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Functional Reconstitution of an ABC Transporter in Nanodiscs for Use in Electron Paramagnetic Resonance Spectroscopy

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Abstract: Electron paramagnetic resonance (EPR) spectroscopy is a powerful biophysical technique for study of the structural dynamics of membrane proteins. Many of these proteins interact with ligands or proteins on one or both sides of the membrane. Membrane proteins are typically reconstituted in proteoliposomes to observe their function in a physiologically relevant environment. However, membrane proteins can insert into liposomes in two different orientations, and surfaces facing the lumen of the vesicle can be inaccessible to ligands. This heterogeneity can lead to subpopulations that do not respond to ligand binding, complicating EPR spectral analysis, particularly for distance measurements. Using the well-characterized maltose transporter, an ATP binding cassette (ABC) transporter that interacts with ligands on both sides of the membrane, we provide evidence that reconstitution into nanodiscs, which are soluble disk-shaped phospholipid bilayers, is an ideal solution to these problems. We describe the functional reconstitution of the maltose transporter into nanodiscs and demonstrate that this system is ideally suited to study conformational changes and intramolecular distances by EPR.

Transmembrane (TM) signaling involves the interaction of membrane-spanning proteins with ligands or proteins on both sides of the membrane. The well-characterized maltose transporter ($MalFGK_2$) from *E. coli*, an ATP binding cassette (ABC) transporter, is one such system. The interaction of a periplasmic maltose binding protein (MBP) with the TM subunits (MalF-MalG) of the transporter stimulates the ATPase activity of the MalK dimer at the cytoplasmic surface of the membrane.[1] Biophysical studies of purified membrane proteins are traditionally performed either in detergent micelles or in proteoliposome vesicles.[2] However, each has a weakness. The insertion of proteins into liposomes generates heterogeneity in orientation and in accessibility to ligands, while detergent micelles can be poor membrane mimics. Work by Grote et al.[3] using electron paramagnetic resonance (EPR) spectroscopy illustrates how 50% of the population of $MalFGK_2$ can fail to respond to the addition of a nucleotide to proteoliposomes, presumably because the nucleotide-binding sites face the lumen. $MalFGK_2$ displays an MBP-independent ATPase activity in detergent that is not characteristic of the reconstituted transporter.[4] We demonstrate here that nanodiscs[5,6] provide an ideal solution to these problems and are nicely suited for EPR spectroscopy in the investigation of the structural dynamics of multispanning TM proteins.

A nanodisc consists of two membrane scaffold proteins (MSPs), modeled after the serum apoprotein A-1, encircling a patch of phospholipid bilayer. Plasmids are commercially available encoding MSPs of different sizes, ranging from 9.8 to 17.0 nm in diameter.[7] Incorporation of the TM region of a membrane protein into the lipid patch renders the protein soluble in aqueous solution in the absence of detergent and exposes both hydrophilic surfaces to solution. The $MalFGK_2$ transporter was reconstituted into nanodiscs following the procedures outlined (Supporting Information) to determine whether the characteristic ATPase activity seen in proteoliposomes is faithfully recapitulated in nanodiscs.

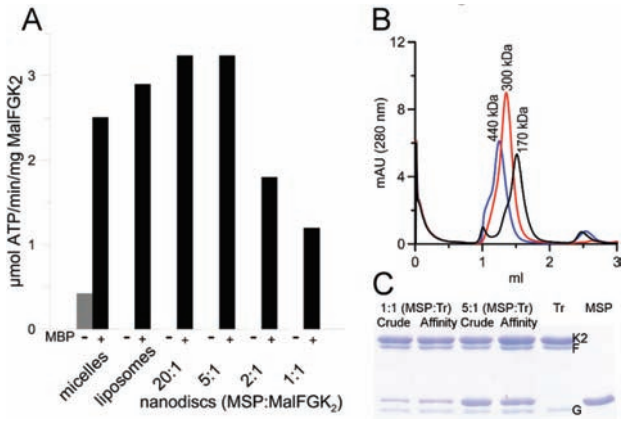

Figure 1. Characterization of the maltose transporter in nanodiscs. (A) ATPase activities at 25 °C of $MalFGK_2$ in detergent micelles, liposomes, or nanodiscs. Nanodiscs were prepared as described in the text, and liposomes as described in the Supporting Information. Each bar is an average of 2−3 determinations, which varied by ≤8%. (B) Size-exclusion chromatography of purified (50:1 lipids/MSP) nanodiscs (ratio MSP/$MalFGK_2$ 1:1, blue, and 5:1, red) and empty nanodiscs (black). Column was calibrated using MW standards (see Supporting Information). (C) Coomassie-stained SDS-PAGE of nanodiscs (Tr:$MalFGK_2$, MSP: membrane scaffold protein, Crude: crude nanodiscs, Affinity: affinity-purified nanodiscs). Each lane contains approximately equal amounts of transporter.

Optimization of nanodisc assembly was achieved by varying the molar ratios of MSP, transporter, and lipids. Soybean phospholipids, previously found to be suitable for reconstitution of $MalFGK_2$,[8] were solubilized with cholate, a detergent commonly used in the nanodiscs literature.[7] *N*-Dodecyl-β-D-maltoside (DDM), a gentle nonionic detergent routinely used for stabilization of membrane proteins,[9] worked equally well (data not shown). Pure lipids and *E. coli* phospholipids have also been used in nanodiscs.[7,10] Initially, nanodiscs were prepared using a 120:1 molar ratio of lipids/MSP with MSP(monomer)/transporter ratios varying from 1:1 to 20:1. A high MSP/transporter ratio increases the likelihood of incorporating just one transporter per nanodisc,[10,11] while a lower ratio reduces the amount of empty nanodiscs and economizes on the use of MSP. Nanodiscs were taken for assay directly following detergent removal by biobeads (crude nanodiscs). Reconstitution into nanodiscs was as efficient as that in proteoliposomes, as judged by the similar recovery of ATPase activity and, most importantly, the lack of MBP-independent ATPase activity that is seen in detergent solution (Figure 1A). Tight coupling between MBP docking and ATP hydrolysis ensures that the substrate maltose is transported each time ATP is hydrolyzed, and incorporation into nanodiscs appears

to recapitulate this important regulatory aspect. Higher activities were obtained with the 20:1 and 5:1 MSP/transporter ratios, while activities using the 2:1 and 1:1 ratios vary from 30% (Figure 1A) to 75% (data not shown) of maximum in different experiments.

To determine the number of transporters incorporated per nanodisc, size exclusion chromatography (SEC) of nanodiscs that had been affinity purified using the polyhistidine tag on $MalFGK_2$ was performed. Major peaks corresponding to 300 kDa for the 1:1 and 2:1 MSP/transporter ratios and 440 kDa for the 5:1 and 20:1 ratios were seen (Figure 1B or not shown). Given that empty nanodiscs (prepared using the same protocol but without transporter) eluted at 170 kDa, these sizes are consistent with one $MalFGK_2$ (173 kDa) being reconstituted per nanodisc at the higher MSP/transporter ratios and two $MalFGK_2$ being reconstituted per disk at the lower ratios. Previous work has shown that the fraction of MSP that aggregates and elutes in the void volume (seen as a small peak at an elution volume of 1 mL for empty nanodiscs in Figure 1B and as a shoulder on the 440 kDa peak) can be minimized by optimization of the lipid/MSP ratio for different sized MSP disks and membrane proteins.[12] A 50:1 ratio of lipid/MSP was optimal for our work using MSP1E3D1 and $MalFGK_2$ at the 5:1 MSP/transporter ratio, as indicated by the more symmetric elution peak for this preparation. ATPase activities in nanodiscs varied by less than 40% in the 50:1 to 120:1 lipid/MSP range used in our experiments, and MBP-independent ATPase activity was never apparent (data not shown).

Both crude nanodiscs and affinity-purified nanodiscs were subjected to SDS-PAGE (Figure 1C). In agreement with the SEC data in Figure 1B, quantitation of the intensity of the MalK and MalF bands on the gel (MW of FK_2 is 140 000) relative to that of MSP (MW of MSP dimer is 65 000) revealed a molar ratio of approximately one transporter per nanodisc for discs prepared at the 5:1 molar ratio of MSP/transporter and approximately two transporters per nanodisc for discs prepared at the 1:1 ratio. The diameter of the nanodiscs formed by the MSP1E3D1 protein used in these experiments is reported to be 12.1 nm in diameter, which is sufficient to accommodate two transporters, as judged from the crystal structure.[13] The lower ATPase activity of nanodiscs containing two transporters as compared to those containing one may reflect crowding within the nanodisc that restricts the rate of protein conformational change. For $MalFGK_2$, nanodiscs prepared using a 5:1 MSP/transporter ratio and a 50:1 lipid/MSP ratio appear optimal for obtaining a uniform population of nanodiscs containing just one transporter.

Crude nanodiscs containing a transporter spin-labeled at cysteine substitutions V16C and R129C in MalK were concentrated to 10−15 mg of $MalFGK_2$/mL for use in EPR. These spin labels move closer together upon closure of the nucleotide-binding interface in $MalFGK_2$.[14] When spin labels are within 7−20 Å of each other, magnetic dipolar interactions cause a decrease in central peak amplitude of a continuous wave (CW) EPR spectrum, accompanied by line broadening.[15] In the absence of added ligand (Figure 2, black), the EPR spectrum showed no evidence of dipolar coupling, indicating that the spin labels were >20 Å apart. Addition of MBP and the nonhydrolyzable ATP analogue AMP-PNP/Mg^{2+} induced a strong broadening of the spectrum (Figure 2, blue), consistent with a new distance of ∼8 Å,[16] and closing of the nucleotide-binding interface. A higher percentage of transporter responded to the ligands in nanodiscs (Figure 2B, ∼73%) compared to liposomes (Figure 2A, ∼63%). EPR line shapes and the results of the distance analyses did not appreciably vary using nanodiscs made with any of the different MSP/transporter or lipid/MSP ratios employed in this study, indicative of the robustness of nanodiscs for cwEPR.

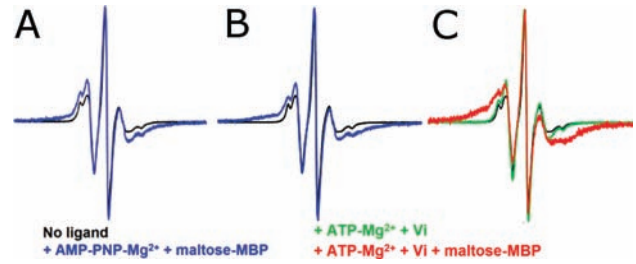

Figure 2. EPR spectra of the spin-labeled mutant (V16C/R129C) transporter. Experimental spectra normalized to maximum amplitude. (A) Proteoliposomes prepared using a 50:1 (w/w) lipid/transporter ratio. Ligand concentrations: ATP or AMP-PNP 10 mM, $MgCl_2$ 20 mM, maltose 1 mM, MBP 200 μM. (B and C) Nanodiscs prepared using a 5:1 MSP/transporter and a 50:1 lipid/MSP ratio. Ligand concentrations: ATP or AMP-PNP 10 mM, $MgCl_2$ 10 mM, maltose 1 mM, MBP 200 μM, and Vi 500 μM. Additional details are provided in the Supporting Information.

However, conditions generating just one transporter per nanodisc might be more advantageous for pulsed-EPR techniques, including double electron−electron resonance (DEER), where intermolecular distances up to 70 Å can be detected.[17]

Use of vanadate, which acts as a transition state analogue for phosphate and stably traps ADP, the product of ATP hydrolysis, in the nucleotide-binding site, increased the fraction of the transporters in the closed (∼8 Å) conformation to ∼85% (Figure 2C, red). Consistent with a role of MBP in stimulation of ATP hydrolysis, closure of the nucleotide-binding interface and vanadate trapping did not occur in the absence of MBP (Figure 2C, green). This high percent closure provided evidence that both nucleotide-binding sites along the dimer interface were closed following incubation with vanadate, even though vanadate trapping is thought to occur in just one site.[18] The use of nanodiscs, as compared to liposomes, increased the fraction of transporters undergoing ligand-induced conformational changes, presumably because of improved accessibility to ligand binding sites. This characteristic is important because a significant proportion of noninteracting spins can hamper calculation of accurate distances.[19]

In summary, nanodiscs provide a suitable membrane environment for the functional reconstitution of this multispanning transmembrane transporter and have proven to be far more versatile than traditional proteoliposomes. They are easily prepared and brought to the high protein concentrations necessary for EPR and provide a uniformity that is especially useful for the myriad of membrane proteins that interact with ligands, including ABC transporters, membrane receptors, ligand-gated ion channels, solute carrier proteins, and TRAP transporters.

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Supporting Information Available: Experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Davidson, A. L.; Shuman, H. A.; Nikaido, H. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 2360–4.
(2) Seddon, A. M.; Curnow, P.; Booth, P. J. *Biochim. Biophys. Acta* **2004**, *1666*, 105–17.
(3) Grote, M.; Polyhach, Y.; Jeschke, G.; Steinhoff, H. J.; Schneider, E.; Bordignon, E. *J. Biol. Chem.* **2009**, *284*, 17521–6.
(4) Reich-Slotky, R.; Panagiotidis, C.; Reyes, M.; Shuman, H. A. *J. Bacteriol.* **2000**, *182*, 993–1000.

(5) Nath, A.; Atkins, W. M.; Sligar, S. G. *Biochemistry* **2007**, *46*, 2059–69.
(6) Borch, J.; Hamann, T. *Biol. Chem.* **2009**, *390*, 805–14.
(7) Ritchie, T. K.; Grinkova, Y. V.; Bayburt, T. H.; Denisov, I. G.; Zolnerciks, J. K.; Atkins, W. M.; Sligar, S. G. *Methods Enzymol.* **2009**, *464*, 211–31.
(8) Chen, J.; Sharma, S.; Quiocho, F. A.; Davidson, A. L. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 1525–30.
(9) Prive, G. G. *Methods* **2007**, *41*, 388–97.
(10) Boldog, T.; Li, M.; Hazelbauer, G. L. *Methods Enzymol.* **2007**, *423*, 317–35.
(11) Denisov, I. G.; Baas, B. J.; Grinkova, Y. V.; Sligar, S. G. *J. Biol. Chem.* **2007**, *282*, 7066–76.
(12) Denisov, I. G.; Grinkova, Y. V.; Lazarides, A. A.; Sligar, S. G. *J. Am. Chem. Soc.* **2004**, *126*, 3477–87.
(13) Oldham, M. L.; Khare, D.; Quiocho, F. A.; Davidson, A. L.; Chen, J. *Nature* **2007**, *450*, 515–21.
(14) Orelle, C.; Ayvaz, T.; Everly, R. M.; Klug, C. S.; Davidson, A. L. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 12837–42.
(15) Klug, C. S.; Feix, J. B. *Methods Cell. Biol.* **2008**, *84*, 617–58.
(16) Altenbach, C.; Oh, K. J.; Trabanino, R. J.; Hideg, K.; Hubbell, W. L. *Biochemistry* **2001**, *40*, 15471–82.
(17) Zou, P.; McHaourab, H. S. *Biophys. J.* **2010**, *98*, L18–20.
(18) Sauna, Z. E.; Kim, I. W.; Nandigama, K.; Kopp, S.; Chiba, P.; Ambudkar, S. V. *Biochemistry* **2007**, *46*, 13787–99.
(19) Banham, J. E.; Baker, C. M.; Ceola, S.; Day, I. J.; Grant, G. H.; Groenen, E. J.; Rodgers, C. T.; Jeschke, G.; Timmel, C. R. *J. Magn. Reson.* **2008**, *191*, 202–18.

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