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THE BINDING BEHAVIOUR AND CONFORMATION OF *Rhodobacter sphaeroides* TSPO IN DDM AND DPC DETERGENTS

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ABSTRACT

We have studied the binding behavior of *Rhodobacter sphaeroides* translocator protein (RsTSPO) to its natural ligand Protoporphyrin IX (PPIX) in n-dodecyl-ß-D-maltoside (DDM) and n-dodecylphosphocholine (DPC) detergents. RsTSPO was chosen as the model for mammalian TSPO. The result of intrinsic tryptophan fluoroscence quenching showed that RsTSPO has higher affinity to bind to PPIX in DDM (K_d = 1.2 µM) compare to DPC (K_d = 18.6 µM). We have also studied the conformation of RsTSPO by utilizing Nuclear Magnetic Resonance (NMR) and circular dichroism (CD). NMR chemical shift analysis confirmed that the protein was successfully reconstituted into the lipids in its native α-helical conformation, which is further supported by secondary structure analysis by circular dichroism. Intrinsic tryptophan fluorescence quenching experiments indicated that the RsTSPO was expressed and purified in a functional state.

Key word: translocator protein, Protoporphyrin IX, fluorescence quenching, binding

INTRODUCTION

The 18 kDaTranslocator protein (TSPO) is an integral membrane protein of five transmembrane helices. Membrane proteins are proteins associated with a membrane bilayer, and they typically comprise about 25% of all proteins encoded for by the genome[1]. TSPO in mammals was discovered by Braestrup*et al.*[2] as a secondary receptor for diazepam. TSPO was then known as a peripheral benzodiazepine receptor (PBR). In bacteria, TSPO was known as a tryptophan rich sensory protein (TspO)[3].Due to the multiple functions of this protein, Papadopoulos *et al.*[4] in 2006 suggested new nomenclature: Translocator Protein (TSPO).

Eukaryotic TSPO is primarily located in the outer mitochondrial membrane bilayer. TSPO is particularly enriched at the sites of outer/inner mitochondrial contact[5]. The carboxyl end of TSPO is located on the outside of the mitochondrion and the amino terminal is inside the mitochondrion[6].TSPO comprises up to 2% of the outer mitochondrial membrane protein[7]. At low levels, TSPO is also expressed in plasma and nuclear membranes[8]. Endogenous ligands for TSPO include protoporphyrin IX (PPIX), hemin and cholesterol. Protoporphyrin IX is the biosynthetic precursor of heme in mammals and chlorophyll or bacteriochlorophyll in plants or bacteria.

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TSPO has been associated with several health conditions, namely post-ischemic heart reperfusion injury, cancers and neurodegenerative diseases. TSPO has become a potential drug and imaging target for the above conditions. We started our investigation of TSPO ligand binding and structural characterization with the *Rhodobacter sphaeroides* TSPO (RsTSPO) homolog for a number of reasons. The TSPO protein is highly evolutionary conserved[4, 9]from bacteria to eukaryotes. Moreover, *Rhodobacter* is one of the closest ancestors of mitochondria[10].

A suitable environment is critical for the isolation and purification of every membrane protein. An integral membrane protein such as TSPO is not soluble in water, thus detergents and lipids are commonly used to solubilize membrane proteins in aqueous media. In this research, we investigated the behaviour of RsTSPO in two different detergents, the n-dodecyl-ß-D-maltoside (DDM) and n-dodecylphosphocholine (DPC) using protoporphyrin IX (PPIX) as the ligand. We also investigated the conformation of RsTSPO in both detergents using circular dichroism (CD) and NMR.

METHODOLOGY

Materials. Chemicals were purchased from Fisher and Sigma Aldrich. Dodecyl maltoside (DDM) and n-dodecylphosphocoline (DPC) (Anagrade) were purchased from Anatrace. Protoporphyrin IX (PPIX) was purchased from Frontier Scientific. Lysozyme was purchased from Sigma Aldrich. Ampicillin and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Gold Biotechnology. Chloramphenicol was purchased from Acron Organics.Complete EDTA free Protease inhibitor was purchased from Roche. Both ^{15}N labelled ammonium hydroxide and ^{13}C labelled glucose were purchased from Cambridge Isotope Laboratories, Inc. Bio-Beads SM-2 were purchased from Bio-Rad Laboratories.

Methods. *Protein expression and purification*. *E.coli*BL21 (DE3) pLysS containing pET23(a) plasmidwas grown overnight in 25 mL Luria broth. The overnight culture (5 mL) was then transferred in to 1 L of LB and grown to optical density at 600 nm (OD_{600}) of 0.7. The protein expression in the culture was then induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.4mM final concentration). Cells were then grown for another 6 hours while shaking. Cells were harvested by centrifugation (20 min 4,400 × g, 4 °C). Cell pellets were resuspended in Tris buffer (20 mM Tris and 200 mM NaCl, pH 7.50) and immediately frozen in liquid nitrogen. Cell pellets were stored at -80°C until cell lysis. Before cell lysis, complete EDTA free protease inhibitor cocktail and lysozyme were added to the resuspended pellet. Cells were lysed by sonication and by passing through French Press three times. The cell lysate was centrifuged (20 min, 8,000 × g, 4 °C) to separate cell debris.

Membranes containing TSPO were collected by ultracentrifugation (2 h, 170,000 × g, and 4 °C). For isotopically labeled cells growing, after the cell reach the OD_{600} of 0.7, the cell was then removed to minimal media which contain ^{15}N labelled ammonium hydroxide and ^{13}C labelled glucose and grown for another 11 hours before harvesting.

Extraction and purification of TSPO. Membranes were resuspended in Tris buffer(20 mM Tris and 200 mM NaCl, pH 7.50) supplemented with 2 % of either DDM (equal to 196 × CMC) or DPC (equal to 57 × CMC). Protein was extracted by gently shaking the solution for 6 h at 4 °C. Solubilized proteins and the membranes were separated by ultracentrifugation (2 h, 170,000 × g,4°C). Theprotein was then concentrated with Amicon Ultra centrifugal filter (30 kDa molecular weight cutoff) and filtered with Sterile Milex Filter unit (0.22 µm). Extracted protein was purified with AKTA FPLC system using GE health His Trap 1 mL FF Ni-affinity column. Unbound proteins were washed off the column using Tris buffer(20 mM Tris and 200 mM NaCl, pH 7.50)containing either 0.1 % DPC (equal to 2.6 × CMC) or 0.025 % DDM (equal to 2.5 × CMC) and supplemented with 50 mM imidazole. TSPO was eluted with Tris buffer pH 7.50 containing either 0.1 % DPC or 0.025 % DDM and supplemented with 500 mM imidazole. After purification with the Ni-affinity column, the sample fractions containing the protein was passed through Superdex75 size exclusion column using Tris size exclusion buffer (20 mM Tris and 30 mM NaCl, pH 7.50) containing either 0.1 % DPC or 0.025 % DDM. Purified protein then was run on 12% SDS-PAGE (sodium dodecyl sulfate poly acrylamide gel electrophoresis) and stained using Coomassie Blue to visualize the protein.

TSPO ligand binding studies. TSPO binding studies were carried out with purified, detergent solubilized protein (0.1 % DPC or 0.025 % DDM). All experiments in the binding studies were done with excitation wavelength at 285 nm and emission at 290-800 nm at room temperature (20 °C). Excitation and emission slits were set to 2.5 nm and 5 nm, respectively, with both excitation and emission filter were set to auto. After each addition of the ligand, the solution was incubated for 5 minutes either in dark or in ambient light before measuring fluorescence. We used a Varian Cary Eclipse Fluorometer and a Varian Cary UV-vis spectrophotometer. Fluorescence intensity (290-800 nm) and absorbance spectra (200-800 nm) were measured for every titration point. Fluorescence intensity was plotted against ligand concentration. Percent quenching was calculated using integration for every titration point.

Conformational study using circular dichroismn. Freshly purified protein with concentration of 2.5 µM in both detergents was used for this study. We used Jasco Varian Circular Dichroism at the near and far UV region to study the conformation of TSPO. The result was calculated using K2D program.

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NMR Study. Freshly purified high concentration protein (100 – 600 μM) were used for NMR studies. Data was analyzed using Sparky.

RESULT AND DISCUSSION

Expression and purification of TSPO. We have successfully expressed and purified RsTSPO in *E.coli* in both DDm and DPC detergents.

Binding study. RsTSPO solubilized in the nonionic detergent (DDM) exhibited greater TSPO fluorescence quenching than RsTSPO solubilized in the zwitter ionic DPC (Figure 1), which is line with the previously reported results[11]. TSPO in DDM also exibit higher affinity to bind PPIX (Kd is 1.26 μM) compare to TSPO in DPC (Kd is 18.66 μM).

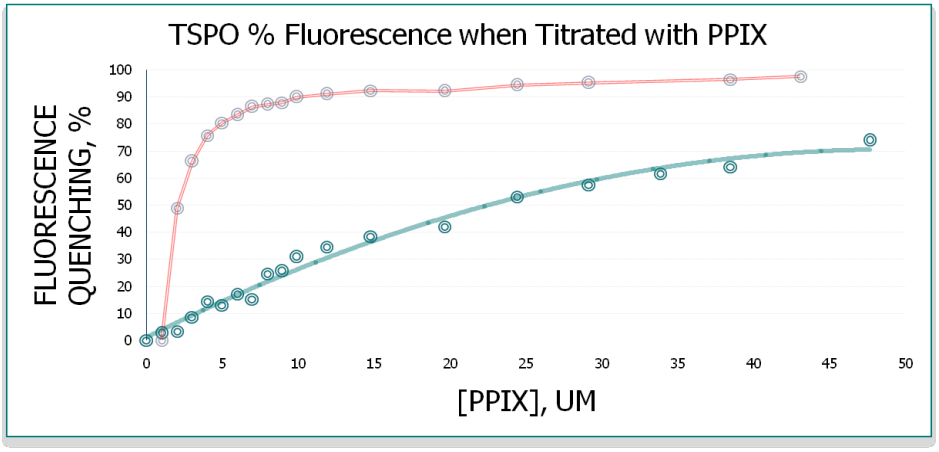

Figure1. Dose-response curve when TSPO in DPC was titrated with PPIX (blue) and TSPO in DDM was titrated with PPIX (red).

Conformational studies. Both circular dichroism and NMR studies of RsTSPO purified in DDM confirmed the α-helical conformation of RsTSPO which indicates that the protein was in its native conformation.

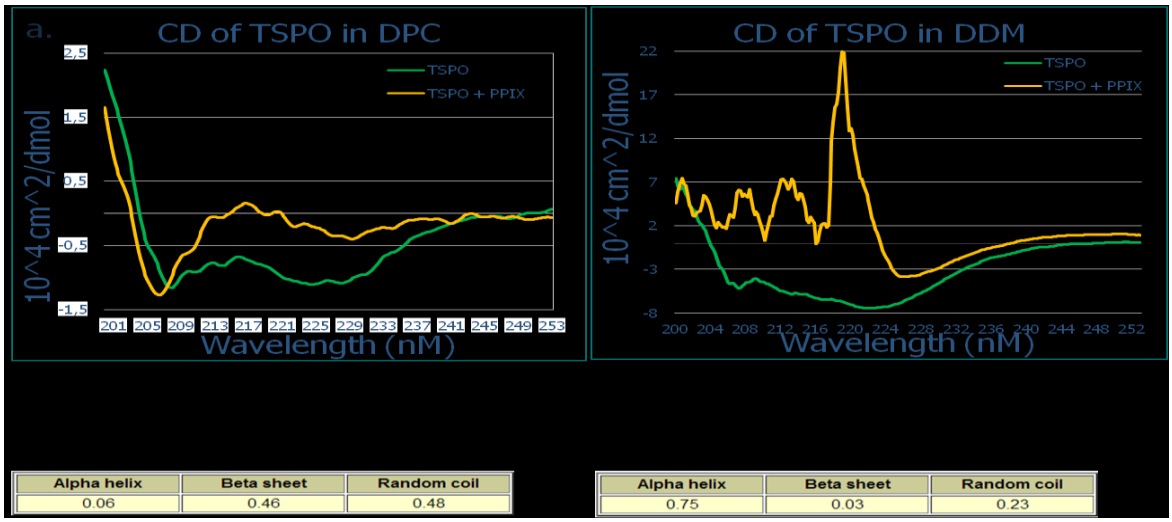

Figure 2. TSPO tertiary structure analysis using CD. Data was interpreted using K2D program.

CONCLUSION AND PROSPECT

From the result of this study, we can conclude that RsTSPO has higher affinity to bind to its natural ligand, PPIX, in DDM than DPC. This is in line with the conformational studies which indicated the healthier protein in DDM compare to DPC.

As a drug and imaging target, TSPO is an important protein to study. More information about the binding behavior of this protein with more ligands is critical.

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REFERENCES

1. Voet, D.; Voet, J. G., Biochemistry. 4th ed.; John Wiley & Sons, Inc: New Jersey, 2011.
2. Braestrup, C.; Squires, R. F., Specific benzodiazepine receptors in rat brain characterized by high-affinity (^{3}H)diazepam binding. *Proceedings of the National Academy of Sciences of the United States of America* **1977,***74* (9), 3805-3809.
3. Yeliseev, A. A.; Kaplan, S., A Sensory Transducer Homologous to the Mammalian Peripheral-type Benzodiazepine Receptor Regulates Photosynthetic Membrane Complex-formation in *Rhodobacter sphaeroides*. *Journal of Biological Chemistry* **1995,***270* (36), 21167-21175.
4. Papadopoulos, V.; Baraldi, M.; Guilarte, T. R.; Knudsen, T. B.; Lacapere, J. J.; Lindemann, P.; Norenberg, M. D.; Nutt, D.; Weizman, A.; Zhang, M. R.; Gavish, M., Translocator protein (18 kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. *Trends in Pharmacological Sciences* **2006,***27* (8), 402-409.
5. Taliani, S.; Pugliesi, I.; Da Settimo, F., Structural Requirements to Obtain Highly Potent and Selective 18 kDa Translocator Protein (TSPO) Ligands. *Current Topics in Medicinal Chemistry* **2011,***11* (7), 860-886.

6. Bernassau, J. M.; Reversat, J. L.; Ferrara, P.; Caput, D.; Lefur, G., A 3D Model of The Peripheral Benzodiazepine Receptor and Its Implication in Intra Mitochondrial Cholesterol Transport. *Journal of Molecular Graphics* **1993,***11* (4), 236-244.
7. Miller, W. L., Steroid hormone synthesis in mitochondria. *Molecular and Cellular Endocrinology* **2013,***379* (1-2), 62-73.