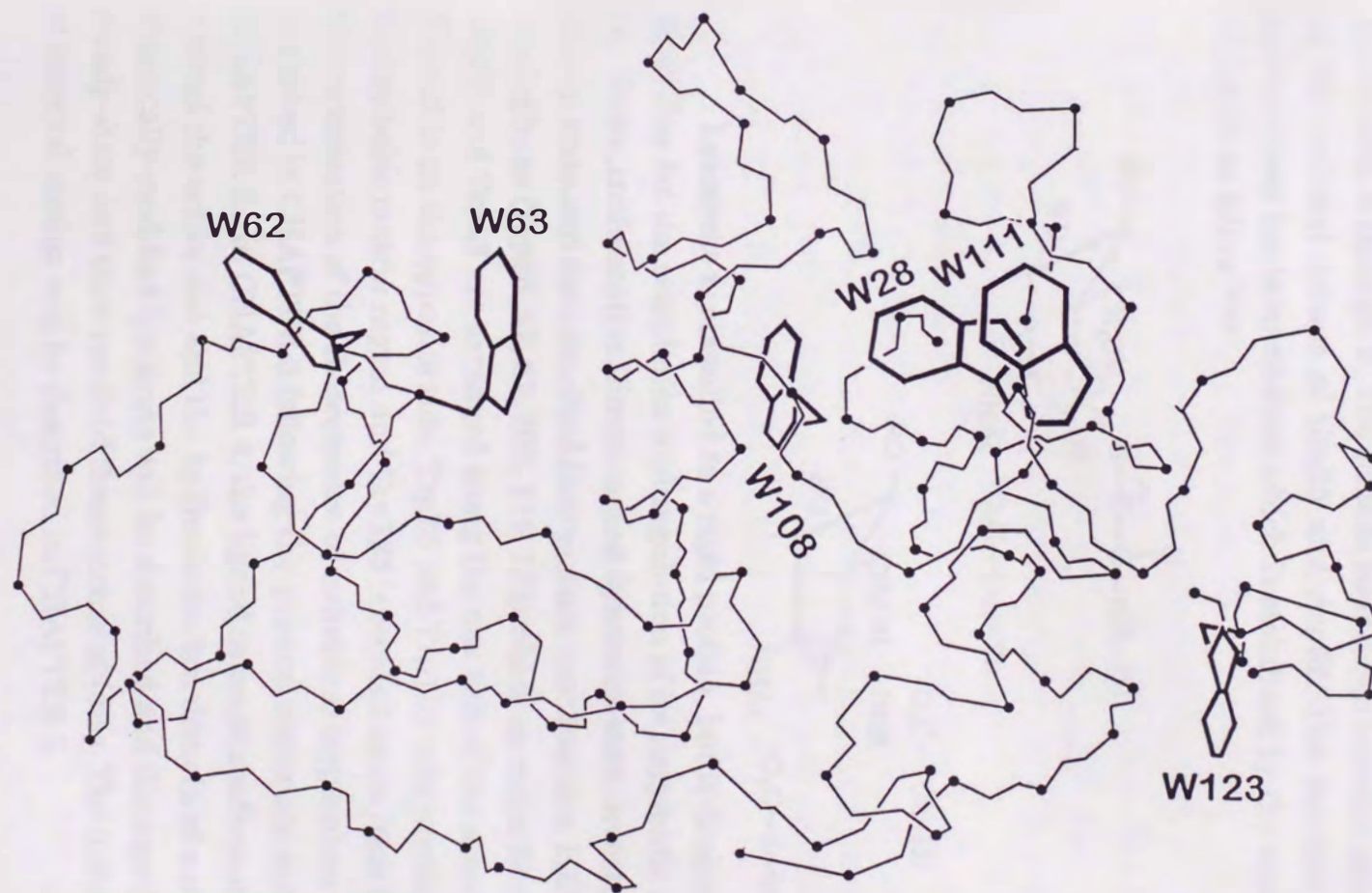
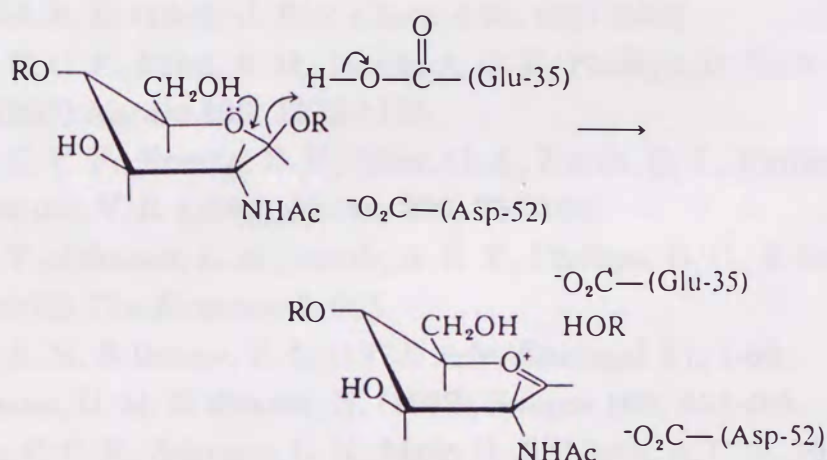


Fig. 1.3 Crystallographic Structure of Hen Egg-white Lysozyme.

order for reaction to occur, the substrate must bind across sites D and E of the six subsites. Small polysaccharides avoid the strain in the D subsite by binding the A, B, and C sites, i.e., trimers and tetramers bind nonproductively in A, B, and C and in A, B, C, and D, respectively. However, the favorable binding energy of occupying site E and F causes hexamer to bind productively in subsites, A through F. The scissile bond lies between sites D and E, close to the carboxyl groups of Glu35 and Asp52. The reaction proceeds via a carboxonium ion intermediate which is stabilized by the ionized carboxylate of Asp52 as follow.⁸⁻¹⁵⁾



Lysozyme is classified to a rigid protein, but it doubtless exhibits flexibility for the completion and regulation of its enzymatic action. Through my thesis, conformation, function and dynamics were investigated by the steady-state and time-resolved fluorescence spectroscopy. HEWL contains six tryptophans (Trp28, 62, 63, 108, 111, 123) which as major fluorescence probe, Trp62 and Trp63 are arranged along the one side of the active site and Trp108 is on the opposite side. Trp28 and Trp111 take positions at the hydrophobic matrix region and Trp123 is located apart from the others. The characterization of the fluorescence of individual tryptophan residues was described in CHAPTER 3 following the general materials and methods (CHAPTER 2). In CHAPTER 4, the ligand induced conformational change around the active site and the hydrophobic box domain of native and chemically-modified lysozyme will be described and discussed based on our steady-state and time-resolved fluorescence studies. The molecular dynamics of internal motion will be described in CHAPTER 5.

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CHAPTER 2

Materials and Methods

2.1 Materials.

HEWL [EC3.2.1.17] recrystallized five times was purchased from Seikagaku Kogyo Co. (Tokyo, Japan) and used after desalting by a cation-exchange chromatography. A (1-4)-linked trimer of *N*-acetyl-D-glucosamine ((NAG)₃) was purchased from Sigma. CM-Toyopeal 650M, a cation-exchange resin, was obtained from Tosoh (Tokyo, Japan) and a column of Wakosil 5C18-200 (4.6×250 mm) was from Wako Pure Chemical Institute (Osaka, Japan). Chitin-coated Celite which is an affinity absorbent for lysozyme were prepared according to the method of Yamada et al.¹⁾ Other chemicals were of analytical grade and used without further purification.

2.2 Preparation of Kyn62-lysozyme.

Preparation of Kyn62-lysozyme was described in ref. 2). Lysozyme were oxidized by ozone in aqueous solution according to the methods of Sakiyama and Natsuki.³⁾ Oxidized lysozyme was chromatographed on a chitin-coated cellulose column under the conditions described by Imoto and Yagishita.⁴⁾ The unabsorbed materials in fraction were collected, dialyzed against deionized water at 4°C and lyophilized. The lyophilized materials (100 mg) were dissolved in 10 ml of cold 0.025M HCl and the mixture was immediately frozen below -70°C. The frozen solution was kept at -10°C for 24 hours. When the reaction was complete, the frozen solution was thawed and mixed with 10 ml of 0.2 M sodium phosphate buffer containing 2 M NaCl, pH 6.4. The resulting weakly acidic solution was applied to a chitin-coated cellulose column equilibrated with 0.1 M sodium phosphate buffer containing 1 M NaCl, pH 6.4. The adsorbed materials were eluted with 0.1 M Acetic acid, collected, dialyzed against deionized water at 4°C, and lyophilized. Crude Kyn62-lysozyme thus obtained was purified by ion exchange chromatography on Bio Rex 70 using essentially the same buffer system as Imoto et al.⁵⁾ Pure Kyn62-lysozyme was collected, desalted and lyophilized.

2.3 Mutant Lysozyme.

Two mutant lysozymes, W62Y- and W108Y-lysozyme in which Trp62 and Trp108 were replaced with tyrosine, respectively, were prepared

following the method described previously.⁶⁾ Each yeast *Saccharomyces cerevisiae* AH22 transformant implanted the mutagenic primers used for site-directed mutagenesis to replace Trp62 and Trp108 with tyrosine, respectively, was cultivated at 30°C for 125 hours. The mutant lysozymes secreted in the culture supernatant was isolated by cation-exchange chromatography (CM-Toyopeal 650M). The lysozyme eluted with a gradient of 0-0.5 M NaCl in phosphate buffer (pH 7.0) was collected, dialyzed against distilled water, and then lyophilized.

2.4 Steady State Fluorescence Spectrum.

HEWL and mutant lysozymes were dissolved in sodium acetate buffer (pH 5.5) of which ion strength was adjusted to 0.1. Steady state fluorescence spectrum was recorded on a SLM C-1000 fluorescence spectrophotometer (SLM, USA). Every emission spectra were strictly corrected for the excitation-detection response of the instrument and for stray light.

2.5 Steady State Fluorescence Anisotropy

Steady state fluorescence anisotropy (r_s) was measured based on equation (2.1).

$$r_s = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + 2GI_{\perp}} \quad (2.1)$$

where $I_{\parallel}$ and $I_{\perp}$ were the intensities of the parallel and perpendicular components, respectively, and G was the grating factor. The $I_{\parallel}$ and $I_{\perp}$ were detected simultaneously with T-format configuration of SLM C-1000. One Glan Taylor polarizers was set vertical at the excitation side and the other two polarizers vertical and horizontal against the vertical excitation, respectively. G -factor was obtained by measuring the intensity ratio of $I_{\parallel}$ and $I_{\perp}$ against the horizontal excitation.

2.6 Time-Resolved Fluorescence Measurements

Time-resolved fluorescence measurements were made by the method of time-correlated single photon counting (Fig. 2.1) which involves measuring the time of arrival of a single photon emitted by the sample after a given excitation flash. In a traditional configuration, the time is measured by sending an electrical pulse, at a time correlated with the time of excitation of

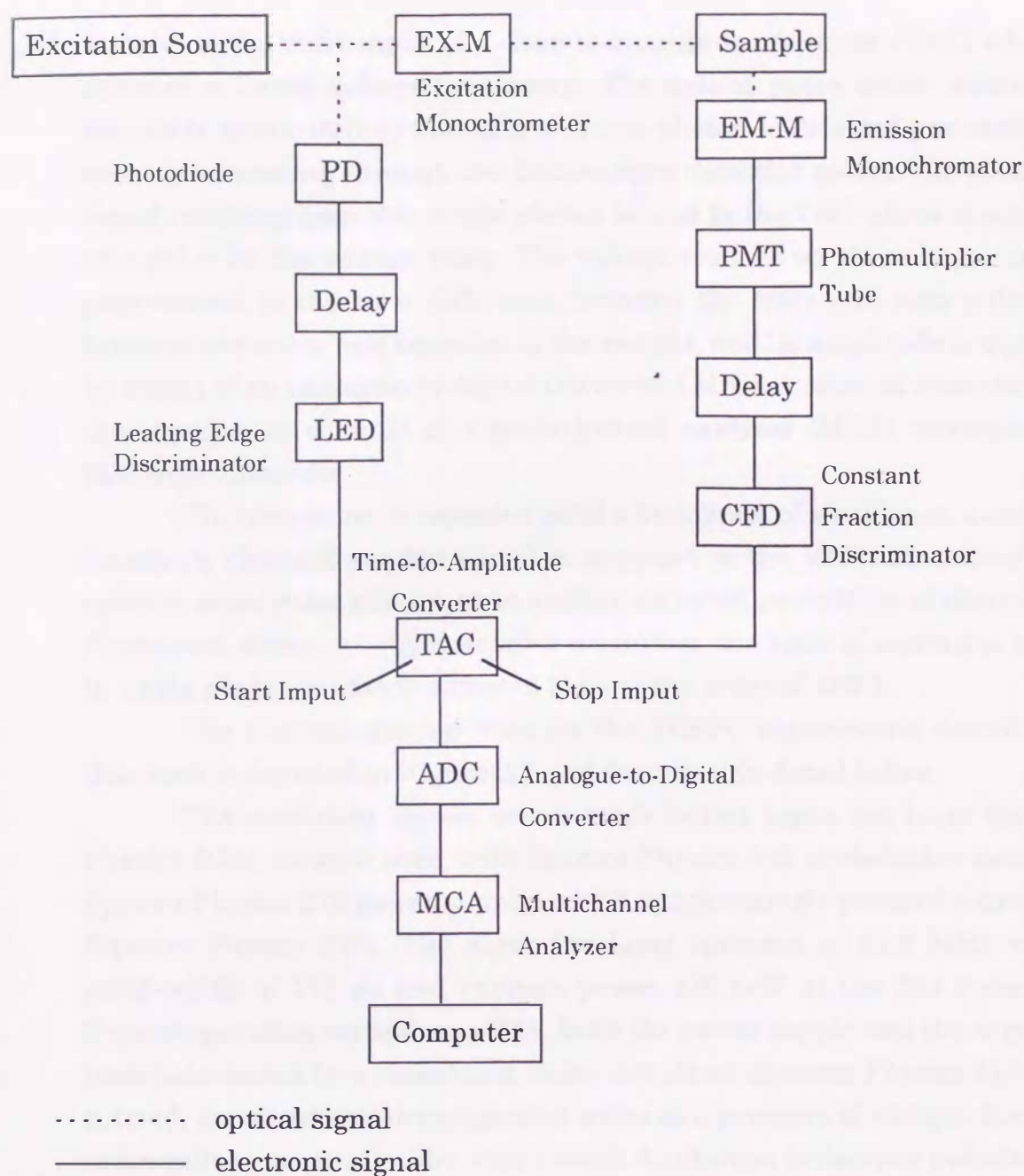


Fig. 2.1 Schematic diagram of basic instrumentation required for time-correlated single photon counting (TCSPC) experiments.

sample, to the start input of a time-to-amplitude converter (TAC) where it initiates a linear voltage-time ramp. The optical pulse which excites the sample is attenuated so that only a single photon is detected per excitation event after passing through the fluorescence detection optics. The electronic signal resulting from this single photon is sent to the TAC where it acts as a stop pulse for the voltage ramp. The voltage reached on the voltage ramp is proportional to the time difference between the start and stop pulses, or between excitation and emission of the sample, and its amplitude is digitized by means of an analogue-to-digital converter (ADC). A count is then stored in the appropriate channel of a multichannel analyzer (MCA) corresponding that time difference.

This procedure is repeated until a histogram of a sufficient number of counts vs. channel number (time) is acquired in the MCA. Statistically, in order to avoid pulse pile up, or to archive an equal probability of observing a fluorescent photon at any time after excitation, the ratio of excitation pulses to single photon events is adjusted to be of the order of 100:1.

The instrumentation used for the TCSPC experiments described in this work is depicted in Figure 2.2 and described in detail below.

The excitation source was a mode-locked argon ion laser (Spectra Physics 2030, ceramic bore, with Spectra Physics 342 mode-locker head and Spectra Physics 270 power supply) which synchronously pumped a dye laser (Spectra Physics 375). The argon ion laser operated at 81.6 MHz with a pulse-width of 175 ps and average power 470 mW at the 514.5 nm line. Typical operating current was 30A. Both the power supply and the argon ion laser tube cooled by a closed loop water circulator (Spectra Physics 314) with filtered, deionized and deoxygenated water at a pressure of <35 psi. flow rate of 3.5 gallons/minute (in line with a small diaphragm to dampen pulsations of the pump), such that the temperature of the water returning from laser was <32°C. The immediate area around the brewster angle mirrors in the ion laser cavity was purged by air passing through a closed loop system containing molecular sieve (to remove ozone), desiccant (to remove water vapors which could deposit on the mirrors).

The dye solution in the dye laser circulated at a pressure of 100 psi (dye laser circulator, Spectra Physics 376B) and was kept at a temperature of <30°C (in order to avoid temperature changes of the circulating dye with time), by passing through a heat exchanger connected to Haake water

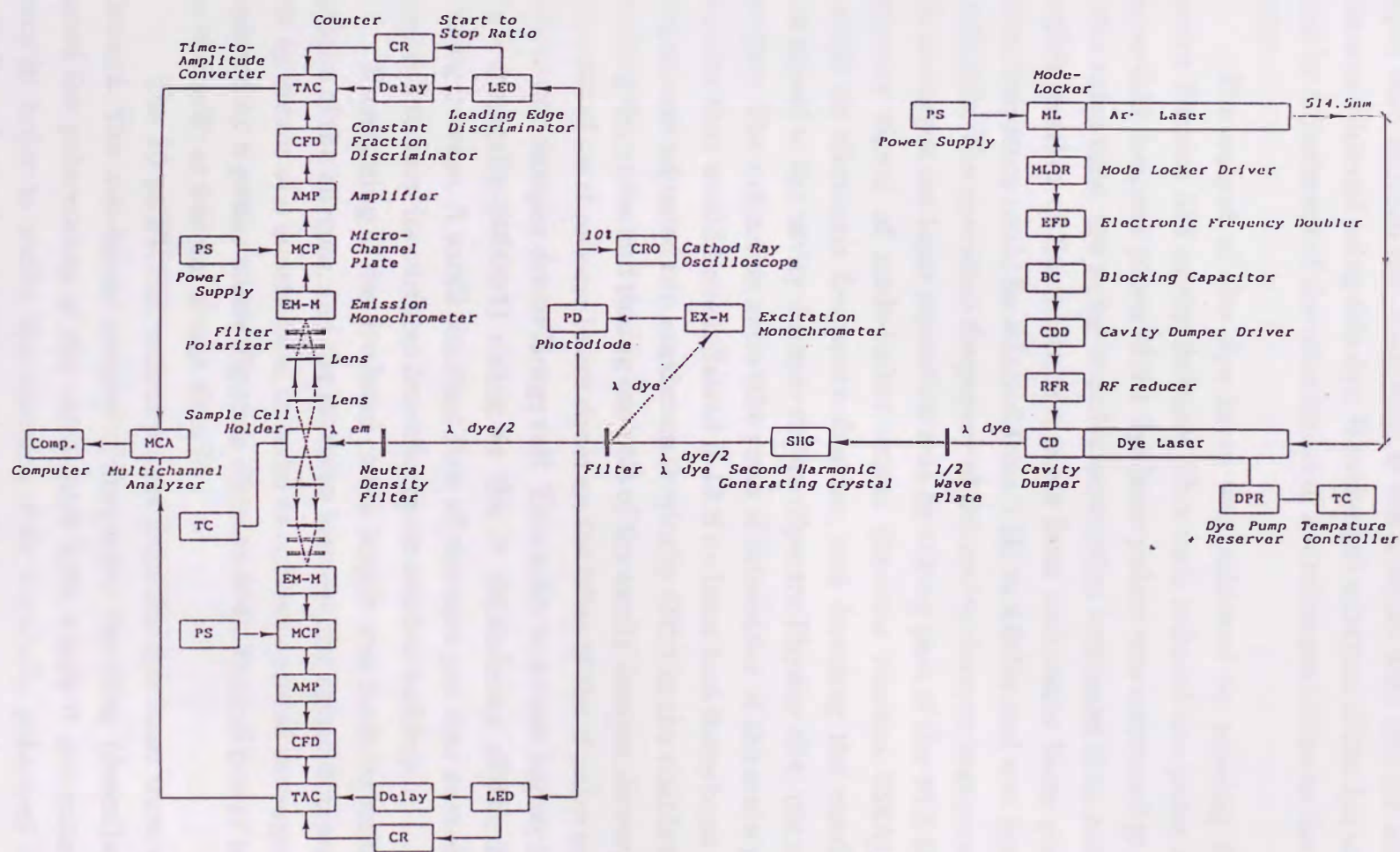


Fig. 2.2 Schematic Diagram of the Instrumentation Used for the Time-resolved Fluorescence Experiments.

←----- Optical Signal, ←----- Electronic Signal

circulator. Rhodamine 6G in ethylene glycol (2 mM) provided a tunable range of 570-640 nm. This could be frequency doubled to give ultraviolet light output from 285-320 nm. Excitation of the samples with 295 nm and 320 nm light was achieved using this dye. Wavelength selection of the dye output was made by adjustment of the orientation of a birefringent filter in the dye laser cavity.

The output of the dye laser was achieved by passing through a Spectra Physics 344 cavity dumper. This unit reduced the pulse repetition rate so that the peak power of the dye laser pulses was increased to 1.5-2 kW. At the same time, the pulse to pulse separation increased thus avoiding the possible overlap of fluorescence signals from successive laser pulses. The output frequency could be selected from 1 Hz to 4 MHz and was typically set at 816 kHz. The operation frequency of the cavity dumper was synchronized with the argon ion laser repetition rate by taking part of the 40.8 MHz radio frequency signal of mode-locker driver (Spectra Physics 452A), passing through an electronic frequency doubler, and directing the resulting 81.6 MHz signal to the cavity dumper driver (Spectra Physics 454) via a blocking capacitor. The extinction ratio (the ratio of intensities of the main pulse and the pulse that would have followed it 12.3 ns later had there been no cavity dumper) was adjusted to a maximum (typically 500:1 in the visible region) by adjusting the pulse and timing controls of the cavity dumper driver, and also by the use of an rf reducer to cut down on the lobes of the rf pulse coming out of the cavity dumper driver Bragg cell. This ratio was even higher in the UV region (typically 30000:1) owing to the I^2 dependency of the frequency doubling process. A small constant flow of nitrogen gas was passed over the Bragg cell to keep its surfaces free of organic residue buildup.

Fine tuning of the dye laser cavity length was made by adjusting the positions of the mirrors, and the distance between the cavity dumper and dye laser by means of a quartz tube, in order to achieve optimal average power as measured by a power meter (Spectra Physics 404). Typical power at 590 nm was >50 mW; at 640 nm it was 40 mW.

The 20 ps FWHM output pulses from the dye laser were vertically polarized. The non-linear process of frequency doubling (described below) rotated the polarization of the ultraviolet light which it generated by 90°. Hence in order to excite the samples with vertically polarized light, the vertically polarized output from the dye laser was passed through a half-

wave plate (Special Optics R8 9012) to rotate the polarization direction to horizontal before passing through an angle-tuned second harmonic generating crystal (Cleveland Crystal KDP1, 25 mm length) to give the desired UV light of vertical polarization. The light at the fundamental wavelength which passed through the crystal was filtered from the UV output by a 7-54 Corning filter (295, 320 nm), a UG1 Schott filter (350 nm). The filter was also used to reflect the visible light onto the slits of a monochromator (Jobin Yvon H-10) and onto a silicon avalanche photodiode (Texas Instruments TIED 56, operating at 90V DC). 10% of the resulting electrical signal (Tektronix Signal Pickoff CT-3) was directed to a cathode ray oscilloscope (Tektronix 7904 with Amplifier 7A19 triggered internally by a 7B92 dual time base) in order to monitor the performance of the laser system. Fine adjustment of the laser output was made when necessary by adjusting the phase and timing controls of the cavity dumper driver.

The remaining 90% of the signal from the photodiode was passed into a 100 MHz leadingedge discriminator (Ortec 436) whose output passed through delay lines (Ortec 425) and then was used as the stop input signal for the biased time-to-amplitude converter (TAC, Ortec 457). This is the opposite configuration to that described earlier in the general description of the TCSPC method, where the signal correlated with the excitation pulse is used as the start pulse for the TAC. In the conventional configuration, the TAC was not able to register all excitation events and hence not all signal photon events. This was because the time between successive excitation pulses was 1.2 μ s (1/816 kHz) while the TAC conversion time was $\sim$ 13 μ s. Even if no stop pulse was detected, as would happen 99 times out of 100 because of the 100:1 ratio of the excitation events to emission events, the dead time of the TAC was still $\sim$ 6 μ s. By having the detected single photons initiate the voltage ramp and the delayed excitation pulse terminate the ramp, since the ratio of detected photons to the number of excitation pulses is $\sim$ 100:1, as mentioned earlier, there is 0.12 ms between successive start pulses and all the detected photons would be registered.

The UV light pulses, after passing through the filters mentioned above, passed through a neutral density filter which had constant thickness but different densities along its 125 mm length. In this way, the intensity of the UV pulses could be attenuated in order to achieve the proper counting statistics mentioned earlier. Following this, the light entered a thermostatted

cell compartment (Polyscience 90 Temperature Controlle), which could held up to four 1 cm cuvettes, whose rotation was controlled by a stepping motor (Slo-Syn). The emission was collected at right angles through a collimating lens, and through a second lens which focused the fluorescence on to the slits of a monochromator (Jobin Yvon H-10) with either 4 nm (0.50 mm slits) or 2 nm (0.25 mm slits) resolution. Immediately in front of the monochromator were positioned a Glan Taylor polarizer set at 54.7° (magic angle) to eliminate time distortions due to rotational relaxation of the fluorophore, and a cut-off filter to either block (295 nm, 2 microscope slides) or pass through (for measurement of the instrument response profile) any scattered light. The monochromator was placed on a precision labjack (Newport Research 270) so that its height could be adjusted according to height variation of the UV beam, which resulted from different orientation angles of the doubling crystal.

The fluorescent photon which passed through the monochromator was detected by a proximity-type microchannel plate (MCP) detector (Hamamatsu R1564V-01, operating at -3.2 kV) whose output was amplified (Electronic Navigation Industries 500AP amplifier, 27 dB), passed into a constant fraction discriminator (Tennelec TC 453), and directed to the start output of the TAC. The dark count rate was usually 40 Hz. The ratio of stop to start pulses was measured by a counter (Ortec 9315) using of the negative output of the TAC for the number of start pulses and an output of the excitation pulse leading edge discriminator for the number of stop pulses. The stop:start ratio was generally set at 100:1 for the collection of the sample fluorescence decay and instrument response profiles (defined below).

The positive output of the TAC was read into an analogue-to-digital converter (ADC) (Tracor Northern TN-1211, 50 MHz) and recorded in 1024 or 2048 channels (channel width 3.5 ps/channel to 84.8 ps/channel) of a multichannel analyzer (MCA) (Tennecomp) which was controlled by a digital 1104 computer. Usually at least 16000 counts were collected in the maximum channel of the sample decay curve. A blank was measured for each sample for the same accumulation time, as controlled by a real time clock in the MCA. The instrument response profile (FWHM ~ 80 ps) was determined by measuring the line from distilled water at the excitation wavelength.

All fluorescence intensity/time profiles were acquired sequentially and were stored on 8" floppy disks, converted to RT-11 format on a PDP 1134 computer, and transferred to an IBM 3270 VM computer for data analysis.

2.7 Fluorescence Anisotropy Decay

The fluorescence anisotropy decay measurement was performed using the same instrument as the fluorescence decay measurement. But the parallel $I_{\parallel}(t)$ and perpendicular $I_{\perp}(t)$ component were measured separately by setting Glan-Taylor polarizer vertically and horizontally against vertical excitation. $I_{\parallel}(t)$ and $I_{\perp}(t)$ were deconvoluted and used to calculate $r(t)$ using equation (2.1). G-factor at the wavelength region 300 nm to 400 nm was corrected using azanaphthen in ethanol solution as a fluorescence standard. Adequacy of the fluorescence anisotropy decay was judged by the non-linear least square curve fitting based on Marquardt-Levenberg algorithm.⁷⁾

2.8 Fluorescence Decay Data Analysis

Curve Fitting and Quality of fit

The measured sample fluorescence decay profile, D_s , is a convolution of the impulse function L (i.e. the time profile of the excitation pulse), the true sample fluorescence response function F_s , and an instrument response function H (i.e. the time distortion of an optical signal by the detection and electronic systems), and given by equation 2.2.

$$D_s = L * F_s * H \quad * = \text{convolution} \quad (2.2)$$

F_s is the function of the interest and is generally found to be a sum of exponentials consistent with the physical model of fluorescence decay. In order to arrive at F_s , the convolution $L * H$ (equation 2.3) must be known, and can be obtained by measuring the response of the system to scattered light by directing the excitation pulses onto distilled water under identical optical arrangements.

$$D_i = L * H \quad (2.3)$$

Typical sample fluorescence decay profile D_s and instrument response profile (scatterer curve) D_i at channel width of 21.5 ps/channel are shown in Fig. 2.3.

The desired function F_s is determined by convolving the scatterer curve D_i with trial function forms of F_s (using a weighted nonlinear least squares method and the Marquadt algorithm)^{8,9)} until there is good agreement with the measured sample fluorescence decay profile D_s . This procedure assumed that the instrument response function H is independent of wavelength, since the scatterer curve D_i is measured at the excitation wavelength λ_{ex} , while the sample fluorescence decay profile D_s is measured at an emission wavelength λ_{em} . Equations 2.2 and 2.3 are more precisely

written:

$$D_s = L \cdot F_s \cdot H_{em} \quad (2.4)$$

$$D_i = L \cdot H_{ex} \quad (2.5)$$

It has been found that the transit time spread of microchannel plate detectors is practically invariant with wavelength of incident light ranging from 300 to 800 nm. If it is assumed that any other possible mechanisms giving rise to the instrument response function are also independent of wavelength (i.e. $H_{ex} = H_{em}$), then the above convolution procedure will yield the correct function form of F_s .

The best fit between the convolved curve $D_i \cdot F_s$ and the sample fluorescence decay curve D_s was found when the weighted sum of squares of differences or residuals (WSSR) between the experimental points $e(t_i)$ and the calculated points $c(t_i)$ (equivalent to the chi-square value χ^2) was minimized (equation 2.6).

$$WSSR = \chi^2 = \sum_{i=1}^n w_i [e(t_i) - c(t_i)]^2 \quad (2.6)$$

n = number of data points, channels

w_i = weighting appropriate to data in channel i

Since the counting statistics of the TCSPC method is Poissonian, the correct weighting factor w_i is the reciprocal of the variance σ_i^2 , which is equal to the number of counts in channel i (equation 2.7).

$$w_i = 1 / \sigma_i^2 = 1 / e(t_i) \quad (2.7)$$

So,

$$WSSR = \chi^2 = \sum_{i=1}^n \{ [e(t_i) - c(t_i)]^2 / e(t_i) \} = \sum_{i=1}^n [r(t_i)]^2 \quad (2.8)$$

$r(t_i)$ = weighted residual

In the convolution procedure, the whole sample fluorescence decay profile D_s was fitted, but the statistical parameters used to judge the quality of the fit were calculated from the point corresponding to 50% of the rising edge of D_s . These statistical parameters included:

1. Sigma value
2. Serial Variance Ratio
3. Inspection of weighted residuals / root mean square of the residuals.

The sigma value is the square root of the reduced chi-square χ_v^2 (equation 2.9)

$$\Sigma = (\chi_v^2)^{1/2} = \{\chi^2 / (n_2 - n_1 + 1 - p)\}^{1/2} \quad (2.9)$$

where χ^2 is the chi-square or WSSR defined earlier, and the term in the denominator represents the number of degree of freedom. n_1 and n_2 are the start and stop channels used for determination of the statistical parameters and p is the number of variable parameters in the trial function F_s (for a sum of three exponential terms, $p=6$). Sigma should be close to a value of one for Poisson distributed data.

The serial variance ratio (SVR, equation 2.10) is a measure of the correlation between residuals and is thought to be more sensitive than the sigma value to discrepancies between the calculated and experimental decay curves.

$$SVR = \frac{\sum_{i=n_1+1}^{n_2} [r(t_i) - r(t_{i-1})]^2}{\sum_{i=n_1}^{n_2} [r(t_i)]^2} * \frac{(n_2 - n_1)}{(n_2 - n_1 - 1)} \quad (2.10)$$

For a good fit, SVR should be in the range of 1.8~2.0.

A more subjective test (but quite instructive with experience) of the quality of a fit to the sample fluorescence decay profile, D_s , is an inspection of a plot of the weighted residuals $r(t_i)$ divided by the root mean square of the residuals (equivalent to the sigma value). Such plots for a single, double triple and quadruple exponential fit to the fluorescence decay curves in Fig. 2.3, are shown in Fig. 2.4-1,2,3, and 4. The points should be randomly distributed about zero, and the majority of them should fall in the range -1 to 1 (equivalent to the standard deviation). Based on this criterion alone, it can be seen that the fluorescence decay curve in Figure 2.3 is best fit by a sum of four exponential terms. The values of the sigma and SVR parameters for the single, double, triple, and quadruple exponential fits confirm this conclusion.

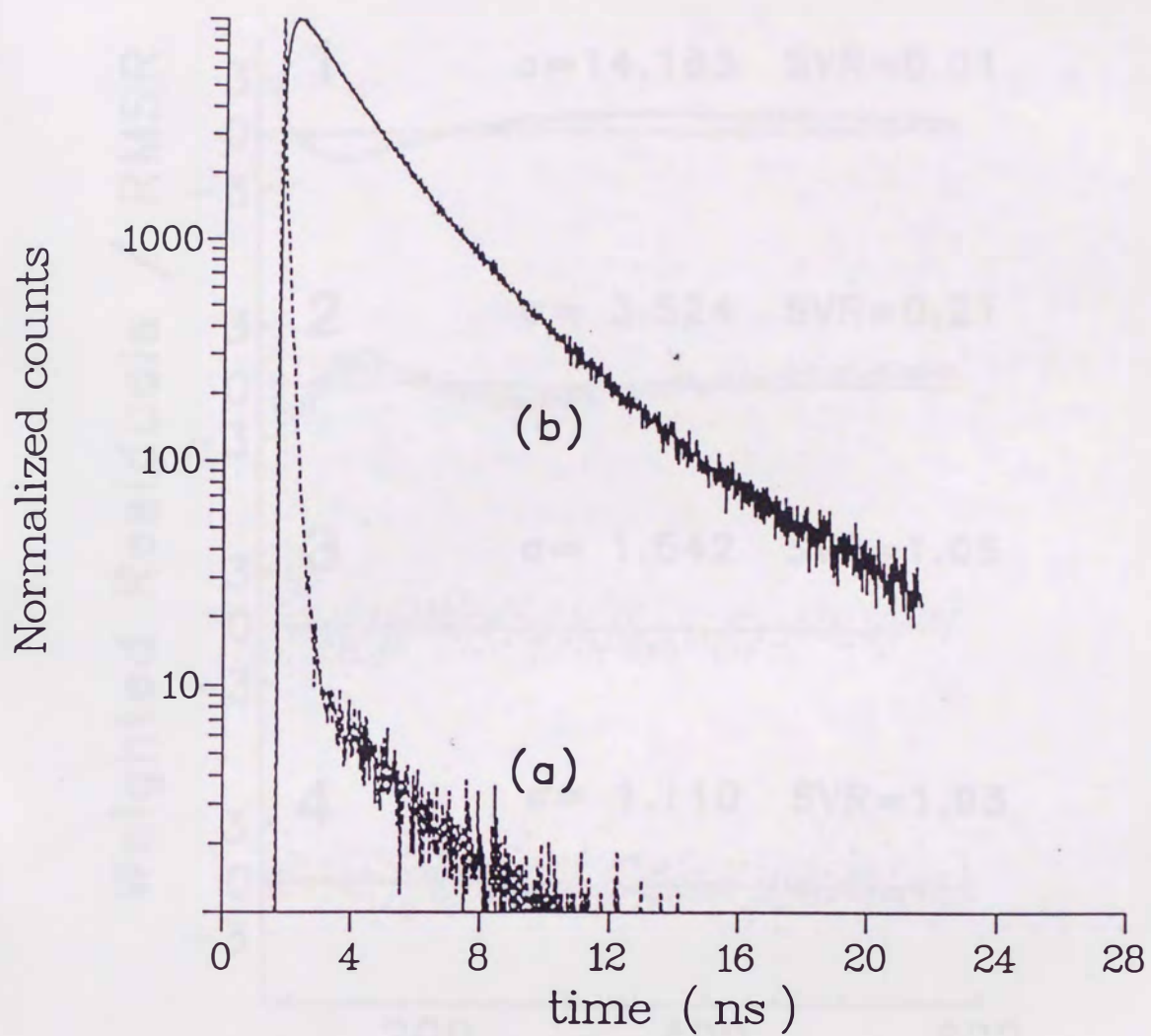


Fig. 2.3 Fluorescence decay profile (b) of HEWL in acetate buffer, pH 5.5 ($\lambda_{\text{ex}}=295$ nm, $\lambda_{\text{em}}=320$ nm) measured at a channel width of 21.5 ps/channel, with the corresponding instrument response profile (a).

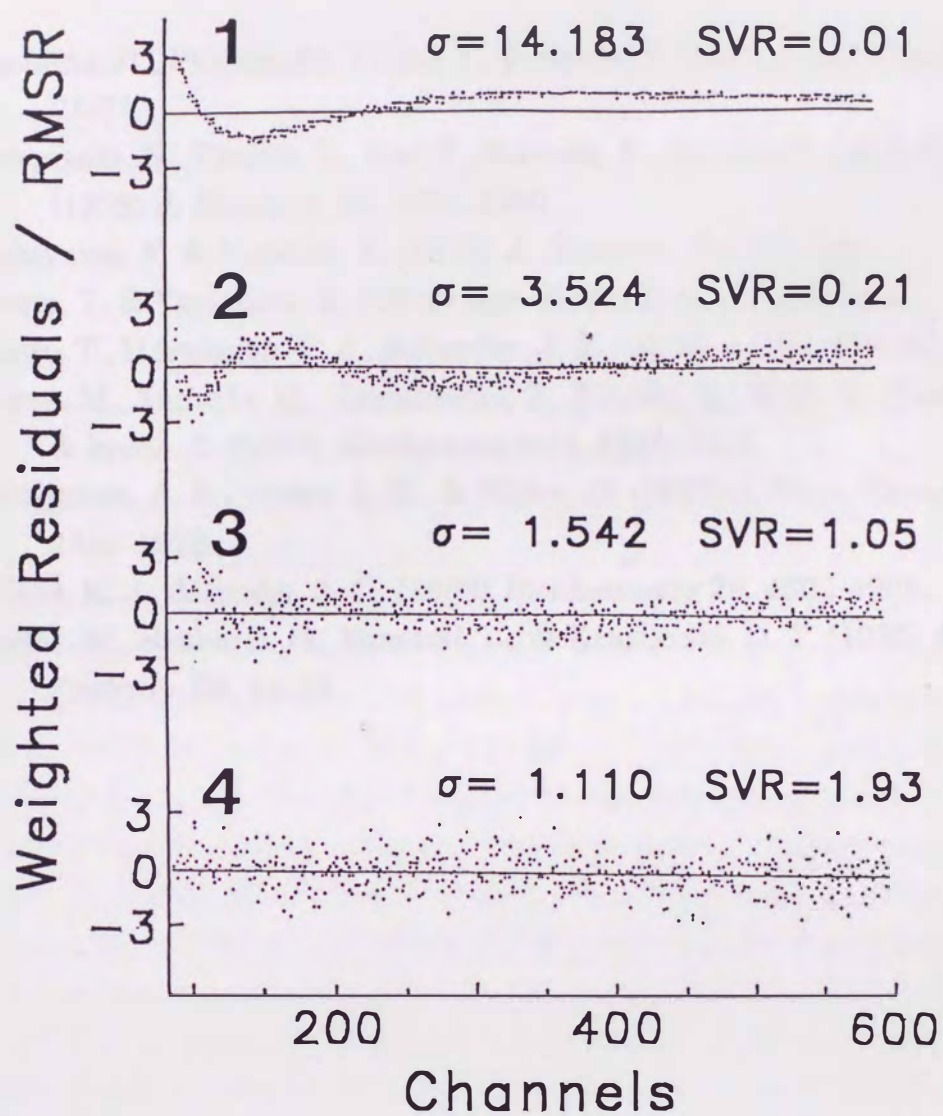


Fig. 2.4 Plots of weighted residuals/root mean square of residuals for a single-exponential fit (1), a double-exponential fit (2), a triple-exponential fit (3), and a quadruple-exponential fit (4), to the fluorescence decay profile in Fig. 2.3.

2.8 References

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CHAPTER 3

Characteristic Fluorescence Properties of Individual Tryptophan Residue.

3.1 Introduction

The fluorescence spectroscopy is one of the most powerful method to study inter-relationship between conformation, dynamics, and function in protein. Taking advantage of the high spectroscopic sensibility and excellent time-resolution power, function associating properties such as internal dynamics, conformational change, and heterogeneity potentially kept in the flexible structure of protein can be cleared.¹⁻³⁾ Using this method, hen egg-white lysozyme (HEWL) is an interesting subject to examine the correlation between ligand induced conformational change and enzymatic action. Because two most dominant fluorophores, Trp62 and Trp108, are arranged around the binding site and play important roles for binding with substrate and for stabilizing the structure of HEWL, respectively. Under a concept that such peculiar arrangement of tryptophan residues can reflect how HEWL interacts with ligand or substrate in their fluorescence properties, many studies have conducted on the fluorescence spectroscopy of HEWL. Indeed, it has been reported that significant conformational change were induced around the active site on the interaction with the ligand such as trimer of *N*-acetyl-D-glucosamine ((NAG)₃).⁴⁾ But it should be noted that some serious obscurities are inevitably included in previous works because of the spectral overlap of some tryptophyl fluorescence in HEWL. Although the Trp62 and Trp108 contribute almost 80% to the total fluorescence of HEWL,⁵⁾ HEWL contains other two fluorescent tryptophan residues, Trp28 and Trp111, at the hydrophobic matrix box region. If the characteristic fluorescence of these four tryptophan residues are individually differentiated with each other, the local conformation and its corresponding response to the interactions with the ligand or substrate could be more cleared. Recently, we have discriminated the fluorescence of Trp62 and Trp108 from one of Trp28 and Trp111 using quenching resolving method.⁶⁾ Although the technique and knowledge shown in it is useful to analyze differentially the conformation of HEWL at the binding site and the hydrophobic matrix box region, closer characterization are required for the fluorescence properties of Trp62 and Trp108 arranged at the different position along the binding site. It is still now difficult to distinguish the fluorescence of tryptophan residues belong to the same

domain through usual fluorescence spectroscopic techniques. However, the recent development of the mutagenetic technique, fortunately, enabled to investigate separately the fluorescence of the individual tryptophan residue in multitryptophan-containing proteins.^{7,8)} The tryptophan residue or its analogue designated by site directed mutagenesis can provide valuable information on the local conformation and interaction with ligand of protein through the steady-state and time-resolved fluorescence spectroscopic studies on it.^{9,10)} In this chapter, we used two mutant lysozymes W62Y-, W108Y-lysozyme, in which Trp62 or Trp108 are replaced with tyrosine residue, respectively, to obtain the characteristic fluorescence of Trp62 or Trp108. For discriminating Trp62 or Trp108 with tryptophan residues at the hydrophobic matrix, we used fluorescence quenching resolving techniques. In the quenching resolved fluorescence spectrum, the fluorescence of HEWL was resolved into sub-bands according to the collisional frequency and/or the quenching cross section to the quencher molecule according to each location in protein. Because the knowledge of the fluorescence lifetime is essential to decide the collisional fluorescence quenching constant and to characterize the individualities of each tryptophan residue in lysozyme, the fluorescence decay kinetics as well as the fluorescence spectra of the native and mutant lysozymes were precisely measured through the whole spectral regions.

3.2 Materials and Methods

Materials.

Native and mutant lysozymes were prepared as described in CHAPTER 2.

Measurement of Steady State and Time-resolved fluorescence.

Instruments and methods of steady state and time-resolved fluorescence measurements were described in CHAPTER 2.

Quenching-resolved Fluorescence Spectrum.

Collisional quenching constant (k_q) of acrylamide against the fluorescence of native and mutant lysozymes were determined using well-known Stern-Volmer equation,

$$(I_0/I) - 1 = K_{SV} \cdot [Q] \quad (3.1)$$

$$K_{SV} = k_q \cdot \tau \quad (3.2)$$

where I_0 and I are the fluorescence intensities in the absence and at the presence of the quencher $[Q]$, respectively. τ and K_{SV} are the fluorescence lifetime and Stern-Volmer constant, respectively. The fluorescence

intensities at various wavelengths were measured adding the freshly prepared solution of acrylamide without changing the concentration of the lysozymes. K_{sv} was decided from the slope and k_q was calculated from K_{sv} and τ .

Assuming a gaussian line shape;

$$F(\sigma) = a \cdot \exp\{-b \cdot (\sigma - \sigma_0)^2\} \quad (3.3)$$

for a tryptophyl fluorescence band, where a and b are constants giving the fraction and band width, respectively, and σ and σ_0 are wavenumber and one giving the maximum, respectively. The normalized fluorescence spectra of native, W62Y-, and W108Y-lysozyme were shared according to the value of k_q . The curve fitting was performed with a microcomputer (IBM type2410) based on the algorithm of Marquardt-Lebenberg.¹¹⁾

3.3 Results

Fluorescence Spectrum.

Fig. 3.1 shows the normalized fluorescence spectra of HEWL and mutant lysozymes in 0.1 M sodium acetate buffer (pH 5.5). While the fluorescence maximum of HEWL was seen at 342 nm, W108Y-lysozyme in which Trp108 was changed to tyrosine also showed a maximum at this wavelength. The fluorescence spectrum of W62Y-lysozyme in which Trp108 was the most dominant fluorophore deflected 6 nm to the shorter wavelength side to give a maximum at 336 nm. When the fluorescence quantum yields of W62Y- and W108Y-lysozyme were measured using L-tryptophan as a fluorescence standard (0.14), they were 0.053 and 0.047, respectively, while one of HEWL was 0.070.

As well known, the fluorescence of HEWL shifts to the blue side and its intensity is reduced at pH 5.5.¹²⁾ Trimer of *N*-acetyl-D-glucosamine ((NAG)₃) modified the fluorescence spectra of mutant lysozymes when interacting with the lysozymes. The fluorescence intensity of W62Y-lysozyme was enhanced at the shorter wavelength side in the complex with (NAG)₃, keeping the maximum wavelength unchanged as shown in Fig. 3.2. The fluorescence of W108Y-lysozyme responded similarly to one of the native lysozyme to the interaction with (NAG)₃, the fluorescence maximum shifted to the shorter wavelength side and the intensity at the maximum wavelength was reduced about 10%.

Fluorescence Decay Kinetics

The fluorescence decay of HEWL and mutant lysozymes were

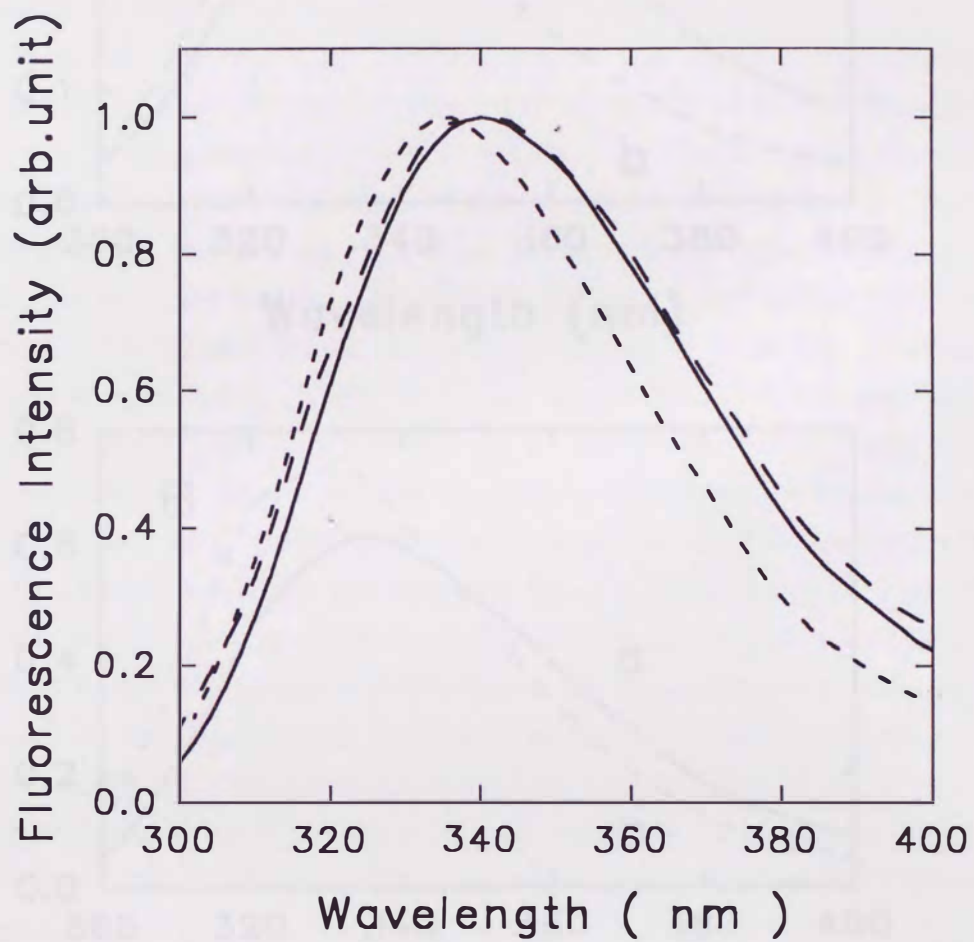


Fig. 3.1 The fluorescence spectra of HEWL and mutant lysozyme in 0.1 M acetate buffer (pH 5.5).

——, HEWL; ----, W108Y-lysozyme; ·····, W62Y-lysozyme. Excitation wavelength, 300 nm.

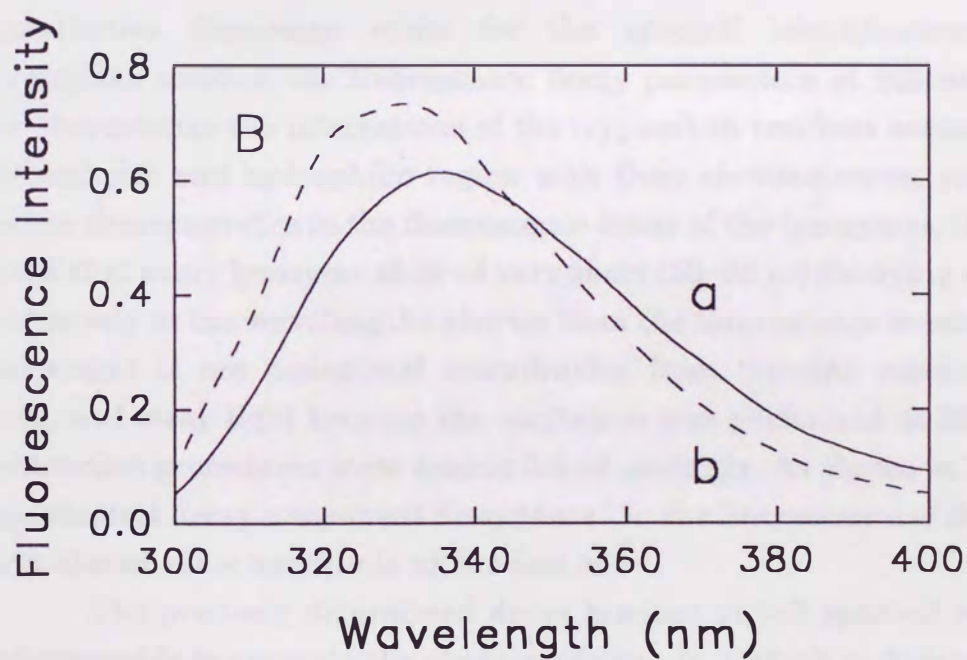
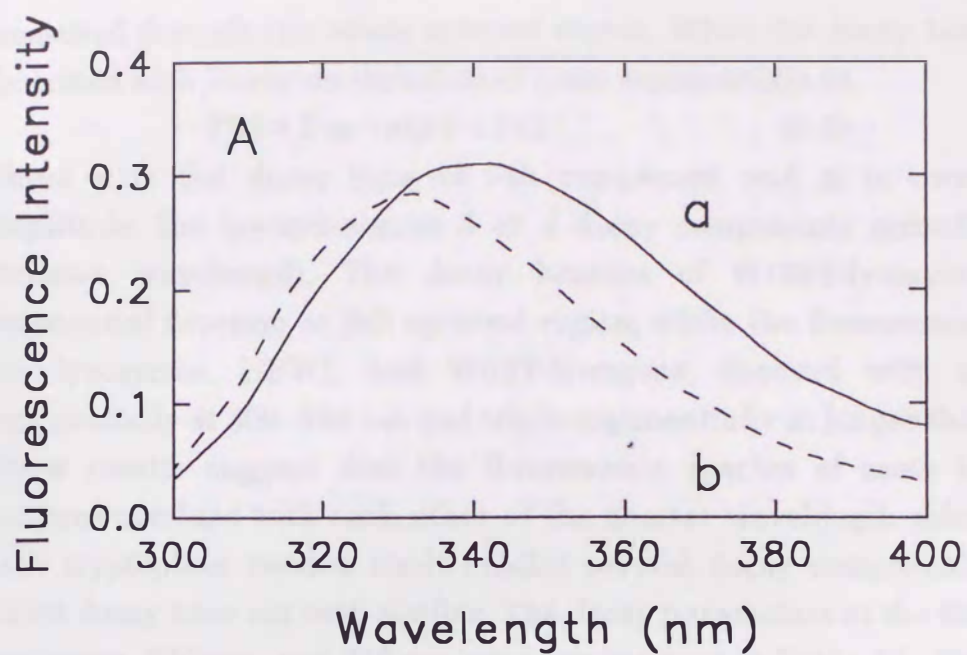


Fig. 3.2 The effect of (NAG)₃ on the fluorescence spectra of W108Y-lysozyme (A) and W62Y-lysozyme (B).

—, free enzyme; - - - - - , lysozyme-(NAG)₃ complex.

The molar ratio of enzyme/trisaccharide, 1/100; Excitation wavelength, 300 nm.

measured through the whole spectral region. When the decay kinetics were described with linear combination of some exponentials as,

$$F(t) = \sum \alpha_i \cdot \exp(-t / \tau_i) \quad (3.4)$$

where τ_i is the decay time of i -th component and α_i is corresponding amplitude, the lysozymes gave 3 or 4 decay components according to the emission wavelength. The decay kinetics of W108Y-lysozyme was 3-exponential function at full spectral region, while the fluorescence of other two lysozymes, HEWL and W62Y-lysozyme, decayed with quadruple-exponentially at 300–360 nm and triple-exponentially at longer than 360 nm. These results suggest that the fluorescence spectra of some tryptophan residues overlaps with each other at the shorter wavelength side, and that each tryptophan residue itself exhibit several decay components some of which decay time are very similar. The decay parameters at the fluorescence maximum, 320 nm, and 340 nm were summarized in Table 3.I. Although the quantitative discussion waits for the spectral identification of each tryptophan residue, the fluorescence decay parameters at 320 nm and 380 nm characterize the interactions of the tryptophan residues arranged at the hydrophobic and hydrophilic region with their circumstances, respectively. As one characteristics in the fluorescence decay of the lysozymes, it should be noted that every lysozyme showed very short (50–80 ps) decaying component exclusively at the wavelengths shorter than the fluorescence maximum. This component is not emissional contribution from tyrosine residue nor the undesired stray light because the excitation was performed at 300 nm and subtraction procedures were accomplished perfectly. As shown in Table 3.II, this shortest decay component disappeared in the fluorescence of the complex with almost same amplitude as the free one.

The precisely determined decay kinetics at full spectral regions are indispensable to estimate the average lifetime (τ_{av}) which is defined as,

$$\tau_{av} = \sum \alpha_i \cdot \tau_i \quad (3.5)$$

and is essential to apply the fluorescence quenching experiments. At the wavelength longer than 350 nm, the average lifetimes of HEWL were similar to one of W108Y-lysozyme and they were always longer than those of W62Y-lysozyme. When Trp108 was replaced with tyrosine τ_{av} at the shorter wavelengths were prolonged and contrarily the replacement of Trp62 with tyrosine shortened them.

Fluorescence Quenching by Acrylamide.

Although the fluorescence of the lysozymes were quenched by

Table 3.I Fluorescence decay parameters of HEWL and mutant lysozymes.

	Wavelength (nm)	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_4 (ns)	α_1	α_2	α_3	α_4	τ_{av} (ns)	σ	SVR
HEWL	320	3.21	1.21	0.40	0.05	0.09	0.28	0.35	0.28	0.78	1.05	1.96
	340	3.41	1.46	0.46	0.04	0.12	0.32	0.29	0.27	1.02	1.02	2.00
	380	3.61	1.58	0.43	-	0.21	0.58	0.21	-	1.76	1.03	1.97
W62Y	320	2.90	1.17	0.42	0.05	0.06	0.31	0.37	0.26	0.71	1.02	2.00
	340	3.62	1.43	0.47	0.04	0.06	0.34	0.36	0.24	0.88	1.02	1.96
	380	3.86	1.51	0.56	-	0.15	0.54	0.31	-	1.57	1.01	1.98
W108Y	320	2.85	0.74	0.08	-	0.13	0.54	0.32	-	0.10	1.10	1.81
	340	3.61	1.57	0.55	-	0.19	0.36	0.45	-	1.50	1.00	2.00
	380	4.26	1.97	0.49	-	0.23	0.50	0.27	-	2.10	1.02	2.00

Excitation wavelength, 300 nm

Table 3.II Fluorescence Decay Parameters of Lysozyme-(GlcNAc)₃ Complex.

	Wavelength (nm)	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	α_1	α_2	α_3	τ_{av} (ns)	σ	SVR
HEWL	320	1.92	0.80	0.22	0.17	0.72	0.11	0.93	1.03	1.94
	340	2.28	0.98	0.42	0.14	0.60	0.26	1.02	1.03	2.00
	380	2.54	1.02	0.34	0.15	0.63	0.22	1.10	1.07	1.82
W62Y	320	1.77	0.71	0.05	0.17	0.63	0.20	0.76	1.05	2.00
	340	1.93	0.71	0.06	0.19	0.64	0.17	0.83	1.05	1.98
	380	2.28	0.71	—	0.29	0.71	—	1.17	1.00	1.90
W108Y	320	1.14	0.38	—	0.53	0.47	—	0.78	1.03	2.00
	340	1.51	0.43	—	0.46	0.54	—	0.93	1.01	1.96
	380	2.38	0.45	—	0.45	0.55	—	1.32	1.02	1.93

Excitation wavelength, 300 nm

acrylamide, the quenching efficiency was dependent on the emission wavelength. When I_0/I was plotted as a function of the concentration of acrylamide based on the usual Stern-Volmer equation, the fluorescence of W108Y-lysozyme showed straight lines with various slopes giving Stern-Volmer constant (K_{sv}). As shown in Fig. 3.3, K_{sv} were dependent on the emission wavelength and they were longer at the longer wavelength. Although data are not shown, Stern-Volmer plot for the fluorescence quenching of W62Y-lysozyme also exhibited linear relation between I_0/I and the concentration of acrylamide. Stern-Volmer constant allows us to estimate the collisional fluorescence quenching constant (k_q) through a relation, $K_{sv} = k_q \cdot \tau_{av}$. The estimated k_q of HEWL, W108Y- and W62Y-lysozyme were shown in Fig. 3.4. W108Y-lysozyme gave the constant k_q (3.2/ns) at the wavelength range from 360 nm to 400 nm and decreased gradually as the wavelength was shorter. Although k_q of W62Y-lysozyme was also constant 360 to 400 nm, their values ($k_q \approx 0.9/\text{ns}$) were much smaller than one of W108Y-lysozyme. At the wavelengths around 320 nm, the apparent collisional quenching constants of two mutant lysozymes were almost same ($k_q \approx 2.0/\text{ns}$). It is interesting to recognize that W62Y-lysozyme shows smaller k_q at longer wavelength side than at shorter wavelengths. Because this result means that the tryptophyl fluorescence at the hydrophobic matrix box is more sensitive to the quenching action of acrylamide.

In the complexes of every lysozymes with $(\text{NAG})_3$, the fluorescence collisional quenching constants were suppressed. The fluorescence of W108Y-lysozyme became almost free from the quenching action by acrylamide at the shorter wavelength side, while the collisional quenching rate at the longer wavelengths was reduced from 3.2/ns to 1.5/ns. Although the change in the fluorescence quenching efficiency of W62Y-lysozyme by the interactions with $(\text{NAG})_3$ was not so drastic as W108Y-lysozyme at the shorter wavelength side, the quenching rate was also suppressed to about 50% around 320 nm.

Quenching Resolved Fluorescence Spectrum.

Because the collisional fluorescence quenching rate of W108Y-lysozyme were independent on the emission wavelength at 360~400 nm, indicating that the corresponding fluorescence is attributable to single tryptophan, an emission band with gaussian line shape was obtained fitting it to the fluorescence spectrum of W108Y-lysozyme at this wavelength region (curve b in Fig. 3.5-A). The maximum wavelength of this band was found at

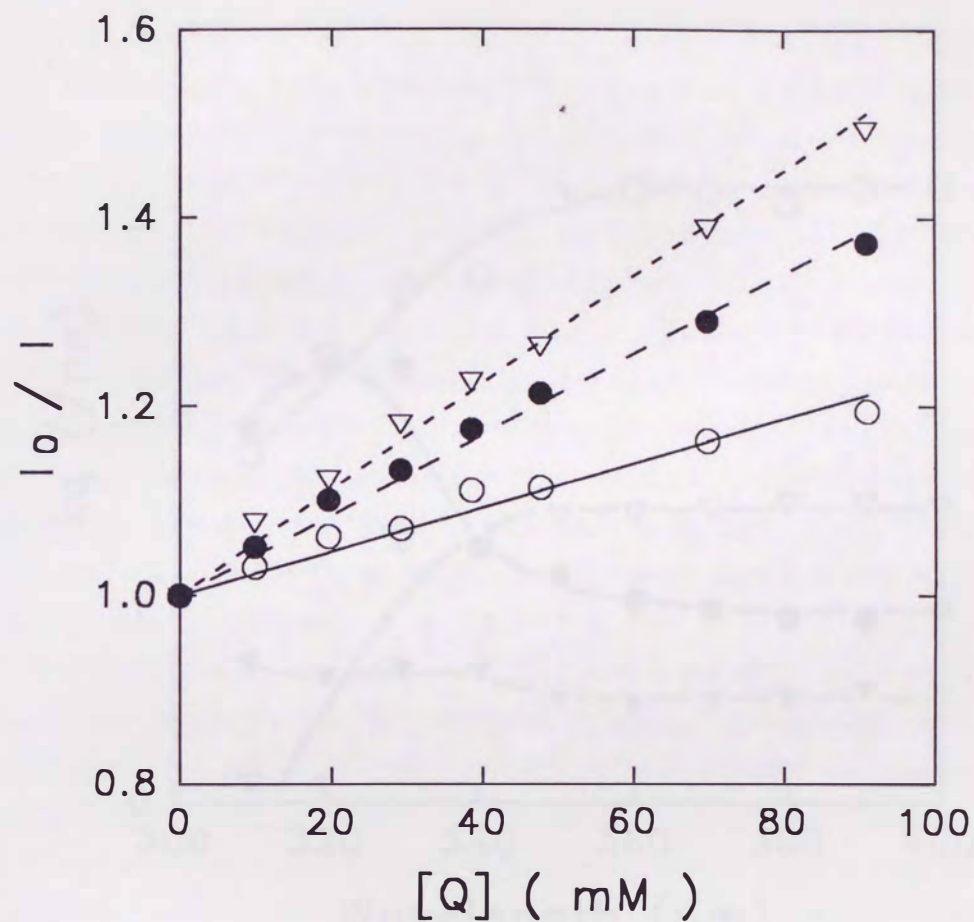


Fig. 3.3 Stern-Volmer plot for the fluorescence quenching of W108Y-lysozyme monitored at 320 nm ($-O-O-$), 340 nm, ($-●-●-$), and 360 nm ($--\nabla--\nabla--$). The concentration of W108Y-lysozyme, 10 μM ; $[Q]$, the concentration of acrylamide used as fluorescence quencher.

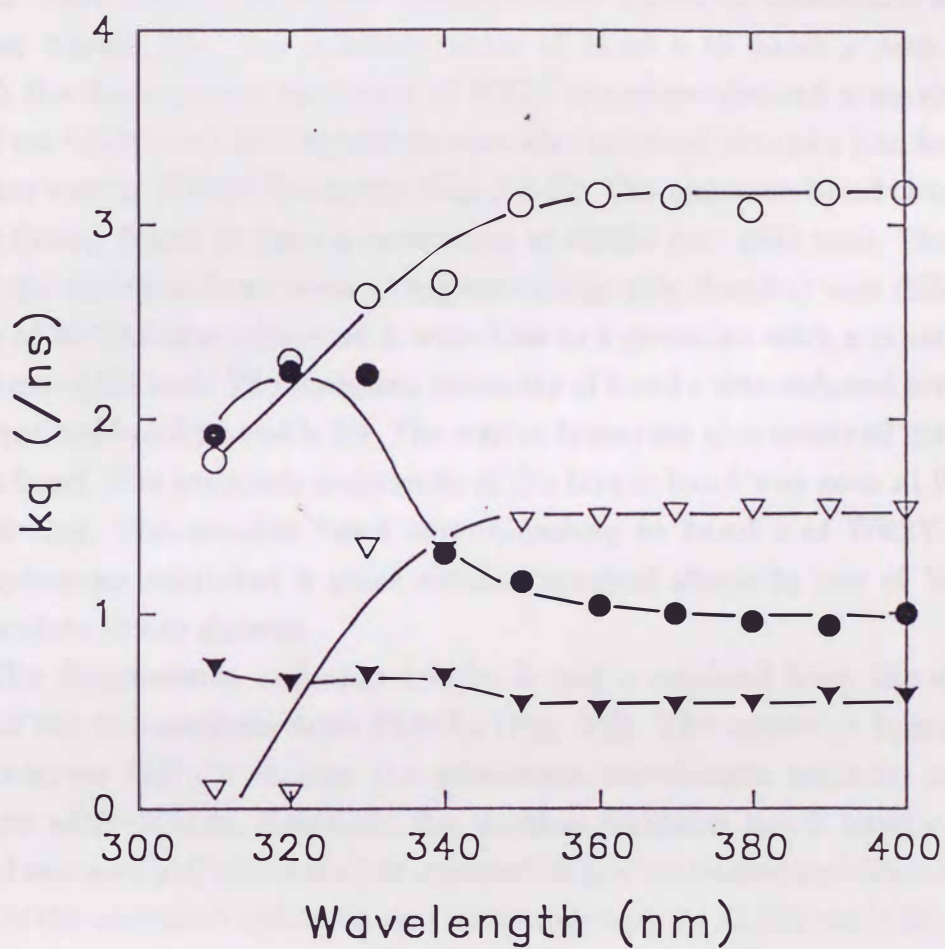


Fig. 3.4 Wavelength dependence of the collisional fluorescence quenching constant of mutant lysozyme and their complex with (NAG)₃.
 -○-○-, W108Y-lysozyme; -●-●-, W62Y-lysozyme; -▽-▽-, W108Y-lysozyme-(NAG)₃ complex; -▼-▼-, W62Y-lysozyme-(NAG)₃ complex. k_q was calculated with Stern-Volmer constant (K_{sv}) and average lifetime.

28400 cm^{-1} (352 nm) and was consistent with the fluorescence maximum of N-acetyltryptophanamide of which spectral line shape also could be approximated with a gaussian band (data is not shown). Another emission band (curve c) was obtained subtracting curve b from the total fluorescence emission (curve a) of W108Y-lysozyme. The line shape of curve c deviated from a gaussian to give a maximum and shoulder at 30300 cm^{-1} (330 nm) and 31300 cm^{-1} (320 nm), respectively. Compared the emission intensities at the maximum wavelength, the intensity ratio of band b to band a was 0.47. Although the fluorescence spectrum of W62Y-lysozyme showed a maximum at 29200 cm^{-1} (336 nm), this spectrum was also resolved into two bands with the similar way to W108Y-lysozyme (Fig. 3.5-B). The emission band obtained by curve fitting (band b) gave a maximum at 29200 cm^{-1} (342 nm). The line shape of the emission band seen at higher energy side (band c) was different from one of W108Y-lysozyme and it was close to a gaussian with a maximum at 30800 cm^{-1} (325 nm). The emission intensity of band c was reduced lower to give the ratio of band c/band b 1/3. The native lysozyme also resolved into two emission band. The emission maximum of the larger band was seen at 28700 cm^{-1} (348 nm). The smaller band corresponding to band c of W62Y- and W108Y-lysozyme exhibited a quite similar spectral shape to one of W62Y-lysozyme (data is not shown).

The fluorescence emission bands, b and c resolved from the entire spectra of the interactions with (NAG)₃ (Fig. 3.6). The emission band b of W62Y-lysozyme did not change the maximum wavelength position on the interaction with (NAG)₃. However, the another emission band, band c, was enhanced two and half times and its spectral shape was deformed drastically. Because of the exclusive enhancement of the intensity at 31300 cm^{-1} (320 nm), the maximum wavelength was shifted from 30300 cm^{-1} (330 nm) to 31300 cm^{-1} (320 nm). On the other hand, the band b of W62Y-lysozyme shifted the maximum wavelength 10 nm to the blue side. Smaller but clear spectral deformation was induced in the emission band c on the complex of W62Y-lysozyme with (NAG)₃. Then, the distinct change in the emission intensity of band c, itself, was not found.

3.4 Discussion

The characteristic fluorescence properties of Trp62 and Trp108 in HEWL were identified first time in the present work. A peculiar photophysical property of tryptophan residue demonstrates that the emission

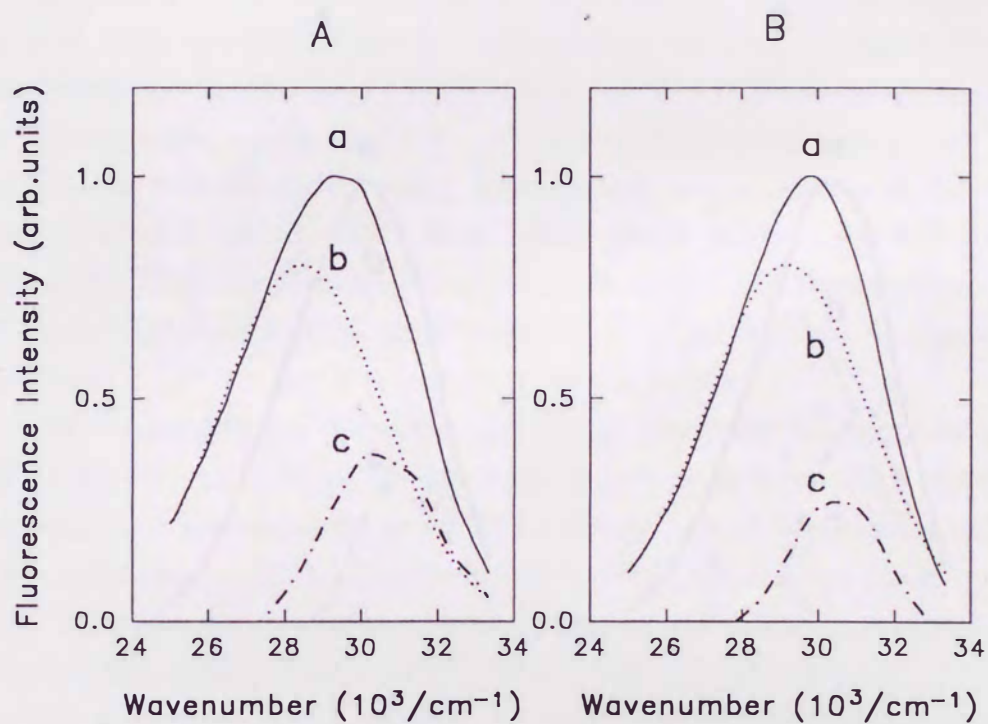


Fig. 3.5 The quenching resolved fluorescence of W108Y-lysozyme (A) and W62Y-lysozyme (B).

a, full spectrum of the lysozyme; b, an emission band obtained with curve fitting; c, an emission band obtained with curve a – curve b (Trp28 and Trp111). See the text. Concentration of the mutant lysozyme, 10 μ M; excitation wavelength, 300 nm.

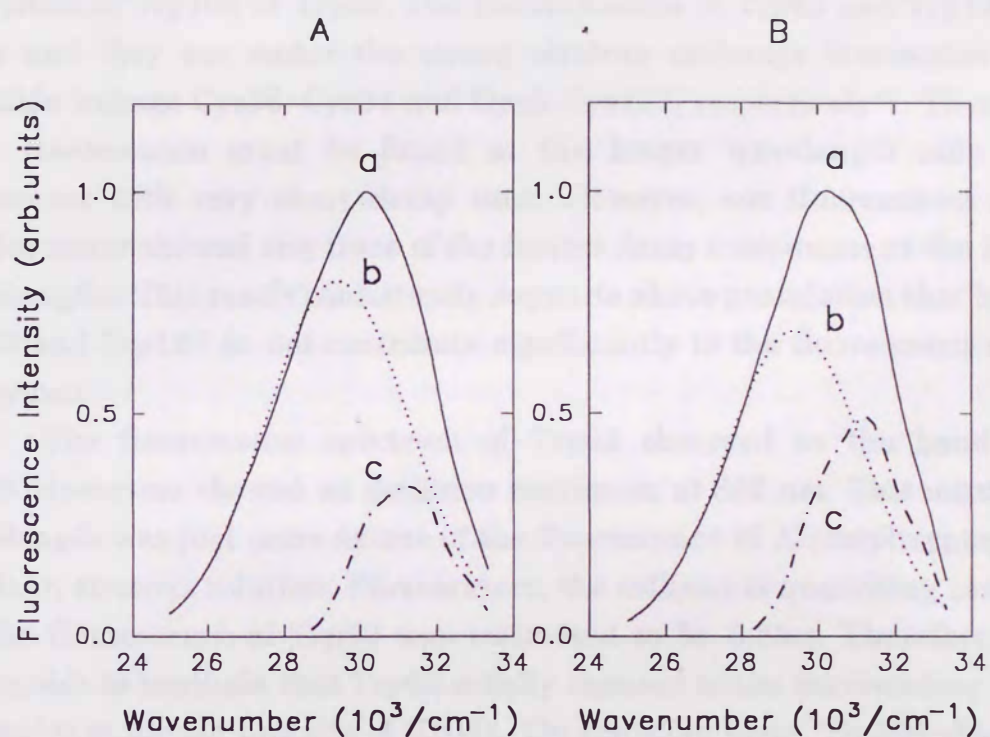


Fig. 3.6 The quenching resolved fluorescence of W108Y-lysozyme (A) and W62Y-lysozyme (B) with $(\text{NAG})_3$.

a, full spectrum of the lysozyme; b, an emission band obtained with curve fitting; c, an emission band obtained with curve a – curve b (Trp28 and Trp111). See the text. Concentration of the mutant lysozyme, 10 μM . The molar ratio of lysozyme/ $(\text{NAG})_3$, 1/100. Excitation wavelength, 300 nm.

band b of W108Y- and W62Y-lysozyme correspond to the fluorescence of Trp62 and Trp108, respectively. Because Trp62 and Trp108 are surrounded with polar circumstances in the lysozymes, their fluorescence spectra deflect to the lower energy side. Although it is generally accepted that Trp63 and Trp123 are non-fluorescent,¹³⁾ this postulation should be strictly reconfirmed again. Because these tryptophan residues also located at the hydrophilic parts of HEWL and the traces of their fluorescence may bring serious obscurities into the characterization of the fluorescence spectroscopic properties of Trp108 or Trp62. The circumstances of Trp63 and Trp123 are polar and they are under the strong electron exchange interaction with disulfide linkage Cys76-Cys94 and Cys6-Cys127, respectively¹³⁾. Therefore, their fluorescence must be found at the longer wavelength side as a component with very short decay time. However, our fluorescence decay studies never showed any trace of the fastest decay component at the longer wavelengths. This result consistently supports above postulation that both of Trp63 and Trp123 do not contribute significantly to the fluorescence of the lysozymes.

The fluorescence spectrum of Trp62 observed as the band b of W108Y-lysozyme showed an emission maximum at 352 nm. This maximum wavelength was just same as one of the fluorescence of *N*-acetyltryptophan-amide in aqueous solution. Furthermore, the collisional quenching constant for the fluorescence of Trp62 was estimated to be 3.2/ns. Therefore, it is reasonable to conclude that Trp62 is fully exposed to the surrounding water molecules at the binding site of HEWL. On the other hand, Trp108 showed a fluorescence maxima at 342 nm, indicating that this residue is also surrounded with polar circumstances. But this residue would not be exposed to water molecules, because the collisional fluorescence quenching constant of Trp108 was smaller ($k_q = 0.9/\text{ns}$). Miura et al. reported a specific raman line attributed to interacting Trp108 with neighboring amino acid residues in the cage of the peptide main chain as expected by X-ray structure of HEWL.¹⁴⁾

The fluorescence band c in W108Y- and W62Y-lysozyme, deviated from a single gaussian line shape, would be composed of the emissional contributions from Trp28 and Trp111. The maximum and shoulder observed at 330 nm and 320 nm are attributable to the emission from Trp111 and Trp28, respectively. Because X-ray crystallographic structure demonstrate that Trp28 is more densely packed than Trp111 in the hydrophobic matrix box of HEWL.¹⁵⁾

As shown in the quenching resolved fluorescence spectra of mutant lysozymes and their complex with (NAG)₃, the spectral overlap of the fluorescence of Trp62 or Trp108 with one of Trp28 and/or Trp111 was limited within 300 nm~360 nm. Therefore, the fluorescence decay kinetics observed at the wavelengths longer than 360 nm exclusively reveals the interactions of Trp62 or Trp108 with each surroundings in the mutant lysozymes. Both of Trp62 and Trp108 showed three components in their fluorescence decay kinetics. According with Willis and Szabo, the heterogeneous fluorescence decay exhibited by protein and peptide mostly originates in the conformer of the indole moiety of tryptophan residue interacting with the neighbors in different ways.³⁾ The characteristic fluorescence decay components of mutant lysozymes would correspond to such conformers of Trp62 or Trp108. The reduction of the decay component from three to two in the fluorescence decay kinetics of Trp62 and Trp108 by the binding with (NAG)₃, probably reflect such a redistribution of the these conformer as only two conformer would be stabilized in the lysozyme-ligand complex. Because of the large spectral overlap, the characteristic fluorescence decay kinetics of Trp28 or Trp111 could not be uniquely determined. But it is not unreasonable to consider that the fluorescence lifetime of Trp28 and Trp111 are very short. Because the fluorescence decay times of mutant lysozymes were shorter at the shorter wavelength side in spite of the spectral overlaps with the fluorescence of Trp62 or Trp108 exhibiting longer decay times. Although we have to keep in mind the difference in the transition type between Trp28/Trp111 and Trp62/Trp108, the fluorescence transition of Trp28 or Trp111 may be attributed to $^1L_b \rightarrow A$, while one of Trp62 and Trp108 is $^1L_a \rightarrow A$, the shorter lifetimes of Trp28 and Trp111 result from the strong interactions of these residues with the hydrophobic matrix box to quench their fluorescence. Similar type of the short decay component was reported for the fluorescence decay of the tryptophan residue in hydrophobic matrix box of calcium-dependent membrane binding protein.²⁾

Characterization of individual fluorescence of Trp62, Trp108, and Trp28/Trp111 allowed us to discuss the local conformational change induced by the interactions of the lysozymes with (NAG)₃ by analyzing their modified fluorescence properties. The blue shift of the fluorescence maximum of Trp62 demonstrates that the polarity at the binding cleft of HEWL, at least in the vicinity of Trp62, is reduced. Trp62 is fully hydrated with surrounding water molecules in the free state but it is bound to (NAG)₃ through a hydrogen bond

in the presence of the ligand. This exclusion of water molecules by the trisaccharide is, probably, the source for the spectral shift of Trp62. The participation of the hydrogen bond of Trp62 is confirmed in other fluorescence and NMR spectroscopic studies.^{16,17)} Trp108 did not shift its fluorescence maximum position on the interaction with (NAG)₃. This result suggests any local conformational change leading to the change in the polarity is not induced in the vicinity of Trp108. We previously showed that Trp108 had no participation in the spectral shift of HEWL analyzing the fluorescence spectrum of Kyn62-lysozyme in which Trp62 was chemically replaced with kynurenine.¹⁶⁾ The spectral response of W62Y-lysozyme to the interaction with (NAG)₃ was consistent with one of Kyn62-lysozyme.

The shortening of the average lifetime of Trp62 or Trp108 on the interaction of the lysozymes with the ligand supports a model for the conformational change in HEWL which was originally proposed by Lehrer and modified by Yamashita et al.^{4,6)} According to this model, the carboxyl group of Glu35 approaches closer to Trp108 and, at the same time, Trp62 flips its indole moiety to Trp108, when (NAG)₃ binds with HEWL. The protonated carboxyl group is one of the most efficient quenching group in protein and it quenches the fluorescence of these two tryptophan residues.

The fact that alterations of the fluorescence properties of Trp62 and Trp108 concomitantly accompany the changes in the fluorescence of Trp28 and/or Trp111 consistently demonstrates that some interacting conformational changes were also induced at the hydrophobic matrix box region corresponding to the lysozyme-(NAG)₃ interactions. In spite of the non-spectral shift in the fluorescence of Trp108, the large emission enhancement was seen at the blue side in the entire fluorescence spectrum of W62Y-lysozyme. The emission intensity of the band c increase in the quenching resolved spectra of the complex of W62Y-lysozyme with (NAG)₃. Furthermore, the average fluorescence lifetime of W62Y-lysozyme at 320 nm was also prolonged. All of these results support that the excited singlet state of Trp28 and/or Trp111 was stabilized to enhance their fluorescence. Hitherto, it has been considered that the structure of HEWL is rigid based on the crystallographic structure, especially, its hydrophobic matrix box is densely packed to seal the internal motion in it. Nevertheless, it is also confirmed that the fluctuational motion of eosin molecule keeps large freedom at the hydrophobic matrix box of HEWL in the physiological condition.¹⁸⁾ Our fluorescence quenching experiments showed that the collisional fluorescence

quenching constant for Trp28/Trp111 was rather larger, indicating the internal motion at the hydrophobic matrix box of HEWL was more active at the hydrophobic matrix box in the free enzyme. The enhancement of the fluorescence of Trp28 and Trp111 by the interaction with (NAG)₃, probably, results from the suppression of such fluctuational motions at the hydrophobic matrix box. Because the collisional fluorescence quenching constants were remarkably lowered by the interaction of HEWL with the ligand.

More deeper insight into the band shapes of Trp28/Trp111 suggests that (NAG)₃ may bring about some distortion of the conformation at the hydrophobic matrix box of HEWL interacting with the lysozymes. Because the maximum wavelength of band c was switched from 330 to 320 nm on the binding of the mutant lysozymes with (NAG)₃. This spectral change demonstrates that Trp28 is exclusively liberated from the interaction with the hydrophobic surroundings and its fluorescence state of Trp28 is more stabilized than Trp111.

The indole moiety of Trp108 in W62Y-lysozyme is hydrophilic and buried in the peptide main chain. Therefore, the replacement of this residue to tyrosine with the lower hydrophobicity and smaller molecular size may give some effect on the molecular packing at the hydrophobic matrix box of HEWL. Although the apparent value of the quenching constant estimated at 320 nm were quite similar for two mutant lysozymes, W108Y-lysozyme would give smaller k_q for the fluorescence of Trp28/Trp111 than W62Y-lysozyme. Because the fluorescence of Trp28/Trp111 in the former overlaps with the fluorescence of Trp62, while one in the latter mutant with fluorescence of Trp108. When k_q of Trp28/Trp111 of W108Y- and W62Y-lysozyme were estimated using the emissional fraction of Trp28/Trp111 to Trp62 or Trp108 at 320 nm, they were 1.3/ns and 2.5/ns, respectively. Considering the difference in the fluorescence spectral shape of Trp28/Trp111 and their response to the interaction with (NAG)₃ between W62Y- and W108Y-lysozyme, this fact suggests that the replacement of Trp108 with tyrosine give more densely packed structure to the hydrophobic matrix box of HEWL.

The quenching resolved fluorescence spectra of mutant lysozymes and their complex with (NAG)₃ consistently showed that (NAG)₃ changed not only the conformation around binding site, but also its binding effect extended to the hydrophobic matrix box region of HEWL. Such long ranged effect of the enzyme-ligand interaction must be very interesting to consider the relationship between the ligand or substrate induced conformational

change and the enzymatic action.

3.5 Conclusion

The characteristic fluorescence properties of tryptophan residues in hen egg-white lysozyme (HEWL) and their responses to the interaction with ligands were investigated through the time- and quenching-resolved spectroscopy of two mutant lysozymes, W62Y- and W108Y-lysozyme, in which Trp62 and Trp108 were replaced with tyrosine residue, respectively. The quenching resolved fluorescence spectrum of these mutant lysozymes showed that Trp62 and Trp108 gave the fluorescence maximum at 352 nm and 342 nm, respectively, and that the fluorescence of the other two fluorescent tryptophan residues of HEWL, Trp28 and Trp111, was observed as an unresolved emission band with a maximum and shoulder at 320 nm and 330 nm. The intrinsic collisional fluorescence quenching constant of the mutant lysozyme suggest that Trp62 was fully exposed to the solvent molecule but Trp108 was sealed in the cage of the main chain although the circumstances of these two residues were polar. It was also shown that the fluorescence of Trp28 and Trp111 were considerably influenced by the fluctuational motion at the hydrophobic matrix box of HEWL.

The fluorescence of each tryptophan residue was individually modified in the complex of lysozyme with trimer of *N*-acetylglucosamine indicating that the ligand binding effect on the HEWL conformation was not limited to the binding site but it extended to the hydrophobic matrix region.

3.6 References

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CHAPTER 4

Ligand-induced Conformational Change in Lysozyme

4.1 The Steady State and Time-resolved Fluorescence Studies on the Lysozyme-Ligand Interaction.

4.1.1 Introduction

Hen egg-white lysozyme (HEWL) is an interesting subject to examine the correlation between the enzymatic activity and ligand or substrate induced conformational change through fluorescence spectroscopy, because two tryptophan residues, Trp62 and Trp108, working as the major fluorophore, are arranged around the binding site. Since the fluorescence of these residues may provide valuable information on the interactions with substrate or inhibitor, a number of studies have been done on the fluorescence spectroscopy of HEWL. But it should be noted that most of them suffer from lack of reliability because of putting the importance on the spectral analysis without decay analysis or include some uncertainties due to inadequate instruments. Therefore, many delicate but important conformation change induced in HEWL have been overlooked. Recently a high-performance excitation and detection system has been developed and knowledge of the photophysical properties of tryptophan residues has been accumulated. The fluorescence spectroscopy has been one of the most powerful methods to detect subtle conformational changes and their dynamics in HEWL. At our convenience, the crystallographic structure of HEWL is established and, therefore, the location of the tryptophan residue working as the intrinsic fluorophore has been confirmed.¹⁾ This knowledge allows us to interpret the fluorescence data along with the three-dimensional structure of HEWL. As described in CHAPTER 2, HEWL has six tryptophan residues. Although these tryptophan residues must be intrinsic fluorescence probes for studying the conformational in HEWL, the excited singlet states of Trp63 and Trp123 are extremely destabilized under the electron exchange interactions with disulfide linkages, Cys76-Cys94 and Cys6-Cys127, adjacent to them, respectively.²⁾ Therefore, it is reasonable to consider that these tryptophan residues do not contribute significantly to the fluorescence of the lysozyme. In a pioneering work on the fluorescence spectroscopic studies of HEWL, Imoto et al. reported that 80% of the fluorescence yield of HEWL was

due to Trp62 and Trp108.³⁾ Their results imply that the modifications of the fluorescence properties of HEWL by some perturbation may be related mostly to the conformational alterations around the binding site. But, paradoxically speaking, the other 20% of the total fluorescence, which can be attributed to Trp28 and Trp111, must be able to provide information on the hydrophobic matrix box region. If it is possible to differentiate the fluorescence of these tryptophan residues, we can trace the conformations at the hydrophobic matrix box and at the active site playing essential roles in structural stabilization and in the catalytic reaction of HEWL. When trying to do so, a tryptophan residue had a favorable photophysical property, the polarity dependence of the spectral distribution. The fluorescence band of a tryptophan residue surrounded with polar groups is deflected to the lower energy side because of the larger dipole-dipole interaction in the excited singlet state. Indeed, a raman spectroscopic study showed that for Trp108 as well as Trp62, a tryptophan residue at the binding site was surrounded in the polar circumstances.⁴⁾ Therefore, the fluorescence of these residues are expected to be more dominant at longer wavelengths. On the other hand, the emissional contributions from Trp28 and Trp111 should deviate to the shorter wavelength side. Such a peculiar fluorescence property of the tryptophan residue enabled us to trace separately the ligand-induced conformational change at two sites, one hydrophilic binding site, the other hydrophobic matrix box, by paying attention to the spectral distribution of the fluorescence of HEWL.

Kyn62-lysozyme is an active derivatives of HEWL, in which Trp62 of HEWL is specifically replaced with kynurenine. Comparing its fluorescence with one of the native lysozyme, it is possible to differentiate Trp108 with Trp62.

We examines here the ligand-induced conformational change of HEWL, confirming the steady-state and time-resolved fluorescence properties of HEWL, Kyn62-lysozyme and their complexes with tri-*N*-acetyl-D-glucosamine ((NAG)₃).

4.1.2 Materials and Methods

The preparation method of Kyn62-lysozyme was described previously in CHAPTER 2.

Tri-*N*-acetyl-D-glucosamine ((NAG)₃) was purchased from Sigma

Chemical Co. The native and Kyn62-lysozyme were dissolved in sodium acetate buffer (pH 5.5) or potassium phosphate buffer (pH 8.0) of which ion strength were precisely adjusted to 0.1. Concentrations of HEWL and the modified lysozyme solution were measured spectrophotometrically using the molar extinction coefficients $\epsilon=3.9\times10^4\text{M}^{-1}\text{cm}^{-1}$ at 280 nm and $\epsilon=4.7\times10^3\text{M}^{-1}\text{cm}^{-1}$ at 360 nm, respectively.⁵⁾

4.1.3 Results

Fluorescence spectra at room temperature.

The fluorescence spectrum of HEWL on the excitation at 295 nm has a broad band with a maximum at 342 nm regardless of the pH of the solution. At pH 5.5, progressive reductions of the emission intensity and the blue shifts of the maximum were seen with increasing the concentration of (NAG)₃ (Fig. 4.1.1). When HEWL was saturated with (NAG)₃, the emission intensity was reduced to 70% and the maximum wavelength shifted to 332 nm. The fluorescence difference spectra between the absence and at the presence of (NAG)₃ have a small positive maximum at about 320 nm and a broad negative maximum at 363 nm (Fig. 4.1.1 insertion).

In the phosphate buffer at pH 8.0, the blue shift of the fluorescence maximum accompanied a large enhancement of the emission intensity (Fig. 4.1.2). The difference spectra between HEWL and HEWL-(NAG)₃ complex were composed of a large positive part with a maximum at 323 nm and a broad negative one with a maximum at 374 nm.

Fluorescence spectra of Kyn62-lysozyme and its complex with (NAG)₃ at pH 5.5 and 8.0 are shown in Figs. 4.1.3 and 4.1.4, respectively. When Kyn62-lysozyme was excited at 295 nm, it gave two distinct maxima at 336 nm and 450 nm. The latter, which is not seen in the fluorescence spectrum of HEWL, is attributed to the emission of kynurenine and the former is due to one of the tryptophan residues. The binding of (NAG)₃ partially quenched the fluorescence of tryptophan residues and, on the contrary, enhanced the emission intensity of kynurenine at pH 5.5. The increase in the emission at around 320 nm, which was recognized in the fluorescence of HEWL-(NAG)₃ complex, was also clear. However, it should be noted that the tryptophyl fluorescence maximum of Kyn62-lysozyme, upon the binding with (NAG)₃, did not show any shift at all, in spite of the change in the fluorescence intensity. The spectral change created by the ligand is obviously shown in the

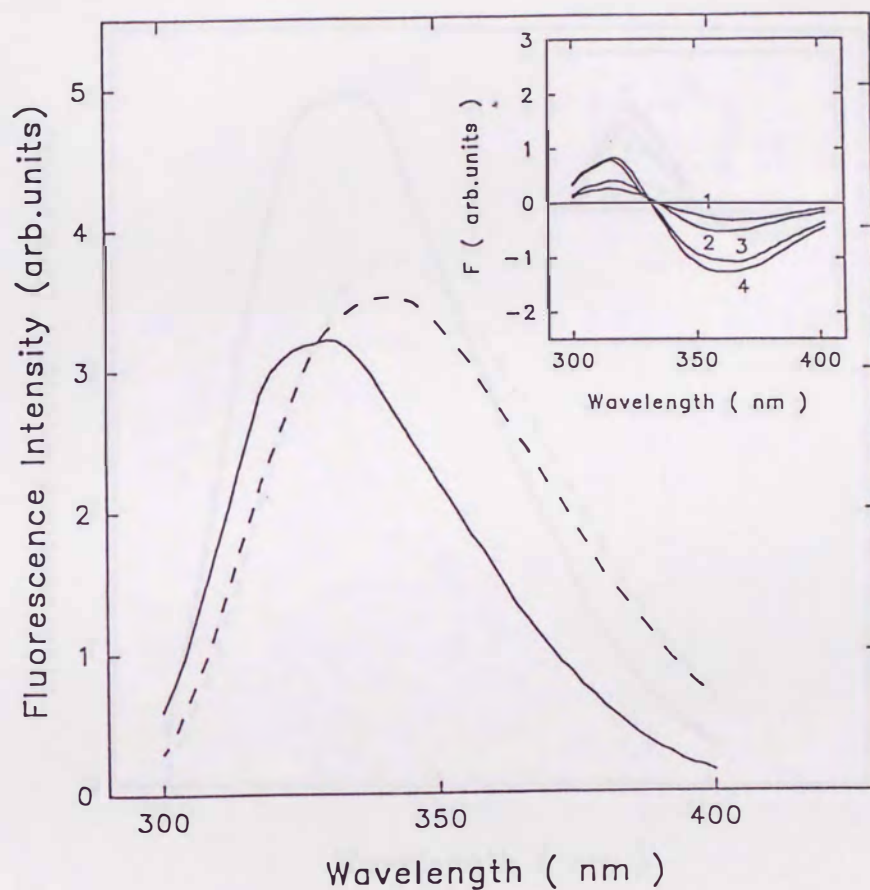


Fig. 4.1.1 Effects of $(\text{NAG})_3$ on the Fluorescence spectrum of HEWL at pH 5.5.

Concentration of HEWL, $10 \mu\text{M}$; temperature, 25°C ; excitation wavelength, 295 nm ; bandpass of excitation/emission, $5 \text{ nm}/5 \text{ nm}$; broken line, free HEWL; solid line, HEWL- $(\text{NAG})_3$ complex in the presence of $200 \mu\text{M}$ of $(\text{NAG})_3$.

Insertion, fluorescence difference spectra between in the absence and at the presence of $(\text{NAG})_3$; Curves 1, 2, 3, and 4 are at the presence of 10, 50, 100, and $200 \mu\text{M}$ of $(\text{NAG})_3$, respectively.

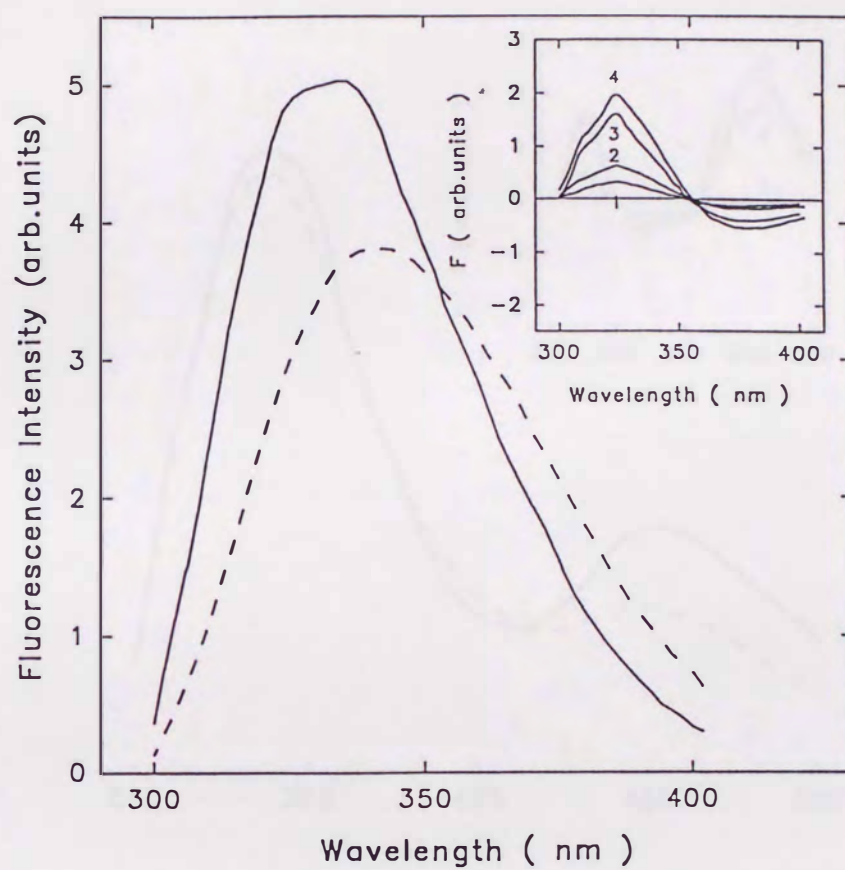


Fig. 4.1.2 Effects of $(\text{NAG})_3$ on the fluorescence spectrum of HEWL at pH 8.0.

The conditions are the same as in Fig. 4.1.1 except pH of the buffer solution. See the legends of Fig. 4.1.1.

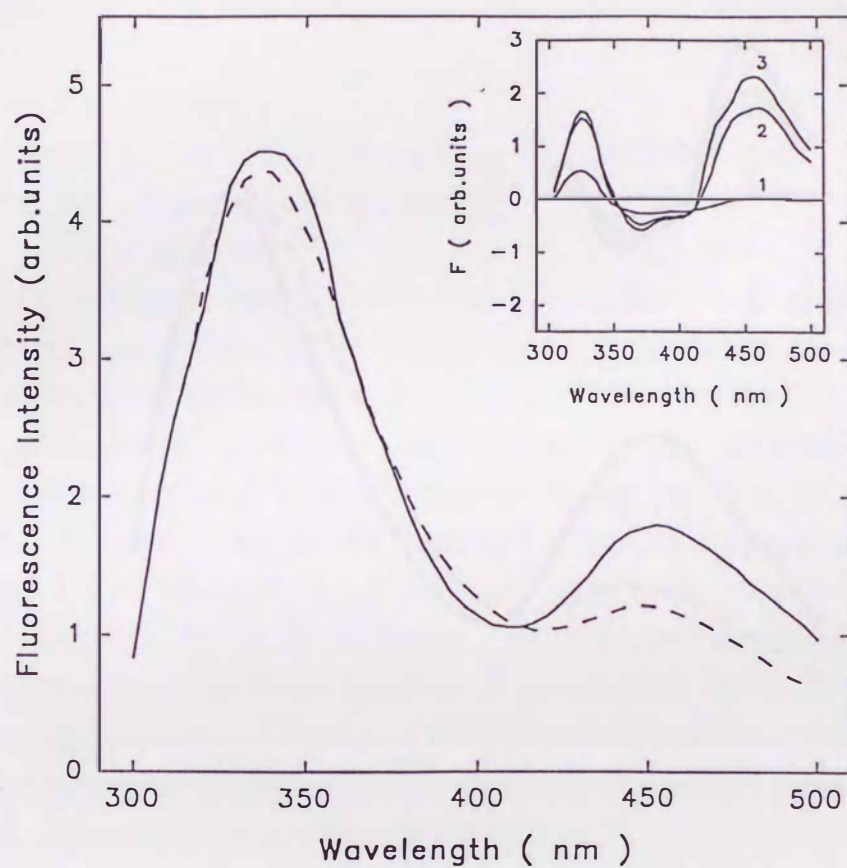


Fig. 4.1.3 Effects of $(\text{NAG})_3$ on the Fluorescence spectrum of Kyn62-lysozyme at pH 5.5.

Concentration of Kyn62-lysozyme, $5 \mu\text{M}$; temperature, 25°C ; bandpass of excitation/emission, $5 \text{ nm}/5 \text{ nm}$; broken line, free Kyn62-lysozyme; solid line Kyn62-lysozyme- $(\text{NAG})_3$ complex in the presence of $200 \mu\text{M}$ of $(\text{NAG})_3$.

Insertion, fluorescence difference spectra between in the presence and at the absence of $(\text{NAG})_3$; Curves 1, 2, and 3 are in the presence of 10, 100, and $200 \mu\text{M}$ of $(\text{NAG})_3$, respectively.

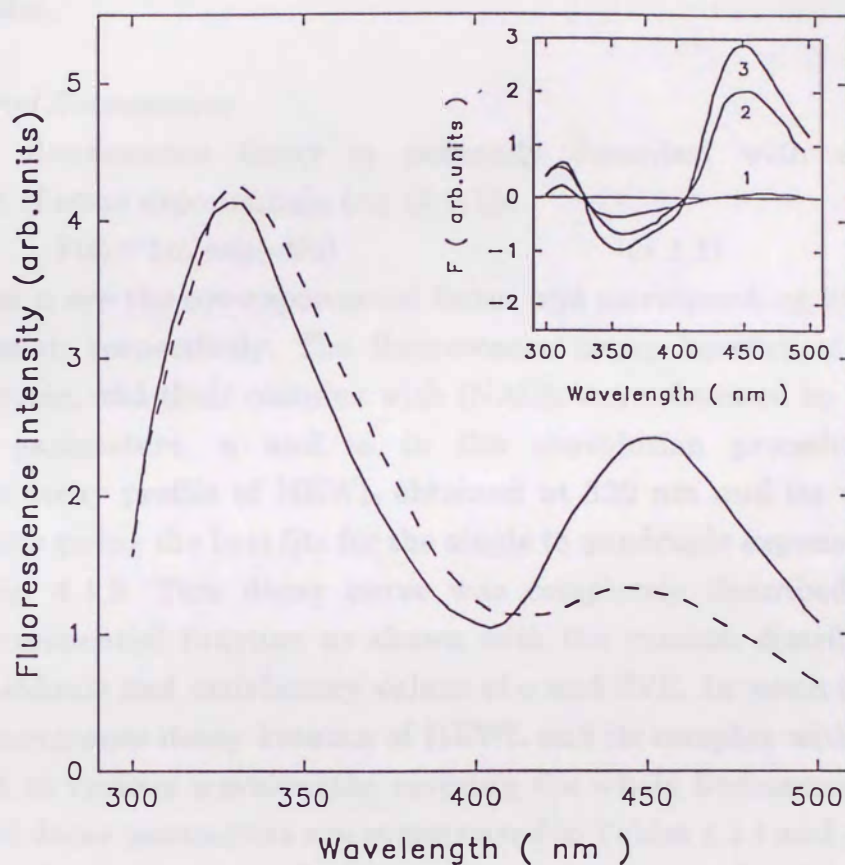


Fig. 4.1.4 Effects of $(\text{NAG})_3$ on the fluorescence spectrum of Kyn62-lysozyme at pH 8.0.

The condition are the same as in Fig. 4.1.3 except pH of the buffer solution. See the legend of Fig. 4.1.3.

difference spectra between Kyn62-lysozyme and its complex with (NAG)₃. The positive maximum at 450 nm, which was not seen in HEWL, indicates the enhancement of the emission intensity of kynurenine by the binding of (NAG)₃. Within the wavelength region of the tryptophyl fluorescence (300–400 nm), the difference spectra of Kyn62-lysozyme showed also a bipolar structure similarly with HEWL, although they depended on the pH of the buffer solution.

Time-resolved fluorescence

The fluorescence decay is generally described with a linear combination of some exponentials (eq. (4.1.1)).

$$F(t) = \sum \alpha_i \exp(-t/\tau_i) \quad (4.1.1)$$

where α_i and τ_i are the pre-exponential factor and corresponding lifetime of i -th component, respectively. The fluorescence decay kinetics of HEWL, Kyn62-lysozyme, and their complex with (NAG)₃ were obtained by deciding the decay parameters, τ_i and α_i in the convolution procedure. The fluorescence decay profile of HEWL obtained at 320 nm and its weighted residuals plots giving the best fits for the single to quadruple exponential are given in Fig. 4.1.5. This decay curve was completely described with a quadruple-exponential function as shown with the random distribution of weighted residuals and satisfactory values of σ and SVR. In much the same way, the fluorescence decay kinetics of HEWL and its complex with (NAG)₃ was decided at various wavelengths covering the whole fluorescence band. The obtained decay parameters are summarized in Tables 4.1.I and 4.1.II. At the wavelengths longer than the fluorescence maximum, the fluorescence decay of HEWL were described with triple-exponential kinetics, but a clear participation of the fast decay component were recognized at the shorter wavelength side and, therefore, the quadruple-exponential is the best function for the fluorescence decay of HEWL at this wavelength region. A characteristics in the fluorescence decay kinetics of HEWL was the wavelength dependence of the decay time of each component. The decay times were more prolonged the longer was the emission wavelength.

The average fluorescence lifetime of HEWL defined as $\tau_{av.} = \sum \alpha_i \tau_i$ was plotted against various emission wavelengths (Fig. 4.1.6). The resulting curves at pH 5.5 and 8.0 increased quite similarly to the wavelength although the average lifetimes were larger a little at pH 8.0 than at pH 5.5. (NAG)₃,

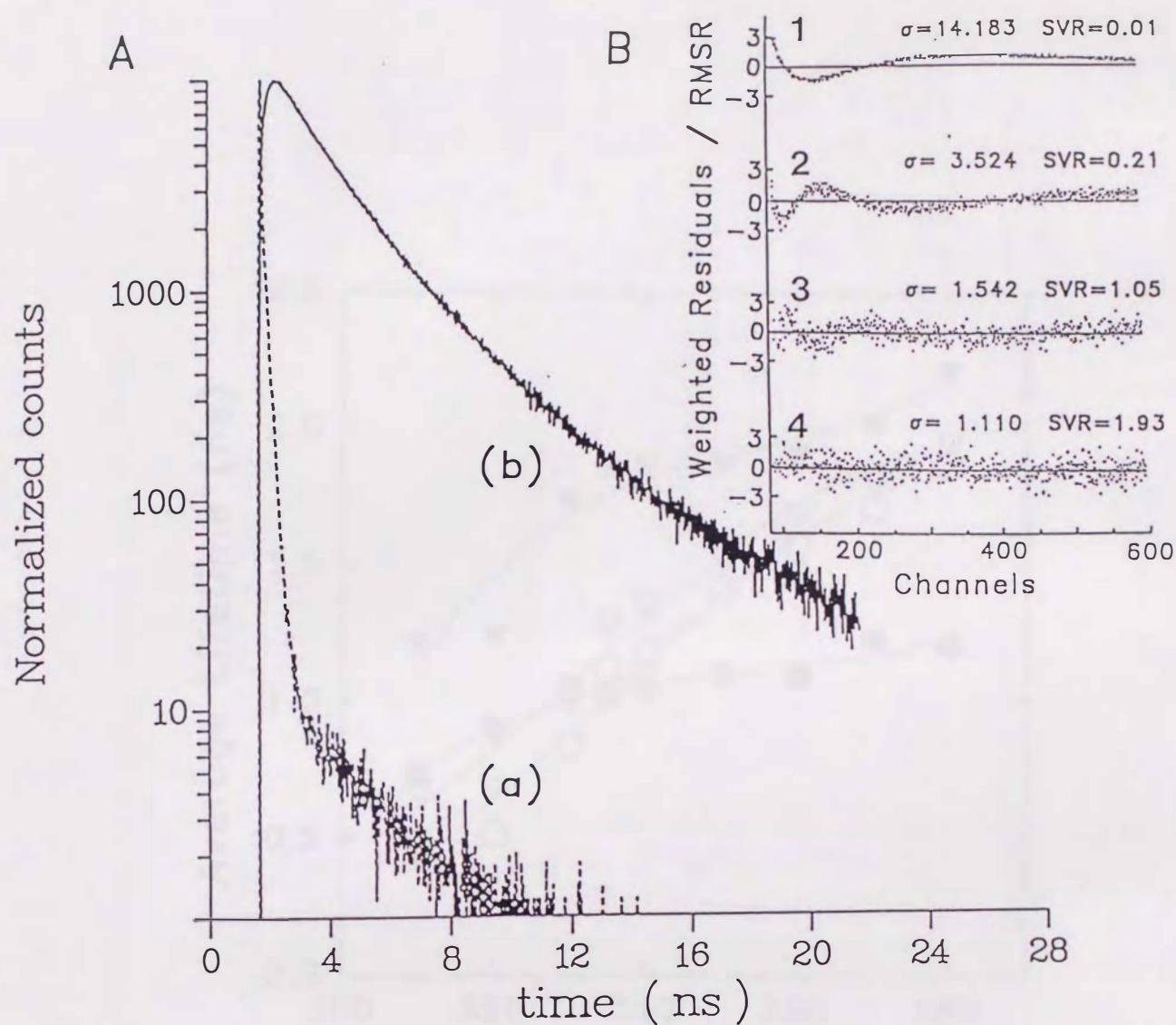


Fig. 4.1.5 Fluorescence decay profile (A) and the weighted residual plots of HEWL (B) at pH 5.5.

Excitation wavelength, 295 nm, emission wavelength, 320 nm; excitation pulse width, 15 ps; channel width of the multichannel analyzer, 21.5 ps. a, fluorescence decay profile; b, excitation pulse.

1, 2, 3, and 4 in (B), the weighted residual plots giving the best fit for the single-, double-, triple-, and quadruple-exponential function, respectively. SVR and σ are serial variance ratio and sigma value, respectively. (See the text.)

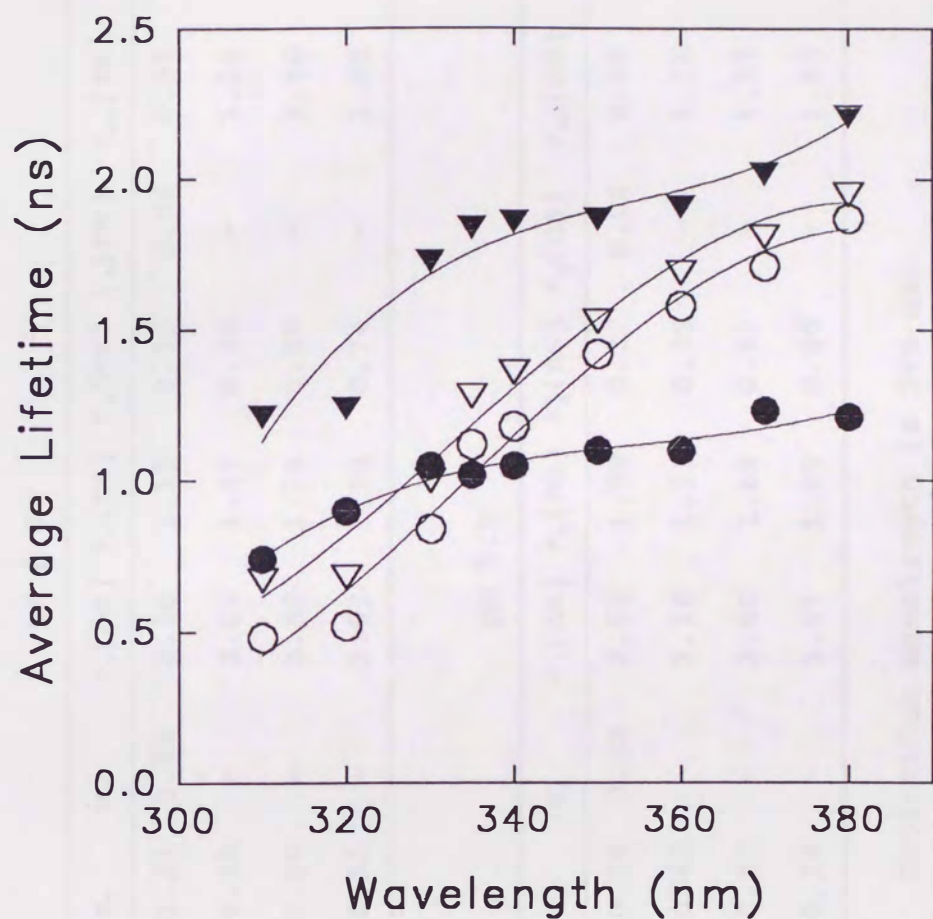


Fig. 4.1.6 Wavelength dependence of the average fluorescence lifetime of HEWL.

Average lifetime was calculated with the decay parameters based on the definition, $\tau_{av} = \sum \alpha_i \tau_i$.

—○—○—, free HEWL at pH 5.5; —●—●—, HEWL-(NAG)₃ complex at pH 5.5; —▽—▽—, free HEWL at pH 8.0; —▼—▼—, HEWL-(NAG)₃ complex at pH 8.0.

Table 4.1.I Fluorescence Decay Parameters of HEWL

pH 8.0											
Wavelength(nm)	α_1	α_2	α_3	α_4	$\tau_1(\text{ns})$	$\tau_2(\text{ns})$	$\tau_3(\text{ns})$	$\tau_4(\text{ns})$	$\tau_{av}(\text{ns})$	SVR ^a	σ^b
310	0.11	0.18	0.23	0.48	2.30	1.15	0.39	0.06	0.68	2.00	1.16
340	0.17	0.50	0.33	-	3.01	1.47	0.36	-	1.36	1.96	1.07
360	0.15	0.57	0.28	-	3.52	1.76	0.57	-	1.69	2.00	1.03
380	0.14	0.64	0.22	-	3.93	1.94	0.74	-	1.95	2.00	1.03

pH 5.5											
Wavelength(nm)	α_1	α_2	α_3	α_4	$\tau_1(\text{ns})$	$\tau_2(\text{ns})$	$\tau_3(\text{ns})$	$\tau_4(\text{ns})$	$\tau_{av}(\text{ns})$	SVR ^a	σ^b
310	0.04	0.20	0.36	0.40	2.63	1.09	0.37	0.06	0.48	1.96	1.16
340	0.14	0.43	0.42	-	3.16	1.31	0.38	-	1.16	2.00	1.16
360	0.16	0.47	0.37	-	3.60	1.69	0.57	-	1.57	2.00	1.07
380	0.17	0.54	0.29	-	3.87	1.85	0.65	-	1.85	2.09	1.06

Excitation wavelength is 295 nm.

a, b, Serial variance ratio and reduced chi-square, respectively. (See text.)

Table 4.1.II Fluorescence Decay Parameters of HEWL-(GlcNAc)₃ Complex

pH 8.0											
Wavelength(nm)	α_1	α_2	α_3	α_4	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_4 (ns)	τ_{av} (ns)	SVR ^a	σ^b
310	0.48	0.17	0.35	-	2.13	0.74	0.05	-	1.25	2.00	1.12
340	0.77	0.23	-	-	2.16	0.84	-	-	1.86	2.00	1.06
360	0.77	0.23	-	-	2.18	0.91	-	-	1.89	1.96	1.10
380	0.82	0.18	-	-	2.29	1.14	-	-	2.23	1.81	1.15

pH 5.5											
Wavelength(nm)	α_1	α_2	α_3	α_4	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_4 (ns)	τ_{av} (ns)	SVR ^a	σ^b
310	0.06	0.41	0.32	0.22	2.16	1.00	0.48	0.03	0.74	2.00	1.08
340	0.16	0.58	0.26	-	2.16	0.99	0.55	-	1.06	2.00	1.06
360	0.14	0.53	0.33	-	2.37	1.14	0.57	-	1.12	2.00	1.07
380	0.19	0.53	0.28	-	2.40	1.11	0.63	-	1.22	2.03	1.03

Excitation wavelength is 295 nm.

a, b, Serial variance ratio and chi-square, respectively. (See text.)

greatly modified these average lifetime-wavelength curves by interacting with HEWL. at pH 5.5, the average lifetime were shortened at wavelengths longer than 335 nm and prolonged at the shorter side than that wavelength. On the other hand, they were enormously increased at pH 8.0, especially in the wavelength region 300 nm to 350 nm.

The fluorescence decay of Kyn62-lysozyme was more complicated than one of the native lysozyme and it was described with 4-exponential kinetics through the whole fluorescence spectral region. The lifetime of Kyn62-lysozyme was shorter than one of HEWL as expected from its lower fluorescence quantum yield. The fluorescence decay parameters of Kyn62-lysozyme and ligand binding effects on them are given in Table 4.1.III. The fluorescence decay property of Kyn62-lysozyme was also changed by the bound (NAG)₃. The tendency, found in the fluorescence of HEWL at pH 5.5, was also clear in Kyn62-lysozyme.

Quenching resolved fluorescence spectrum

The collisional quenching constant of acrylamide for the fluorescence of HEWL at pH 5.5 was constant ($k_q=1.4\times10^9\text{M}^{-1}\text{s}^{-1}$) at wavelengths from 420 nm to 350 nm and gradually increased to give $2.5\times10^9\text{M}^{-1}\text{s}^{-1}$. This wavelength dependence of k_q suggests that the fluorescence of HEWL is composed of the emissional contribution from two groups with different k_q . Because k_q was constant from 400 nm to 350 nm, indicating only the tryptophans with quite similar collisional frequency and quenching cross sections contributed to the fluorescence of HEWL, the fluorescence spectrum of these tryptophan residues was resolved assuming the gaussian type line shape and the curve fitting procedure. The obtained spectrum ranged from 400 nm to 300 nm and showed a maximum at 348 nm (Fig. 4.1.7 curve b). The subtraction of the curve b from the total fluorescence of HEWL (curve a) gave an another fluorescence band with a maximum at 328 nm (curve c). Although the latter band also kept the gaussian type line shape, indicating a homogenous emission source, it deviated to the shorter wavelength side and diminished perfectly at 350 nm. The intensity ratio of these two bands (c/b) was estimated to be 1:3.75. The quenching resolved spectrum for the HEWL-(NAG)₃ complex was obtained in a similar way. The maximum wavelength of the bands occupied longer wavelengths was same to one of the free HEWL. But the fluorescence band positioned at the shorter wavelength side were

Table 4.1.III Fluorescence Decay Parameters of Kyn62-lysozyme
and Its Complex with (GlcNAc)₃ at pH 5.5.

Wavelength(nm)	α_1	α_2	α_3	α_4	$\tau_1(\text{ns})$	$\tau_2(\text{ns})$	$\tau_3(\text{ns})$	$\tau_4(\text{ns})$	$\tau_{\text{av}}(\text{ns})$	SVR ^a	σ^b
310	0.03	0.08	0.48	0.42	2.10	0.66	0.16	0.04	0.21	1.95	1.05
	0.03	0.09	0.50	0.38	2.15	0.72	0.20	0.03	0.24	2.00	1.05
340	0.03	0.11	0.47	0.38	3.18	1.09	0.22	0.07	0.35	1.97	1.03
	0.03	0.12	0.27	0.57	2.92	1.09	0.33	0.16	0.40	2.00	1.14
380	0.03	0.15	0.36	0.47	4.54	1.45	0.31	0.09	0.51	1.82	1.12
	0.03	0.15	0.51	0.31	4.10	1.23	0.27	0.06	0.44	1.97	1.05

Excitation wavelength is 295 nm, Lower row at each wavelength is Kyn62-lysozyme-(GlcNAc)₃ complex. a, b, Serial variance ratio and chi-square, respectively. (See text.)

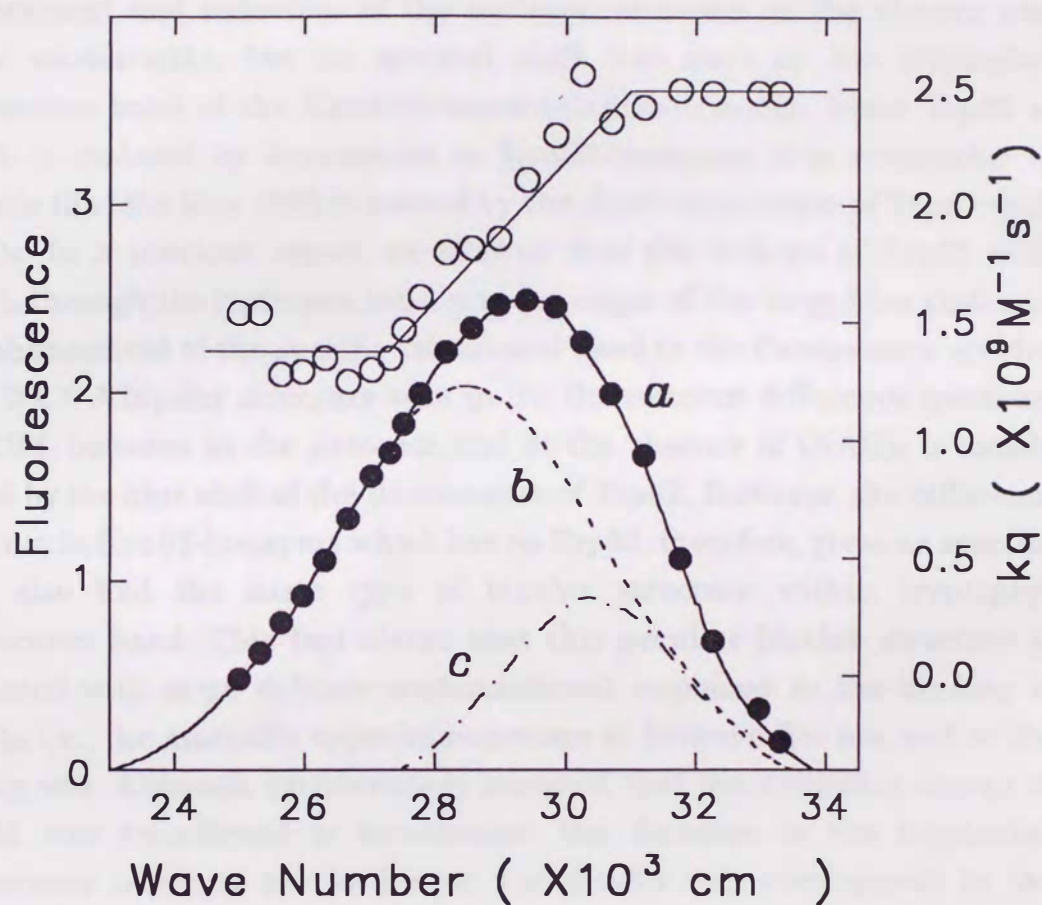


Fig. 4.1.7 Quenching resolved fluorescence spectrum of HEWL at pH 5.5. Fluorescence band of HEWL (—●—●—, Curve a) was resolved according with k_q (—○—○—). k_q was estimated with Stern-Volmer constant (K_{sv}) and the average lifetime (τ_{av}). Curve b was the fluorescence spectrum of Trp62 and Trp108 obtained by fitting a gaussian type spectrum to the fluorescence band of HEWL at 400 nm-360 nm, and Curve c was one of Trp28 and Trp111 obtained by subtraction of quenched Curve b from Curve a. See text.

greatly enhanced (Fig. 4.1.8-c). The line shape of this band did not give an adequate fit to the single gaussian band and tailed to the lower energy side.

4.1.4 Discussion

HEWL showed a blue shift in the fluorescence spectrum forming a complex with (NAG)₃, regardless of pH of the buffer solution. Clear were the enhancement and reduction of the emission intensity at the shorter and longer wavelengths, but no spectral shift was seen in the tryptophyl fluorescence band of the Kyn62-lysozyme-(NAG)₃ complex. Since Trp62 of HEWL is replaced by kynurenine in Kyn62-lysozyme, it is reasonable to conclude that the blue shift is caused by the direct interaction of Trp62 with (NAG)₃. In a previous report we showed that the linkage of Trp62 with (NAG)₃ through the hydrogen bond was the origin of the large blue shift and the enhancement of the specific vibrational band in the fluorescence spectra of HEWL.⁶⁾ A bipolar structure seen in the fluorescence difference spectrum of HEWL between in the presence and at the absence of (NAG)₃ is mostly caused by the blue shift of the fluorescence of Trp62. However, the difference spectrum in Kyn62-lysozyme which has no Trp62, therefore, gives no spectral shift, also had the same type of bipolar structure within tryptophyl fluorescence band. This fact shows that this peculiar bipolar structure is associated with more delicate conformational responses to the binding of (NAG)₃, i.e., the mutually opposite responses at hydrophobic box and at the binding site. Although we previously reported that the excitation energy of Trp108 was transferred to kynurenine, the decrease in the tryptophyl fluorescence intensity at the longer wavelength side corresponds to the conformational change around Trp108 to promote the energy transfer to kynurenine.^{7,8)} The enhancement of its fluorescence intensity at the shorter wavelengths suggests that some conformational alteration is induced also at the hydrophobic matrix box region where Trp28 and Trp111 are arranged. The hydrophobic matrix box of HEWL is known to retain more fluctuational freedom than that expected from its compact structure given in X-ray structure.⁹⁾ The binding of (NAG)₃ would restrict this fluctuational motion to stabilize the fluorescence state of Trp28 and/or Trp111. Such specific ligand-binding effects on the hydrophobic matrix box are also probable in HEWL, although it is kept out of sight because of the large emissional overlap by the large spectral shift of Trp62.

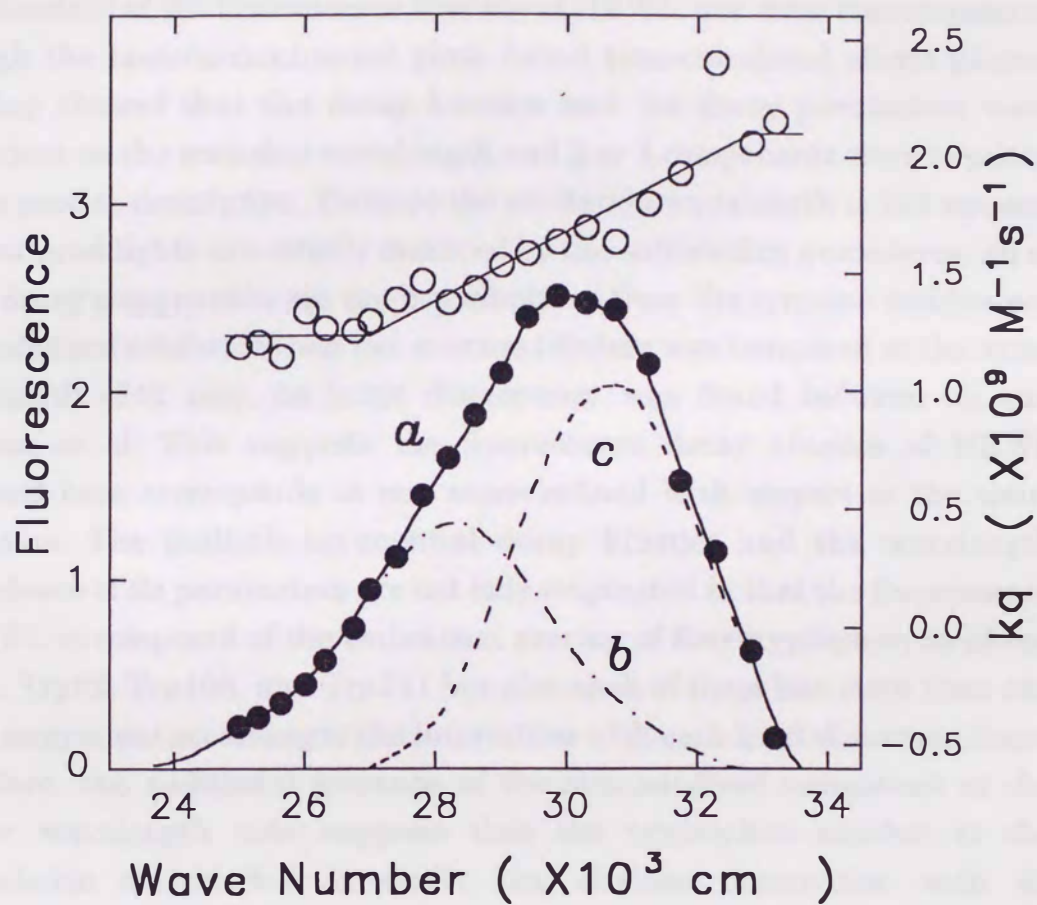


Fig. 4.1.8 Quenching resolved fluorescence spectrum of HEWL-(NAG)₃ complex at pH 5.5.

Fluorescence band of HEWL-(NAG)₃ complex (—●—●—, Curve a) was resolved according with k_q (—○—○—). Curve b, the fluorescence spectrum of Trp108; Curve c was obtained by the subtraction of Curve b from Curve a. See the legend of Fig. 4.1.7.

When the protein structure is discussed on the basis of the fluorescence spectroscopy, the knowledge on the exact fluorescence lifetime is essential. Therefore, we confirmed the fluorescence decay kinetics of HEWL. Formoso and Forster reported that it decayed with a double exponential kinetics with $\tau_1=2.26$ ns and $\tau_2=0.4$ ns and corresponding amplitude, 0.44 and 0.56 at the fluorescence maximum.¹⁰⁾ Although their result has been adopted as a standard of the fluorescence lifetime of HEWL, our close measurements through the laser/microchannel plate based time-correlated single photon counting showed that the decay kinetics and the decay parameters were dependent on the emission wavelength and 3 or 4 components were required for the precise description. Because the excitation wavelength is 295 nm and the scattered lights are strictly removed by the subtraction procedures, all of these decay components are not contributions from the tyrosine residue nor the undesired artifact. When the average lifetime was compared at the same wavelength (342 nm), no large discrepancy was found between us and Formoso et al. This suggests the fluorescence decay kinetics of HEWL proposed here corresponds to one more refined with respect to the time-resolution. The multiple-exponential decay kinetics and the wavelength dependence of its parameters are not only originated in that the fluorescence of HEWL is composed of the emissional overlap of four tryptophan residues, Trp28, Trp62, Trp108, and Trp111 but also each of them has more than one decay component according to the interaction with each kind of surroundings. Therefore, the additional presence of the shortest-lived component at the shorter wavelength side suggests that the tryptophan residue at the hydrophobic matrix box is under the stronger interaction with its surroundings, probably due to conformational fluctuation. The reduction of the decay component at pH 8.0 might be rationalized by the restriction of these fluctuational motions under the interaction with (NAG)₃.

Unfortunately, it is now impossible to assign a given decay component to a tryptophan residue. But the average lifetime based on the precise fluorescence decay parameters at various wavelengths allow us to derive the "quenching resolved fluorescence spectrum". In this spectrum, the fluorescence spectrum of the tryptophan residue with a given quenching rate is extracted from the total fluorescence of HEWL. Frequently, the Stern-Volmer constant, K_{sv} , have been used as a criterion for the conformational change.¹¹⁾ But its value depends on the lifetime of the fluorophore shown as

$K_{SV} = \tau \cdot k_q$. Therefore, the collisional quenching rate, k_q , is rather intrinsic for differentiating the tryptophan residue in HEWL. The fluorescence spectrum of HEWL was resolved into the emission band with maximum at 348 nm and one with a maximum at 328 nm. Based on the photophysical properties of the tryptophan residue, the former band is the emission from the residues in the hydrophilic region, Trp62 and Trp108, and the latter corresponds to one from Trp28 and Trp111. As far as we know, these are the first fluorescence spectra of tryptophan residues located at the binding site and hydrophobic region in native HEWL. The band intensity ratio of these emission, estimated to be 1:3.75, was quite consistent with the value reported by Imoto et al.³⁾ Considering the ligand-induced conformational change site to site through the fluorescence spectroscopy of the tryptophan residue, it is important to note that Trp28 and Trp111 give no contribution at longer wavelengths than 350 nm. The fluorescence emission at longer wavelengths is due to Trp62 and Trp108. The quenching-resolved fluorescence spectrum of HEWL-(NAG)₃ complex demonstrates that the emission of Trp62 is largely deflected to the shorter wavelength side and it overlaps with the emissions of Trp28 and Trp111. Although the mixed spectrum of Trp28, Trp111, and Trp62 could not be resolved, the maximum of the interacting Trp62 with the ligand is anticipated to be at 340 nm judged from the deviation from the gaussian line shape. The polar circumstances of Trp108 are also kept in the HEWL-(NAG)₃ because the spectral distribution of Trp108 was free from effects of the ligand binding.

The average lifetime at the specified wavelength characterizes the fluorescence state of a tryptophan residue that is most dominant at the corresponding wavelength. On the formation of the lysozyme-ligand complex, Trp108 did not change the fluorescence spectral distribution as shown in the quenching resolved spectra. Because Trp62 shifted its maximum to the blue side and its low energy edge is 370 nm at most, it is reasonable to consider that the exclusive emission at the longer wavelength than 370 nm is one of Trp108 in the HEWL-ligand complex. The decrease of average lifetime at 380 nm suggest that the fluorescence state of Trp108 is greatly destabilized at pH 5.5. As expected from the fluorescence difference spectrum, the average lifetime at the emission wavelength corresponding to Trp62 was also reduced. These results demonstrate that the conformational alteration is induced near the active site by the binding of (NAG)₃ to quench the fluorescence of Trp62

and Trp108 at pH 5.5. Lehrer reported analyzing a fluorescence titration curve in which an unionized carboxyl group acted as a quencher for the fluorescence of the tryptophan residues around the binding site.¹²⁾ According to X-ray analysis, Glu35 is arranged near Trp108 for the source of carboxyl group.¹⁾ Our results clearly demonstrate that it approaches closer to Trp108 and Trp62 by the binding of (NAG)₃. On the other hand, at pH 8.0, the average lifetime of Trp108 and Trp62 were rather prolonged by the ligand binding. The fluorescence quenching efficiency of ionized carboxyl group is not as high as the unionized one,¹³⁾ but an ionized carboxyl group is also strong quencher for the fluorescence of tryptophan residue. If Glu35 came closer to these tryptophan residues regardless of pH, their fluorescence lifetimes should be shorted also at pH 8.0. The prolonged lifetime suggests these residues are rather kept apart from Glu35.

At the wavelength from 300 nm to 350 nm, the fluorescence spectra of the tryptophan residues both in hydrophobic and active site overlapped each other. Therefore, it is difficult to know only the conformational change at the hydrophobic matrix box. But our spectral and time-resolved analysis consistently suggest that the effects of the ligand binding extend to the hydrophobic matrix box region and they restrict the fluctuational motions around Trp28 and Trp111 to quench their fluorescence. Because the fluorescence difference spectrum of Kyn62-lysozyme had a bipolar structure at pH 5.5 where the fluorescence of Trp108 was largely quenched. Furthermore, the average lifetime of HEWL at wavelengths from 300 nm to 340 nm corresponding to the emission band of Trp28 and Trp111 were prolonged by the binding of the ligand, in spite of the remarkable destabilization of Trp62 and Trp108 at pH 5.5. Unfortunately, our results here cannot show whether the degree of the restricting effects depend on the pH.

Our time-resolved and steady state fluorescence studies of HEWL and its active derivative showed that interacting (NAG)₃ induced a conformational alteration around the binding site of HEWL and its effect extends to also the hydrophobic matrix region. Such a wide-ranging protein-ligand interaction is interesting to consider in the biological meanings of conformational flexibility in proteins.

4.1.5 Conclusion

The conformational change of hen egg-white lysozyme (EC 3.2.1.17) induced by the interaction with tri-*N*-acetyl-D-glucosamine were investigated by steady state and time-resolved fluorescence spectroscopy. To identify more clearly the conformation of hen egg-white lysozyme interacting with the ligand, the fluorescence decay kinetics of the lysozyme and its complex with the ligand were precisely measured at their full spectral regions. The spectral analysis based on the time-resolved studies showed that the binding of the ligand affected not only the Trp62 directly linked to the ligand but its influence was extended to the vicinity of Trp108 and further to the hydrophobic matrix region. Near the binding site, the intramolecular distance between Trp108 and Glu35 was expanded or contracted depending on the pH of the buffer solution. On the other hand, the interaction of Trp28 and/or Trp111 with their surroundings was reduced by restriction of fluctuational motions at the hydrophobic matrix box region.

4.1.6 References

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4.2 Steady-State and Time-resolved Fluorescence Studies on the Ligand Induced Conformational Change in an Active Lysozyme Derivative; Kyn62-Lysozyme.

4.2.1 Introduction

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.¹⁾ The catalytic reactions of enzymes are initiated through initial complex formation with its appropriate substrate. Occasionally their function is regulated through the interaction with a specific activator or supressor. 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) is a model enzyme whose biochemical properties are well understtod and its crystallographic structures has been established. According to the X-ray analysis, HEWL is considered to have a relatively rigid structure.²⁾ 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.³⁾ This contradiction demenstrates 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 high sensitivity and ability to uncover subtle change in protein structure. Such studies have provided information on the protein conformational changes occurring in bioactive proteins in their complexies with ligands⁴⁾; the conformational heterogeneity demendtrated in proteins and peptides⁵⁾; and motional freedom found in some proteins.⁶⁾ Fluorescence studies of proteins usually take advantage of the spctroscopy of tryptophan residue. This fluorescent amino acid is found in limited quantities in proteins and its photophysical properties make it a useful probe for monitering 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 fluorophores⁷⁾ 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 single tryptophan residue, the local conformation and interactions with the surroundings can be localized as being in the segment containing the tryptophan residue.^{8,9)} 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-hydroxy-tryptophan, which has an extended red shifted absorption spectrum, into a oncomodulin mutant using site directed mutagenesis.¹⁰⁾ 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 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 afterwards.¹¹⁾ 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.¹²⁾ 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 this section, 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.

4.2.2 Materials and Methods

Materials.

Native and Kyn62-lysozyme were prepared as described in Chapter 2.
Evaluation of Energy Transfer Rate and Distance between Energy

Donor/Acceptor Pair.

The distance between the energy donor and acceptor (r) according to the following equation.

$$(R_0/r)^6/\tau_d^0 = k_e \quad (4.2.1)$$

R_0 is the critical distance which gives 50% efficiency of energy transfer and is expressed by eq. 4.2.2,

$$R_0^6 = 8.785 \times 10^{-25} \cdot \kappa^2 \cdot \Phi_0 \cdot n^{-4} \cdot J \quad (4.2.2)$$

$$J = \int \epsilon_A(\lambda) F_0(\lambda) \lambda^4 d\lambda / \int F_0(\lambda) d\lambda \quad (4.2.3)$$

where J is spectral overlap integral, n is the refractive index of the medium, κ^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 κ^2 for the estimation of R_0 . Because this approximate value, $\kappa^2 = 2/3$, is adequate only when donor/acceptor pairs have isotropic rotations on the time scale of the fluorescence lifetime, the upper ($\langle \kappa^2 \rangle^{\max}$) and lower ($\langle \kappa^2 \rangle^{\min}$) limit were calculated for the orientation factor based on Dale.¹³⁾ 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. $\langle \kappa^2 \rangle^{\max}$ and $\langle \kappa^2 \rangle^{\min}$ are then given by,

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

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

4.2.3 Results

The absorption spectrum (Fig. 4.2.1) 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 296 and 280 nm, similar to that of native HEWL.

Figure 4.2.2 shows the fluorescence spectra of the native and Kyn62-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

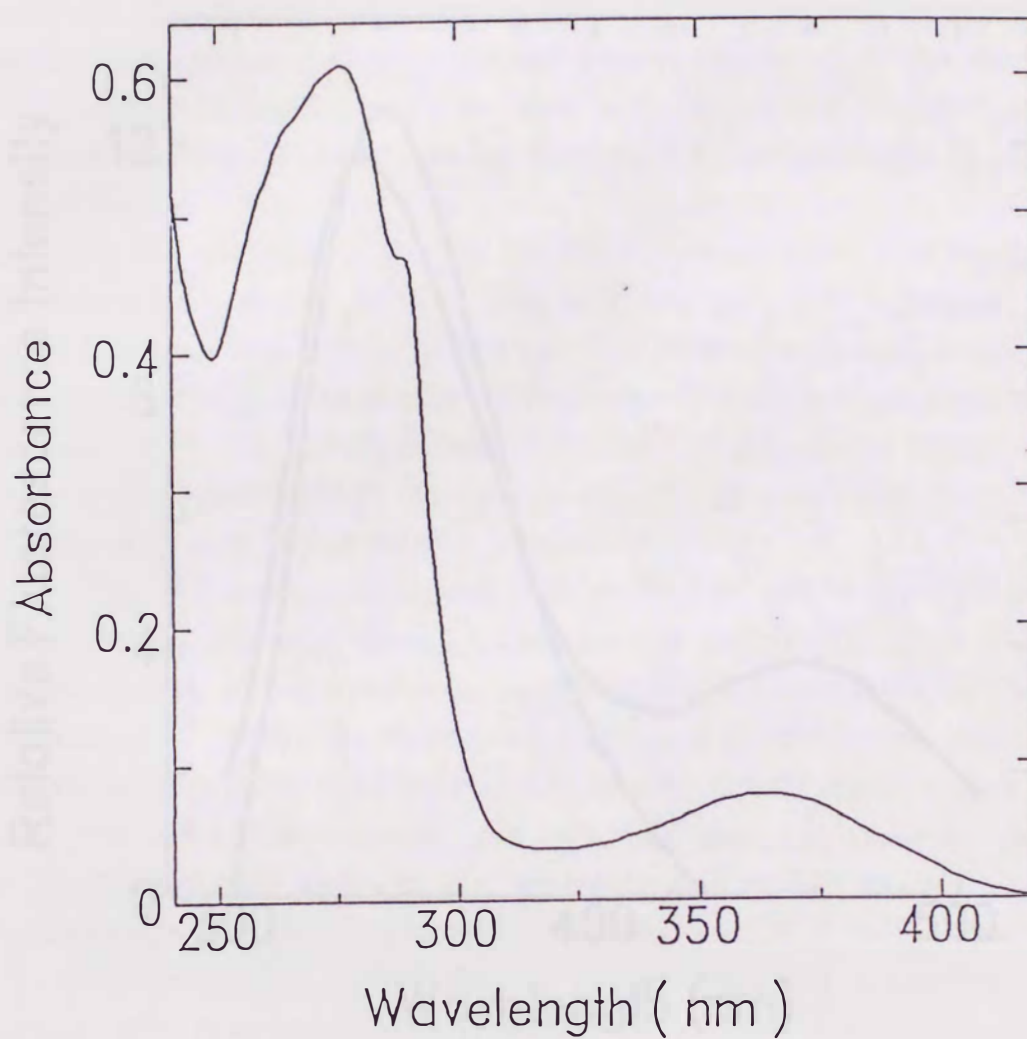


Fig. 4.2.1 Absorption spectrum of Kyn62-lysozyme.

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

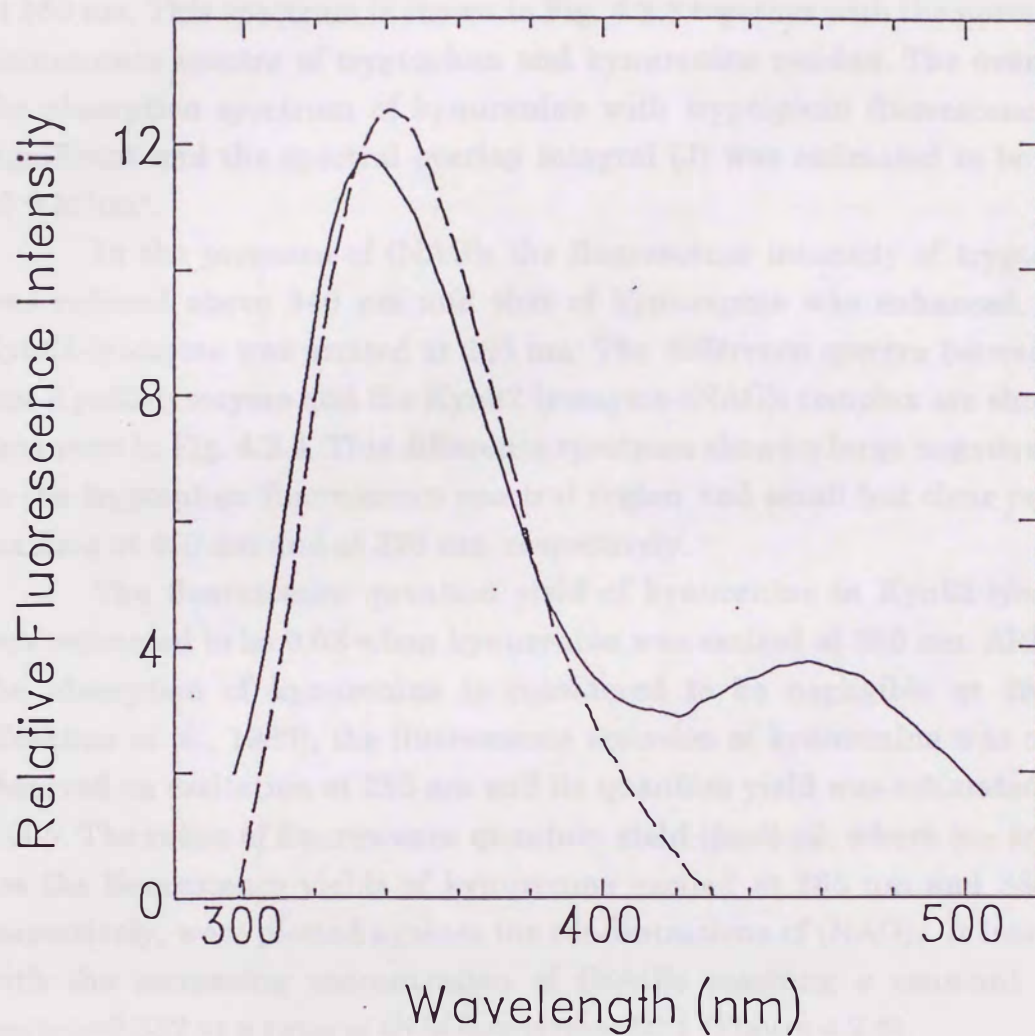


Fig. 4.2.2 Fluorescence spectra of native lysozyme (-----) and Kyn62-lysozyme (—). The concentration of native lysozyme and Kyn62-lysozyme were 10^{-6} M. Excitation wavelength, 285 nm.

residue in Kyn62-lysozyme was obtained by extrapolating the absorption line from 420 nm to 330 nm to the shorter wavelength side. It showed a maximum at 350 nm. This spectrum is shown in Fig. 4.2.3 together with the normalized fluorescence spectra of tryptophan and kynurenine residue. The overlap of the absorption spectrum of kynurenine with tryptophan fluorescence was significant and the spectral overlap integral (J) was estimated to be $4.92 \times 10^{-15} \text{ M}^{-1} \text{ cm}^3$.

In the presence of $(\text{NAG})_3$ 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- $(\text{NAG})_3$ complex are shown in the insert in Fig. 4.2.4. 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 fluorescence quantum yield (ϕ_{285}/ϕ_{360}), where ϕ_{285} and ϕ_{360} are the fluorescence yields of kynurenine excited at 285 nm and 360 nm, respectively, were plotted against the concentrations of $(\text{NAG})_3$. It increased with the increasing concentration of $(\text{NAG})_3$ reaching a constant value $\phi_{285}/\phi_{360}=0.352$ at a ratio of $(\text{NAG})_3/\text{enzyme}=80:1$ (Figure 4.2.5).

The fluorescence decay parameters of Kyn62-lysozyme and its complex with $(\text{NAG})_3$ measured at the emission maxima of kynurenine are summarized in Table 4.2.I. 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 pre-exponential factor. This is evidence for the energy transfer from tryptophan to kynurenine in the fluorescence of the Kyn62-lysozyme- $(\text{NAG})_3$ 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

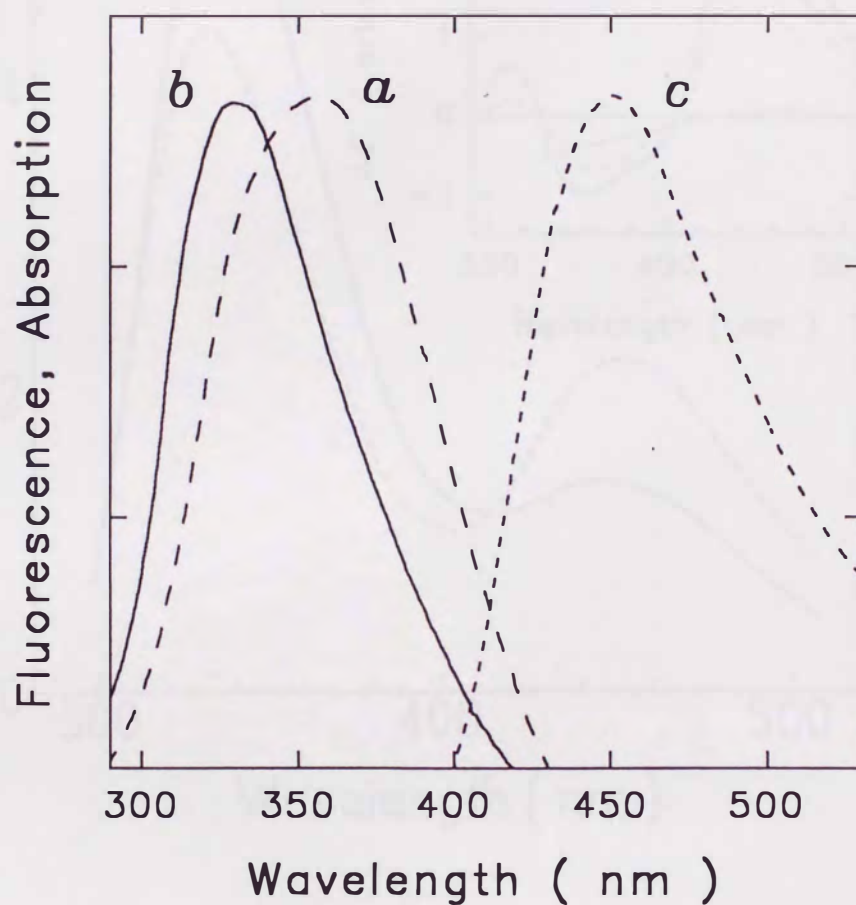


Fig.4.2.3 The spectral overlap of the kynurenyl absorption band (curve a) with the tryptophyl fluorescence band (curve b). Curve c, fluorescence band of kynurenine residue in Kyn62-lysozyme. Curve a was obtained extrapolating the lowest absorption band of Kyn62-lysozyme to the shorter wavelength side.

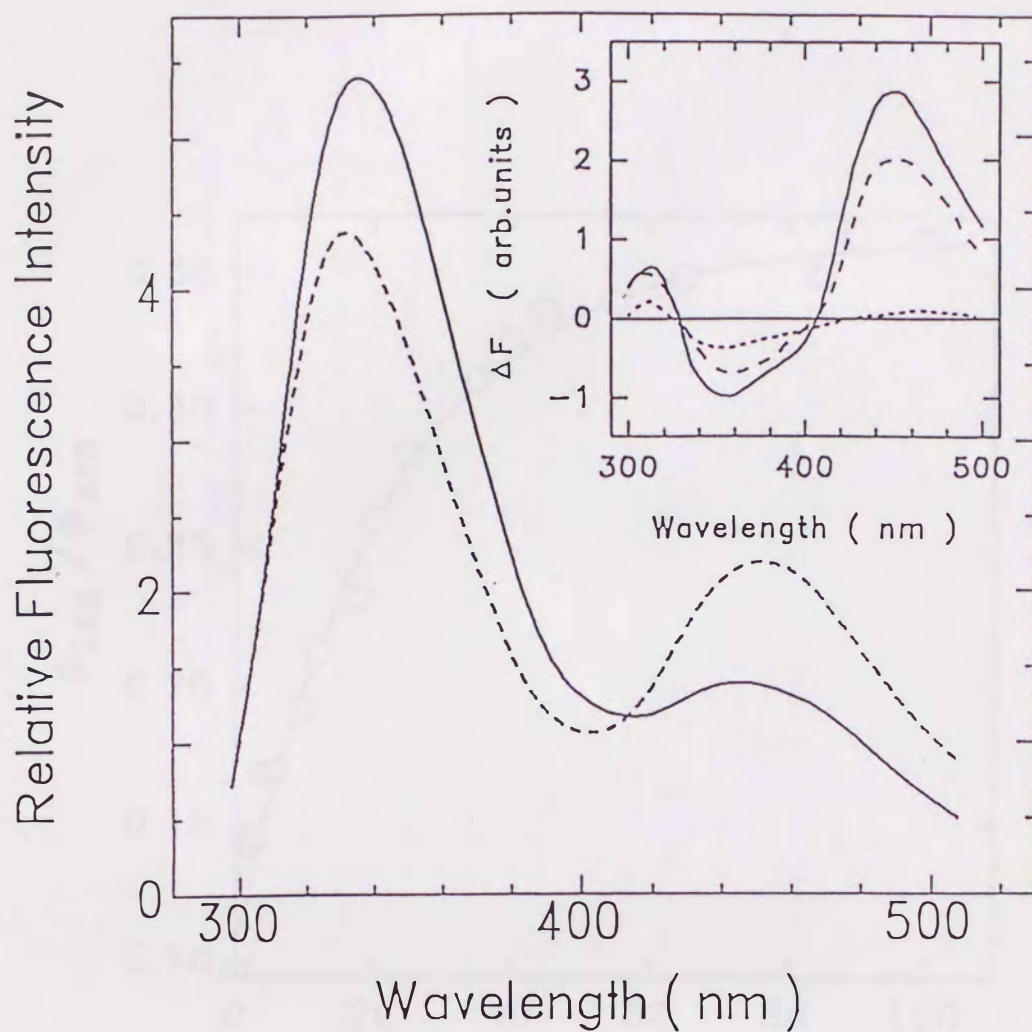


Fig. 4.2.4 The effect of $(\text{NAG})_3$ on the fluorescence spectra of Kyn62-lysozyme. Concentration of Kyn62-lysozyme was 2.5×10^{-6} M. (—) Kyn62-lysozyme, (---) in the presence of 250×10^{-6} M of $(\text{NAG})_3$. Inset: the fluorescence difference spectra between at the absence and in the presence of $(\text{NAG})_3$. The reference was Kyn62-lysozyme.
 (—) Kyn62-lysozyme + 250×10^{-6} M of $(\text{NAG})_3$
 (---) Kyn62-lysozyme + 50×10^{-6} M of $(\text{NAG})_3$
 (- - - - -) Kyn62-lysozyme + 5×10^{-6} M of $(\text{NAG})_3$

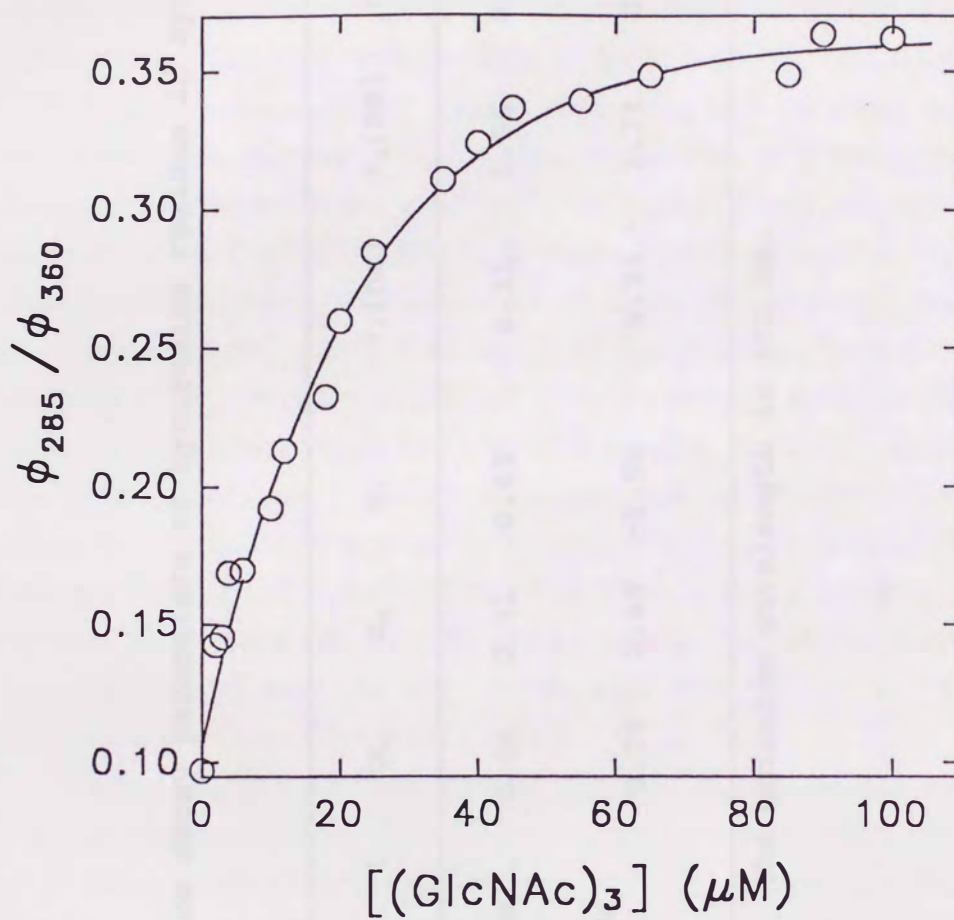


Fig. 4.2.5 The effect of (NAG)₃ on the ratio of the fluorescence quantum yield of kynurenine in Kyn62-lysozyme excited at 285 nm (ϕ_{285}) to one excited at 360 nm (ϕ_{360}). Emission was measured at 450 nm. The concentration of Kyn62-lysozyme was 10^{-6} M.

Table 4.2.I. Fluorescence decay parameters of kynurenine residue in Kyn62-lysozyme.

	α_1	α_2	α_3	$\tau_1(\text{ns})$	$\tau_2(\text{ns})$	$\tau_3(\text{ns})$
Kyn62-lysozyme (Ex=320nm)	0.06	0.31	0.63	6.31	1.95	0.36
Kyn62-lysozyme + (GlcNAc) ₃ (Ex=285nm)	0.36	2.49	-1.58	4.24	1.71	0.15

The emission wavelength is 450 nm.

used. As the temperature increased the decay time corresponding to the negative amplitude decreased.

The fluorescence decay of the tryptophan residues ($\lambda_{em}=340$ nm) of Kyn62-lysozyme were more complicated and best described by four exponential decay components (Table 4.2.II). This is obviously the consequence of combination of the fluorescence of each of the other tryptophan residues remaining in the protein. These decay times represent 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.¹⁴⁾ The DAS of the tryptophan residues are shown in Fig. 4.2.6. 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 (NAG)₃ decreased the relative emission of the τ_1 , τ_2 and τ_3 components at wavelength >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 pre-exponential 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 $\tau_{av}=\sum\alpha_i\tau_i$, was plotted against the emission wavelength, it increased linearly with the wavelength from 310 to 360 nm to reach a constant value, $\tau_{av}=0.50$ ns (Fig. 4.2.7). This τ_{av} -wavelength curve was different in the (NAG)₃ complex (Fig. 4.2.7, 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 wavelength >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 (NAG)₃. Time-resolved anisotropy measurements showed that the dynamics of the enzyme and enzyme-ligand complex reflected both the overall rotational motion of the enzyme and of the local segmental motion of the kynurenine residue (Table 4.2.III). The 4.20 and 4.50 ns correlation times may be assigned to the overall rotational

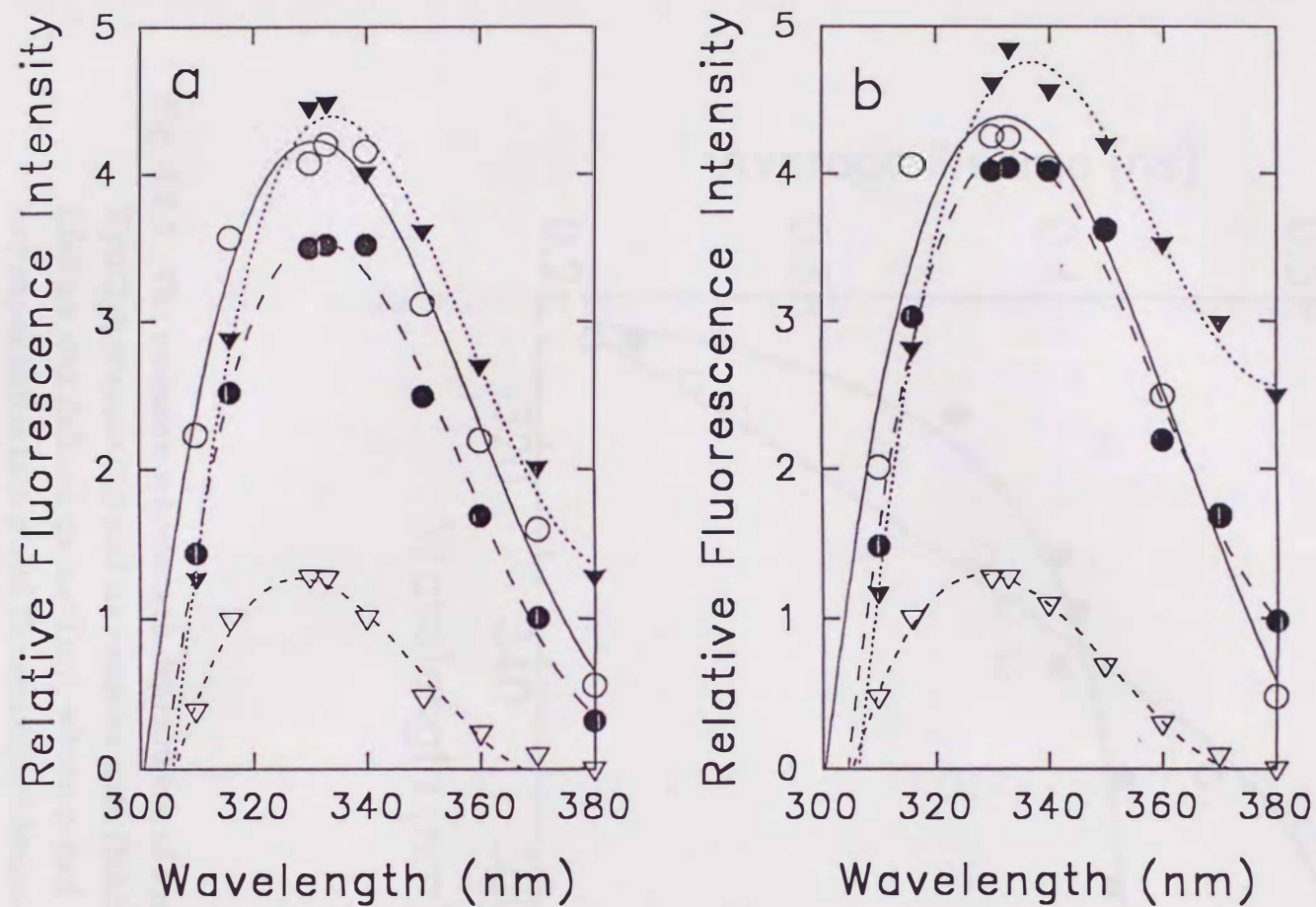


Fig. 4.2.6 The fluorescence decay associated spectra of Kyn62-lysozyme (b) and its complex with (NAG)₃ (a).

●, 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).

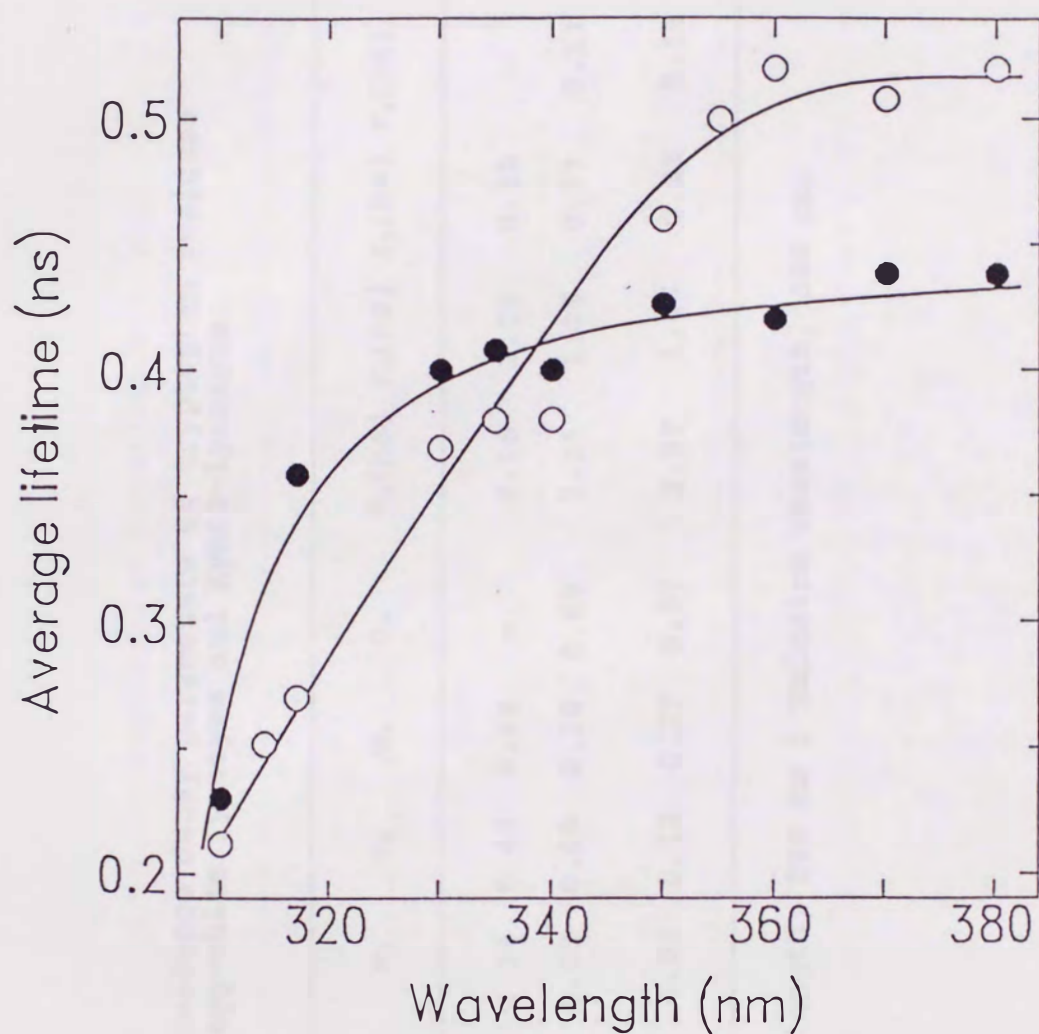


Fig. 4.2.7 The emission wavelength dependence of average lifetime of Kyn62-lysozyme (○) and its complex with (NAG)₃ (●). The average lifetime was defined as, $\tau_{av} = \sum \alpha_i \tau_i$, where α_i and τ_i are normalized pre-exponential factor and fluorescence lifetime of *i*-th component, respectively.

Table 4.2.II Fluorescence decay parameters of tryptophan residues
in hen egg-white lysozyme and Kyn62-lysozyme.

	SVR	σ	α_1	α_2	α_3	α_4	$\tau_1(\text{ns})$	$\tau_2(\text{ns})$	$\tau_3(\text{ns})$	$\tau_4(\text{ns})$	$\tau_{\text{av}}(\text{ns})$
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

Excitation wavelength, 295 nm ; Emission wavelength, 340 nm.

Table 4.2.III The dynamical parameters of segmental motions of kynurenine and tryptophan residue in Kyn62-lysozyme.

	β_1	β_2	β_3	$\phi_1(\text{ps})$	$\phi_2(\text{ns})$	$\phi_3(\text{ns})$	r_0
Kynurenine (free)*	0.16	0.13	0.03	150	1.40	4.20	0.32
Kynurenine (complex)*	0.13	-	0.18	170	-	4.50	0.31
Tryptophan (free)#	0.23	-	0.06	<20	-	4.44	0.29
Tryptophan (complex)#	0.24	-	0.08	<20	-	4.56	0.32

*, Excitation wavelength, 320 nm; Emission wavelength, 450 nm.

#, Excitation wavelength, 295 nm; Emission wavelength, 340 nm.

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 ($\lambda_{\text{ex}}=295$ nm) gave a value of 0.1, in the presence or absence of the 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.

4.2.4 Discussion

The fluorescence emission band of tryptophan has a high degree of overlaps 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 tryptophan residue. The fluorescence intensities of tryptophan and kynurenine residues complementary decreased and increased on the binding of (NAG)₃. 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 Kyn62-lysozyme-(NAG)₃ complex even when kynurenine was directly excited at 360 nm.¹⁵⁾ Furthermore, the tryptophyl fluorescence of HEWL which contained no energy acceptor was quenched by the presence of (NAG)₃ at pH 5.0.³⁾ 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 the formation of Kyn62-lysozyme-(NAG)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. (4.2.4, c).

$$\phi_{360} = k_r^K / (k_r^K + k_i^K) \quad (4.2.4, a)$$

$$\phi_{285} = k_e / (k_f^T + \sum k_i^T + k_e) \cdot k_f^K / (k_f^K + \sum k_i^K) \quad (4.2.4, b)$$

$$\phi_{285} / \phi_{360} = k_e \cdot \tau_d \quad (4.2.4, c)$$

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

According to Förster formalism,¹⁶⁾ the energy donor-acceptor distance (r) and other parameters related to the energy transfer in Kyn62-lysozyme are evaluated by using eq. (4.2.1)~(4.2.3) and (4.2.4, c). The parameters decided experimentally are summarized in Table 4.2.IV. 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 the 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 the negative pre-exponential factor in the decay kinetics of kynurenine. According to the well known energy donor corresponds to the decay time of the component with negative pre-exponential factor in the fluorescence decay kinetics of the energy acceptor.¹⁷⁾ Theoretically, the latter must be valid for the lifetime of 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 ($\kappa^2=2/3$) 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 state enzyme were estimated to be 23.8 Å 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 Å-21.2 Å or 22.9 Å-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 (NAG)₃. However, it should be noted that the lower limit of the donor-acceptor distance in the free Kyn62-lysozyme is shorter

Table 4.2.IV

The 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_o^{RDA} (Å)	16.5	16.5
R_o^{max} (Å)	19.8	20.7
R_o^{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 pre-exponential factor.

than the upper limit in the complex with (NAG)₃. This fact demonstrates a possibility that the donor-acceptor pair in Kyn62-lysozyme might exclusively change the mutual orientation on the interaction with (NAG)₃ to increase κ^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 state are almost completely quenched by the Dexter-type electron exchange interactions with the adjacent disulfide linkages (Cys76-Cys96, Cys6-Cys127).¹⁸⁾ The fluorescence properties of Kyn62-lysozyme and the complex with (NAG)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 (NAG)_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 the (NAG)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 atmosphere¹⁹⁾ 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 indole ring of tryptophans, were 19.32, 5.73, 13.09, 18.63 and 27.13 Å, respectively.²⁰⁾ 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.

Unfortunately, our results can not 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 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 the cleavage of the indole ring of Trp62 and, therefore, it may be connected to the peptide chain with the longer arm.

The active site of HEWL is composed of six subsites (subsite-A, -B, -C, -D, -F). Trp62 is a constituent residue of the subsite-C and it binds directly with (NAG)_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 62-kynurenine is also linked to (NAG)₃. Based on 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 of 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 (NAG)₃ or the binding of itself with other amino acid residue such as Leu56.²¹⁾ 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/mole) in the Arrhenius plot for the energy transfer rate (Fig. 4.2.8), 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 studies with NMR spectroscopy. But a loosely packed conformation was drawn for Kyn62-

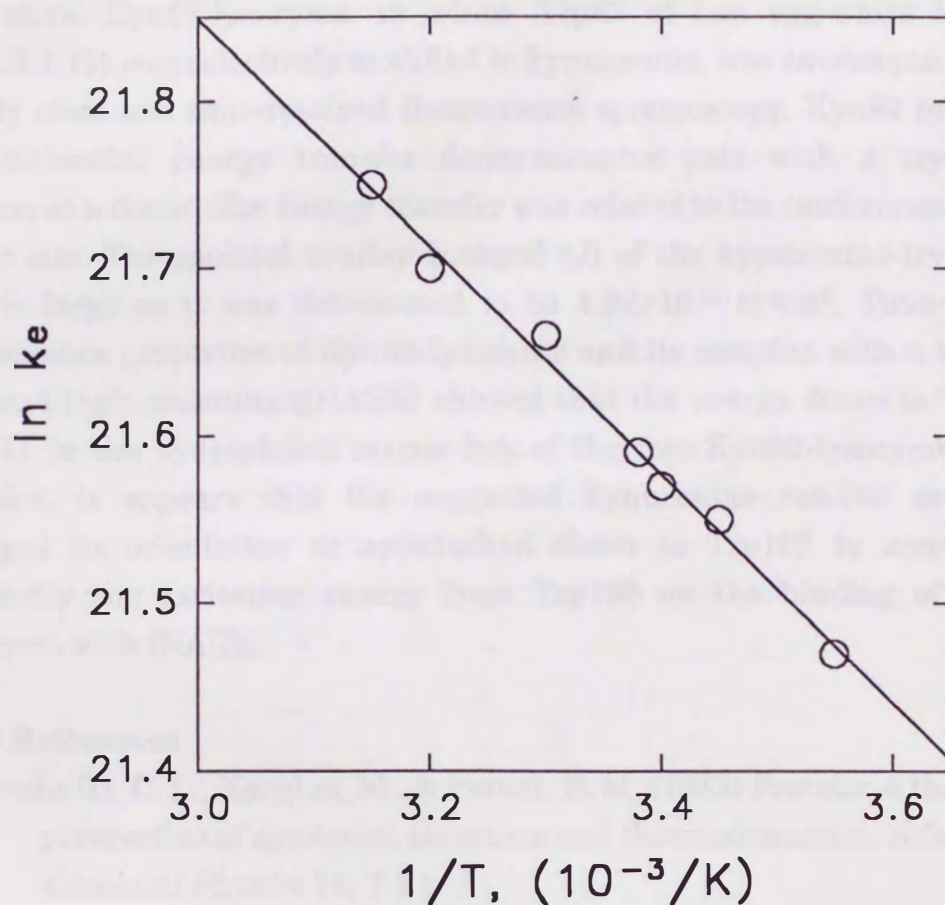


Fig. 4.2.8 Arrhenius plots of the energy transfer rate (k_e) in Kyn62-lysozyme-(NAG)₃ complex. k_e was evaluated with the fluorescence lifetime of energy donor and ϕ_{235}/ϕ_{360} at various temperatures using eq. (4,c) (see text).

lysozyme and the complex with oligosaccharide. Such a conformation is rather interesting because the potential flexibility of protein conformation must be more vividly exhibited in physiological conditions.

4.2.5 Conclusion

The ligand induced conformational change of an active lysozyme derivative, Kyn62-lysozyme, in which Trp62 of hen egg-white lysozyme (EC3.2.1.17) was selectively modified to kynurenine, was investigated by the 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 $4.92 \times 10^{-15} \text{ M}^{-1} \text{ cm}^3$. Time-resolved fluorescence properties of Kyn62-lysozyme and its complex with a trimer of *N*-acetyl-D-glucosamine ((NAG)₃) showed 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 suggested 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 (NAG)₃.

4.2.6 References

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