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Motions and structures of chromatophore of a photosynthetic bacterium (*Chromatium vinosum*) as revealed from carbon-13 and phosphorus-31 NMR

Tsunenori Nozawa [a,*], Masaru Ohta [a], Masahiro Hatano [a], Hidenori Hayashi [b], Mitsuo Tasumi [b] and Keizo Shimada [c]

[a] *Chemical Research Institute of Non-aqueous Solutions, Tohoku University, Sendai 980,* [b] *Department of Chemistry, Faculty of Science, University of Tokyo, Bunkyo-ku, Tokyo 113, and* [c] *Department of Biology, Faculty of Science, Tokyo Metropolitan University, Fukazawa, Setagaya-ku, Tokyo 158 (Japan)*

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Carbon-13 enriched-chromatophore was prepared from a ^{13}C-enriched culture of a photosynthetic bacterium (*Chromatium vinosum*) with a purpose to investigate the dynamic structures of photosynthetic membranes and proteins by ^{13}C-NMR spectra which were observed in a relatively resolved state at 75.46 MHz (under a 7.05 T static magnetic field). This was in clear contrast with phosphorus-31 NMR spectra of chromatophore with broad band signals. The ^{13}C-NMR was assigned to signals essentially from lipids (mostly phospholipids with a small amount of galactolipids). Linewidths of ^{13}C-NMR signals of an intact chromatophore were narrowed on addition of detergents such as Triton X-100, sodium dodecyl sulfate (SDS), and lauryl-*N*,*N*-dimethylamine *N*-oxide (LDAO). Particularly the line-narrowing effect by Triton X-100 was remarkable among them. High resolution ^{31}P-NMR spectra of phospholipid in a chromatophore were observed only in the presence of detergents. Spin–lattice relaxation times (T_1's) of the lipid ^{13}C-NMR signals in chromatophore indicate a low mobility of the polar head groups of the lipids, which was interpreted to indicate the presence of the interactions between the polar head groups of the lipids with proteins.

Introduction

The photosynthetic apparatus of a phototrophic bacterium *Chromatium vinosum* is localized in a system of intracytoplasmic membranes [1] which, upon mechanical disruption, give rise to vesicles referred to as 'chromatophores' [2]. Chromatophore contains most of the light-harvesting (antenna) and reaction center bacteriochlorophyll (BChl)-proteins [3]. Dynamic structures of the membranes are of particular importance in relation to the organization of photosynthetic BChl proteins. Structures and motions of phospholipids in chromatophore have been investigated by electron-spin resonance (ESR) spectra [4,5], and by using broad band ^{31}P-NMR spectra [6].

In a previous paper [7] we applied a solid-state high-resolution (^{13}C cross-polarization/magic angle spinning)-NMR to chromatophore of a purple photosynthetic bacterium, where motions of both

* To whom correspondence should be addressed.

Abbreviations: BChl, bacteriochlorophyll; B890, an antenna bacteriochlorophyll-protein complex that absorbs at 890 nm; CMC, critical micellar concentration; CTAB, cetyltrimethylammonium bromide; DSS, sodium-2,2-dimethyl-2-silapentane-5-sulfate; LDAO, lauryl-*N*,*N*-dimethylamine *N*-oxide; *N*, the number of protons directly attached to a particular carbon; NMR, nuclear magnetic resonance; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; RC, reaction center; SDS, sodium dodecyl sulfate; S/N, signal-to-noise ratio; T_1, spin–lattice relaxation time; T_2, spin–spin relaxation time; τ_c, correlation time; Triton X-100, α-[4-(1,1,3,3-tetramethylbutyl) phenyl]-ω-hydroxypoly(oxy-1,2-ethanediyl) (*p-t*-octylphenylpolyoxyethylene ether).

proteins and lipids were discussed. However, the ^{13}C cross-polarization/ magic angle spinning NMR can be applied only for freeze-dried or frozen samples up to now, the motions in native states can be investigated only by NMR of solution states. In an accompanying paper (Ref. 24) effects of detergents on an antenna BChl-protein have been investigated by ^{13}C-NMR and CD spectroscopies, where the ^{13}C-NMR signals of a B890 protein (the antenna BChl protein which absorbs maximally at 890 nm) were observed. The results were discussed on the basis of the amino acid sequence data of the B890 protein [8]. On the other hand the solution NMR of chromatophore predominantly gave ^{13}C-NMR signals from the membrane lipids even for a ^{13}C-enriched sample. However, since interactions between constituents in membranes and proteins may be reflected on dynamic behavior of membranes (phospholipids), we explored the interactions from the dynamic structures of a photosynthetic membrane on the basis of the lipid ^{13}C- and ^{31}P-NMR by using detergents as perturbants. Sicne the resolutions of the lipid ^{13}C- and ^{31}P-NMR were high enough to identify respective resonances, we were able to examine the dynamic structures of the constituents. The revealing interaction of polar head groups of phospholipids with proteins was suggested to be an important factor for the organization of photosynthetic membrane and protein systems.

Materials and Methods

Materials. C. vinosum was cultured anaerobically under continuous illumination with a 60 W incandescent lamp at 10 cm distance for 3 or 4 days at 30°C in the medium reported previously [9,10], except $NaHCO_3$ which was replaced by $NaH^{13}CO_3$. Chromatophore was prepared as described previously [11]. Harvested cells were suspended in a 50 mM buffer of 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) buffer containing 5 mM thioglycolic acid and exposed to sonic oscillation of 20 kHz for 10 min by a Branson, Model 185 sonifier. About 2 g (wet weight) of cell was dissolved in 20 ml of the same Tris buffer and was subjected to 5 times of 2 min of sonication (40–50 W) in an ice bath with 2 min cooling times between each run. After removal of debris by low-speed centrifugation (15000 × *g*, 15 min), a crude chromatophore preparation was collected by high-speed centrifugation (100000 × *g*, 80 min). The crude chromatophore preparation was resuspended in 20 ml of the same Tris buffer and subjected to sonic oscillation (40–50 W) of 20 kHz for 4 min (2 times of 2 min) in an ice bath. The chromatophore preparation was prepared by low-speed centrifugation followed by high-speed centrifugation from the crude chromatophore preparation. During all procedures the temperature was kept at 0–4°C. Phospholipids were extracted from chromatophore with dichloromethane-methanol (2 : 1, v/v) and purified by gel filtration on Sephadex LH60 (Pharmacia).

$NaH^{13}CO_3$ (95% enrichment) was purchased from the Commissariat à l'Énergie Atomique, Saclay, France. Triton X-100, SDS, and dipalmitoylphosphatidylethanolamine and dipalmitoylphosphatidylglycerol were purchased from Sigma Chemicals Co. Ltd. LDAO was obtained from Fluka Chemicals Co. Ltd.

Methods. ^{13}C- and ^{31}P-NMR spectra were obtained on a Bruker CXP-300 FT NMR spectrometer in conjunction with a Bruker broad band (BB) probe which covers a 30–125 MHz range. The observations were carried out under internal ^{2}H-lock using deuterated solvents with 10 mm tubes. Broad band decoupling of 2 W was applied to all measurements. For ^{13}C-NMR an observation bandwidth of 31.25 kHz was used with a flip angle of 30° (a pulse-width of 10 μs), a recycle time of 1.0 s and an accumulation of 10000 transients. A spin-lattice relaxation time (T_1) was observed by a conventional invertion recovery method [12]. Interpulse times were varied from 100 ms to 5 s with a recycle delay of 10 s. A 90° pulse was 30 μs and the number of accumulation was 1000. For ^{31}P-NMR an observation bandwidth of 6024 Hz was used with a flip angle of 30° (a pulse-width of 9 μs), a recycle time of 1.0 s and an accumulation of 3600 transients. A Bruker temperature controller (B-VT-1000) was employed and calibrated by measuring the frequency differences between the ^{1}H-NMR resonances of ethyl alcohol [13]. The chemical shifts are referred to sodium-2,2-dimethyl-2-silapentane-5-sulfate (DSS) for ^{13}C-

NMR, and 85% orthophosphoric acid for ^{31}P-NMR. The high-magnetic field sides of the NMR were plotted on the right side in all spectra.

Results

^{13}C-NMR spectra of chromatophore in buffer solutions

Fig. 1 shows a ^{13}C-NMR spectrum of a ^{13}C-enriched chromatophore in a 5 mM Tris $^{2}H_2O$ buffer solution. The ^{13}C-NMR spectrum with an excellent signal-to-noise ratio (S/N) can be obtained only for a ^{13}C-enriched chromatophore. From comparison of this spectrum with that of the extracted lipid in deuterated chloroform-methanol (1 : 1, v/v), it was found that the spectrum mainly displays phospholipid carbon resonances. Assignments of the signals were given in Fig. 1. In the magnetic field higher than 40 ppm relative to DSS the alkyl groups from the fatty acids of the lipids display the ^{13}C signals. The glycerol backbones and the polar head groups gave signals between 40–75 ppm. The signals at 78 and 102 ppm come from galactolipids which are minor components of the lipids of the photosynthetic bacterium. Resonances in the range of 120–140 ppm are assigned to aromatic and olefinic carbons of the fatty acid chains. Peaks in the region from 170 to 180 ppm are due to carbonyl carbons of the fatty acid chain.

Addition effects of detergents were observed for Triton X-100, SDS, LDAO, and a typical result was shown for Triton X-100 in Fig. 2. Triton X-100 in a concentration of 0.8 mM (0.05%, v/v) gave a significant effect on the linewidths of the ^{13}C-NMR signals of chromatophore (Fig. 2). On the other hand SDS shows somewhat weaker addition effects (not shown). SDS in a concentration of 7 mM (0.2%, v/v) induced a narrowing of the linewidths. The addition effect of LDAO was intermediate between those of Triton X-100 and SDS (not shown). In Fig. 3 the detergent effects on the linewidths were compared for the apparent linewidth of the methylene resonance of the fatty acid. The sharp effect of Triton X-100 was remarkable as compared with those of SDS and LDAO.

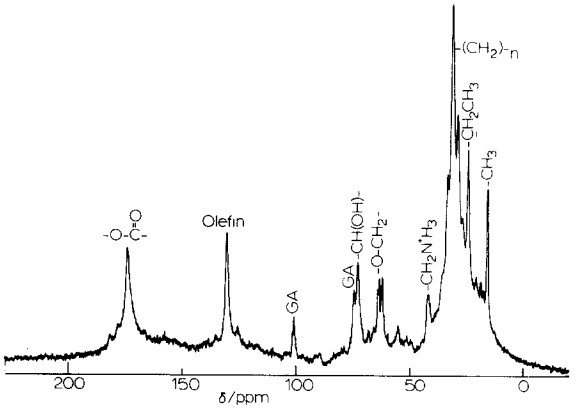

Fig. 1. ^{13}C-NMR spectrum of ^{13}C-enriched intracytoplasmic membrane (chromatophore) of *C. vinosum* at 303 K in a $^{2}H_2O$ buffer with 5 mM Tris and 0.5 mM thioglycolic acid. Concentration: $A_{800} = 15$; flip angle = 30°; recycle time = 1.0 s; number of transients = 10000; observed bandwidth = 31250 Hz. The observation was done under ^{2}H-lock. A broad band decoupling of 2 W was applied. The chemical shifts are referred to DSS. A line broadening of 10 Hz was applied to the spectrum. GA means galactolipid.

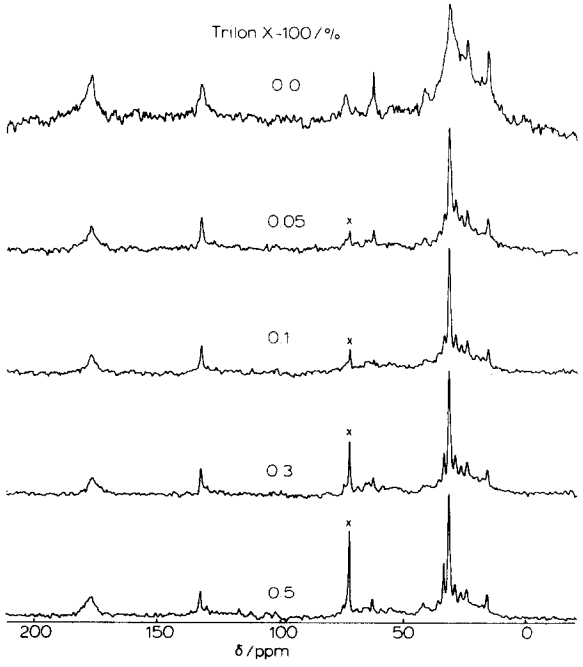

Fig. 2. Addition effect of Triton X-100 on the ^{13}C-NMR spectrum of ^{13}C-enriched intracytoplasmic membrane (chromatophore) of *C. vinosum* at 303 K in a $^{2}H_2O$ buffer with 5 mM Tris and 0.5 mM thioglycolic acid. Other experimental conditions are the same as those in Fig. 1. Signals with x were derived from Triton X-100.

Spin-lattice relaxation times for respective carbon signals in chromatophore were obtained by the invertion recovery method. The lipids of *C. vinosum* are mainly composed of phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) [14]. A typical result was shown in Fig. 4 for PE in chromatophore. A similar result was obtained for PG in chromatophore (not shown). The horizontal axis represents NT_1 values, where N is the number of the protons directly bound to a particular carbon. The NT_1 value is related to the motional behavior of the carbon atom, because the relaxations are mostly determined by the interactions with the attached protons [15]. The vertical axis expresses types of carbon atom. The T_1 values of the polar head group carbons on the bottom. The variation of NT_1 values of the presence of detergents is also included in Fig. 4.

^{31}P-NMR spectra of chromatophore in buffer solutions

^{31}P-NMR spectra of chromatophore of *C. vinosum* were observed in the absence and presence of Triton X-100, LDAO, SDS, and cetyltrimethylammonium bromide (CTAB). A typical result was shown in Fig. 5 for Triton X-100. In the absence of detergents no peak was observed in the chemical shift range of phospholipids. On addition of detergents, the -0.4 and -1.0 ppm signals which can be attributable to PE and PG, respectively, began to appear. This assignment was derived from the comparison of the ^{31}P-NMR signals for the extracted lipids with those of authentic samples of PG and PE in deuterated chloroform-methanol (1 : 1, v/v). It was seen that the concentrations of detergents which made ^{31}P-NMR of PG and PE appear, were dependent on the kind of detergents. The concentration was the lowest for Triton X-100, the highest for SDS, and the intermediate for LDAO and CTAB. Also it is interesting to point out that Triton X-100 made both PG and PE signals appear equally, and LDAO somewhat preferentially worked on PG, while in the case of CTAB, the ^{31}P-NMR signal of PE appeared first, and in the case of SDS that for PG emerged first. In any case ^{31}P-NMR signals of both PE and PG are present in the nearly same magnitudes on addition of enough detergents.

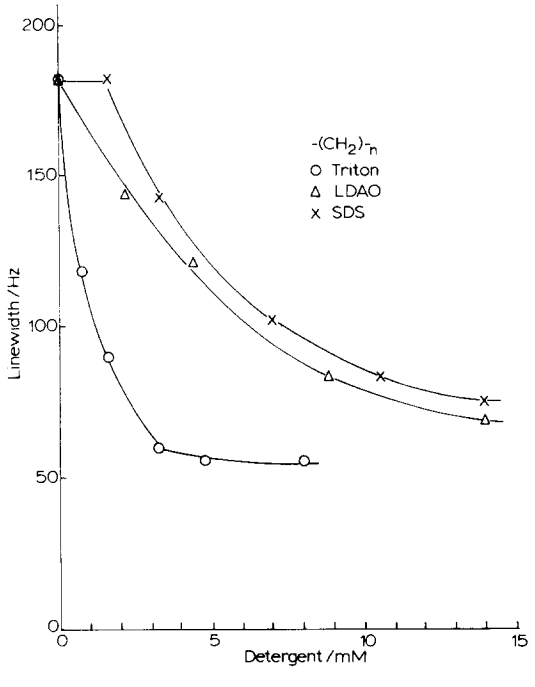

Fig. 3. Variation of the linewidth on the concentration of detergents for the ^{13}C-NMR signal of the methylene carbon of the fatty acid at 29 ppm.

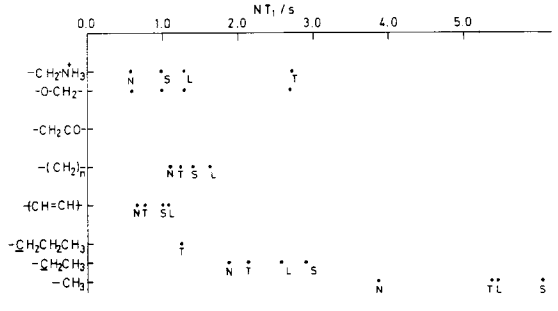

Fig. 4. Spin–lattice relaxation time (T_1) of phosphatidylethanolamine in ^{13}C-enriched chromatophore of *C. vinosum*, and their dependence on the detergents. N is the number of the protons directly bound to the particular carbon. The T_1 values were measured by the invertion recovery Fourier transform NMR method. The 90° pulse was 30 μs, the interpulse times were varied from 100 ms to 5 s. The 1000 transients were accumulated for each interpulse time with a recycle delay of 10 s. The letters N, T, L and S represent no addition, in the presence of Triton X-100, LDAO and SDS, respectively.

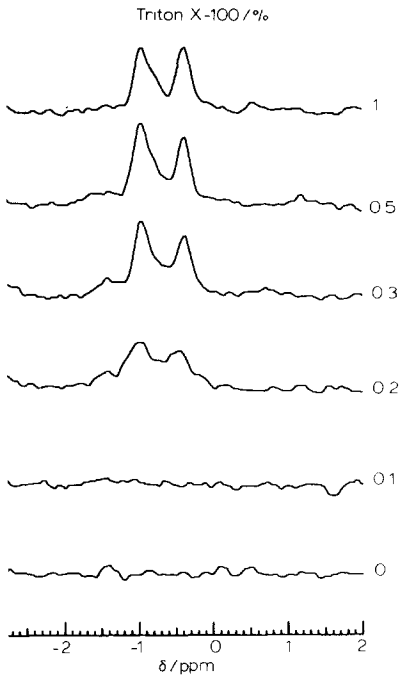

Fig. 5. Addition effect of Triton X-100 on ^{31}P-NMR spectrum of chromatophore of *C. vinosum* at 303 K in a $^{2}H_2O$ buffer with 5 mM Tris and 0.5 mM thioglycolic acid. Concentration: $A_{800} = 15$; flip angle = 30°; recycle time = 1 s; number of transients = 3600; observed bandwidth = 6024 Hz. The observation was done under ^{2}H-lock. A broad band decoupling of 2 W was applied. The chemical shifts are referred to 85% orthophosphoric acid. A line broadening of 10 Hz was applied to the spectra.

Discussion

Motions of lipids and linewidths of ^{13}C- and ^{31}P-NMR spectra in the absence and presence of detergents

To our knowledge this paper will be the first report on a solution ^{13}C-NMR of a photosynthetic membrane. The low NMR sensitivity of the ^{13}C nucleus precluded the solution ^{13}C-NMR observation of the photosynthetic membrane with ^{13}C of natural abundance. We have succeeded in preparing a ^{13}C-enriched chromatophore from a ^{13}C-enriched culture of *C. vinosum*. Somewhat unexpectedly, the solution ^{13}C-NMR of the ^{13}C-enriched chromatophore yielded relatively resolved signals. This is clearly in contrast with a solution ^{31}P-NMR of an intact chromatophore, where a broad signal with a chemical shift anisotropy of about 30 ppm was observed (not shown). The large chemical shift anisotropy of the ^{31}P nucleus as compared with that of the ^{13}C nucleus induced a broad ^{31}P-NMR spectrum for an intact chromatophore in a bilayer membrane [6,17]. On the other hand the small chemical shift anisotropy of the ^{13}C nucleus and the relatively fast local molecular motions yielded relatively resolved ^{13}C-NMR signals for the intact chromatophore. The obtained solution NMR predominantly gave the ^{13}C-NMR signals from the membrane lipids even for the ^{13}C-enriched chromatophore. This may be caused by slow local molecular motions of membrane proteins which make the NMR signals broad. In fact we could observe ^{13}C-NMR signals of proteins from an isolated ^{13}C-enriched B890 (the results in the accompanying paper: Ref. 24). Solution ^{13}C-NMR spectra of chromatophore with enough detergents are very similar to those of an aqueous dispersion of the extracted phospholipids with detergents (not shown). This also supports that only the lipid ^{13}C signals were observed by the solution ^{13}C-NMR of chromatophore, and that the membrane proteins still associate together to prevent free movements of their constituent proteins. The ^{13}C-NMR signals of the intact chromatophore have rather broad linewidths as compared with normal solution ^{13}C-NMR signals. An apparent spin–spin relaxation time (T_2^*) value was evaluated from the linewidth ($\Delta\nu$) [19,20] of the ^{13}C signal by the first part of the following relation

$$2\pi\Delta\nu = \frac{1}{T_2^*} = \frac{N\gamma_H^2\gamma_C^2\hbar^2}{5r_{HC}^2}\tau_C \qquad (1)$$

where N is the number of protons directly attached to the particular carbon, γ_H, and γ_C are the gyromagnetic ratios for ^{1}H and ^{13}C nuclei, r_{HC} is the proton–carbon distance, τ_c is the correlation time of motions. In this equation only the dipolar contribution to the spin–spin relaxation rate is considered. For the methylene carbons in the fatty acid chain of the lipid the apparent linewidth of 200 Hz yields the T_2^* value of 1.6 ms, which gives the rotational correlation time of $3 \cdot 10^{-8}$ s, from the second part of Eqn. 1. On the other hand a rigid-body tumbling rate can be calculated from

the Stokes–Einstein relation by use of a diameter of chromatophore:

$$\tau_c = \frac{4\pi\eta R^3}{3k_B T} \quad (2)$$

(η, the viscosity of the chromatophore solution; R, the radius of chromatophore; k_B the Boltzmann constant; T, an absolute temperature). With $\eta_{H_2O} = 1.347$ cP at 283 K [17] and $R = 50$ nm [2], the tumbling correlation time (τ_c) of chromatophore was estimated to be $1.7 \cdot 10^{-4}$ s. This value is 4 orders of magnitude too large as compared with that estimated from the linewidth of the ^{13}C-NMR signal from Eqn. 1, which indicates that the overall tumbling motion is not enough to explain the observed T_2^* value, and that there exist active local molecular motions that control the spin–spin relaxation time of the fatty acid methylene carbons of the lipid in chromatophore. We can exclude the possibility that the lipids which gave the resolved ^{13}C-NMR are the minor components with much smaller radii, and the vesicles with 50 nm radius still gave a broad ^{13}C-NMR band underneath the resolved signals, because the absolute NMR intensity did not change much in the absence and presence of excess detergents, and because even if the radius is 10 nm, the τ_c value by the tumbling motion is $1.36 \cdot 10^{-6}$ s, which is still too large as compared with the value estimated from the T_2^* value.

Addition of detergents rapidly decreased the linewidths of the ^{13}C-NMR signals of chromatophore. The effect of Triton X-100 was remarkable as compared with those of SDS and LDAO. One of the reasons for this may exist in the values of the critical micellar concentration (CMC), which are 0.24 and 10 mM for Triton X-100 and SDS, respectively [21]. In addition to this the following two reasons will be considered. Triton X-100 appeared to have solubilized ability for both PE and PG, while SDS and LDAO preferentially for PE and PG, respectively, as revealed from the ^{31}P-NMR results. Also Triton X-100 seemed to have ability to weaken the polar head group interactions of the lipids with themselves and/or with proteins as discussed in the detergent effects on NT_1 values. The linewidth (50 Hz) of the ^{13}C signals of the methylene carbons in the presence of Triton X-100 gives the T_2^* value of 6.4 ms, which yields the correlation time of $1.2 \cdot 10^{-9}$ s. The detergent will make the lipid bilayer into micelles. As stated above the micelles with a radius of 10 nm should have the tumbling correlation time of $1.36 \cdot 10^{-6}$ s, which is critically different from that estimated from the linewidth. This indicates again that extra motions in the fatty acid chains other than a tumbling motion control the spin–spin relaxation time, and that the micellar size difference among detergents used cannot be a critical factor for the linewdith discussion.

Molecular motions of the glycerol backbones and the polar head groups in lipids can be explored directly from ^{31}P-NMR spectra. An intact chromatophore of *C. vinosum* gave a broad ^{31}P-NMR band with the chemical shift anisotropy of about 30 ppm (not shown). The chemical shift anisotropy of a ^{31}P-NMR signal of *R. rubrum* G-9 chromatophore was determined to be 25 ppm [6]. In the absence of detergents no high resolution peaks were observed in the chemical shift region for PE and PG. Addition of 3.2 mM of Triton X-100 made the ^{31}P-NMR signals appear for PE and PG, 6.9 mM of SDS for PE first, 5.5 mM CTAB for PG first, and 8.7 mM LDAO for PG relatively preferentially. In the presence of enough detergents a ^{31}P-NMR linewidth (36 Hz) gave the correlation time of about $1.6 \cdot 10^{-6}$ s [6]. This agrees with those estimated from the Stokes–Einstein equation with $R = 10$ nm ($1.36 \cdot 10^{-6}$ s). Thus, the T_2^* values of the ^{31}P nuclei in the presence of enough detergents can be interpreted mainly by the overall tumbling motion. This is in contrast with the results for the ^{13}C nuclei, where the local molecular motions other than the overall tumbling motion are the main mechanism for the relaxations. These results indicate that the individual motion of the polar head group in vesicles is restricted as compared with that of the fatty acid chain, and the polar head group essentially moves with vesicles as a whole.

Motions of lipids from spin–lattice relaxation times (T_1's) of chromatophore

Even for the ^{13}C-enriched samples, the dipole-dipole interaction between C–H is the main relaxation mechanism as is usual for ^{13}C-NMR, because of the longer C–C distances as compared with those of C–H and the smaller γ_C value than

the γ_H one. The NT_1 value is inversely proportional to the correlation time of rotation. A larger NT_1 value, therefore, corresponds to a smaller correlation time which means a faster molecular motion. Fig. 4 showed that in the native chromatophore the polar head group showed a very small NT_1 value as compared with that of the solubilized phospholipids systems [16,22,23]. Thus, the phospholipid in a micelle or aqueous dispersion, the polar head groups had the relatively larger NT_1 values than the glycerol backbones. This means that the rotational motions of the polar head groups are restricted in the native chromatophore. Therefore lipid and protein interactions are considered as the main factors for the smaller NT_1 values of the polar head group carbons. The effect of Triton X-100 which selectively breaks the interactions between the lipid polar head group and the proteins are particularly of note. On the other hand the SDS and LDAO had rather smaller effects for the polar head group interaction. The main lipid components of chromatophore of *C. vinosum* are phosphatidylglycerol and phosphatidylethanolamine. Taking account of the detergent effects on the ^{31}P-NMR spectra, we can conclude that the anionic detergent SDS weakly interacted with the anionic lipid PG, and the cationic detergent CTAB favorably interacted with the anionic lipid PG. The zwitter type detergent LDAO somewhat preferentially worked on PG. The electrostatic type effects worked on the selective action of detergents on the phospholipid are particularly of note.

In conclusion, dynamic structures of chromatophore in a photosynthetic bacterium (*C. vinosum*) were elucidated by detergent effects on ^{13}C- and ^{31}P-NMR spectra. Presence of interactions of lipid polar head groups with proteins were suggested from comparison of linewidths and NT_1 values of the lipid carbon signals of chromatophore in the absence and presence of detergents. The interactions were diminished by detergents, and peculiar effects of Triton X-100 was discovered in these systems.

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