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Oligomerization State of the Plasma Membrane Proteolipid Apoprotein Purified from the Bovine Kidney, Probed by the Fluorescence Polarization

Quae Chae* and Sang Rye Nam

Department of Chemistry, Chungbuk National University, Cheongju 360-763
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In order to investigate the oligomerization state of the plasma membrane proteolipid apoprotein purified from the bovine kidney, fluorescence polarization experiment was carried out in the two different solvent systems, i.e., water and organic solvent(chloroform-methanol). The molecular volumes of the proteins estimated from the Perrin equation, were to be 45,258A^3 and 17,608A^3 in water and organic solvent, respectively. These values indicate that a trimerization is possibly occurring in the aqueous environment. As an auxiliary experiment for the calculation of the molecular volume using Perrin equation, fluorescence quenching constants (K_q) with the quencher acrylamide and fluorescence lifetimes (τ_F) of the intrinsic fluorophore tryptophan residue were estimated in the two different solvent systems. K_q in water was 18.21M^{-1} and it was 46.24M^{-1} in organic solvent. Fluorescence lifetimes of tryptophan residue were calculated to be 2.80 nsec. in water and 3.81 nsec. in organic solvent, respectively.

Introduction

Proteolipid is a kind of hydrophobic membrane-bound proteins. They are characterized by their solubility in chloroform-methanol, and contain covalently bound fatty acid residues, probably attached in ester linkage to serine or threonine hydroxyl groups. By the way, the apoprotein whose lipid components are almost removed can be obtained by the routine methods. The extraction of proteolipids is possible by the chloroform-methanol solvent mixture. They are readily soluble in chloroform-methanol (2:1, v/v) solvent system and insoluble in water, but a water soluble form can be prepared by several methods[1-3]. The organic solvent soluble form of proteolipid and the water soluble one can be interconvertible each other.

The proteolipids in the plasma membrane have all been related with the ion channel formation for transporting K^+ ions. To understand the structure-function relationship of these proteins, a few physico-chemical studies have been carried out. Circular dichroic analysis for these proteins must be

*To whom correspondence should be addressed

one of the significant studies carried out so far. According to the CD study[4], the helical contents of the proteins were changed by variation of the solvent system, i.e., from organic solvent to water. Their interpretation was that these results were not only due to the solvent effect but also due to the different states of oligomerization in the two solvent systems. As a key concept for the interpretation, they proposed a hypothesis of monomer-oligomer transition in going from the organic solvent soluble protein to the water soluble one[4]. Several biochemists believe that the proteolipid proteins in membrane bilayer exist as a hexamer structure[4-6], and they assume a kind of channel formation that facilitates ion transport. However, any critical experimental evidences to support the above idea have not been presented yet. The background to propose the concept of monomer-oligomer transition is mainly based upon the fact that some membrane bound proteins to transport the ions exist in the oligomerized form in the membrane bilayer[7]. For instance, the ion transporting proteins in the mitochondrial and purple membrane are found to be the oligomerized forms in their membrane bilayer[8].

When we assume that the biological membrane environments are at least more similar with the aqueous one than the organic solvent system, it would be valuable work to observe any molecular aggregation phenomenon of the protein in the two different solvent systems as a model study. In the present study, we employed the fluorescence polarization technique which is very sensitive to monitor the molecular size of the macromolecules and found the obvious evidence to probe that the hypothesis of monomer-oligomer transition was quite feasible.

Experimental

Materials. Sephadex LH-60, 2-mercaptoethanol, Coomassie brilliant blue and Fiske-Subbarow reagent were purchased from Sigma Chemical Co. and used without further purification. Folin-Ciocalteau reagent, sodium dodecyl sulfate, bromophenol blue, acrylamide and glycerol were purchased from Junsei Chemical Company Ltd. Sucrose, chloroform and methanol were all reagent grade. Bovine kidney was purchased from the urban slaughterhouse (Cheongju, Korea) and was free from the extraneous tissue by dissection. It was cut into pieces of the desired size, and they were packed in aluminum foil and sored at −50°C until use.

Preparation of the Plasma Membrane. For the extraction and purification of the proteolipid protein from the plasma membrane of bovine kidney, plasma membrane was first prepared by following the precedure of Sapirstein *et al.*[4]. The tissue of bovine kidney was homogenized in the solution of 0.25 M sucrose after the following steps; the tissue was blending and filtered through two layers of cheesecloth. The homogenate was centrifuged at 10,000 g for 15 min. and supernatant was used for crude plasma membrane. The supernatant(S_1) was centrifuged at 15,000 g for 15 min. and the supernatant(S_2) was centrifuged at 25,000 g for 30 min. The resultant pellet was used for crude plasma membrane isolation. The pellet was carefully suspended in 0.32 M sucrose. The care which should be taken for suspending the pellet is the clean removal of the pinkish overlying plasma membrane fraction from the dense brown mitochondrial pellet. The plasma membrane was homogenized in a glass-teflon homogenizer, and the centrifugation, resuspension processes were repeated until a membrane fraction which was free of the mitochondrial pellet was obtained.

Extraction and Purification of the Proteolipid. Membranes were extracted with 15 volumes of chloroform: methanol mixture(2:1, v/v). After filtration of the extract, partition with 0.2 volume of H_2O was carried out. The phases were separated by centrifugation and lower phase was placed at 4°C for several hours for the exclusion of more H_2O. The steps to remove H_2O from the organic solvent phase were required to maintain the stability of the protein during concentration. The extract was then concentrated in a rotary evaporator. Further concentration to 5% by weight was carried out with nitrogen gas bubbling.

The concentrated sample was acidified with 0.01 N HCl and 1% acetic acid to dissociate the protein moiety from the acidic lipids[9]. The acidified sample was then loaded to Sephadex LH-60 column(2 × 90cm) equilibrated with chloroform-methanol-acetic acid (2:1:0.03, v/v/v). The sample after the first Sephadex LH-60 column chromatography was rechromatographed on a neutral Sephadex LH-60 column to remove acetic acid.

Preparation of the Water Soluble Form

The water soluble form of proteolipid protein was prepared by the method of Sherman and Folch[1]. 10 m*l* of proteolipid protein solution in chloroform-methanol was placed in the 50 m*l* test tube. Water aliquots (25μl) were added to the surface of the solution with one minute interval. During the additions of water aliquots, chloroform and methanol were removed by nitrogen gas bubbling until water remained alone. If it is successfully prepared, a clean and transparent solution is obtained. The solution is stored at 4°C and can use it for one month.

Determination of the Proteolipid Apoprotein and Phospholipid. Protein determination was carried out by Lowry method[10]. Bovine serum albumin was used as a standard protein and absorbance at 750 nm was checked for the determination of protein content. Phospholipid determination was performed by phosphorus assay using Fiske-Subbarow reagent[11]. Absorbance at 830 nm was compared with the standards containing 0.5-6.0μg phosphorus.

Measurement of Fluorescence Polarization and Quenching. Fluorescence emission polarization was measured by the spectrofluorometer (Hitachi) equipped with the Glan-Thomson polarizers. For the calculation of polarization value (P), we used the Azumi equation[12] expressed as following

$$P=\frac{I_{EE}-I_{EB}(I_{BE}/I_{BB})}{I_{EE}+I_{EB}(I_{BE}/I_{BB})}$$

where I_{EE}, I_{EB}, I_{BE} and I_{BB} are the fluorescence intensities obtained by the horizontal(E) and perpendicular(B) combinations of excitation and emission polarizers. It is possible to get four different combinations. Fluorescence of tryptophan residue of the protein was measured at 20°C. Since fluorescence polarization value of the molecule is sensitive to the viscosity of the solvent, the viscosities of the two samples (in water and organic solvent) were controlled to be identical by addition of glycerol.

Fluorescence quenching experiment was carried out by titrating samples (1.25 m*l*) with 5μl aliquots of 5 M acrylamide

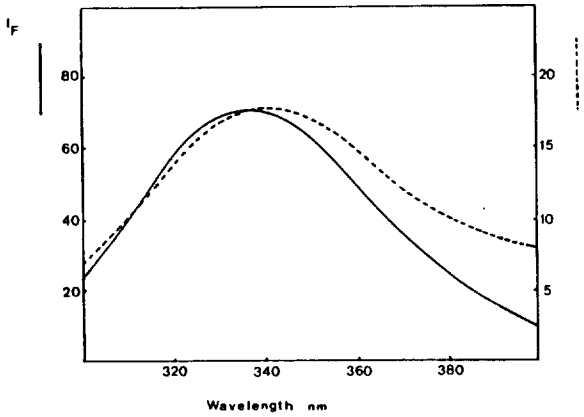

Figure 1. Fluorescence emission spectra of the plasma membrane proteolipid protein in water (solid line) and in chloroform-methanol (dotted line). Exciting wavelength is 280 nm which is the absorption maximum of tryptophan residue.

solution. Final concentration of acrylamide was 0.2 M and fluorescence emission of tryptophan residue was monitored at 340 nm. Data were analyzed by the modified Stern-Volmer equation that describes quenching data when both dynamic and static quenching are operative[13]

$$F_o/F = 1 + (K_q + \frac{1}{K_{diss}})\ [Q] + \frac{K_q}{K_{diss}}\ [Q]^2$$

where K_q is the quenching constant of collision of tryptophan with quencher and K_{diss} is the dissociation constant of their complex, respectively. At low concentration of quencher, $[Q]^2$ term becomes close to zero and K_{diss} becomes very large. In this condition the equation is deduced to be $F_o/F = 1 + K_q[Q]$, which is the normal Stern-Volmer equation representing the collisional quenching process.

Results and Discussion

Figure 1 shows fluorescence emission spectra of proteolipid protein, purified from bovine kidney, in water and chloroform-methanol systems, respectively. In the water system, the emission maximum was slightly blue shifted in comparison with the one in organic solvent system. Fluorescence intensity of the sample in water was much higher than the one in organic solvent. From these two fluorescence parameters, it is sure that the environment of the fluorophore(tryptophan residue(s)) is changed by varying solvent systems. Upon observing the changes of two parameters, blue shift of emission maximum and higher fluorescence quantum yield in water system, it is certain that the hydrophobic environmental effect on the tryptophan residue(s) is more significant in water system than the case in organic solvent system. The environmental change of tryptophan residue(s) must be induced by the conformational change of protein which is possible through the interaction between the protein and solvent molecules. But it is hard to say now how this conformational change of protein can be related with the oligomerization process in water.

Fluorescence quenching experiment was carried out and Stern-Volmer plots are shown in figure 2. Fluorescence of tryptophan residue was quenched by the quencher acrylamide, which is the specific fluorescence quencher for indole

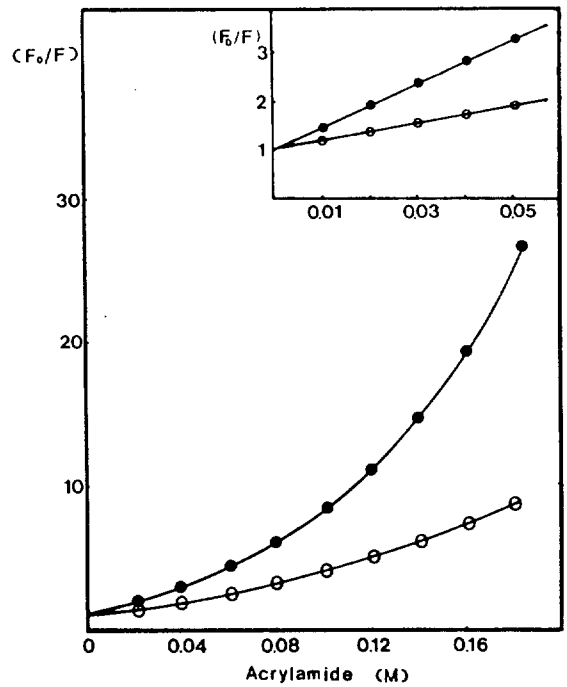

Figure 2. Stern-Volmer plots for the tryptophan fluorescence quenching by acrylamide in water (-○-○-) and in chloroform-methanol (-●-●-). Fluorescence intensities were measured at the wavelength of 340 nm with excitation at 280 nm. Inset shows the Stern-Volmer plot at the low concentration of the quencher.

derivatives, in water and organic solvent systems, respectively. The hyperbolic shapes of the quenching curves suggest us that the quencher acrylamide acts to cause a decrease in fluorescence through both types of quenching processes, i.e., dynamic and static quenching. However, the straight lines(inset) were obtained at low concentration of quencher. This indicates that only dynamic quenching is efficient at low concentration of quencher and the quenching constants (K_q) can be estimated from the slopes of these straight lines. The calculated quenching constants are 18.31 M^{-1} and 46.24 M^{-1} in water and organic solvent, respectively. A higher fluorescence quenching is observed in organic solvent system, comparing with the one in water. This result suggests us that the accessibility of quencher to the fluorophore (tryptophan residue) is better in organic solvent system than in water. In other words, tryptophan residue fluorescing predominantly is somehow buried in water environment and the accessibility by the quencher is then lowered. The same information about environment of the tryptophanyl residue(s), observed in the fluorescence spectral analysis of figure 1, was also revealed in the quenching experiment. Another important objective to carry out the quenching experiment is to obtain fluorescence lifetime (τ_F) of tryptophan residue in the proteolipid protein because a direct measurement of the lifetime was not possible in the nation. For the estimation of fluorescence lifetime from the quenching constant(K_q), the quenching rate constant(k_q) must be calculated at first. Under assumption that diffusion dominates the quenching process in our sample, we used the Debye equation ($k_q = \frac{8RT}{3\eta}$) to calculate k_q in the two different solvent systems. As we expected, k_q in organic solvent system was fairly higher than

Table 1. The various fluorescence parameters needed to calculate the molecular volume of the proteolipid protein using Perrin equation.

Sample \ Parameters	$K_q(M^{-1})$	$k_q(M^{-1}S^{-1})$	τ_F(nsec.)	A_o	V(Å^3)
Proteolipid in water	18.31	6.54×10^9	2.80	0.14	45258
Proteolipid in chloroform-methanol(2:1, v/v)	46.24	1.22×10^{10}	3.81	0.14	17608

K_q : Fluorescence quenching constant, $K_q = \tau_F.k_q$
k_q : Fluorescence quenching rate constant, $k_q = 8RT/3\eta$
τ_F : Fluorescence lifetime of tryptophan residue in the proteolipid proteins
A_o : Limiting anisotropic value
V : Molecular volume of the proteolipid proteins

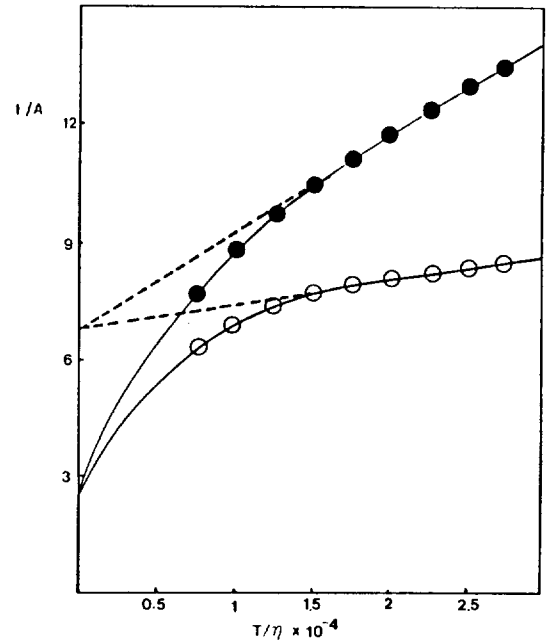

Figure 3. To generate the Perrin plot, the temperature was maintained constant at 20°C while the viscosity was varied by the addition of glycerol to aqueous solution (-○-○-) and organic solvent system (-●-●-) of the proteolipid protein.

the one in water (table 1). From these two values (K_q and k_q), fluorescence lifetimes (τ_F) of the tryptophan residue of the protein in the two different solvent systems were calculated (table 1) and were used to get the molecular volumes in the Perrin equation. When we compare the calculated lifetime value (τ_F = 2.80 nsec) of tryptophan residue of the proteolipid protein in water with the one in text[14], measured in the other proteins, our values are believed to be the right ones.

Fluorescence polarization is known to be the useful technique to determine the molecular size of the macromolecules becaues the polarization value is directly related with the molecular rotation. Molecular rotation is then correlated with the molecular size and shape. In our work we assume that the shape of the proteolipid protein is more or less globular and the overall shapes of the protein are not changed much by changing the solvent systems. Since nobody exactly

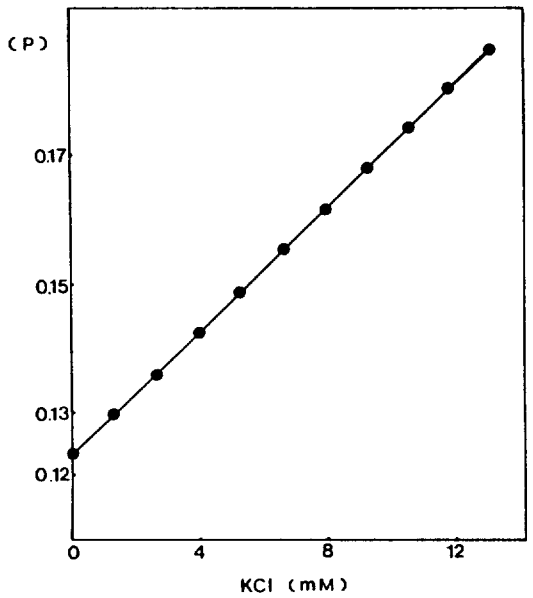

Figure 4. Plot for fluorescence polarization versus KCl concentration. Sample was the proteolipid protein in water.

knows the molecular shapes of the proteolipid protein and we know the usual membrane-bound proteins are almost globular shapes, the shape factor can be neglected in the calculation process of the molecular volume. One other factor to be considered here is the viscosity of the solvent. We made the viscosity of the solution be exactly identical by addition of glycerol to aqueous and organic solvent systems. Figure 3 shows the typical anisotropy plots versus the change of solution viscosity. The plots generate the curvature instead of the straight line. This result implies that there is some limited flexibility in the attachment of the fluorescent moiety to a macromolecule[15]. Extrapolation of the linear portion of the plots to $T/\eta = 0$ gives an apparent value for the limiting anisotropy ($A_o = 0.142$) and this value is used to determine the molecular volume in Perrin equation. The relationship between polarization(P) and anisotropy(A) is expressed as

$$(1/P-1/3)^{-1}=3A/2$$

and Perrin equation is expressed to be

$$(1/P-1/3)=(1/P_o-1/3)\left(1+\frac{\tau_F kT}{V\eta}\right)$$

$$1/A=1/A_o(1+\tau_F kT/V\eta)$$

where P: polarization value, P_o: limiting polarization value, τ_F: fluorescent lifetime, k = Boltzman constant, T: absolute temperature, V: molecular volume and η: viscosity of the solution. From the Perrin equation expressed by anisotropy, we get the equation: slope = $\frac{\tau_F k}{A_o V}$ The values of slopes estimated from the linear part of the plots in figure 3 are 0.6×10^{-4} and 2.1×10^{-4} in water and organic solvent systems, respectively. Using the values of τ_F obtained from the quenching experiment, the values of the limiting anisotropy(A_o), Boltzman constant and the values of slopes in the anisotropy plot, molecular volumes of the proteolipid protein in water and organic solvent are then estimated to be 45,258 Å^3 and 17,608^3, respectively. The volume of the protein in water is about 2.6 times bigger than one is organic solvent. The result

suggests that the molecular aggregation is certainly occurring in water environment and a trimerization of the proteolipid protein is likely forming by changing the solvent from chloroform-methanol to water. As we know that the main biological function of the plasma membrane proteolipid protein is the K^+ ion transport, we tried to investigate the K^+ ion effect to the molecular aggregation in aqueous system. Figure 4 shows the polarization plot versus KCl concentration in aqueous system. Polarization value was enhanced linearly as the K^+ ion concentration increases. This result is very interesting in connection with the oligomerization state and K^+ ion transport in the biological membrane. Nonetheless, any conclusive remarks can not be presented at the moment and further study on this subject should be carried out.

Summarizing the fluorescence spectral analysis and quenching experiment, tryptophan environment is more hydrophobic and buried in case of the water soluble form of proteolipid. Fluorescence polarization data present the strong evidence for oligomerization in aqueous system. In addition to the above results, the fact that a hydrophobic interaction is the predominant molecular interaction between the subunits of the proteins is known in general. Upon considering the results and the fact, we can carefully mention about the oligomerization process and state of the proteolipid in water as following. During the formation of the water soluble proteolipid, most of the exposed domain of the protein becomes hydrophilic to maintain the water solubility. But a certain part of the domain keeps the hydrophobic character for the formation of oligomer through the hydrophobic interaction. This hydrophobic domain may contain tryptophan residue or not. Even though tryptophan residue is located in this domain, it is still buried by other subunit and reveal a hydrophobic character after the oligomerization process. However, it is not easy to draw the exact molecular topography of the oligomer and more experimental evidences are required.

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Synthesis of an Intermediate for Hirsutene

Jong-Gab Jun*

Department of Chemistry, Hallym University, 1 Okchon-Dong, Chunchon 200-702

Bradford P. Mundy

Department of Chemistry, Montana State University, Bozeman, MT 59717, U.S.A.

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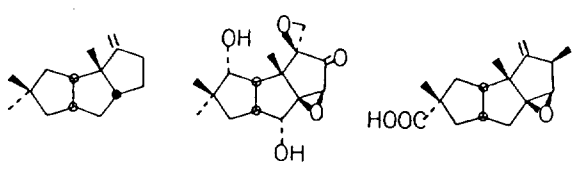
2,7,7-Trimethyl-cis-1,5-dicarboethoxybicyclo[3.3.0]octan-2-ol(**5**) is synthesized as a synthetic intermediate for hirsutene by use of dianion methodology.

Introduction

Hirsutene(**1**), the biogenetic precursor of coriolin(**2**) and hirsutic acid(**3**), is a tricyclic sesquiterpene hydrocarbon isolated from *Coriolus consers*[1].

In addition to the synthetic interest elicited by the skeletal features of these terpenoids, there exist a number of remarkable physiological properties associated with the coriolin-type sesquiterpenes.[2] The antibiotic and antitumor



1 **2** **3**

activities of coriolin and hirsutic acid dictate an efficient synthetic approach to these compounds, particularly in view of