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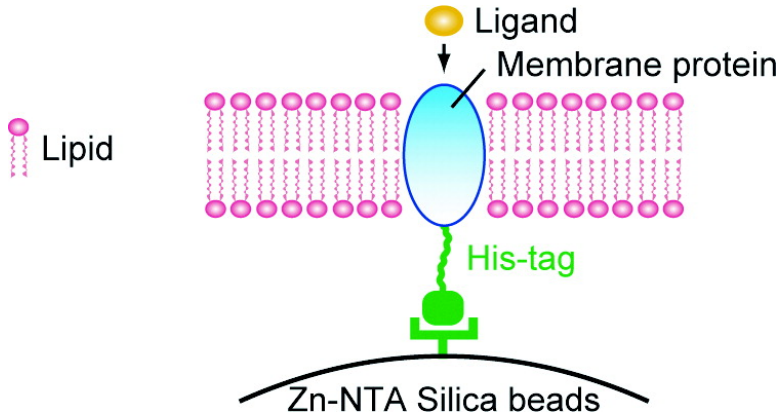

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Bead-linked Proteoliposomes: A Reconstitution Method for NMR Analyses of Membrane Protein−Ligand Interactions

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Abstract: Structural information about the interactions between membrane proteins and their ligands provides insights into the membrane protein functions. A variety of surfactants have been used for structural analyses of membrane proteins, and in some cases, they yielded successful results. However, the use of surfactants frequently increases the conformational instability of membrane proteins and distorts their normal function. Here, we propose a new strategy of membrane protein reconstitution into lipid bilayers on affinity beads, which maintains the native conformation and function of the protein for NMR studies. The reconstituted membrane proteins are suitable for NMR analyses of interactions, by using the transferred cross-saturation method. The strategy was successfully applied to the interaction between a potassium ion channel, KcsA, and a pore-blocker, agitoxin2 (AgTx2). This strategy would be useful for analyzing the interactions between various membrane proteins and their ligands.

Introduction

Protein−protein interactions are essential in many biological processes, such as signal transduction, cellular recognition, and the immune response. In particular, membrane proteins, which represent 20−30% of the total proteins encoded by the human genome,[1] play important roles through their interactions with intra- and extracellular ligands and the transmission of information across membranes. Even with the recent expansion of structural genomics[2−5] and the general appreciation of their biological significance, little is known about the structures and functions of membrane proteins. This is mainly due to practical problems in handling membrane proteins. One common problem is the poor stability of membrane proteins, under conventional experimental conditions. Although surfactant-solubilized membrane proteins have been used in structural analyses,[6] the solubilization of membrane proteins frequently causes conformational instability, followed by aggregation or denaturation.[7,8] Furthermore, the instability renders many membrane proteins biochemically intractable and precludes high-resolution structure determinations.

It is generally accepted that membrane proteins maintain their native conformation more stably in lipid bilayers than in a surfactant-solubilized environment,[9−11] and some membrane proteins are known to require specific phospholipids to maintain their structure and function.[12,13] Therefore, in molecular biological analyses, biomembranes where membrane proteins of interest are inherently expressed or engineered to be expressed have been used. In contrast, several membrane-protein-reconstitution techniques for investigating the functions of membrane proteins, such as liposome incorporation[14] and reconstitution on chips[15,16] and on beads,[17] have been utilized to artificially reconstitute membrane proteins into lipid bilayers. However, primarily due to the heterogeneity of the reconstituted samples, containing lipid bilayers and membrane proteins, it is difficult to apply these strategies to structural analyses using X-ray crystallography. Although NMR, another powerful tool for structural analyses of biomolecules, tolerates targeting-system heterogeneity, it has not been recognized as an ideal tool for structural analyses of membrane proteins. The main drawback is its low

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sensitivity, which necessitates the use of large amounts of purified membrane proteins, and the molecular-weight limitations in NMR measurements. In a conventional NMR analysis, the molecular weight of the target protein has to be less than 150 kDa[18,19] and the protein concentration should ideally be more than 1 mM. Although several surfactant-solubilized membrane proteins have been successfully analyzed,[20−23] membrane proteins reconstituted in lipid bilayers obviously exceed the molecular weight limitations. Furthermore, the low concentrations achieved by conventional reconstitution strategies are insufficient for carrying out an extensive structural analysis.

We recently proposed a novel NMR method, termed transferred cross-saturation (TCS), for identifying contact residues in a large protein complex.[24] In this method, the molecular weight limitation is overcome by properly transferring information about the contact residues on the ligand proteins in the complex, from the ligand proteins in the bound state to those in the free state, under a fast exchange process. We have already demonstrated the effectiveness of this method for investigating the protein interactions of extremely large proteins, liposomes, and insoluble biomolecules.[25−27] However, the development of a sample preparation strategy that ensures the incorporation of a sufficient amount of membrane protein into lipid bilayers has remained elusive.

Here, we propose a novel strategy that reconstitutes membrane proteins linked to beads, where the membrane proteins are embedded in lipid bilayers. In combination with the TCS method, the bead-linked proteoliposomes facilitate the identification of the contact residues on ligand proteins interacting with a wide variety of membrane proteins.

In the present paper, we prepared bead-linked proteoliposomes, composed of a potassium channel, KcsA, embedded in the lipid bilayer (KcsA-proteoliposomes), and used them to investigate the interaction between KcsA and its pore-blocking peptide, agitoxin2 (AgTx2). Analyses of the KcsA-proteoliposomes with the TCS method allowed us to successfully identify the KcsA binding surface on AgTx2.

Materials and Methods

Materials. Ni−NTA silica beads were purchased from QIAGEN. All phospholipids, including the fluorescently labeled phospholipids, were obtained from Avanti Polar Lipids as chloroform solutions. Oregon green 488 maleimide (OGM) was purchased from Molecular Probes Inc. Other chemicals were obtained from Wako Chemicals, Nacalai Tesque, and Dojindo. Expression, purification, and stable-isotope labeling of KcsA and AgTx2 were carried out as described.[26]

Preparation of Zn−NTA Beads. NTA silica beads were utilized as the base of the KcsA-proteoliposomes. After removal of the prechelated Ni^{2+} ions from the Ni−NTA silica beads with 0.3 mM ethylenediaminetetraacetic acid (EDTA) and extensive washing with H_2O, the beads were charged with Zn^{2+} ions by an incubation in 100 mM $ZnSO_4$. The excess Zn^{2+} ions were removed with H_2O to create the Zn−NTA beads.

Preparation of Lipid Solutions. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), mixed with 0.2% (by weight) 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(Lissamine Rhodamine B Sulfonyl) (rhodamine-DOPE), was dried by rotary evaporation using a round-bottomed flask to yield a thin lipid film. The film was then thoroughly dried under a vacuum for at least 3 h. Buffer A, containing 20 mM Tris-HCl (pH 8.0) and 150 mM KCl, was added to the lipid film and vortexed vigorously under a nitrogen atmosphere, which typically yielded a 16 mg/mL lipid solution. The lipid solution was solubilized by adding 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), at final concentrations of 120 mM for POPE and 85 mM for POPC.

Construction of the KcsA-Proteoliposomes. The purified KcsA solution (20 μM) was incubated with the Zn−NTA beads in a 1.5 mL tube for 2 min. After mild centrifugation, the supernatant was removed. These processes were repeated until the desired amount of KcsA was immobilized on the beads. After the immobilization procedure, *n*-dodecyl-β-D-maltoside (DDM) was exchanged for CHAPS. This exchange procedure might be important for the successful organization of the phospholipid bilayers on the beads, because the higher critical micelle concentration (CMC) of CHAPS (6−10 mM) would be more advantageous for efficient dialysis than the lower CMC of DDM (<0.2 mM). Immediately after the exchange procedure, the KcsA-immobilized beads were mixed with five bed volumes of lipid solution and transferred into a 3.5 K molecular weight cutoff dialysis membrane. The surfactants were removed by dialysis for 12 h at room temperature against buffer A. The residual unbound phospholipids and surfactants were removed by a washing procedure.

Analysis of the Protein to Lipid Composition in the KcsA-Proteoliposomes. The quantity of KcsA in the KcsA-proteoliposomes was calculated from the 280 nm absorbance of the KcsA (ϵ = 34 850 M^{-1} cm^{-1}) solution, before and after the immobilization procedure. The amount of the phospholipids was calculated from the fluorescent intensity of rhodamine−DOPE. The surface area of the beads was analyzed by the multiple point Brunauer−Emmett−Teller (BET) method, using the ASAP2400 analyzer (Micromeritics Instruments). The surface area occupied by the KcsA molecules on the beads was calculated from the dimensions of KcsA. The approximate area occupied by one lipid molecule in the bilayer membrane was estimated to be 60 $Å^2$.[28]

Analyses of Lipid Bilayers in the KcsA-Proteoliposomes. The organization of the lipid within the KcsA-proteoliposomes was analyzed by differential scanning calorimetry (DSC) and ^{31}P NMR. The conditions of the ^{31}P NMR experiments are mentioned below, in the NMR Spectroscopy section. DSC measurements were performed with VP-DSC (MicroCal Inc.) and DSC3300SA (Bruker AXS Inc.) scanning calorimeters. Three samples (the KcsA-proteoliposomes, the Zn−NTA silica beads without the reconstitution procedure, and the liposomes) were analyzed. For the analyses of the KcsA-proteoliposomes and the Zn−NTA silica beads, the same amounts of Zn−NTA silica beads were used as the reference. The beads were suspended in buffer C (20 mM acetate (pH 6.0) and 150 mM KCl) at 35 μL of beads/mL of buffer. When the pure liposomes were analyzed, a 20 mM POPE liposome

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solution containing 0.2% rhodamine−DOPE was dispersed in buffer C, and that buffer without phospholipids was used as the reference.

Ligand-Binding Properties of the KcsA-Proteoliposomes. The binding of AgTx2 to KcsA in the KcsA-proteoliposomes was analyzed by confocal laser scanning microscopy (CLSM, Olympus). For this purpose, the AgTx2_D20C mutant was conjugated with a thiol-reactive fluorescent reagent, OGM, (AgTx2_OGM). Two types of KcsA-proteoliposomes, which contained the same amount of the V84K or R64D mutant of KcsA and the pure Zn−NTA silica beads, were utilized in the experiment. They were all equilibrated with buffer D (20 mM Tris-HCl (pH 8.0), 5 mM KCl, 140 mM NaCl) and were subsequently incubated with a sufficient quantity of AgTx2_OGM (0.1 mM) for a few minutes. After a moderate washing procedure, the beads were directly observed by CLSM.

NMR Spectroscopy. We utilized a Bruker DRX 400 spectrometer for the ^{31}P NMR measurements and an Avance 600 spectrometer for the TCS experiments. NMR samples containing the KcsA-proteoliposomes were fully packed in a 5 mm symmetrical microtube matched with D_2O (Shigemi). In the^{31}P NMR measurements, ^{31}P (162.02 MHz) spectra were acquired using a small flip angle ^{31}P pulse (2 μs) and a relaxation delay of 70 ms between scans. The TCS experiments were carried out as described in the previous study.[24] The total number of scans was 344 and the measurement time was 48 h, with a relaxation delay of 2.6 s and a saturation delay of 0.4 s. A 1 mM concentration of ^{2}H, ^{15}N-labeled AgTx2 in complex with 20 mol % (0.2 mM/bead volume) of the nonlabeled wild-type or V84K mutant of KcsA, reconstituted with POPE or POPC, was dissolved in buffer E (2 mM MES (pH 6.0), 200 mM NaCl, and 5 mM KCl) composed of 20% H_2O/80% D_2O. We selectively saturated the KcsA-proteoliposomes by irradiation of the aliphatic proton resonances, using a WURST-2 broadband decoupling scheme.[29] The maximum rf amplitude was 0.17 kHz for WURST-2 (the adiabatic factor $Q_0 = 1$). The saturation frequency was set at 0.8 ppm. Since the saturation scheme is highly selective for the aliphatic proton region, the irradiation of the KcsA-proteoliposome/AgTx2 complex affected neither the intensities of the AgTx2 amide protons nor the water resonance. All spectra were processed with the programs XWINNMR and nmrPipe.[30]

Results

Preparation of the KcsA-Proteoliposomes. We designed bead-linked KcsA-proteoliposomes, in which the channels linked to the beads were surrounded by lipid bilayers (Figure 1). The KcsA protein, containing a decahistidine tag at the N-terminus, was expressed in *Escherichia coli*. Then, the channels were solubilized by DDM and purified by Ni−NTA affinity chromatography. Immediately after the purification, the KcsA protein was immobilized by virtue of the affinity between the N-terminal decahistidine tag and the metal-chelating affinity beads. While Ni^{2+} and Co^{2+} ions are widely used for this purpose, these paramagnetic ions cause significant line-broadening of the NMR signals. Thus, we prepared Zn−NTA affinity beads from commercially available Ni−NTA silica beads, by exchanging the chelating metal ion from Ni^{2+} to diamagnetic Zn^{2+}. The noncovalent interaction allowed stable immobilization of the channel on the Zn−NTA beads. The maximum amount of protein absorbed on the Zn−NTA beads was identical with that on the Ni−NTA beads (data not shown).

Protein Composition of the KcsA-Proteoliposomes. The quantity of the KcsA incorporated into the proteoliposomes was calculated from the difference in the absorbance of the KcsA solutions at 280 nm before and after the incubation with the

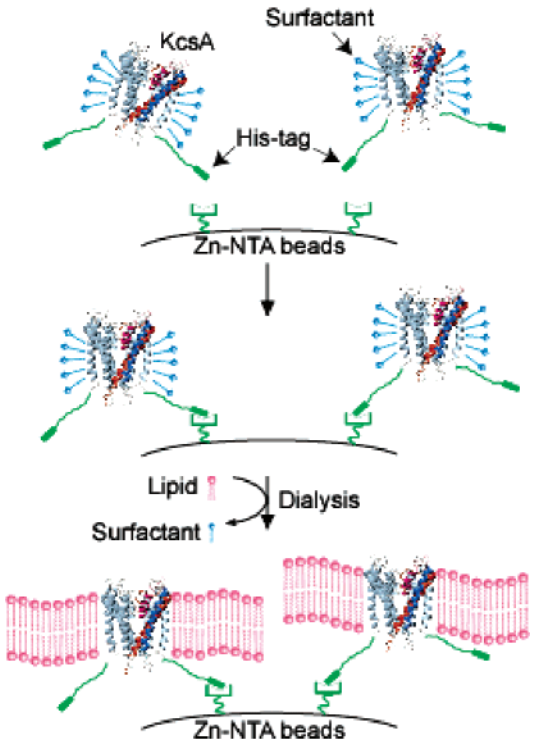

Figure 1. Schematic representation of the preparation of KcsA-proteoliposomes. Purified KcsA, harboring a decahistidine tag at its N-terminus, was used in this study. *n*-Dodecyl-β-D-maltoside (DDM)-solubilized KcsA was incubated with Zn−NTA silica beads to immobilize KcsA noncovalently, but firmly, on the surface of the porous silica beads. The KcsA-immobilized beads were mixed with a surfactant-solubilized lipid solution containing 0.2% rhodamine−DOPE. The lipids became organized during the dialysis procedure and formed a lipid bilayer around the KcsA molecule.

Zn−NTA beads. The KcsA was firmly immobilized on the Zn−NTA beads during the reconstitution procedure, in which the beads were exposed to a high-concentration lipid−surfactant mixture. A 15 mg amount of KcsA was immobilized/mL of Zn−NTA beads, corresponding to 0.2 μmol of KcsA/mL of beads.

Analysis of the Lipid Incorporated in the KcsA-Proteoliposomes. To determine the amount of phospholipids incorporated into the KcsA-proteoliposomes, a lipid solution, containing 0.2% rhodamine−DOPE, was used in the experiments. We resolubilized the incorporated phospholipids with a solubilization buffer, containing 200 mM DDM and 800 mM imidazole, and measured the fluorescence intensity of the rhodamine−DOPE in the solution. The quantity of phospholipids within the proteoliposomes was proportional to the amount of KcsA immobilized on the beads (Figure 2). On the basis of the amount of absorbed phospholipids, about 150 POPE molecules were estimated to surround each KcsA molecule in the proteoliposome. By comparing the surface area of the porous Zn−NTA beads and the area occupied by the KcsA and phospholipids, we estimated that at least 35% of the bead surface was covered with these components.

The phospholipids incorporated into the KcsA-proteoliposomes were directly observed by confocal microscopy (Figure 3). The KcsA-proteoliposomes, containing 0.2% rhodamine−DOPE, exhibited an intense and uniform fluorescence, indicative of the phospholipid incorporation (Figure 3B,E). On the other hand, we could detect only dim fluorescence from the control

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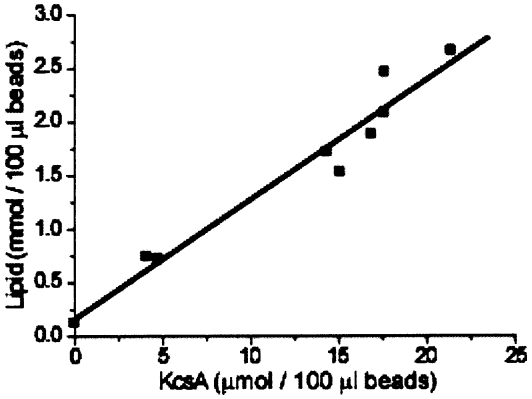

Figure 2. KcsA-dependent lipid acquisition on proteoliposomes. The quantities of KcsA and phospholipids acquired within the KcsA-proteoliposomes were determined, as described in the Materials and Methods section. The solid line shows a linear least-squares fitting of the data.

beads without KcsA (Figure 3H). All of the cross sections of the confocal microscopy exhibited almost the same fluorescence intensity. These results demonstrate that the KcsA and lipid molecules had free access to the inside of the porous beads during the reconstitution period. Neither lipid vesicles nor other structures with a diameter greater than 0.1 μm were observed on either the surface or inside of the beads. These data are consistent with the idea that the bead-linked KcsA is surrounded by phospholipid molecules, with few irregularities. The transmitted microscopic views indicated that the beads possess a diameter of 20−40 μm.

Analysis of the Lipid Organization in the KcsA-Proteoliposomes. To confirm the formation of lipid bilayers in the KcsA-proteoliposomes, DSC analyses and ^{31}P NMR studies were performed. In the presence of lipid bilayers, a DSC thermotropic peak appears at the temperature where a cooperative gel to liquid-crystalline phase transition occurs. The DSC thermogram of the KcsA-proteoliposomes exhibited a thermotropic peak at 15.8 °C (Figure 4A), while the beads without the lipid exhibited no thermotropic peak (data not shown). A ^{31}P NMR measurement of the KcsA-proteoliposomes yielded a broad asymmetric peak with a low-field shoulder, which is a characteristic of bilayer phase phospholipids[32] (Figure 4B). The ^{31}P NMR spectrum also revealed the randomized orientation of the phospholipids in the KcsA-proteoliposomes, relative to the magnetic field. These two physiochemical analyses confirmed the presence of lipid bilayers in the KcsA-proteoliposomes.

Ligand-Binding Properties of the KcsA-Proteoliposomes. To confirm the binding activity of KcsA in the KcsA-proteoliposomes, the binding of AgTx2 was examined by confocal microscopy. AgTx2 is a pore-blocker of eukaryotic voltage-gated potassium channels, and it also binds to the prokaryotic potassium channel, KcsA. AgTx2 binds to the extracellular pore region of the potassium channel, which is opposite from the N-terminus of KcsA, where the channel was immobilized on the beads. In this study, we prepared two kinds of KcsA-proteoliposomes, containing mutants of KcsA with higher (R64D) or lower (V84K) AgTx2-binding affinity than that of the wild type. Each of them was incubated with

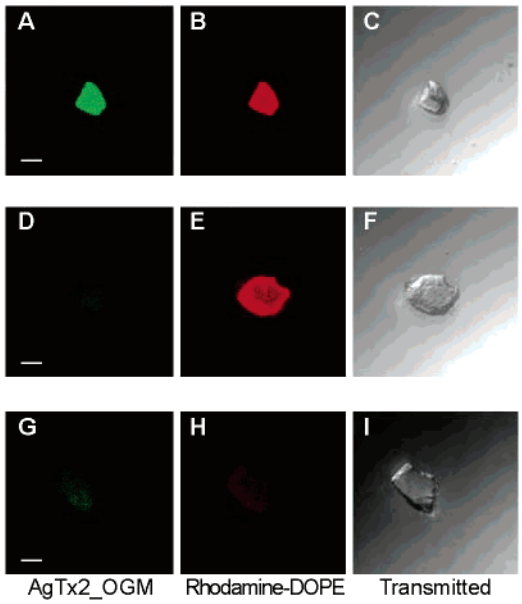

Figure 3. Confocal microscopy of fluorescently labeled KcsA-proteoliposomes. KcsA-proteoliposomes, containing mutants of KcsA with high (R64D, A−C) or low (V84K, D−F) AgTx2 binding affinity, were made as described in Figure 1. The dissociation constants of AgTx2 for the R64D and V84K mutants of KcsA, which were immobilized on the chip under surfactant-solubilized conditions, were 0.48 ± 0.05 μM and 0.42 ± 0.03 mM, respectively. Control beads (G−I) were prepared by an identical procedure, except the KcsA immobilization step was skipped. They were all incubated with fluorescently labeled AgTx2 (AgTx2_OGM) and directly analyzed by confocal microscopy after a mild washing procedure. The AgTx2_D20C mutant conjugated with *N*-ethylmaleimide retained an affinity for KcsA similar to that of the wild-type AgTx2, as determined in the previous observation.[31] Therefore, AgTx2_OGM was prepared from AgTx2_D20C by conjugation with OGM. The dissociation constant of AgTx2_OGM for the R64D mutant of KcsA (0.51 ± 0.06 μM) was almost the same as that for the wild-type AgTx2 (0.48 ± 0.05 μM), as expected. The central column shows fluorescence from the phospholipids acquired in KcsA-proteoliposomes (B, E) or control beads (H). Phospholipids were visualized by virtue of the presence of 0.2% fluorescently labeled phospholipids, rhodamine−DOPE, present in the mixture. The figures on the left display AgTx2 binding to KcsA-proteoliposomes (A, D) or control beads (G), which were visualized by AgTx2_OGM. The figures on the right show the transmitted images of each sample. All samples were analyzed with an Olympus CLSM. Scale bars in A, D, and G correspond to 20 μm. All of the mutants utilized in this study (KcsA_R64D, KcsA_V84K, and AgTx2_D20C) were generated according to the QuikChange (Stratagene) protocol. All of the dissociation constants were calculated by SPR analyses, using a BIAcore 2000 instrument (Biacore AB) equipped with an NTA sensor tip at 25 °C. The running buffer for the determination of the binding affinity contained 50 mM Tris-HCl (pH 8.0), 50 μM EDTA, 100 mM NaCl, 50 mM KCl, 0.1% DDM, and 0.05% NaN_3. The KcsA K^+ channel (6000 RU) was immobilized on the NTA sensor tips and was eluted with running buffer containing 0.3 M EDTA after each experiment. The obtained data were analyzed with the BIAevaluation 3.1 software. All data were corrected for the resonance obtained using a flow cell only charged with Ni^{2+} ions.

fluorescently labeled AgTx2 (AgTx2_OGM), which retained the equivalent KcsA-binding affinity (see more details in the Figure 3 legend). After a moderate washing procedure, the fluorescence from AgTx2_OGM on the KcsA-proteoliposomes was directly observed by confocal microscopy. Control beads without KcsA were prepared and treated in the same way. In contrast to the intense fluorescence of AgTx2_OGM on the KcsA_R64D proteoliposomes, almost no fluorescence was observed on the KcsA_V84K proteoliposomes and the control beads (Figure 3A,D,G). The amount of AgTx2_OGM bound to each bead was consistent with the relative binding affinity of AgTx2 to each KcsA mutant. These results indicate that

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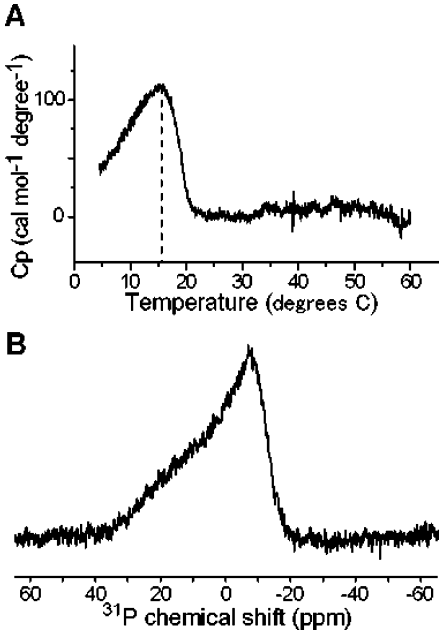

Figure 4. Physiochemical characterizations of the lipid organization in KcsA-proteoliposomes. (A) Differential scanning calorimetry (DSC) thermogram of KcsA-proteoliposomes. The heating range was between 3 and 60 °C, and the heating rate was 60 °C/h. Multiple scans were performed on each sample to check for the reproducibility of the DSC curves. The KcsA-proteoliposomes exhibited a thermotropic peak at 15.8 °C. (B) ^{31}P NMR spectrum of KcsA-proteoliposomes at 303 K. Spectra were collected with 2 K points, 600 K scans, and a spectrum width of 150 ppm, centered at 0 ppm. A high-field peak with a low-field shoulder, which is characteristic of bilayer phase phospholipids, was observed.

AgTx2_OGM specifically bound to KcsA in the proteoliposomes. Furthermore, no fluorescence was observed for the KcsA_V84K proteoliposomes, suggesting that AgTx2 does not bind to membranes alone.

Determination of the KcsA-Binding Surface on AgTx2 by the TCS Method. To identify the surface on AgTx2 that interacts with KcsA in the proteoliposomes, we utilized TCS phenomena.[24−27] Figure 5A shows an outline of the TCS experiment performed in this study. The residues on AgTx2 that bound to surfactant-solubilized KcsA were successfully determined by TCS experiments in our previous study.[26] The TCS experiment was performed as described in the report, except that KcsA-proteoliposomes were used, instead of surfactant-solubilized KcsA.[26] Irradiation of the complex with a frequency corresponding to aliphatic proton resonances affects the entire KcsA-proteoliposome but not the deuterated AgTx2, since no aliphatic protons exist in the molecule. The saturation in the channel is not limited within the molecule but is transferred to the residues at the interface of AgTx2 bound to the channel, through cross-saturation phenomena. Thus, the contact residues on AgTx2 can be identified by comparing the intensities of the amide signals originating from the free AgTx2 molecule in ^{1}H−^{15}N heteronuclear single quantum coherence (HSQC) spectra, with and without the irradiation.

The irradiation of the aliphatic proton resonances would cause direct saturation of the phospholipid resonances as well as indirect saturation by spin diffusion via KcsA. Therefore, there is a possibility that the binding site on AgTx2 to the membrane in the proteoliposome would be detected by the saturation of the phospholipids. However, in this study, the control experiment using the KcsA_V84K mutant (see below) revealed that the effect of the lipid binding by AgTx2 was negligible.

Figure 5B shows a ^{1}H−^{15}N HSQC spectrum observed for AgTx2 in the presence of the KcsA-proteoliposomes. In comparison to the spectrum obtained in our previous study,[26] the present spectrum retained almost identical chemical shifts for each signal. Thus, we could easily assign each resonance, based on the established assignments. These results are also consistent with the conclusion that only the signals originating from the free AgTx2 were observed in the HSQC spectra of the AgTx2/KcsA-proteoliposome mixture. The line width of each signal was extremely broad in this study, because of the magnetic heterogeneity of the sample containing the silica beads and the accelerated relaxation arising from the huge apparent molecular weight of AgTx2 in complex with the KcsA-proteoliposomes.[33,34] The irradiation applied to the complex resulted in selective intensity losses for the free AgTx2 resonances (Figure 5C upper panels). On the basis of the spectra with and without irradiation, we calculated the intensity reduction ratios for each resonance.

The affected residues in this study were V2, C8, T9, G10, Q13, I15, K19, G22, M23, F25, G26, K27, M29, R31, K32, H34, and C35 (Figure 5D). In comparison with the residues affected in the previous studies using the surfactant-solubilized KcsA (V2, T9, G10, S11, Q13, I15, R24, F25, G26, K27, C28, M29, N30, R31, K32, H34, T36, and K38),[26] most of the residues, V2, T9, G10, Q13, I15, F25, G26, K27, M29, R31, K32, and H34, were successfully detected. However, S11 and N30 were not observed here, because of the significant line broadening resulting from the extremely large molecular weight of the complex, and some residues (R24, C28, T36, and K38) were not identified on the binding surface, because of low signal-to-noise ratios for the resonances from these residues. Due to the accelerated spin diffusion, C8, K19, G22, M23, and C35 were affected only in the present experiment.

The TCS experiments were also performed with the KcsA_V84K-proteoliposomes, which have a low affinity for AgTx2, under the same experimental conditions. As shown in the lower panels of Figure 5C, no significant signal intensity reduction was observed, suggesting that AgTx2 does not bind to the channel. This observation is in good agreement with the microscopic measurements discussed in the previous section. The results of the TCS experiments were also consistent with those of extensive site-directed mutagenesis studies.[26,31,35]

Discussion

We have successfully established a novel strategy that is optimized for structural analyses of the interactions between membrane-integrated proteins and their ligands. In the strategy demonstrated here, the preparation of the bead-linked proteoliposomes and the utilization of the TCS method were both essential. We applied the strategy to the analysis of the interaction between KcsA and its pore-blocking peptide, AgTx2, and successfully identified the surface on AgTx2 that interacts with KcsA, incorporated into a phospholipid bilayer. This is

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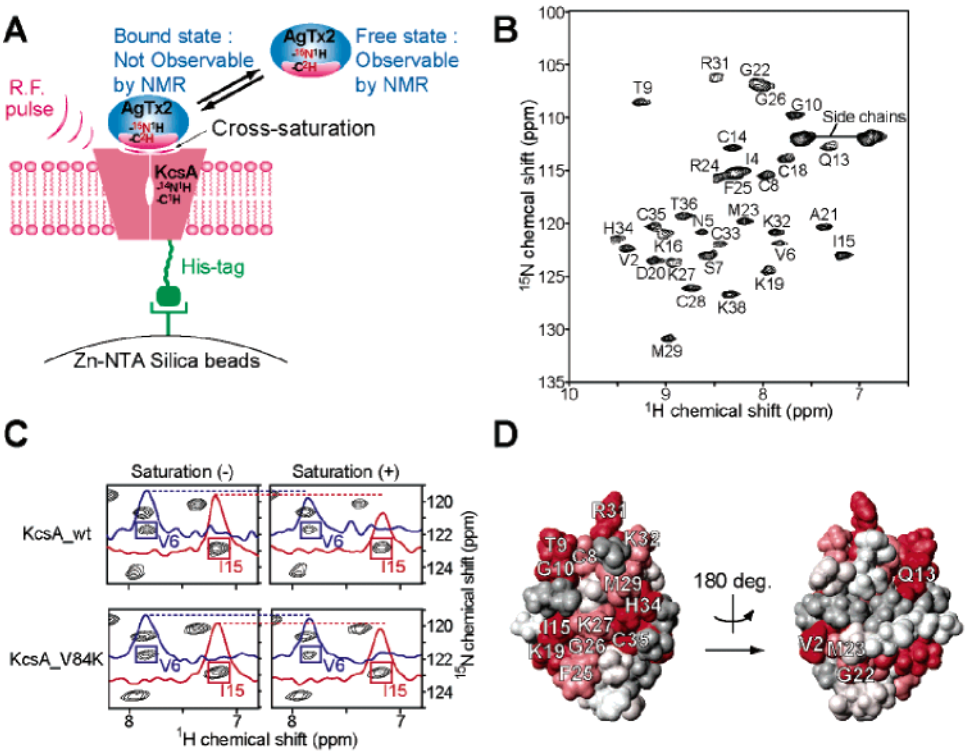

Figure 5. TCS experiments using KcsA-proteoliposomes. (A) Schematic representation of TCS experiments for analyzing the interaction between KcsA-proteoliposomes and AgTx2. The pulse scheme and the decoupling pulse used for the experiments were previously described.[24] (B) ^{1}H−^{15}N HSQC spectrum of the uniformly ^{2}H- and ^{15}N-labeled AgTx2 in the presence of KcsA-proteoliposomes. (C) Cross sections and portions of ^{1}H−^{15}N HSQC spectra observed for the labeled AgTx2, with (right) and without (left) irradiation. The upper panels show the results observed for KcsA_wt-proteoliposomes, and the lower ones show those for KcsA_V84K-proteoliposomes. 1D slices of an interface residue (I15) and a residue on the opposite side of the interface (V6) are shown for comparison. The experiments were performed at 303 K. (D) Mapping of the residues affected by the irradiation on the AgTx2 surface. The color gradient from red to white indicates the signal reduction ratio. The left and right figures are 180° rotations about the vertical axis relative to each other. The front surface in the left figure corresponds to the KcsA-binding surface on AgTx2. Molecular graphics images were prepared with the program MOLMOL.[36]

the first successful determination of the binding surface on a ligand to an integral membrane protein embedded in a lipid bilayer.

The utilization of the bead-linked proteoliposomes has a distinct advantage, as compared to conventional strategies, since the native conformation and the correct orientation of the membrane proteins with respect to the outside and inside of the membrane are guaranteed in the bead-linked proteoliposome system. It is known that phospholipids in membranes play a crucial role in some interactions between ligands and membrane proteins. For example, the full expression of the function of pituitary adenylate cyclase activating polypeptide (PACAP) requires its binding to phospholipids.[37] Therefore, one of the most robust advantages of this strategy using bead-linked proteoliposomes is the increased applicability to the membrane protein−ligand interactions that require phospholipids for their normal function. While, in some cases, the surfactant and lipid compositions might need to be considered, we would basically be able to use all types of phospholipids and a variety of surfactants for the successful preparation of the proteoliposomes.

Two physiochemical experiments, DSC measurements and ^{31}P NMR experiments, demonstrated the presence of the lipid bilayers in the KcsA-proteoliposomes. However, in the DSC experiments, the thermotropic peak from the KcsA-proteoliposomes appeared at a lower temperature than that observed for the pure POPE liposomes. This result might be a consequence of the organization of the KcsA-proteoliposomes, which contain a considerable amount of the KcsA protein, relative to the phospholipids, and/or a trace amount of surfactant, DDM and/or CHAPS, derived from the preparation procedures. On the basis of the results from the two physiochemical experiments and the coverage area of the KcsA-proteoliposomes, calculated from the acquired quantities of KcsA and phospholipid molecules, we propose the existence of a disk bicellelike architecture, that is, a planar particle consisting of a regular phospholipid bilayer with surfactants to cover the rim on the beads.[38]

The temporary solubilization of membrane proteins in the purification step is a potential disadvantage of this strategy, since the solubilization of membrane proteins by surfactants could cause time-dependent inactivation, through the formation of aggregates.[7] However, the membrane protein immobilization on the beads would effectively suppress the aggregate formation, by preventing physical contact between the membrane proteins.[39] It should be noted that affinity beads are used for efficient protein folding in protein purification.[40] By simultaneously achieving both the purification and immobilization on the

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affinity beads, we can minimize the amount of the time that the membrane proteins are solubilized in the surfactants.

Approaches toward analyzing membrane protein−ligand interactions, using cell or membrane fractions, are sometimes advantageous, since they do not require surfactants. However, the purity and quantity of the membrane proteins are not necessarily sufficient, which could lead to nonspecific ligand-binding, and the orientation of the membrane proteins in these samples is usually uncontrollable. This was quite true in our preliminary attempts to incorporate KcsA into liposomes by a conventional reconstitution method, the dilution/dialysis method.[41] The utilization of the bead-linked proteoliposomes is superior in terms of concentrating the membrane protein without aggregation and regulating its orientation, thus minimizing nonspecific interactions.

A variety of membrane proteins could potentially be reconstituted by using affinity beads optimized for each membrane protein. In this study, the His-tag at the N-terminus of KcsA was used to immobilize the protein on the bead. The use of specific antibodies that recognize target membrane proteins would be another way of anchoring the membrane proteins on the affinity beads.

As an approach oriented toward analyses of membrane protein−ligand interactions, Meyer et al. demonstrated that the saturation transfer difference (STD) NMR technique, which is useful for screening compound mixtures for binding activity, is also effective for investigating ligand−protein interactions, using membrane proteins embedded in liposomes[42] or cell surfaces.[43] In some cases, the utilization of proteins immobilized on beads conferred a critical advantage in STD NMR.[33,34] In our present study, we proposed and demonstrated the utility of an appropriate strategy for analyses of membrane protein−ligand interactions, by combining the TCS method with membrane protein-immobilization on beads.

The intramolecular motion of a membrane protein immobilized within a proteoliposome could be restricted, which may affect its activity, such as the gating activity of KcsA. At present, we do not know whether the inherent dynamics of the membrane protein are fully retained; however, optimization of the linker between the membrane proteins and the beads would be a strategy to cope with this problem.

The signal reduction rate of AgTx2 was higher in the present experiments, as compared to that of the surfactant-solubilized KcsA. In our previous study, using the surfactant-solubilized KcsA, 1.0−2.5 s of irradiation had been appropriate, whereas 0.4 s of irradiation produced a sufficient reduction of the signal intensity in the present study. This short irradiation time is reasonable, when we consider the effect of partially restricted molecular tumbling in the bead-linked KcsA-proteoliposomes, leading to increased dipole−dipole interactions. It is known that the resonances arising from compounds bound to beads with flexible linkers remain relatively narrow but are broader than those obtained in the solution phase.[44] This observation suggests that the motion of the bound compounds is partially restricted but not totally inhibited. As for the KcsA-proteoliposomes in this study, a long linker with a length of about 100 Å between the N-terminus of the KcsA molecule and the bead surface would allow some lateral and rotational motion of the KcsA in the bead-linked proteoliposomes. Thus, the shorter optimal saturation period (60% reduction) would be appropriate. However, slight spin diffusion effects were observed in the experiments. Two residues (G22 and M23) that were categorized as saturated residues were not located on the binding surface determined in this study.

Regarding the quality of the observed $^{1}H-^{15}N$ HSQC spectra, each signal exhibited remarkable line broadening and a few signals were difficult to separate, even for the 38 amino acid peptide, AgTx2. These problems mainly resulted from the magnetic heterogeneity of the bead-containing sample as well as the increased relaxation rate, due to the huge molecular weight of the complex. The line widths of the AgTx2 signals were broadened from 25 Hz in solution to about 110 Hz in the presence of Zn−NTA beads, as pointed out in a recent study.[34] Nevertheless, we successfully identified the binding surface of AgTx2, because of the improved spectral resolution with the 2D NMR method. High-resolution magic angle spinning (HR-MAS) techniques are reportedly quite effective for eliminating the magnetic heterogeneity.[33] These techniques could be applied for the further development of the strategy proposed here.

For the ligand to membrane protein ratio, we used a ratio of 5:1. A higher ligand to membrane protein ratio would be helpful for reducing the amount of membrane protein required, as pointed out by Meyer et al.[45] However, in our case, the higher ratio (50:1) gave us less relative reduction.

In this study, we successfully prepared KcsA-proteoliposomes and used the TCS method to determine the surface on AgTx2 that bound to the KcsA-proteoliposomes. This strategy, using the bead-linked proteoliposomes combined with the TCS method, has a crucial advantage for structural analyses of the interactions of membrane-integrated proteins. This strategy may be particularly beneficial for studying membrane proteins that require specific phospholipids. Furthermore, it would be effective for investigations of the interactions between ligand-activated multicomponent receptors and signal transduction molecules.

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Acknowledgment. Abbreviations: TCS, transferred cross-saturation; HSQC spectrum, heteronuclear single quantum coherence spectrum; DDM, *n*-dodecyl-*β*-D-maltoside; AgTx2, agitoxin2; DSC, differential scanning calorimetry.

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