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Studies of Protein Conformation by Fluorescence Measurements

(I) Evaluation of Internal Motion of The Polypeptide Chain of Taka-amylase A, Especially in Partially Folded States Abbreviations

DNS : 1-dimethylaminonaphthalene-5-sulfonyl group DNS Taka-amylase A : Conjugates of Taka-amylase A with 1-dimethylaminonaphthalene-5-sulfonyl chloride RCM DNS Taka-amylase A : reduced and carboxymethylated DNS Taka-amylase A DTNB : 5,5'-dithiobis-(2-nitrobenzoic acid) nsec : nanosecond or 10⁻⁹sec

SUMMARY

Accumulated results indicate that the native structure of a protein molecule can be reconstructed from its randomly coiled state under an appropriate condition. The conformations of both native and randomly coiled states of a protein molecule have been extensively studied, while those of possible intermediate states between native and randomly coiled states have not. In our laboratory, the renaturation, namely the refolding of a protein polypeptide chain from an unfolded state, has been extensively studied, especially on Taka-amylase A(an α amylase produced by Aspergillus oryzae). The conformation of the polypeptide chain of Taka-amylase A before reformation of disulfide bonds has been studied with special interest, because the state should simulate the state of a polypeptide newly synthesized in a living cell ("nascent protein"). No information about the degree of internal motion allowed to the polypeptide of Taka-amylase A could be obtained in previous hydrodynamic and spectrophotometric studies. In the present study, the degree of internal motion was , therefore, studied by fluorescence depolarization method using covalently bound 1-dimethylaminonaphthalene-5-sulfonyl group as a fluorescent probe. The fluorescence depolarization of solutions of DNS

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Taka-amylase A and its derivatives was measured as a function of temperature between 5°C and 50°C. Apparent rotational relaxation times were calculated using Weber's equation. Quoted and mostly unreliable values had been used for the values of fluorescence lifetime in the equation. To discuss with confidence, we measured the fluorescence decay courses ofthome of our samples using a nanosecond light source, and directly estimated their fluorescence lifetimes. Each of the observed decay courses could be assumed to represent a single exponential term. The obtained results suggested that the fluorescence lifetimes of the derivatives could be calculated from the lifetime of a standard sample and the relative fluorescence yield. The rotational relaxation time of DNS Taka-amylase A was 88 nsec which was twice of that calculated assuming a rigid anhydrous sphere, and was natural for an actual globular protein with molecular weight of about 52,000. Rotational relaxation times of reduced DNS Taka-amylase A wayebetween 195 nsec and 154 nsec, which were ingood agreement with the value expected for a swollen partially unfolded molecule with an effective volume estimated from the previous hydrodynamic study. It is to be noted that this result indicates that the internal motion of the polypeptide chain is almost completely restricted at least during

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the fluorescence lifetime. RCM DNS Taka-amylase A had a relaxation time of 123 nsec which suggested that the internal motion of the polypeptide chain was less restricted than that of reduced DNS Taka-amylase A, probably due to the presence of carboxymethyl groups. With RCM DNS Taka-amylase A, the effect of degree of labeling was studied. The rotational relaxation time decreased from 123 nsec for DNS content of 1.1 to 61 nsec for that of 2.5. The result can be interpreted, if the polypeptide chain of RCM DNS Taka-amylase A has more unfolded structure than the reduced one. The polypeptide chain of RCM DNS Taka-amylase A in 8M urea, on the other hand, was shown to have smaller kinetic unit of internal rotation than those of reduced or RCM DNS derivatives of Taka-amylase A in the buffer solution. Namely, the fluorescence depolarization plot ($1/P\ vs.\ T/\eta$) showed a nonlinear curve that was concave to the ordinate from the low temperature, and had an initial slope suggesting a rotational relaxation time as low as 11 nsec.

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INTRODUCTION

From many chemical and physical studies, most proteins have been shown to have unique amino acid sequences and to be folded into a definite three dimensional structures. It was also made clear that the three dimensional structures of proteins were stabilized by various intramolecular interactions (disulfide bonds, hydrogen bonds, salt linkages, hydrogen bonds and hydrophobic interactions etc.). The three dimensional structures are complex as exemplified by structures of myoglobin, lysozyme, ribonuclease and chymotrypsin analized by X-ray diffraction studies. Then the following question arises : How does a newly-synthesized protein polypeptide fold into such a complex It was now generally assumed three dimensional structure ? that the particular three dimensional configuration of the protein is the one that is thermodynamically the most stable and was simply a function of the order of the amino acids as stated by To make sure of this hypothesis, denaturation and re-Crick. naturation processes of many proteins have been extensively White and Isemura ration = 1 et al. proved that the native studied. conformation of ribonuclease and Taka-mylase A can be fully recovered after conversion of these proteins to a randomly coiled state with all of its disulfide bonds cleaved. After these

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works, similar studies were carried out on lysozyme and B.subtilis a-amylase by Imai et al. and Imanishi et al. And complete folding was observed for each of these proteins. These findings have led to the conclusion that the native conformation of most proteins, including the location of disulfide bridges, is uniquely determined by the sequence of amino acids in the protein polypeptide chains. Then the sequence of events during the refolding process attracted interests. Takagi and Isemura found from hydrodynamic and spectrophotometric studies of Taka-amylase A and its derivatives that reduced Taka-amylase A was partially refolded but further refolding was required the reformation of disulfide bonds through reoxidation. On this basis, the following two schemes of the refolding of denatured protein were assumed : (1) The effective volume of the denatured protein decreased markedly before the formation of disulfide bonds. This might be perhaps the reflection of hydrophobic interactions. (2) The slow process such as the formation of proper disulfide bonds In the present study, we wanted to estimate the followed. flexibility of the polypeptide chain of the protein at the primary step of the second process more quantitatively. In this experiment we used Taka-amylase A because of the following reasons : (1) This enzyme is well known to be dispersed in mono-molecular

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state in solution at neutral pH in high concentration. (2)The denatured Taka-amylase A molecule can be folded into the "native" structure in proper conditions. In this study, we determined the rotational relaxation times of the polypeptide chains of DNS Taka-amylase A, reduced DNS Taka-amylase A and RCM DNS Taka-amylase A by the fluorescence depolarization method. Perrin developed the theory of the polarization of fluorescence of solutions on an assumption that the emitting molecules carry rigidly bound linear oscillators of absorption and emission and that the molecular rotations are described by Einstein's 12 Weber used this method to calculate the rotational equation. 3 - 1 4 Recently, Gill et al. relaxation time of bovine serum albumin. applied this method to the study of the synthetic polypeptide chains with internal flexibility. The principle of this method and application to the studies of the protein structure will be described in detail later. In this study, we intended to estimate the extent of the rotational motion of the polypeptide chains of the various states of Taka-amylase A by using the fluorescence depolarization method. Taka-amylase A was conjugated with the fluorescent dye, 1-dimethylaminonaphthalene-5-sulfonylchloride (DNS-Cl), and disulfide bonds of this conjugate were cleaved by reduction with mercaptoethanol in 8M urea, pH 8.0.

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The polarization of fluorescence of these conjugates were measured as the function of temperature. On the other hand, in the previous study, we noticed that the fluorescence intensity of reduced DNS Taka-amylase A and RCM DNS Taka-amylase A increased remarkably as compared with that of DNS Taka-amylase A. We concluded that this phenomena might arise from the decrease of the interaction between the dye residue and the solvent molecules. This suggested that the fluorescence lifetime of RCM DNS Taka-amylase A might be different from that of DNS Taka-In the present study, we determined the absolute amylase A. fluorescence lifetimes of the conjugates by using a nanosecond fluorescence decay time apparatus. From the measurement of the fluorescence decay time and fluorescence spectra of these conjugates, it was found that the fluorescence lifetimes were proportional to the relative fluorescence yields of these conjugates. From the rotational relaxation times of the polypeptide chain of DNS Taka-amylase A and its derivatives obtained from this experiment, it was indicated that the rotational motion of the polypeptide chains of reduced and RCM DNS Takaamylase A might be restricted by intramolecular noncovalent bonds in spite of the absence of intrachain disulfide bonds. The above-mentioned two assumptions concerning the refolding

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process into the "native-like" three dimensional conformation were confirmed and it was concluded that the reformation of the disulfide bonds seemed to be a necessary requirement for further refolding into the "native-like" conformation.

HISTORICAL

The fluorescence depolarization method is concerned with the rotational motion of the polypeptide chain of the protein Detailed description concerning the principle of molecule. this method will be available in the following section. Generally, a fluorescent molecule is introduced into the sidechains of some amino acid residues by a mild chemical reaction. If the dye binds rigidly on the protein molecule, the relaxation time calculated from this method is expected to indicate the rotational motion of the total protein molecule, and we can get the molecular On the other hand, volume assuming a spherical molecular shape. if the protein molecule has some internal rotational freedom, the value of the relaxation time is expected to offer the information about the local motion of the segment of the polypeptide chain. From these reason, this method has been used to study the shape of a protein molecule, assciation and dissociation phenomena and interactions between enzyme proteins and substrates.

Introduction of The Fluorescent Molecule into A Protein Molecule

As mentioned above, it is necessary that the native protein structure is not affected by the introduction of the fluorescent molecule. From this point of view, it may be better to use the

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intrinsic fluorescent amino acid residues of the protein molecule, that is, tyrosine, tryptophan and phenylalanine. But in this case, these residues are not suitable for this experiment from the following reasons : (1) These amino acid residues have their absorption and emission spectra in the ultraviolet region and their absorption and emission spectra overlap largely. This suggests the large probability of energy transfer with radiationless transfer which leads to a fluorescence depolarization as well as to a change of fluorescence lifetime. (2) Their fluorescence quantum yields are not so high that it is difficult to measure fluorescence in low concentration of protein. The fluorescence depolarization measurements are desirable to be carried out in a low protein concentration to avoid unfavorable effects due to intermolecular interactions. (3) It is rather questionable whether the emission oscillators of these residues oriented randomly in the protein molecule. From these reasons, the fluorescence depolarization method is applied to the study of protein conformation by measuring the fluorescence of a dye which emits in the visible region and bound to the protein molecule covalently or noncovalently. But above fluorescent amino acid residues may be useful in the study of energy transfer

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in synthetic polypeptides by measurements of fluorescence polarization. Fluorescent molecules used in the fluorescence depolarization method are as follows : fluorescein isocyanate (FIC), fluorescein isothiocyanate (FITC), 1-dimethylaminonaphthalene-5-sulfonylchloride (DNS-Cl) and mercuric chloride of anthracene. The structural formulas of these fluorescent molecules are summarized in Fig.1. α - Amino, ε -amino or sulfhydryl groups are mentioned as the reactive sidechains of the protein. These groups react as follows

$$-NCO + \begin{pmatrix} -NH_{2} \\ -SH \end{pmatrix} \longrightarrow \begin{pmatrix} -NHCONH- \\ -NHCOS- \end{pmatrix}$$

$$-NCS + \begin{pmatrix} -NH_{2} \\ -SH \end{pmatrix} \longrightarrow \begin{pmatrix} -NHSNH- \\ -NHCSS- \end{pmatrix}$$

$$-SO_{2}C1 + \begin{pmatrix} -NH_{2} \\ -SH \end{pmatrix} \longrightarrow \begin{pmatrix} -SO_{2}NH- \\ -SO_{2}S- \end{pmatrix}$$

$$-HqC1 + -SH \longrightarrow -HqS-$$

As the other reactive groups of the amino acid side chains, the imidazole imino, aliphatic hydroxyl, phenolic hydroxyl and carboxyl groups are mentioned and as the other fluorescent substances, rhodamine B and acridine orange and others are.

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(2) FLUORESCEIN ISOTHIOCYANATE



(3) 1-DIMETHYLAMINONAPHTHALENE- (4) ANTHRACENE MERCURY CHLORIDE 5-SULFONYLCHLORIDE

Fig.l

The introduction of the fluorescent molecule into the protein has been investigated in the field of immunology in relation with the fluorescent antibody technique.

The Application of The Polarization of Fluorescence Method to The Study of The Protein Structure

At first, in 1952, this method was applied to the study He determined the rotational of the protein structure by Weber. relaxation times of ovalbumin and bovine serum albumin. At that time, it was difficult to obtain the fluorescence lifetime and he used the fluorescence lifetime calculated from 23 the rotational relaxation time of ovalbumin determined by Oncley with dielectric dispersion method. The rotational relaxation time of 1.27 x 10^{-7} sec for bovine serum albumin determined by Weber was consistent with the value determined by Oncley. 24 In 1957, Steiner and McAlister measured the fluorescence lifetimes of fluorescein, anthracene and 1-dimethylaminonaphthalene-5-sulfonylconjugates of egg white lysozyme, ovalbumin and bovine serum albumin by the phase shift method. The fluorescence lifetimes of these conjugates were different from each other but the rotational relaxation time of each protein molecule was not

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affected by the variation of the kinds of the fluorescent This result indicated that the dye molecules were molecules. bound on the protein molecule rigidly. But at that time, the column chromatography, with which the number of labeling of the dye could be made even, was not available. Therefore, the effect of the number of the dye on the fluorescence lifetime The values of the fluorescence could not be exactly considered. lifetimes obtained by Steiner and McAlister have been used without suspicion for the calculation of the rotational relaxation time. Recently, as the development of the method of the fluorescence lifetime measurement, it became obvious that the fluorescence lifetimes of the dye protein conjugates varied with the number of labeling of the dye or by the change 25 in polarity of the dye moiety. Wahl showed the above effects with DNS conjugates of lysozyme, and Chen with DNS conjugates These effects should be further of bovine serum albumin. investigated in future. Wahl measured the decay of the fluorescence, while Chen investigated the relation between fluorescence lifetimes, fluorescence yields, rotational relaxation times and the number of labeling. In 1968, Weltman and Edelman examined the fluorescence depolarization by both thermal change and viscosity change. They suggested in the study of $\gamma\text{-}G\text{-}$

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immunoglobulin that the internal motion of this molecule was This result suggested that the information activated thermally. obtained by thermal change and viscosity change were different to each other. Above examples are concerned with the shape of a molecule in solution. Other examples for structural transition 20 Steiner and Edelhoch examined the dissociare the followings. ation of thyloglobulin in urea and in sodium dodecylsulfate solutions and indicated that this protein obtained large flexibility Moreover, this method was applied to study in those solutions. the rotational motion of trypsin-digested fragment of human macroglobulin and papain-digested fragment of human immunoglobulin. On the other hand, for the study of association, Steiner investigated the interaction between lysozyme and deoxyribonucleic acid. Dandliker and Feigen applied the method for the study of ovalbumin and rabbit antibody interaction. Other examples were monomer-32 3 3 polymer equilibrium of α -chymotrypsin, self association of insulin, the interaction between trypsin and soybean trypsin inhibitor With the interaction between and polymerization study of actin. the enzyme and substrate, Massay studied the interaction between 3 5 3.6 Cvatrecasas, Edelhoch and Anfinsen fumarase and its substrate. investigated the interaction between nuclease of staphylococcus 37 3.8 Knopp, Rawitch and aureus and deoxythymidine-3'5'-diphosphate.

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Weber introduced pyrenebutyric acid which had a long fluorescence lifetime (100 nsec) into macroglobulin and thyroglobulin. They showed that, for these macromolecules, such dye offered the different information from that obtained with DNS conjugates whose lifetimes were nearly 20 nsec. At last, the fluorescence depolarization studies with the flexible polymers must be mentioned. Various synthetic polymers are usually used as flexible polymers. ¹³ For example, Gill studied the internal structure of poly Glu⁵²Lys³³ Tyr¹⁵ having intramolecular crosslinks as a model for the three dimensional conformation of a protein. Teramoto et al. reported the studies of the micro-Brownian motion of a polymer chain of the fluorescent conjugates of polyethyleneimine. These studies suggest that polarization data may serve as a basis for elucidating the rotational motion of a given molecule.

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THEORY

The basic theory of the fluorescence polarization was developed by Perrin and extended and applied by Weber and others. In recent fluorescence depolarization studies, the equations derived by Weber have often been used without regard to the basic concepts on which they are based upon. The derivation of the equations, 35,40,41therefore, will be described essentially according to Weber.

Fluorescence Polarization and Fluorescence Intensity

Fluorescence molecules are randomly oriented in solution. The excited fluorescent molecules return to their ground states accompanying radiation of light, fluorescence, after short interval of their excited state lifetimes ($10^{-7} - 10^{-9}$ sec). This fluorescence is emitted uniformly toward all direction (Fig.2). Fig.3 illustrates a fluorescent solution illuminated by a light beam which propagates along OX axis and the emission is observed along OY axis. The vertical and horizontal components of fluorescence of species i are expressed as $I_{\rm Hi}$ and $I_{\rm Li}$, respectively. Then the polarization P_i of species i is defined by

$$P_{i} = \frac{I_{ni} - I_{i}}{I_{ni} + I_{i}}$$
(1)

Fig.2



Fig.3



For all molecules which are randomly oriented,

$$I_{n} = \sum_{i} I_{ni}$$
(2)

$$I_{\perp} = \sum_{i} I_{\perp i}$$
(3)

From these, the polarization of the fluorescent solution is defined by the equation

$$P \equiv \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$
(4)

The fluorescence intensity is considered to be consisted of three components along OX, OY and OZ axes. If the emitted radiation is non-polarized,

$$\mathbf{I}_{\parallel \mathbf{OZ}} = \mathbf{I}_{\perp \mathbf{OX}} = \mathbf{I}_{\perp \mathbf{OY}} = \mathbf{L}$$
(5)

.

and total intensity is proportional to 3 **(**. The intensity of these components are different between the cases of excitation with polarized light and natural light. If the fluorescent molecule is excited with polarized light, the fluorescence is polarized as described below. Fig.3 shows the random orientation of the molecules. When the solution is excited with polarized light whose electric vector is in the OZ axis, then the intensity components are

$$\mathbf{I}_{\perp OX} = \mathbf{I}_{\perp OY} = \mathbf{I}_{\perp} ; \mathbf{I}_{OZ} = \mathbf{I}_{\parallel}$$
(6)

Then, total intensity is

$$I_{\parallel} + 2I_{\perp} = 3$$
 (7)

This requires

$$I_{n} = \mathbf{1} + \Delta$$
 (8)

$$\mathbf{I}_{\perp} = \mathbf{1} - \frac{1}{2} \Delta \tag{9}$$

where Δ is the correction term.

Therefore, from Eqns.(1), (8) and (9), for molecular species i

.

$$\frac{\Delta i}{2} = 2 \ \mathbf{l} \ \mathbf{i} \ / \ 3 \ (\frac{1}{P_i} - \frac{1}{3}) \tag{10}$$

Setting

$$I_{\mathbf{i}\mathbf{i}} + I_{\mathbf{i}\mathbf{i}} = F_{\mathbf{i}} \tag{11}$$

$$I_{ni} - I_{i} = F_{i}P_{i} \qquad (12)$$

Then

$$F_i P_i = 2 \ \mathbf{l}_i / (\frac{1}{P_i} - \frac{1}{3})$$
 (13)

Defining the contribution of the ith molecular species to the total fluorescence intensity as

$$f_{i} = \mathbf{l}_{i} / \sum_{i} \mathbf{l}_{i}$$
(14)

Eqn.(13) is expressed as

$$F_i P_i = 2 \left(\sum_{i} 1_i \right) \frac{f_i}{\frac{1}{P_i} - \frac{1}{3}}$$
 (15)

And from Eqn. (4)

$$\frac{1}{P} - \frac{1}{3} = \frac{\xi}{\xi} \frac{F_{i}}{F_{i}} - \frac{1}{3} = \frac{\xi}{\xi} \frac{F_{i}P_{i}}{F_{i}} \left(\frac{1}{P_{i}} - \frac{1}{3}\right)$$
(16)

Therefore, from Eqns.(15) and (16)

$$\frac{1}{p} - \frac{1}{3} = \frac{1}{\sum_{i=1}^{r} \frac{1}{\sum_{i=1}^{r} \frac{1}{2}}}$$
(17)

In the case of excitation with natural light

$$I_{\text{NOZ}} = I_{\text{NOY}} = I_{\text{NOY}} ; I_{\text{LOX}} = I_{\text{L}}$$
(18)

And total intensity is expressed as

$$2 I_{11} + I_{\perp} = 3 1$$
 (19)

Eqns.(6) and (7) become

$$I_{\parallel} = \mathbf{1} + \frac{1}{2} \Delta \qquad (20)$$

 $I = \mathbf{1} - \Delta$ (21)

In a similar way to derive Eqn.(17), we obtain

$$\frac{1}{P_{n}} + \frac{1}{3} = \frac{1}{\sum_{i} f_{i} / (\frac{1}{P_{i}} + \frac{1}{3})}$$
(22)

where the subscript n refers to the excitation with natural light. The fluorescence polarization is related with the molecular orientation of the fluorescent molecule as follows.

Polarization of Fluorescence and Molecular Orientation

A fluorescent molecule has an absorption oscillator and an emission one. These are sometimes overlap and sometimes not. At first we consider the former case. As shown in Fig.4, suppose the oscillator of the fluorescent molecule makes an angle θ with OZ axis and ψ with OX axis. Suppose the molecule is excited with polarized light propagating along OX direction and having electric vector along OZ direction, and the emitted fluorescence is observed along OY axis. Then I_{II} and I_L components of the fluorescence intensity is expressed as follows

$$I_{ox} = I_{oy} = I_{\perp} = \sin^2\theta \cos^2\psi$$
(23)

If all molecules are equal, θ , and ψ will take all values between 0 and 2π ,

$$\cos^2 \psi = \frac{1}{2} \tag{24}$$

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Fig.4

And Eqn.(24) becomes

$$I_{\perp} = \frac{1}{2} \sin^2 \theta \tag{25}$$

And

$$I_{oz} = I_{\parallel} = \cos^2\theta \qquad (26)$$

Then

$$\frac{1}{P_{\theta}} - \frac{1}{3} = \frac{I_{u} + I_{\perp}}{I_{u} - I_{\perp}} - \frac{1}{3} = \frac{2/3}{(\frac{-3\cos^{2}\theta - 1}{2})}$$
(27)

From Eqns.(27) and (17)

$$\frac{1}{P} - \frac{1}{3} = \frac{1}{\Sigma f(\theta) / (\frac{1}{P_{\theta}} - \frac{1}{3})} = \frac{2/3}{\theta = \pi/2}$$

$$= \frac{2/3}{\frac{\Sigma f(\theta)}{\Sigma f(\theta)} \frac{(3\cos^2 - 1)}{2}}$$

$$= \frac{2/3}{\frac{2/3}{2}}$$
(28)

It is known that the probability of absorption of light is proportional to $\cos^2\theta$. As the absorption oscillators are randomly oriented, the number making an angle θ with OZ axis is proportional to $\sin\theta$.

Therefore,

$$\cos^{2}\theta = \frac{\frac{\pi^{2}}{\int_{0}^{\pi} \cos^{2}\theta \cdot \cos^{2}\theta \cdot \sin\theta \, d\theta}{\int_{0}^{\pi/2} \cos^{2}\theta \, \sin\theta \, d\theta} = \frac{3}{5} \quad (29)$$

And introducing this value to Eqn.(28)

$$P = \frac{1}{2}$$
(30)

Next we consider such a case in which the oscillators of absorption and emission are not parallel but make an angle of λ (Fig.5). Then the relation between these angles are expressed as

$$\frac{3 \cos^2 \theta - 1}{2} = \frac{3 \cos^2 \delta - 1}{2} \cdot \frac{3 \cos^2 \lambda - 1}{2}$$
(31)

Treating $\cos^2 \delta$ as in the case of Eqn.(29), we obtain

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{5}{3}\right) - \frac{2}{3\cos^2 \lambda - 1}$$
(32)

Eqn.(32) reduces to P= 1/2 for $\lambda=0$. If $\lambda = 90^{\circ}$, then $\cos^2\lambda=0$ and P = -1/3. For a vertically polarized exciting light, the polarization of emitted fluorescence varies between these limiting polarizations. For unpolarized exciting light, it was shown by



Fig.5

Jablonski that the limiting polarization varies between -1/7and 1/3. As shown in Fig.6, unit axis OA fixed to a sphere rotates successively along OB and OC. Angles determined by these directions are δ , λ and θ . ψ is the azimuth of OC about the plane AOB. The relation between these angles is expressed as,

$$\cos\theta = \cos\delta \, \cos\lambda + \, \sin\delta \, \sin\lambda \, \cos\psi \qquad (33)$$
Taking average with a large number of molecules, Eqn.(33) gives,
$$\overline{\cos\theta} = \overline{\cos\delta} \, \overline{\cos\lambda} + \overline{\sin\delta} \, \overline{\sin\lambda} \, \overline{\cos\psi} \qquad (34)$$

Squaring and taking average of Eqn.(33)

$$\overline{\cos^2\theta} = \overline{\cos^2\delta} \overline{\cos^2\lambda} + \overline{\sin^2\delta} \overline{\sin^2\lambda} \overline{\cos^2\psi} + 2 \overline{\sin\delta} \overline{\cos\delta} \overline{\sin\lambda} \overline{\cos\lambda} \overline{\cos\psi}$$
(35)

If all azimuths of OC about The plane AOB are equally probable

$$\overline{\cos\psi} = 0 \quad ; \quad \cos^2\psi = \frac{1}{2}$$

Then Eqn. (35) become

$$\frac{1}{\cos^2\theta} = \cos^2\delta \, \cos^2\lambda \, + \, \frac{\sin^2\delta \, \sin^2\lambda}{2}$$
(36a)



Fig.6

Or
$$\frac{3 \cos^2 \theta - 1}{2} = \frac{3 \cos^2 \delta - 1}{2} \frac{3 \cos^2 \lambda - 1}{2}$$
 (36b)

Polarization and Rotational Relaxation Time

Before showing the relation between polarization and the rotational relaxation time of a fluorescent molecule, we are going to relate Brownian motion with the rotational relaxation 43 time. Einstein showed that when a spherical molecule rotates an angle θ within a short time interval Δt , the angle relates with time Δt as

$$\theta^2 = (2 \text{ RT}/3\eta \text{ V}) \cdot \Delta t$$
 (37)

where V is the molecular volume and R is the gas constant, T is the absolute temperature and η is the viscosity of the solvent. Debye defined the rotational relaxation time as the time that cosine of the rotating angle becomes 1/e. For a sphere, this value is expressed as

$$\rho = \frac{3\eta V}{R T}$$
(38)

Then the rotational relaxation time relates with polarization as follows. Consider the polarization at times 0 and t after the excitation and angles about OZ axis $\theta_0 \text{ and } \theta_t$ respectively. If fluorescent molecules are excited with polarized light, P_0 and P_t are expressed from Eqn.(27) as,

$$\frac{\frac{1}{P_0}}{2} - \frac{\frac{1}{3}}{3} = \frac{\frac{2/3}{3\cos^2\theta_0 - 1}}{\frac{3\cos^2\theta_0 - 1}{2}} ; \quad \frac{\frac{1}{P_t}}{\frac{1}{P_t}} - \frac{\frac{1}{3}}{\frac{3}{2}} = \frac{\frac{2/3}{3\cos^2\theta_0 - 1}}{\frac{3\cos^2\theta_0 - 1}{2}}$$
(39)

Now oscillator OE moves an angle $\alpha(t)$ within time 0 and t due to Brownian movement.

Applying Eqn. (36b)

$$\frac{3 \cos^2 \theta}{2} = \left(\frac{3 \cos^2 \theta}{2} - 1\right) = \left(\frac{3 \cos^2 \theta}{2} - 1\right) \left(\frac{3 \cos^2 \alpha(t) - 1}{2}\right) (40)$$

From Eqns. (39) and (40)

$$\frac{1}{P_{t}} - \frac{1}{3} = \left(\frac{1}{P_{0}} - \frac{1}{3}\right) \left(\frac{3\overline{\cos^{2} \alpha(t)} - 1}{2}\right)$$
(41)

If small changes in orientation characterized by $\cos^2\theta$ occure in mean number \bar{n} during the lifetime of the excited state,

$$\frac{1}{\overline{P}} - \frac{1}{3} = \left(\frac{1}{\overline{P}_0} - \frac{1}{3}\right) \left(\frac{2}{3\cos^2\theta} - 1\right)^n$$

$$\simeq \left(\frac{1}{\overline{P}_{0}} - \frac{1}{3}\right) \left(1 + \frac{3}{2} \sin^{2}\theta \,\overline{n}\right)$$
(42)

Since $n = \tau / \Delta t$ and $\sin^2 \theta = \theta$

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right) \left(1 + \frac{3\tau}{\rho}\right)$$
 (43)

Or

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right) \left(1 + \frac{R T \tau}{\eta V}\right)$$
 (44)

Next we consider the relation between P and P, which are the polarizations of fluorescence excited with polarized light and natural light, respectively. Natural light is considered to be such a light that the electric vector can be devided into In Fig.7, E_0 and E'_0 two equal and perpendicular components. The direction of excitation indicate these electric vectors. light is along OX axis, and fluorescence intensity is observed along OP axis which makes an angle Φ with OY axis. The components of fluorescence intensity are indicated with G $_{\parallel}$ and G $_{\perp}$. When a fluorescent molecule is excited with polarized light whose electric vector is in the OZ axis, G_{\parallel} and G_{\perp} components of the fluorescence intensity do not depend on the angle Φ . On the other hand, when a fluorescent molecule is excited with polarized light whose electric vector is in the OY axis, G_{\perp} depends on the angle Φ . G_{\parallel} and G_{\perp} for natural light excitation



Fig.7

are described as follows

$$G_{\mathbf{I}} = I_{\mathbf{I}} + I_{\mathbf{L}}$$

$$G_{\mathbf{L}} = I_{\mathbf{I}} \sin^2 \Phi + I_{\mathbf{L}} \cos^2 \Phi + I_{\mathbf{L}} \qquad (45)$$

where I_{\parallel} and I_{\perp} are the components of fluorescence intensity in OX, OY and OZ directions contributed from the excitation with two plane polarized light beams which have the electric vector of E_0 and E'_0 , respectively. Then, from the definition the polarization $P_n(\Phi)$ is in the form

$$P_{n}(\Phi) = \frac{G_{n} - G_{\perp}}{G_{n} + G_{\perp}} \frac{(1 - \sigma) \cos^{2} \Phi}{1 + 3\sigma + (1 - \sigma)} \sin^{2} \Phi$$
(46)

where σ indicates I_{ii} / I_{\perp} . Observing from OY direction, $\Phi = 0$, above equation is expressed as

$$P_n = \frac{1 - \sigma}{1 + 3\sigma}$$
(47)

While Eqn.(4) is also indicated as

$$P = \frac{1-\sigma}{1+\sigma}$$
(48)

From Eqns.(47) and (48), eliminating σ , we obtain the relation between P and P_n as

$$P_n = \frac{P}{2 - P}$$
From Eqns.(43) and (44), Weber's equation for excitation with natural light is expressed by

$$(\frac{1}{P_{n}} + \frac{1}{3}) = (\frac{1}{P_{n0}} + \frac{1}{3})(1 + \frac{3\tau}{\rho})$$

$$(\frac{1}{P_{n}} + \frac{1}{3}) = (\frac{1}{P_{n0}} + \frac{1}{3})(1 + \frac{R T\tau}{\rho})$$

$$(49)$$

$$(50)$$

$$\frac{1}{\rho_{\rm h}} = \frac{1}{3} \left(\frac{1}{\rho_{\rm h}} + \frac{1}{\rho_{\rm h}} + \frac{1}{\rho_{\rm h}} \right)$$
(51)

where ρ_a , ρ_b and ρ_c are the rotational relaxation times of the three axes of the molecule and in this time it is necessary that absorption and emission oscillators of the fluorescent molecule are randomly oriented against the three axes of the molecule. For excitation with a natural light, P_{n0} of Eqn.(50) can be derived from the intercept of the graph. If the molecular

state of the particle is not extremely asymmetric, (1/p + 1/3) will vary linearly with the variation of T/η . Therefore, if (1/P + 1/3) varies linearly against T/n, we can calculate the rotational relaxation time of the particle as a spherical molecule and effective volume of the molecule from Eqn. (50). If the internal rotation is present, the rotational relaxation time calculated from Eqn.(50) will be smaller than that expected for a rigid particle of the same size. The ratio of the observed value, ρ_h , to the predicted value, ρ_0 , for a rigid and unhydrated sphere of the same molecular weight is the measure of the deviation from sphere. If the values of ρ_h / ρ_0 are larger than unity, the size of the molecule observed is larger than that of a rigid sphere or asymmetric. Theoretical rotational relaxation time, ρ_0 , is given by

$$\rho_0 \approx \frac{3.\eta V_0}{RT}$$
(52)

where $V_{0}^{}$ is the molecular volume calculated from specific volume, $\bar{v},$ and molecular weight, M, by the equation

$$V_{0} = \overline{V} M \tag{53}$$

General Principles of Fluorescence

It is obvious from Eqns.(43) and (49) that the degree of polarization will clearly depend on the rotational relaxation time, \mathcal{P} , and the lifetime of the emission, \mathcal{T} . And this indicates that the lifetime of the excited state is an important factor determining the rotational relaxation time. The general rule of fluorescence is as follows.

The fluorescent molecule is excited owing to absorption of light from its ground state (S_0) to its first excited state (S_1) or second one (S₂) and so on. Its rate constant is nearly 10^{15} In solution, the excess energy of the high vibrational sec⁻¹. level is transfers from the fluorescent molecule to a solvent molecule through thermal relaxation. And this process is so called internall conversion. This process occurs in 10^{-13} 10⁻¹¹ sec. After this process, the excited molecule emits a photon from the lowest vibrational level of the first singlet excited state. This photon emission is observed as fluorescence. A part of the energy of the excited state transfers to triplet state within the lifetime of first singlet excited state (\sim 10 $^{-8}$ This process is referred as intersystem crossing. sec). These process are schematically shown in Fig.8.

Fluorescence Lifetime and Its Quantum Yield

The fluorescence quantum yield is given by

$$Q = \frac{k_{f}}{k_{f} + k_{q}}$$
(54)

where $k_{\rm f}$ and $k_{\rm q}$ are the rate constants of fluorescence and radiationless energy transfer ,respectively. And the fluorescence lifetime, τ , is expressed as

$$\tau = \frac{1}{k_{f} + k_{q}}$$
(55)

While the lifetime without energy transfer is called natural lifetime and given by

$$\tau_0 = \frac{1}{k_f}$$
(56)

Therefore, from Eqns.(54),(55) and (56)

$$Q = \frac{\tau}{\tau_0}$$
(57)

In another condition where the rate constant of radiationless transfer is $k_{\rm q}^{\prime}$,

$$Q' = \frac{\tau}{\tau_0}$$
(58)

Then the relation of the fluorescence yields and fluorescence lifetimes is expressed as

$$\frac{Q}{Q'} = \frac{k_{f} + k'_{q}}{k_{f} + k_{q}} = \frac{\tau}{\tau'}$$
(59)

The relative fluorescence yield obtained from experiment should be consistent with the ratio of Eqn.(59). We can get τ ', if we know the relative fluorescence yield and the standard lifetime.

Calculation of The Fluorescence Lifetime from The Fluorescence Decay Curve

If a fluorescent solute has a single fluorescence decay component with lifetime, τ , the fluorescence decay are ascribed as

$$I = I_0 e^{-t/\tau}$$
(60a)

where I is the fluorescence intensity at the time t after excitation and I_0 is the initial fluorescence intensity. Eqn.(60a) can be written as

log I = log I₀ - $(\frac{1}{\tau})$ log e · t (60b) By measuring the decay curve of fluorescence, we can obtain the fluorescence lifetime, τ , from Eqn. (60b).

wave F1g.8 process radiation. arrows in which Excited denote process ω photon transitions which do сц С Straight emitted arrow 0r not absorbed; denote emit



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EXPERIMENTAL

Materials

Taka-amylase A was prepared from "Taka-diastase Sankyo" 46 according to the method of Toda and Akabori. β -Mercaptoethanol was purchased from Wako Pure Chemicals and distilled at 51°C and 11 mmHg. 1-Dimethylaminonaphthalene-5-sulfonylchloride (DNS-Cl) was obtained from Calbiochem and used without further purification. This and urea were recrystallized from methanol and 70% ethanol respectively. 5-5'-Dithiobis-(2-nitrobenzoic acid)(DYNB) was obtained from Aldrich Chemicals Co.. Other reagents were of reagent grade.

Preparation of 1-Dimethylaminonaphthalene-5-sulfonyl Conjugates of Taka-amylase A

Taka-amylase A was conjugated with 1-dimethylaminonaphthalene-5-sulfonylchloride (DNS-Cl) essentially according to the method of Weber.

1500 mgs of Taka-amylase A was dissolved in 100 ml. of 0.1M Veronal-HCl buffer, pH 8.0 containing 200 mgs of calcium chloride added for the stabilization of the enzyme. To this solution, 20 mgs of DNS-Cl dissolved in 6 ml. of acetone was added under stirring at 20°C. The reaction was continued for 20 hrs at

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5°C under stirring. After reaction, the unreacted dye and its hydrolyzed product were removed by passage through an Amberlite IRA-400 column (1.5 x 20 cm). This ion exchange column had been equilibrated with 0.1M NaOAc-HOAc, pH 6.0 buffer. Then this effluent was dialyzed against 0.1M NaOAc-HOAc, pH 6.0 containing 10^{-3} M CaCl₂. The dialyzate was applied on a DEAE Sephadex A-50 (3 x 25 cm) equilibrated with the above buffer and eluted by the exponential increase in the concentration of sodium chloride. The fractionated sample was divided into several fractions with different average number of labeling. These fractions were dialyzed against distilled water and then lyophilized.

Preparation of Reduced DNS Taka-amylase A and RCM DNS Takaamylase A

DNS Taka-amylase A (10 mg/ml) was reduced by treating with 0.3M β -mercaptoethanol in 8M urea-0.01M EDTA-0.025M Tris-Cl, pH 8.0 for 5 hrs at 37°C. After reduction, urea and β -mercaptoethanol were removed by passage through a Sephadex G-50 column equilibrated with 0.025M Tris-Cl buffer, pH 8.0. The degree of reduction was followed by measuring the sulfhydryl content with DTNB by the procedure of Ellman.

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Reduction and carboxymethylation were performed as follows. Monoiodo acetic acid was added to the reduced sample to the final concentration of 0.3M and reacted for an hour at 37°C. Monoiodo acetic acid had been dissolved in 1N NaOH and neutralized. After reaction, the reacted solution was filtrated with Sephadex G-50 as described above.

Calculation of The Degree of Labeling

The degree of labeling was calculated from the following equation

$$\frac{A_{330}}{A_{280}} \times \frac{\varepsilon_{280}}{\varepsilon_{330}}$$
 (moles/mole)

where A_{280} and A_{330} are the absorbance of the dye-protein conjugate at 280 mµ and 330 mµ respectively. ε_{280} and ε_{330} are the molar extinction coefficient of Taka-amylase A and DNS group assumes to be 11.4 x 10⁴ cm⁻¹mole⁻¹ and 4.3 x 10³ cm⁻¹mole⁻¹, respectively.

The concentration of a protein was determined spectrophotometrically assuming $E_{\rm l\,cm}^{1\,\%}$ to be 22.1 at 280 m μ .

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Sulfhydryl Content

Sulfhydryl groups were determined by reaction with DTNB 47 according to the method of Ellman.

Measurements of Sedimentation Constants and Molecular Weight

Ultracentrifugal studies were performed in a Hitachi UCA-1 or a Spinco Model E ultracentrifuge machine at 55,000 rpm for determination of sedimentation constants and 12,200 rpm for determination of the molecular weight at 20°C in 0.025M Tris-Cl buffer, pH 8.0. Sedimentation constants were calculated by the following equation

$$S_t = \frac{(dr/dt)}{r\omega^2}$$

where S_t is the sedimentation constant at t°C, ω is the angular velocity of the rotor, r is the distance from the center of the rotor to the boundary position at which the refractive index gradient is a maximum. dr/dt is the sedimentation velocity of the solute. The sedimentation constant of the solute in the water at 20°C ($S_{20,w}$) was corrected by the equation

$$s_{20,w} = s_t \cdot \frac{n_{t,w}n_t}{n_{20w}n_{t,w}} \frac{1 - v\rho_{20,w}}{1 - v\rho_+}$$

where $n_{t,w}$ and $n_{20,w}$ are the viscosity of water at t°C and 20°C. n_t is the viscosity of the solvent at t°C. $\rho_{20,w}$ and ρ_t are the density of water at 20°C and that of the solvent at t°C. \bar{v} is the partial specific volume of the solute. The molecular weight was determined by using Archibald's method. Following equation was used for the calculation of the molecular weight

$$M = \frac{R T}{(1 - \bar{v}\rho_t)\omega^2} \cdot \frac{(dc/dr)_m}{r_m c_m}$$

 r_m is the distance of the meniscus from the center of the rotor and c_m is the concentration of the solute. $(dc/dr)_m$ is the concentration gradient at the meniscus.

Measurement of The Fluoresnce Spectra

Fluorescence spectra were measured with an Aminco-Bowman spectrophotofluorometer and a Hitachi MPF-2A spectrophotofluorometer. All measurements were performed at room temperature ($20 - 25^{\circ}$ C).

Measurement of The Fluorescence Decay

Fluorescence decay times were measured with an apparatus

developed by Bennet and a TRW Inc., nanosecond fluorescence decay time apparatus. The block diagram and schematic of the ⁴⁹ instrument developed by Bennet are shown in Fig.9 and 10, respectively. In this study, Toshiba UV D-2 filter was placed in the incident light path and VY-49 in the fluorescence light path.

Measurement of The Polarization of Fluorescence

Measurements of the fluorescence depolarization were made in a modified Brice-Phoenix Model 200 light scattering apparatus. Ultrahigh pressure marcury lamp Model SH 250 (Ushio Kogyo Co. Ltd.) was used as a light source instead of low pressure Model H-85-A3 (General Electric U.S.A) originally equipped in this apparatus. A constant temperature cell holder (Shimazu Co. Ltd.) was used in this apparatus. Temperature was regulated by circulating ethanol through the cell holder from a thermostat (Haake Model KT62). Small square cell suppoter was attached on the bottom of the cell holder. In this suppoter ethylene glycol was filled to increase heat conductivity. Dry nitrogen gas was passed through the apparatus to avoid the appearance of a dew drop on the optical window. Corning filter



Fig. 9 Block diagram of the timing circuitry



Fig.¹⁰ Scheme of the instrumental arrangement of nanosecond light source fluorometer.

5970 and 3385 (Brice Phoenix Co. Ltd.) were placed in the excitation and emission light path, respectively. This apparatus is shown in Fig.ll. All measurements were performed in 0.025M Tris-Cl buffer, pH 8.0. The rotational relaxation times were calculated from Eqn.(50).

Calculation of The Theoretical Rotational Relaxation Time

The theoretical rotational relaxation times for DNS Takaamylase A, reduced DNS Taka-amylase A, RCM DNS Taka-amylase A and RCM DNS Taka-amylase A in 8M urea solution were calculated from Eqn.(52). The effective volume of DNS Taka-amylase A was calculated by $\bar{v}M$ assuming that the partial specific volume was 0.700 and molecular weight was 51,750 . The effective volumes of reduced, RCM DNS Taka-amylase A and RCM DNS Takaamylase A in 8M urea solution were estimated from the following 10 equation

$$\frac{v_e}{v_e'} = \frac{v}{v} \frac{[n]}{[n]'}$$

where V_e is the effective molecular volume and v is the shape factor. [n] is the intrinsic viscosity. The values of the



Fig.ll The apparatus of polarization measurement

shape factor were assumed to be equal for DNS Taka-amylase A, reduced DNS Taka-amylase A and RCM DNS Taka-amylase A. Therefore, ν'/ν is equal to unity. [n] values obtained by Takagi and Isemura were used for this calculation.

RESULT

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Sedimentation Measurement

Taka-amylase A, reduced Taka-amylase A and RCM Taka-amylase A are known to be unimolecularly dispersed in 0.025M Tris-Cl This molecular dispersion is essential for buffer, pH 8.0. the present study so that the experimental parameters obtained from the measurement of the polarization of fluorescence may reflect the behavior of one molecule. Sedimentation patterns of DNS Taka-amylase A and RCM DNS Taka-amylase A are illustrated Sedimentation patterns showed a single peak under in Fig. 12 Sedimentation constants and molecular weights these conditions. of these conjugates are summarized in Table I with those of Taka-amylase A and RCM Taka-amylase A. Sedimentation constants were similar to those of the non-labeled states. The molecular weight of RCM DNS Taka-amylase A coincided with that of RCM From these results, it may be concluded that Taka-amylase A. In the present there is no aggregation of DNS conjugates. study, in order to avoid the intermolecular interaction, all measurements of the polarization of fluorescence were carried out with protein concentrations less than those in ultracentrifugal studies.

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(a)



(b)

Fig.12 The sedimentation patterns of (a) DNS Taka-amylase A and (b) RCM DNS Taka-amylase A in 0.025M Tris-El buffer, pH 8.0. Protein concentration were (a) 7.65 mg/ml and (b) 6.53 mg/ml.

SAMPLE	S _{20,w} Mwt
Taka-amylase A	3.45 (52,000) ⁵
DNS Taka-amylase A	3.49
RCM Taka-amylase A	2.23
RCM DNS Taka-amyl- ase A	2.23 52,700*

Table I Sedimentation constants and molecular weights

* Molecular weight of RCM DNS Taka-amylase A was obtained at the protein concentration of 3.92 mg/ml.

Fluorescence Spectra and The Relative Fluorescence Yield

Fig.¹³ shows the typical fluorescence spectra of DNS Takaamylase A and RCM DNS Taka-amylase A. The fluorescence intensity of RCM DNS Taka-amylase A was stronger than that of DNS Taka-amylase A and that of reduced DNS Taka-amylase A showed the similar effect. The fluorescence maximum of these derivatives of DNS Taka-amylase A shifted about 20 mµ to the shorter wavelength than that of DNS Taka-amylase A. The same phenomen has been observed by Takagi et al. . In the present study, the effect of the degree of labeling on the fluorescence yield was investigated for DNS Taka-amylase A and RCM DNS Takaamylase A to clear whether the molecular parameters obtained from the polarization method reflect correctly the behavior of one molecule or not. Table II shows the apparent relative fluorescence yields calculated from the comparison of the areas under the curves of the fluorescence spectra of the derivatives of DNS Taka-amylase A. The relative fluorescence yields of DNS Taka-amylase A were not affected with the degree of labeling of the dye and those of RCM DNS Taka-amylase A were not also affected. The relative fluorescence intensity of RCM DNS Takaamylase A decreased with the increase of labeling. The relative fluorescence intensity of reduced DNS Taka-amylase A was the

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Fig.13 Emission spectra of DNS Taka-amylase A (a) and RCM DNS Taka-amylase A (b) in 0.025M Tris-Cl buffer pH 8.0. Wavelength of excitation was 330 mµ.

SAMPLE	Degree of labeling	Relative fluorescence yield
н на селото селото на селото на На селото на	moles/mole	
DNS Taka-amylase A	1.1 - 6.4	1.0
RCM		
DNS Taka-amylase A	1,1	1.5
	1.3	1.4
	2.5	1.3
	6.7	1.3
reduced DNS Taka-amylase A	1.1	1.7
RCM DNS Taka-amylase A in 8M urea soln.	1.3	0.87

Table II Dependence of relative fluorescence yields on the degree of labeling strongest among the derivatives of DNS Taka-amylase A. The fluorescence intensity of RCM DNS Taka-amylase A decreased in 8M urea solution. According to the theory of the quenching of fluorescence discussed in the theoretical section, it must be expected that the changes in the fluorescence yields result in the changes in the fluorescence lifetimes.

Fluorescence Decay

Examples of the fluorescence decay curves of DNS Takaamylase A and RCM DNS Taka-amylase A are shown in Fig.14. The decay of the light source is illustrated in the same figure. It is clear that the lifetime of RCM DNS Takaamylase A is longer than that of DNS Taka-amylase A. The semilogarithm plots of the fluorescence decay according to the Eqn.(60b) are illustrated in Fig.15 . The apparent linearity was obtained between log I and t. This linearity shows that the fluorescence decay can be described in terms of a single fluorescence lifetime. The fluorescence lifetimes of DNS Taka-amylase A calculated from the slope of the plot of log I against time(t) using Eqn.(60b)are summarized in TableIII. The fluorescence lifetime of RCM DNS Taka-amylase A was calculated in the same manner. The lifetimes of DNS Taka-

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Fig.¹⁴ Typical examples of fluorescence decay measurements (A) Change of the nanosecond light source intensity measured by setting a reflector in the sample holder

(B) Fluorescence decay signal from DNS Taka-amylase A containing 0.6 dyes/mole of protein in 0.025M Tris-Cl buffer, pH 8.0.

(C) Fluorescence decay signal from RCM DNS Taka-amylase A containing 0.6 dyes/mole of protein in 0.025M Tris-Cl buffer, pH 8.0.

Excitation and emission filters were Toshiba UY-D 25 and Toshiba VY-45, respectively.



Fig. 15 A semilogarithmic plot of the data of the curve shown in Fig.15. a,DNS Taka-amylase A ; b,RCM DNS Taka-amylase A.

amylase A were constant regardless of the variation of the degree of labeling (Table III). The fluorescence lifetimes of DNS Taka-amylase A and RCM DNS Taka-amylase A of the same degree of labeling are illustrated in Table IV in which the relative fluorescence yields are also listed. The observed lifetime of RCM DNS Taka-amylase A was longer than that of DNS Taka-amylase A by about 14 nsec. And the ratio of the lifetimes of RCM DNS Taka-amylase A to that of DNS Takaamylase A was the same as the ratio of the relative fluorescence yields of these conjugates. It may be concluded that the fluorescence lifetimes of RCM DNS Taka-amylase A are proportional to the relative fluorescence yields. It may be, reasonable, therefore , to assume that lifetimes of the other derivatives of DNS Taka-amylase A are also proportional to their relative fluorescence yields. In this experiment, the fluorescence lifetimes of all the derivatives of DNS Taka-amylase A were corrected with the relative fluorescence yields using Eqn.(59). These values were used for the calculation of the rotational relaxation times of the derivatives of DNS Taka-amylase A.

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Degree of labeling	Fluorescence lifetime,τ,	
(moles/mole)	(nsec)	
0.6	22	
0,8	20	
1,1	22	
3,3	22	
3,5	24	
5,4	20	
	l.	

.

Table III Dependence of fluorescence lifetimes of DNS Taka-amylase A on the degree of labeling

		Degree of labeling (moles/mole)	Fluorescence lifetime (nsec)	Relative fluorescence
DNS	Taka-amylase A	0.6	22	1.0
RCM DNS	Taka-amylase A	0.6	36	1.5
DNS RCM DNS	Taka-amylase A Taka-amylase A	0.6	22 36	1.0 1.5

Table IV Fluorescence lífetimes and relative fluorescence yield of DNS Taka-amylase A and RCM DNS Taka-amylase A The Polarization of Fluorescence and Rotational Relaxation Times The polarization of fluorescence of the conjugates decreased with the increase of temperature of the solution. Fig 16 shows the plotting of the reciprocal of polarization, 1/P against T/n of DNS Taka-amylase A. Up to about 43°C, 1/P varied linearly against T/n. Above that temperature, departure from linearity occurred and the relation of 1/P against T/nbecame concave to the ordinate. Most enzymes are unstable and denatured at high temperature. We may then conclude that the departure from linearity is due to the unfolding of the structure of the enzyme with thermal denaturation. Weber observed this phenomena with DNS bovine serum albumin conjugate. From the slope of the plot of 1/P against T/η and the intercept of this plot at $T/\eta = 0$, the rotational relaxation time for excitation with natural light was calculated using Eqn.(50). The molecular parameters of DNS Taka-amylase A are summarized in Tables V and VI. The experimental values of the rotational relaxation time were compared with those of the theoretical rigid sphere, which were calculated using Eqn.(52). If the ratio of $\rho_{\rm b}$ to $\rho_{\rm o}$ are more than unity, this may be the reflection of the asymmetry of the molecule as described in

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Fig.16 Fluorescence depolarization as a function of temperature of DNS Taka-amylase A containing 1.8 dyes/mole of protein in 0.025M Tris-Cl buffer, pH 8.0. Protein concentration was 0.43 mg/ml.

Degree of labeling	ρ _h	^ρ h ^{∕ρ} 0	v
moles/mole	$x 10^{-9} sec$		$x 10^{-4} cm^{3}$
1.1	88	2.0	7.1
1.3	83	1.9	6.7
1.8	90	2.0	7.2
2,9	87	1.9	7.0
6.4	87	2.0	7.1
	l l		

Table V Dependence of molecular parameters of DNS Taka-amylase A on the degree of labeling

Protein conc.	ρ _h
(mg./ml)	(nsec)
0.2	123
0.4	125
0.6	123
0.8	124
1.2	105

Table VI Dependence of molecular parameters of RCM DNS Taka-amylase A on the protein concentrations Degree of labeling, 1.1 moles/mole of protein.

theoretical section. It is shown in Table V that the rotational relaxation times of DNS Taka-amylase A were not affected by the variation of labeling of DNS groups ranging from 1.1 to 6.4 moles/mole Taka-amylase A. A similar plot of 1/P against T/η for RCM DNS Taka-amylase A is shown in Fig.17. Table VI and Table VII show the dependence of the molecular parameters of RCM DNS Taka-amylase A on the variation of the concentration of the protein and the degree of labeling, respectively. It is shown that the rotational relaxation times of RCM DNS Taka-amylase A were not affected by the variation of the concentration of the protein from 0.2 to 1.2 mg/ml at DNS contents of 1.1 moles/mole Taka-amylase A. And the rotational relaxation times were similar in DNS contents of 1.1 and 1.3 moles/mole Taka-amylase A. While the rotational relaxation times decreased remarkably over DNS contents of 2.5 moles/mole The ρ_h / ρ_0 ratio of RCM DNS Taka-amylase A Taka-amylase A. were smaller than unity in the four conjugates ranging in DNS contents from 1.1 to 6.7 moles/mole@Taka-amylase A. In the case of DNS contents of 1.1 and 1.3, the $\rho_{\rm h}$ / $\rho_{\rm 0}$ ratio were similar to unity, on the other hand, in the case of DNS contents of 2.5 and 6.7, the $\rho_h^{}/$ $\rho_0^{}$ were smaller than the above

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Fig. 17 Fluorescence depolarization as a function of temperature of RCM DNS Taka-amylase A containing 1.3 (a) and 2.5 (b) dyes/mole of protein in 0.025M Tris-Cl buffer, pH 8.0. Protein concentration were 0.2 mg/ml(a) and 0.1 mg/m1(b).

Degree of labeling	ρ _h	ρ _h /ρ ₀	V
(moles/mole)	(nsec)		$(\times 10^{-4} \text{cm}^3)$
1.1	123	0.79	99
1.3	130	0.83	105
2.5	61	0.39	49
6.7	58	0.37	48

 ρ_0 was calculated assuming \bar{v} =0.70 and [n]'/[n]=3.4

Table VII Dependence of molecular parameters of RCM DNS Taka-amylase A on the degree of labeling
It is not clear why the rotational relaxation time conjugates. decreased in the high content of DNS, though the small decrease in the fluorescence yield was observed as the increase in DNS content. This decrease might be resulted from the presence of many kinds of moving units in the molecular state of RCM DNS Taka-amylase A. Fig.18 shows the polarization change for reduced DNS Taka-amylase A and its molecular parameters are summarized in Table VIII. The linear plot of 1/P against T/η indicates that the rotational kinetic unit in the polypeptide chain of this state does not change with the increase of the temperature up to about 40 °C. The rotational relaxation time of reduced DNS Taka-amylase A was the longest of all derivatives. Polarization data for RCM DNS Taka-amylase A in 8M urea solution were plotted in Fig.19. The accelerated depolarization of fluorescence was shown and no transition temperature could be detected. Such curvature has been reported in the studies with DNS labeled poly $Glu_{\frac{52}{53}Tyr^{15}}^{52}$, poly $Glu_{\frac{52}{53}}^{63}$, polyvinylamine and polyaminostyrene. The initial slope of the curve might be the reflection of the behavior of the polypeptide Molecular parameters are listed in Table IX. chain itself.

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Fig. 18 Fluorescence depolarization as a function of temperature of reduced DNS Taka-amylase A containing 1.1 dyes/mole of protein in 0.025M Tris-Cl buffer, pH 8.0. Protein concentration was 0.23 mg/ml.

Degree of labeling (moles/mole)	^p h (nsec)	° _h ∕ °₀	v (x 10 ⁻⁴ cm ³)
0.6	195	1.3	15.7
1.1	190	1.3	15.5
1.3	175	1.2	14.1
2.9	154	1.1	12.4
3.3	158	1.1	12.7
	}		

 ρ_0 was calculated assuming \bar{v} = 0.7 and [n]'/[n] = 3.2

Table VIII Molecular parameters of reduced DNS Taka-amylase A



Fig.¹⁹ Fluorescence depolarization as a function of temperature of RCM DNS Taka-amylase A containing 1.3 dyes/mole of protein in 8M urea (solvent : 0.025M Tris-Cl buffer, pH 8.0) Protein concentration was 0.63 mg/ml.

Degree of labeling	^ρ h	° _h ∕°₀	V
moles/mole	x 10 ⁻⁹ sec		$x 10^{-4} cm^{3}$
1.3	11	0.24	0.86

Table IX Molecular parameters of RCM DNS Taka-amylase A in 8M urea solution

DISCUSSION

Fluorescence depolarization method has been applied to various proteins. When applied to a compact globular protein, this method generally gives a reasonable value of rotational relaxation time. This method, however, gives no unique information, because similar information can be obtained with higher accuracy by other methods, such as viscosity or sedimentation velocity measurement. One of interesting application of this method may be an examination of the rigidity of a protein molecule of which structure is expected to be flexible, as has been described by Weber and Teale. On the other hand, during the course of our studies on protein renaturation, we were interested in the conformation of a protein polypeptide before reformation of disulfide crosslinks. Though information about the degree of unfolding of the polypeptide chain before the disulfide reformation could be obtained $\frac{1}{10}$ in the previous study with Taka-amylase A by hydrodynamic and spectrophotometric methods, we were quite ignorant of the degree of internal motion allowed to the polypeptide. Methods that can give such an information are lacking at the present time. Application of the fluorescence depolarization method to the above object seemed to be only recourse to get the above information.

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Before going into the discussion of obtained results, two problems should be mentioned : (1) As has been reported in a previous paper, the DNS group either in reduced DNS Taka-amylase A or in RCM DNS Taka-amylase A is in a somewhat different environment from that in DNS Taka-amylase A. Our results suggest that the DNS group in either of the two derivatives is interacting with other part of polypeptide, probably hydrophobic part, and is less exposed to the solvent. This means that the movement of the group is restricted by the interaction to some extent. It is expected , if the group is attached to a site of a flexible part, the flexibility might be supressed by the interaction when compared with the same part of the non-labeled protein derivatives. We must keep in mind that we are apt to overestimate the rigidity of the polypeptide in such a case. However, the extent of restriction of the polypeptide chain of RCM DNS Taka-amylase A seems to be not solarge, because no cooperative change of fluorescence intensity that suggests a rigid structure could be observed by the addition of urea. The presumed interaction, on the other hand, eliminates the possibility of lower estimate, because the thermal activation of the rotational motion of the attached DNS group observed in the case of DNS γ -globulin is not likely to occur in such

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a case. (2) Due to the interaction mentioned above, the fluorescence yield of the DNS group in reduced DNS Taka-amylase A or RCM DNS Taka-amylase A is increased markedly as shown in the previous paper.¹⁵ Usually any change in fluorescence yield means that there is a change in the fluorescence lifetime. If there is no loss of the excited energy at the initial phase of the fluorescence decay, the lifetime is proportional to the yield of fluorescence. In the present case, no radiationless energy transfer by the resonance mechanism which cause the rapid decay in the initial phase was likely to occur.

The results shown in Table IV indicate that the increase in the absolute fluorescence lifetime of RCM DNS Taka-amylase A is proportional to the increase in the relative fluorescence yield as compared with DNS Taka-amylase A. When the fluorescence yield varies in different states of the conjugates, the reasonable fluorescence lifetime may be obtained from the relative fluorescence yield and the standard fluorescence lifetime using Eqn. ($\frac{59}{}$) or from the direct measurement of the absolute fluorescence lifetime. The variation of the fluorescence lifetime has never been considered in previous applications of the fluorescence depolarization method to the study of the protein conformation. As shown in Tables II and III, in the

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case of DNS Taka-amylase A, the relative fluorescence yields and fluorescence lifetimes did not change with the increase of labeling. The fluorescence decay curve shown in Fig.15 indicates that the fluorescence of DNS Taka-amylase A is characterized by a single exponential decay. The fact that relative fluorescence yields were independent of the degree of labeling suggests that all vulnerable sites are homogeneous with respect to environment having effect on fluorescence As shown in Fig.¹⁵ and Table II, the characteristics. fluorescence decay curve of RCM DNS Taka-amylase A also consisted of a single decay component at the DNS content of 0.6 moles/mole Taka-amylase A and relative fluorescence yields of this conjugate did not change significantly with the degree of labeling. These results support the use of Eqn. (50) in which a single fluorescence lifetime was assumed.

The rotational kinetic unit of DNS Taka-amylase A was larger than the calculated molecular volume of the anhydrous rigid sphere of Taka-amylase A and the values of ρ_h/ρ_0 were nearly two for DNS Taka-amylase A. This suggests that the fluorescent dye moves with the whole protein molecule and behaves as a single kinetic unit. Indeed ρ_h/ρ_0 ratio of two may suggest the asymmetrical molecular shape, but we must

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mention the study of Steiner and McAlister concerning the ratio. They reported that the ρ_h/ρ_0 ratios of bovine serum albumin, egg albumin, lysozyme, soy bean trypsin inhibitor and G-actin which had been considered to be globular proteins were all The value of $\rho_{\rm h}/~\rho_0$ around two may reflect the nearly two. deviation of actual globular protein molecule from the assumed anhydrous spherical molecule. Therefore, from this point of view, it may be concluded that the shape of Taka-amylase A is nearly spherical. The DNS group is a highly hydrophobic It has been reported that DNS compounds with no group. chemically active group are at first bound to a specific sites with high hydrophobicity. It is , therefore, expected that the group is at first bound to a group of which environment is rich in hydrophobic side chains. If the shape of Takaamylase A is asymmetrical, such a preferential binding will lead to the dependence of observed relaxation times on the Thus the independency of the observed degree of labeling. relaxation times on the degree of labeling also indicates that the Taka-amylase A molecule is spherical. The plot of 1/P against T/n in Fig.16 began to deviate from linearity at This trend is generally observed when a protein about 43°C. molecule begin to unfold by thermal denaturation.

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Furthermore, we have no exact knowledge about the translational and rotational movement in such an unfolded protein molecule as reduced Taka-amylase A. Therefore. in this work, we , at first, investigated the rotational motion of the polypeptide chain of RCM DNS Taka-amylase A of which all cysteinyl residues have been converted to S-carboxymethyl cysteinyl residues as a reference to the rotational motion of the polypeptide chain of reduced DNS Taka-amylase A. As indicated in Table VII, the rotational relaxation time of RCM DNS Taka-amylase A depends on the degree of labeling. At low degree of labeling, the rotational relaxation time was larger than that of DNS Taka-amylase A and at high degree of labeling, it was smaller. The observed relaxation times were compared with that calculated from hydrodynamic effective volume The value of $\rho_{\rm h} / \rho_0$ estimated from viscosity measurement. was nearly one at the low degree of labeling. This result indicates that the volume of rotational kinetic unit of RCM DNS Taka-amylase A is comparable with that estimated from the h Therefore, it may be reasonable to hydrodynamic study. conclude that the internal motion of the polypeptide chain of RCM DNS Taka-amylase A is restricted. On the contrary,

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when the degree of labeling exceeded two, the rotational relaxation time of RCM DNS Taka-amylase A decreased markedly and these values were smaller than the calculated value. This suggests the increase in the internal motion of the polypeptide chain of RCM DNS Taka-amylase A. Dependence of the observed relaxation time on the degree of labeling seems to reflect the heterogeneity of the size of the segments, that is, there may be rotational kinetic units of different sizes having long and short rotational relaxation times in that molecular state. As shown in Fig.17, the polarization of fluorescence decreased markedly above DNS contents of 2.5 moles/mole Taka-amylase A and this decrease of polarization is the important factor of the decrease add the rotational relaxation time. There are two causes for the fluorescence depolarization. One is the decrease of the rotational kinetic unit of the fluorescent molecule and another is the transfer of the excited energy by "concentration depolarization" which is explained by Förster mechanism between DNS groups. However, the latter case is expected to be accompanied by the decrease of the fluorescence As shown in Table II and Fig.17, indeed the polarization vield. was affected by the degree of labeling, but the fluorescence yield

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of RCM DNS Taka-amylase A was not. Therefore, the decrease of ρ_h / ρ_0 at high degree of labeling of RCM DNS Taka-amylase A can not be ascribed to "concentration depolarization".

As shown in Table VIII, the molecular parameters of reduced DNS Taka-amylase A suggests that the molecular shape of this state may be similar to that of RCM DNS Taka-amylase A, since in this cases the value of ρ_h / ρ_0 is nearly one as in the case of RCM DNS Taka-amylase A. However, it is noted that the rotational relaxation time of reduced DNS Taka-amylase A is larger than that of RCM DNS Taka-amylase A. Takagi and Isemura suggested in their hydrodynamic studies of Taka-amylase A and its derivatives that RCM Taka-amylase A was more unfolded than reduced Taka-amylase A. They explained this based on the presence of carboxymethylated sulfhydryl group in the case of RCM Taka-amylase A which was charged in experimental condition. Therefore, the above result observed in this work, may result from the same cause as in the case of nonlabeled With reduced DNS Taka-amylase A, the effect Taka-amylase A. of the degree of labeling of the rotational relaxation time was not studied extensively, since this state was not so stable like RCM DNS Taka-amylase A. From the results obtained at

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the low degree of labeling, it may be concluded that both reduced and RCM DNS Taka-amylase A have the similar internal motion of polypeptide chains. As shown in Fig.17 and Fig.18, the plot of 1/P against T/n showed good linear_ity and the deviation from linear_ity was observed only above 40°C. It may be concluded that these results indicate that RCM DNS Taka-amylase A and reduced one hold rigid structure that could not be destroyed by thermal agitation up to this temperature.

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Gill investigated the relation between the intramolecular covalent crosslinks and the transition temperature of poly $Glu^{52}Lys^{33}Tyr^{15}$ with almost the same molecular weight as Taka-amylase A. He found that the increase in the number of the intramolecular crosslinks were accompanied by the increase in the transition temperature and the rotational relaxation time. His result with the polypeptide without crosslinks is in marked contrast with our data with reduced and RCM DNS Taka-amylase A preparations in that his plot of 1/P against T/η was concave to the ordinate from the beginning and the slope obtained by extrapolation gave a relaxation time as low as 11 nsec. He suggested that the

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increase in the transition temperature indicated that the increase in the rigidity of the conformation of the molecule. This result supports the conclusion of this investigation.

As shown in Fig.19, 1/P did not change linearly against T/n in the case of RCM DNS Taka-amylase A in 8M urea solution and it was difficult to discuss the rotational motion of this conjugate from the data of the polarization. But the rotational kinetic unit which was determined by the extrapolation of 1/P at low temperature to $T/\eta = 0$, is smaller than that of DNS Taka-amylase A and further, than the rotational relaxation time of 11 nsec of the model compound of poly Glu⁵²Lys³³Tyr¹⁵ and has no intramplecular crosslinks. Therefore, it may be concluded that RCM DNS Taka-amylase A in 8M urea solution has no effective force to hold the three dimensional molecular The results of the present study indicate clearly structure. that the polypeptide chains of reduced and RCM DNS Taka-amylase A were restricted by some forces even in the absence of the formation of the disulfide bonds.

Finally, some problems concerned with the fluorescence depolarization method in general will be mentioned. In the present study, it was indicated that a particular fluorescent group could have several lifetimes depending on its environment.

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This point has not been taken into consideration. Fluctuation of fluorescence lifetime depending on environment should be carefully considered in future studies. Wahl and Lami²⁵ indicated that there were two fluorescence lifetimes in DNS conjugates of lysozyme. Further, in the case of RCM DNS Takaamylase A in 8M urea solution, the plot of 1/P against T/n was not linear and we used the polarization data at low temperature. As has been mentioned in the theoretical section, Weber's equation is based on the assumption that the molecule is a rigid sphere. Further studies seem to be necessary to interpret the fluorescence depolarization data with flexible polymer, such as RCM DNS Taka-amylase A in 8M urea solution.

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REFERENCES

- (1) J.C.Kendrew, Brookhaven Symp., <u>16</u>, 216 (1962)
- (2) D.C.Phillips, Proc.Nat.Acad.Sci.U.S., <u>57</u>, 484 (1967)
- (3) G.Kartha, J.Bello, D.Harker, Nature, <u>213</u>, 862 (1967)
- (4) P.B.Sigler, D.M.Blow, B.W.Matthews, R.Henderson, J.Mol.Biol., 35, 143 (1968)
- (5) F.H.C.C rick , On protein synthesis. Symp. Soc. for Exper. Biol., <u>13</u>, 138 - 163.
- (6) F.H.Jr., White, J.Biol.Chem., 236, 1353 (1961)
- (7) T.Isemura, T.Takagi, Y.Maeda, K.Imai., Biochem.Biophys. Research Communs., 5, 373 (1961)
- (8) K.Imai, T.Takagi, T.Isemura, J.Biochem., 53, 1 (1963)
- (9) A.Imanishi, K.Kakiuchi, T.Isemura, J.Biochem., 54, 89 (1963)
- (10) T.Takagi, T.Isemura, Biochim.Biophys.Acta, 130, 233 (1966)
- (11) F.Perrin, J.Phys.Radium, 7, 390 (1926)
- (12) G.Weber, Biochem., 51, 155 (1952)
- (13) T.J.Gill, III, Biopolymers, 3, 43 (1965)
- (14) T,J.Gill, III, G.S.Omenn, J.Am.Chem.Soc., 87, 4188 (1965)
- (15) T.Takagi, Y.Nakanishi, N.Okabe, T.Isemura, Biopolymers, 5, 627 (1967)
- (16) Y.Ueno, T.Ohta, A.Wada, Protein, Nucleic acid, Enzyme, 8,

No.7, 362 (1963)

- (17) A.Pesce, E.Bodenheimer, K.Norland, G.D.Fasman, J.Am.Chem. Soc., 86, 5669 (1964)
- (18) I.Weinryb, R.F.Steiner, Biochemistry, 7, 2488 (1968)
- (19) R.H.Conrad, L.Brand, Biochemistry, 7, 777 (1968)

- (20) R.C.Nairn, Fluorescent Protein Tracing, Williams and Wilkins, Baltimore, Maryland, (1962)
- (21) D.J.R.Laurence, Methods in Enzymology (Eds. S.P.Colowick and N.O.Kaplan) Vol IV, p174, Academic Press, New York, (1957)
- (23) J.L.Oncley, Chem. Rev., 30, 443 (1942)
- (24) R, Steiner, A.J. McAlister, J. Polym. Scien., 24, 105 (1957)
- (25) P.Wahl, H.Lami, Biochem.Biophys. Acta, 133, 233 (1967)
- (26) R.F.Chen, Arch.Biochem.Biophys., 128, 163 (1968)
- (27) J.K.Weltman, G.M.Edelman, Biochem., 6, 1437 (1967)
- (28) R.Steiner, H.Edelhoch, J.Am.Chem.Soc., 83, 1435 (1961)
- (29) H.Metzger, R.L.Parlman, H.Edelhoch, J.Biol.Chem., <u>241</u>, 1741 (1966)
- (30) R.Steiner, Arch.Biochem.Biophys., 46, 291 (1953)
- (31) W.Dandliker, G.Feigen, Fed.Proc., A-11f (1961)
- (32) V.Massey, W.Harrington, B.Hartley, Disc.Faraday.Soc., 20, 24 (1955)
- (33) R.Steiner, A.McAlister, J.Colloid Science, 12, 80 (1957)
- (34) T.Tsao, Biochim.Biophys.Acta., 11, 236 (1953)
- (35) G.Weber, Advances inProtein Chem., (Eds. M.L.Anson, K. Bailey and J.T.Edsall) Vol VIII, p415, Academic Press, New York (1953)
- (36) P.Cuatrecasas, H.Edelhoch, C.B.Anfinsen, Proc. Natl.Acad. Sci., 58, 2043 (1967)
- (37) J.A.Knopp, G.Weber, J.Biol.Chem., 244, 6309 (1969)
- (38) A.B.Rawitch, G.Weber, J.Biol.Chem., 244, 6543 (1969)

- (39) A.Teramoto, M.Morimoto, Y.Nishijima, J.Poly.Sci., <u>5</u>, 1021 (1967)
- (40) G.Weber, Biochem.J., 51, 145 (1952)
- (41) G.Weber, Fluorescence and Phosphorescence Analysis,(Ed. D.M.Hercules), p217, John Wiley and Sons, New York,(1966)
- (42) A.Jablonski, Z.Physik., 96, 236 (1935)
- (43) A.Einstein, Ann.Physik., 19, 371 (1906)
- (44) P.Debye, Polar Molecules, Chemical Catalog Co.New York
 (1929)
- (45) F.Perrin, Compt.Rend., <u>180</u>, 580 (1925) ; ibid.,<u>181</u>,514 (1925)
- (46) H.Toda, S.Akabori, J.Biochem., 53, 102 (1963)
- (47) G.L.Ellman, Arch.Biochem.Biophys., 82, 70 (1959)
- (48) W.J.Archibald, J.Phys. and Colloid Chem., 51, 1204 (1947)
- (49) R.G.Bennet, Rev.Sci.Instr., 31, 1275 (1960)
- (50) G.S.Omenn, T.J.Gill, III, J.Biol.Chem., 241, 4899 (1966)
- (51) M.Frey, P.Wahl, H.Benoit, J.Chim.Phys., 61, 1005 (1964)
- (52) Y.Y.Gottlieb, P.Wahl, J.Chim.Phys., 60, 849 (1963)
- (53) P.Wahl, J.Chim.Phys., 63, 68 (1966)
- (54) G.Weber, F.W.J.Teale, The Proteins (2nd ed.) (Ed. H. Neurath) Vol III, p445, Academic Press, (1965)
- (55) T.Förster, Fluoreszenz Organischer Verbindungen, p221Vandenhoeck und Rupprecht, Göttingen (1951)
- (56) E.A.Stein, J.M.Junge, aEdH.Fisher, J.Biol.Chem., 235, 371
 (1960)

Studies of Protein Conformation by Fluorescence Measurements

(II) Effect of Labeling with DNS Group on The Renaturation of Lysozyme

SUMMARY

Effect of DNS groups on the renaturability of lysozyme has The effect was found to be contrastively been studied. different between the singly labeled lysozyme and the doubly The former could be completely renatured, but the labeled one. latter was refolded to a less ordered and aggregated product. These conformational changes could be followed by measuring fluorescence characteristics of DNS group which was sensitive to an environmental change. Combined with the results of previous study with other proteins, the present result with the doubly labeled lysozyme suggests that the high hydrophobicity of DNS group inhibits the correct renaturation, because the group prefers to be incorporated into the protein interior. The result with the singly labeled lysozyme was presumed to indicate that the above tendency was countered by the unfavorable exposure of previously incopporated intrinsic hydrophobic Thus the renaturability residues to the aqueous environment. of a DNS conjugates of a protein molecule seemed to be affected by the polarity of the site coupled with DNS groups.

Abbreviations

DNS		:	l-dimethylan	nin	onaphthal	Lene-5	sulfonyl group
DNS	lysozyme	:	Conjugates d	of	lysozyme	with	l-dimethylamino-
			naphthalene-	-5-	sulfonyl	chlor	ride

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INTRODUCTION

Since early works on ribonuclease, $1 = \frac{1}{2}$, $1 = \frac{2}{3}$ and Taka-amylase $\frac{4}{3}$, many experimental results have been reported with various proteins indicating that their polypeptide chains can refold to their native conformations starting from their randomly coiled states. Clearly these results indicate the importance of amino acid sequence in determining the mode of folding of the polypeptide chain of a protein molecule under a given condition. Effect of chemical modifications on protein renaturation have been extensively studied especially with ribonuclease. Its refolding capacity has been observed to be conserved after various chemical modifications. They include succinvlation, methylation, dansylation, phthalylation, butyrylation, caproylation 5 and polypeptidylation 6 . 7 8 Many people have taken a chemical modification only as a blocking of a reactive group and have tended to conclude that the side chain with the reactive group was not essential to the refolding process. Such a way of thinking seems to be incorrect from the present knowledge of a globular protein structure. Accumulated results of protein denaturation studies established that hydrophobic interactions, the tendency of hydrophobic amino acid side chains being excluded from direct contacts with aqueous environment, are the most important factor to stabilize the native globular conformation. From this point of view, a chemical modification which significantly changes the polarity of a side chain on protein surface from

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hydrophilic to hydrophobic one is expected to interfer a correct refolding. All chemical modifications reported not to have interfered a correct refolding turned out to be those bringing about no major change in the polarity. An exceptional chemical modification which interfered significantly with the correct refolding was the one made by the introduction of DNS group into amino acid groups of ribonuclease reported by Epstein and They followed the renaturation process Goldberger and White. only by measuring enzymatic activity. White and Epstein et al. 11 suggested the bulkiness and the hydrophobic nature of the DNS group, respectively, as a possible cause of the observed interfering effect. No further discussion has been made on the mechanism of the effect. In a previous paper, we have reported that the DNS group coupled to Taka-amylase A is incorporated into the protein interior in the refolding process probably due to hydrophobic interactions and thus made the proper refolding The change of the medium of DNS group was clearly impossible. reflected on its fluorescence characteristics. In this study, a more through study was carried out on the effect of introduction of DNS groups into lysozyme on its renaturability. The most interesting finding in this study is the fact that the renaturability depends strongly on the degree of labeling. The singly labeled lysozyme molecule was found to be refolded to "native-like" conformation and to regain the enzymatic activity. The doubly labeled

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implications by a hydrophobic group.

EXPERIMENTAL

Four-times recrystallized hen's egg white lysozyme and its substrate, dried cell wall of M.lysodekticus, were purchased from Seikagaku Kogyo Co.. 1-**Di**methylaminonaphthalene-5-sulfonyl chloride (DNS-Cl) was obtained from Calbiochem., and used without further purification. Urea and Tris were recrystallized from 70 percents aqueous ethanol and methanol, respectively. β -Mercaptoethanol was redistilled at 11 mmHg and 51 °C. 5-5'-**D**ithiobis-(-2-nitrobenzoic acid) (DTNB) was obtained from Aldrich Chemicals Co.. Other reagents were of reagent grade.

Concentration of DNS lysozyme , as well as that of non-labeled one, was determined spectrophotometrically assuming $E_{lcm}^{1\%}$ to be 26.9 at 280 mµ². The contribution of DNS group at this wavelength was corrected assuming that it was one half of the absorbance at 330 mµ. The number of DNS groups coupled to lysozyme was calculated using the following equation

$$\frac{A_{330}}{A_{280} - \frac{1}{2}A_{330}} \times \frac{\varepsilon_{280}}{\varepsilon_{330}} \quad (\text{ moles/mole})$$

where A is the absorbance at wavelength indicated, and $\varepsilon_{330}^{}$ is the molar extinction coefficient of DNS group at 330 mµ, and ε_{280} , that of lysozyme at 280 mµ, assumed to be 4.3 x 10³ and 3.85 x 10⁴ M⁻¹cm⁻¹, respectively.

The enzymatic activity of lysozyme was determined by measuring the decrease of optical density at 540 m μ of M.lysodeikticus cell wall suspension in 0.02M phosphate buffer, pH 6.0-1.0 percent NaCl

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at 37 °C according to the method of Jolles.

Fluorescence was measured with a Hitachi model MPF-2A.spectrophotofluorometer. Excitation wavelength was 325 mµ. The absorption and circular dichroic spectra were measured with a Jasco J-10 CD/UV meter.

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Sedimentation velocity was measured with a Hitachi model UCA-1 instrument.

Preparation of 1-dimethylaminonaphthalene-5-sulfonyl conjugate of lysozyme

1-Dimethylaminonaphthalene-5-sulfonyl chloride (DNS-C1) was coupled to lysozyme essentially as described Weber.¹⁵ 920 mgs. of DNS-Cl was dissolved in 5 ml. of acetone, and was added to 100 ml. of 1 percent lysozyme solution in 0.1 M NaHCO3, pH 8.6 in an ice bath under vigorous stirring. Final ratio of protein to DNS-Cl was l : 4 (molar ratio). The reaction was continued in a cold room at 4 °C for 60 hours. Insoluble material was centrifuged off from the reaction mexture. The solution was diluted ten-fold with 0.01M sodium phosphate buffer, pH 6.0 , and freed from by-products by passing through a Sephadex G-25 column (2.5 X 40 cm) equilibrated with 0.01M phosphate buffer, pH 6.0. The eluate was applied on a CM-Sephadex (C-25) column (3 X 50 cm) equilibrated with the same buffer and eluted with a linear increase of the concentration of sodium chloride (gradient, 0.001 M/ml). The effluent was collected in 5 ml. fractions.

Reduction and reoxidation of DNS lysozyme

DNS lysozyme (10 mg/ml) was reduced with 0.3M mercaptoethanol in 8M urea - 2 X 10^{-4} M EDTA - 0.025M Tris-Cl buffer,pH 8.0. The mercaptoethanol was removed from the reaction mixture by passing through a Sephadex G-25 column equilibrated with 8M urea 0.025M Tris-Cl ,pH 8.0. The eluate was diluted with 0.025M Tris-Cl pH 8.0 so that the final concentration of the protein and urea to be 0.0l percent and 2M, respectively. It was necessary to add the dilu**ent** slowly to avoid formation of floc**cule**. The solution

was poured into a 500 ml. beaker and air-oxidized at 20 °C for three days, being stirred occasionally. The reoxidation was followed by measurement of sulfhydryl content with DTNB.¹⁶ After sulfhydryl groups disappeared, the reoxidized solution was dialyzed against 0.02M KCl, pH 3.5 (HCl), and then concentrated with an Amicon model 200 ultrafiltration cell using a Diaflo membrane PM-10. Further fractionation will be described in the text.

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RESULTS

Chromatography of DNS lysozyme

DNS lysozyme was prepared as described in the experimental section, and was applied on a CM-Sephadex column to cut off highly labeled lysozyme. The elution pattern is shown in Fig.l as well as the relative fluorescence intensity, the enzymatic activity decreased with the increase of labeling. Fractions between 105 and 135 were pooled and used for further experiments. The pooled solution showed 80 percents of the original activity and contained 1.3 DNS groups per lysozyme molecule.

Conjugation and protein structure

DNS lysozyme thus obtained showed a single symmetrical sedimentation peak with S_{20,w} value of 2.0S (protein concentration, 4.9 mg/ml ; 0.1 M KCl, pH 6.0) which was identical with that for non-labeled lysozyme (1.9 S at the same condition). DNS lysozyme also showed the same denaturation curve as native one as shown in Fig.7. Fig.2 shows the optical rotatory dispersion of DNS lysozyme in comparison with that of native lysozyme. It is to be noted that DNS lysozyme is a little less levorotatory than native lysozyme. The change is opposite to the direction expected for unfolding of the protein molecule.

Reactivation through reoxidation

Both reduced lysozyme and reduced DNS lysozyme were reoxidized in the same conditions. Time course of the recovery of their



Fig.1 Chromatogram of DNS lysozyme on CM-Sephadex C-25 : **absorbance** at 280 mµ (\bigcirc) ; relative fluorescence intensity at 520 mµ (\bigcirc); number of fluorescent residues per protein molecule (\triangle) and relative activity (\square). Fraction showed with an arrow (\longleftarrow) was pooled.



Fig.2 Optical rotatory dispersion of native lysozyme (\square) and DNS lysozyme (\bigcirc).

TABLE I

	S _{20,w}
NATIVE	1 96*
Lysozyme	1,00
DNS Lysozyme	2.0

* A.J.SOPHIANOPOULOS et al., J.Biol.Chem., 237,1107(1962)

enzymatic activity are shown in Fig.3. The reactivation was markedly inhibited by the coupling of DNS group.

Fractionation of reoxidized DNS lysozyme

Reoxidized DNS lysozyme as well as reoxidized lysozyme was applied to a Sephadex G-75 column. As shown in Fig.4b, two distinct peaks appeared in the elution diagram. The first peak was only observed with reoxidized DNS lysozyme and its position coincided with that of Blue Dextran. The position of When reduced second peak coincided with that of native lysozyme. lysozyme was reoxidized, only one peak was observed at the same position as that of the second peak mentioned above. Comparison with the curve for native lysozyme indicate, however, presence of a small amount of aggregates. Fractions composing each of the two peaks in Fig.4b were pooled and designated as Fr-I and Fr-II, respectively. Fr-I showed no enzymatic activity, but Fr-II showed about 65 percents of that of DNS lysozyme. Fr-I is clearly aggregated and this fact made further characterization of this fraction difficult. It is through this aggregation, however, that we could fractionate the reaction mixture.

Absorption and fluorescence spectra

Fig.5 shows absorption spectra of Fr-I and Fr-II. Fr-I showed higher absorption around 325 m μ than Fr-II. DNS contents were calculated to be 2.3 and 0.9 for Fr-I and Fr-II, respectively.

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Fig.3 Time dependence of the recovery of activity at 37 °C through oxidation on time : lysozyme (\bigcirc) and DNS lysozyme (\bigcirc).



Fig.4 Gel filtration by Sephadex G-75 (1.5 x 88 cm)
of no conjugated lysozyme (a) and conjugated lysozyme(b):
(a) native lysozyme (○) ; reoxidized lysozyme (●),
(b) DNS lysozyme (○) ; reoxidized DNS lysozyme (●).
The effluent was collected in 5ml. fractions. Sephadex
G-75 was equilibrated with 0.02M KCl,pH 3.5. Fr-I and
Fr-II were pooled.

Table II

	DNS / PROTEIN (moles/mole)	Relative Activity (%)
DNS Lysozyme	1.3	100
F-I	2.3	0
F-II	0.9	65


Fig.5 Absorption spectra of reoxidized DNS lysozyme Fr-I and Fr-II in 0.02M KCl, pH 3.5 : Fr-I (a) and Fr-II (b).

As shown in Fig.6, the fluorescence yield of Fr-I was markedly higher than those of Fr-II and DNS lysozyme. Fig.6 also includes the fluorescence spectrum of DNS lysozyme that has been renatured from the denatured state in which disulfide groups are intact. It is clear that the renaturation is complete in this case.

Effect of guanidine

Fig.7 shows the effect of guanidine on the circular dichroism at 230 mµ of lysozyme of various modifications. Native lysozyme, DNS lysozyme and Fr-II of reoxidized DNS lysozyme showed almost same curves with a drastic transition around 3.8M guanidine. On the other hand, the curve of Fr-I changed slowly from the begining with a gradual decrease. It is also to be noted that the magnitude of circular dichroism of Fr-I at zero guanidine concentration is appreciably smaller than those of other three. The effect of guanidine on fluorescence intensity shows apparently contrasting behavior as shown in Fig.8. The effect on Fr-II was not significant and only a small but reproducible transition-like change was observed around 3.8M guanidine. On the other hand, Fr-I showed a large transition-like change around 4.2M.

Solvent perturbation

As shown in Fig.9, the fluorescence of both DNS lysozyme and Fr-II of reoxidized DNS lysozyme was markedly affected by the addition of dioxane. While the fluorescence of Fr-I was hardly



Fig.6 Fluorescence spectra of DNS lysozyme, reoxidized DNS lysozyme Fr-I and Fr-II in 0.02M KCl, pH 3.5 at room temperature : DNS lysozyme (a), Fr-II (b), Fr-I (c) and DNS lysozyme after treating with 6M guanidine, pH3.5 for 4 hrs at 25 °C (d). Excitation wavelength was 325 mµ.



Fig.7 Effect of Gu-HCl on $[\theta]_{230}$ of native lysozyme (\square) DNS lysozyme (\blacksquare), Fr-I (\bullet) and Fr-II (O). Treated with pH 3.5 at 25 °C and equilibrated for about 12 hrs.



Fig.8 Effect of Gu-HCl on the fluorescence intensity of reoxidized DNS lysozyme Fr-I (\bullet) and Fr-II (O). Treated with pH 3.5 at 25 °C and equilibrated for about 12 hrs.



Fig.9 Effect of the addition of dioxane on the relative fluorescence intensity of ε -DNS lysine (Δ),DNS lysozyme (\blacksquare), Fr-I (\odot), Fr-II (\bigcirc) and mixture of Fr-I and Fr-II (\bigcirc) in 2M urea-0.025M Tris-Cl buffer, pH 7.5. Excitation wavelength was 325 mµ and emission maximum was measured.

affected by dioxane. The reoxidized mixture before the fractionation showed an intermediate behavior and ε -DNS lysine was most markedly affected.

Acid dissociation constant of dimethylamino group of DNS group

Fig.10 shows the titration curve of dimethylamino group of DNS group. Since it is well known that a protonated DNS group is not fluorescent, it was assumed that the fluorescence intensity became zero, when the dimethylamino groups were fully protonated. Apparent acid dissociation constants (pK_a 's) were obtained from midpoints of the curves. The values of apparent pK_a of DNS lysozyme and Fr-II of reoxidized DNS lysozyme were 3.0 and that of Fr-I was 1.0.



Fig.10 pH titration of DNS lysozyme, reoxidized DNS lysozyme Fr-I and Fr-II in 0.02M KCl, pH 3.5 : DNS lysozyme (■), Fr-I (●) and Fr-II (**O**). Excitation wavelength was 325 mµ and emission maximum was followed.

DISCUSSION

Most of chemical modifications so far reported did not appreciably interfere with renaturation to a high degree. They include the change of the sign of the side chain groups by modifications such as succinylation, the introduction of bulky group such as poly-DL-alanyl chain and introduction of butyl and caproyl residues which are weakly hydrophobic. 10 On the other hand, White found that DNS ribonuclease retained 100 percents of the original activity , but could regain only 15 percents of activity after reductive denaturation in 8M urea and subsequent reoxidation. He attributed the effect to the bulkiness of this group, but such an interpretation does not work, because introduction of bulky polypeptidyl groups have been shown not appreciably interfere with the renaturation of Epstein et al. suggested that the effect might ribonuclease. be attributed to the hydrophobicity of DNS group. DNS group is highly hydrophobic especially when its dimethylaminogroup ($pK_{a}\simeq~4$) remains in the unprotonated form. $\epsilon\text{-DNS}$ group involves a tetramethylene group which is also hydrophobic. Even if the expected opposite effect due to the relatively polar sulfonyl imino group taken into account, the group such as ϵ -DNS lysyl group which remains to be exposed to an aqueous medium is energetically unstable. Such ϵ -DNS group should prefer to be incoorporated into the interior of the protein molecule which is rich in hydrophobic side chains. In a previ-12 ous study, such a situation could be observed with DNS Taka-

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The enzyme seemed, however, not to be favorable amylase A . for further studies, since its molecular structure is unknown and it is insolble in the pH region where dimethylamino group of DNS group ionizes. We have selected lysozyme, which is free from the above shortcomings and studied the effect of labeling on the renaturability of lysozyme. The results contains interesting implications for the further understanding of the effect of chemical modifications on protein structure. The results of sedimentation and optical rotatory dispersion measurements indicate that DNS lysozyme retains the globular It is to be noted, however, that a slight decrease in nature. levorotatory power suggests that the change of the conformation accompanying the modification is an increase of the globular nature of the protein molecule. However, the result of denaturation curve in Fig.7 indicate that the lysozyme molecule become a little more labile to quanidine. In any case, such a possible minor conformational change will not be discussed in the present study. The most interesting result of the present study is that the renaturability depends markedly on the degree The singly labeled lysozyme molecule could be of labeling. renatured to the native state. On the other hand, the doubly labeled lysozyme molecule could not be renatured and polymerized. The almost integral values of the degree of labeling, and the absence of intermediary fraction strongly indicate that the way

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of refolding is guite different between the singly labeled protein and the doubly labeled one. Presumably lysozyme molecules with more than three labeled DNS groups behave in the same manner as the doubly labeled one. Most of the heavily labeled molecules seem to have been removed in the chromatography shown in Fig.1. We examined various properties of the two fractions and found contrasting difference between them. As will be clear from the denaturation curves in Fig.7, Fr-II showed the same curve as DNS lysozyme before treatment of reductive denaturation. No appreciable difference was observed also in the fluorescence perturbation and the ionization of dimethylamino group. Clearly, the Fr-II is the lysozyme molecule completely renatured to the original conformation. As shown in Fig.6, a minor difference was observed between the fluorescence spectrum of DNS lysozyme and that of Fr-II. Seemingly, the difference is insignificant, since a small pH fluctuation results appreciable change in fluorescence yield at pH 3.5 as is clear from Fig.10. The absolute value of circular dichroism at 230 mu of Fr-I was about one third of native lysozyme. Increase of guanidine concentration caused no cooperative change of the circular dichroism. Clearly, Fr-I is devoid of ordered structures characteristic of globular The elution position of Fr-I indicates that it is protein. aggregated. Let turn our eyes to the local environment of the DNS group coupled to the lysozyme molecule. DNS group coupled

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to various proteins generally show a fluorescence maximum around Presumably the presence of maximum around 540 $\ensuremath{\text{m}}\mu$ 540 mu. represents that the DNS group is exposed to a polar environment. As shown in Fig.6, the DNS group coupled to the lysozyme molecule showed a fluorescence spectrum just like this. The result of the solvent perturbation (Fig.9) and the $\ensuremath{\text{pK}}_{\ensuremath{\text{a}}}$ of the group (Fig.10) also show that the group is exposed to the aqueous The two fractions obtained after the reductive surrounding. denaturation-renaturation treatment, showed very interesting result as shown in Fig.6. There were contrasting difference between the behavior of DNS group of Fr-I and that of Fr-II. The fluorescence characteristics of Fr-II was the same as that of DNS lysozyme before the treatment. Fr-I showed a markedly enhanced fluorescence and had a maximum at 490 mµ. Quite the same change was observed with DNS Taka-amylase A when it was Chen¹⁸ treated in the same way as in the present experiment. clearly showed that such a change occurs when DNS group is transferred from a polar environment to a less polar one. Parker et al. found that the fluorescence quantum yield of ϵ -DNS lysine increased 25 to 30 fold and the emission maximum shifted from 556 to 500 mµ, when it reacted with the rabbit antibody which was specific for DNS group and must have a binding site with high affinity to the hydrophobic portion of the group. This and the above mentioned inspection of Fig.7 - 10 will make

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clear a curious fact. Namely the DNS group of Fr-I seems to be surrounded by a rigid structure. Fig.8 shows a transitionlike decrease of the fluorescence intensity to the same value as that of the group of Fr-II. The middle point of the transition is about 4.2M quanidine. At this quanidine concentration, even the denaturation of native lysozyme is almost completed as shown in Fig.7. The result of solvent perturbation in Fig.9 indicates that the DNS group of Fr-I is inaccessible to the solvent . As shown in Fig.10, the pK value of the DNS group shifted to acidic pH by about 2 units. Such a phenomenon has been observed with phenolic hydroxyl groups of tyrosine residues in the so-called "masked state". Therefore, the unprotonated form of the DNS group of Fr-I seems to be in a masked state. Clearly, the DNS group of Fr-I is surrounded by a kind of structure with a hydrophobic nature, though it is bound to a polypeptide chain almost devoid of ordered structures. A11 lysine residues which are most probable sites of labeling are on the molecular surface and are exposed to the surrounding medium. Introduction of a strongly hydrophobic group such as DNS group must increase the free energy of the protein molecule. The group should prefer to be surrounded by non-polar hydrophobic amino acid side chains which are abundant in the interior of the globular proteins. If the polypeptide chain is not rigidly fixed, the preference of DNS group of a hydrophobic environment to a polar one will cause denaturation. In case of the lysozyme

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molecule, this did not occur due to the rigid structure of the molecule. All but one lysyl residue of the lysozyme molecule are very nearly positioned to disulfide bonds which will restrict the possible conformational change induced by dansylation. Α small change in optical rotatory dispersion suggests an assumption of more compact conformation, but the change is so small that most characteristics of the native lysozyme including its enzymatic activity are preserved. It is desirable to estimate the hydrophobicity of the DNS group. The quantitative value Tanford² estimated hydrophobicities is not, however, available. of amino acid side chains from solubility measurements in several solvents. For transfers of tryptophan, tyrosine and phenylalanine side chains from non-polar to polar solvent, the free energy changes were assigned values of 3000, 2870 and 2650 cals/ residue mole, which are higher than those of other side chains. ε -DNS lysyl side chain contain l-dimethylaminonaphthalene ring, sulfonyl imino group and tetramethylene group. It may safely msist that the dimethylaminonaphthalene ring has a higher hydrophobicity than tryptophan side chain, possibly more than 4 Kcal./ residue mole. The free energy of the native lysozyme molecule has been estimated only 10 - 20 Kcal./mole lower than that of 21 the randomly coiled lysozyme molecule in the absence of denaturant. Therefore, the increase of free energy by about 4 Kcal./mole protein may be enough as a driving force of a minor conformational change. It is to be noted, however, that the incorporation of

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DNS groups does not necessarily lowers the free energy of the Inco**rp**oration of a DNS group may be followed by DNS protein. changes which have contrary contribution to the free energy Exposure of hydrophobic groups originally buried in change. the protein interior may be a major factor. The balance between the above mentioned opposing contributions may determine whether the incorporation of a particular DNS group is favored or not. Fluorescence decay study on DNS lysozyme by Wahl and Lami suggests that the binding site of DNS group on lysozyme is heterogeneous. If the singly labeled DNS lysozyme is modified at a special site, and the above opposing contributions counteract the stabilization by the incorporation of DNS group, the observed renaturation of Fr-II can be interpreted without contradiction. Whether the second site to be labeled is a particular one or not is not clear. However, labeling with DNS groups clearly favors the conformational change observed and both of the two DNS groups are incorporated into the protein interior as is clear from the result of solvent perturbation study (Fig.9). The presence of the two groups in a protein molecule might have allowed the conformational change which have made the incorporation of the first DNS group possible. However, the direct interaction between the two DNS groups is less probable, because such a interaction leads to a decrease in the fluorescence yield contrary to the observed fact. Some hydrophobic residues may be exposed to the molecular surface and lead to formation of intermolecular aggregates whose presence

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made the successful separation of Fr-I from the reaction mixture possible.

In conclusion, the results of the present study represent a marked effect of the introduction of DNS group on the renaturation of lysozyme molecule. Though the present study is concerned only with labeling with DNS groups, the obtained result is believed to give useful suggestions to understand the effect of introduction of hydrophobic groups by covalent linkage.

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1	F.H White. Jr., : J.Biol.Chem., <u>236</u> , 1353 (1961)
2	K.Imai, T.Takagi, T.Isemura : J.Biochem., <u>54</u> , 1 (1963)
3	C.J.Epstein, R.F.Goldberger : J.Biol.Chem., 238, 1380 (1963)
4	T.Isemura, T.Takagi, Y.Maeda, K.Imai : Biochem.Biophys.Research Communs., <u>5</u> , 373 (1961)
5	C.J.Epstein, R.F.Goldberger : J.Biol.Chem., 239, 1087 (1964)
6	C.B.Anfinsen, M.Sela, J.P.Cooke : J.Biol.Chem., 237,1825 (1962)
7	C.J.Epstein, C.B.Anfinsen : J.Biol.Chem., 237, 3464 (1962)
8	A.Frensdorff, M.Wilchek, M.Sela, : European J.Biochem., <u>1</u> , 281 (1967)
9	C.Tanford : Advances in Protein Chem., (Eds. C.B.Anfinsen, M.L. Anson, J.T.Edsall, F.M.Richards) Vol.23, p.122, Academic Press, New York (1968)
10	F.H.White, Jr. : J.Biol.Chem., 239, 1032 (1964)
11 12	C.J.Epstein, F.R.Goldberger, C.B.Anfinsen, Cold Spring Habor Symp. Quant.Biol., 28,439 (1963) T.Takagi, Y.Nakanishi, N.Okabe, T.Isemura : Biopolymers, <u>5</u> , 627 (1967)
13	H.Edelhoch, R.F.Steiner : Biochim.Biophys.Acta., 60, 365 (1962)
14	P.Jollės : "Methods in Enzymology", (Ed. by S.P.Colowick, N.O.Kaplan) Academic Press Inc., New York, Vol5, p.137 (1962)
15	G.Weber : Biochem., <u>51</u> , 155 (1952)
16	G.L.Ellman, : Arch.Biochem.Biophys., <u>82</u> , 70 (1959)
17	D.Lagunoff, P.Ottolenghi : Comptes Rendus des Travaux du Laboratoire, Carlsberg, <u>35</u> , 63 (1966)
18	R.F.Chen : Arch.Biochem.Biophys., <u>120</u> , 609 (1967)
19	C.W.Parker, T.J.Yoo, M.C.Johnson, S.M.Godt, Biochemistry, <u>6</u> , 3408 (1967)

- 20 C.Tanford : J.Am.Chem.Soc., <u>84</u>, 4240 (1962)
- 21 K.C.Aune, C.Tanford : unpublished data
- 22 P.Wahl , H.Lami : Biochim, Biophys.Acta., 133, 233 (1967)