

IKATAN TRANSLOKATOR PROTEIN DARI *Rhodobacter sphaeroides* DENGAN HEMIN DALAM DDM DAN DPC

The Binding Behavior of *Rhodobacter sphaeroides* TSPO with Hemin in DDM and DPC Detergents

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Abstrak

Ikatan antara translokator protein (TSPO) dari *Rhodobacter sphaeroides* (RsTSPO) dengan hemin dalam dua detergen yang berbeda yaitu n-dodecyl- β -D-maltoside (DDM) and n-dodecylphosphocholine (DPC) detergents telah diteliti. RsTSPO dipilih sebagai model untuk TSPO mamalia. RsTSPO di tanam dalam *E.coli* kemudian diekstraksi menggunakan buffer Tris pH 7,5 dan diikuti dengan pemurnian menggunakan Fast Protein Liquid Chromotography (FPLC). Hasil fluoresensi triptofan intrinsik menunjukkan bahwa RsTSPO memiliki afinitas terhadap hemin yang lebih tinggi dalam DDM ($K_d = 0.35 \mu\text{M}$) dibandingkan DPC ($K_d = 0.575 \mu\text{M}$)

Kata kunci : translokator protein, hemin, fluoresensi, ikatan

Abstract

We have studied the binding behavior of *Rhodobacter sphaeroides* translocator protein (RsTSPO) to one of its ligand Hemin in n-dodecyl- β -D-maltoside (DDM) and n-dodecylphosphocholine (DPC) detergents. RsTSPO was chosen as the model for mammalian TSPO. The RsTSPO was expressed in *E.coli* and followed by extraction in Tris buffer pH 7.5 and purification by utilizing FPLC. The result of intrinsic tryptophan fluorescence quenching showed that RsTSPO has higher affinity to bind to Hemin in DDM ($K_d = 0.35 \mu\text{M}$) compare to DPC ($K_d = 0.575 \pm 0.005 \mu\text{M}$).

Key word : translocator protein, hemin, fluorescence quenching, binding

Introduction

The 18 kDa Translocator 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 (Voet, 2011). TSPO in mammals was discovered by Braestrup *et al.* (Braestrup, 1977) 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) (Yeliseev, 1995). Due to the multiple functions of this protein, Papadopoulos *et al.* (Papadopoulos, 2006) 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 (Taliani, 2011). The carboxyl end of TSPO is located on the outside of the mitochondrion and the amino terminal is inside the mitochondrion

(Bernassau, 1993). TSPO comprises up to 2% of the outer mitochondrial membrane protein (Miller, 2013). At low levels, TSPO is also expressed in plasma and nuclear membranes (Olson, 1988). 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.

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 (Papadopoulos, 2006 dan Fan, 2012) from bacteria to eukaryotes. Moreover, *Rhodobacter* is one of the closest ancestors of mitochondria (Bui, 1996).

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- β -D-maltoside (DDM) and n-dodecylphosphocholine (DPC) using hemin as the ligand.

Materials and Methods

2.1 Materials

Chemicals were purchased from Fisher and Sigma Aldrich. Dodecyl maltoside (DDM) and n-dodecylphosphocholine (DPC) (Anagrade) were purchased from Anatrace. Hemin 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.

2.2 Methods

2.2.1 Protein expression and purification

E.coli BL21 (DE3) pLysS containing pET23(a) plasmid was 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 $4,400 \times g$, 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, $8,000 \times g$, 4 °C) to separate cell debris. Membranes containing TSPO were collected by ultracentrifugation (2 h, $170,000 \times g$, and 4 °C).

2.1.2 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 $196 \times CMC$) or DPC (equal to $57 \times CMC$). 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, $170,000 \times g$, 4°C). The protein was then concentrated with Amicon Ultra centrifugal filter (30 kDa molecular weight cutoff) and filtered with Sterile Millex Filter unit (0.22 μ m). 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 $2.6 \times \text{CMC}$) or 0.025 % DDM (equal to $2.5 \times \text{CMC}$) 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.

2.1.3 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.

2.1.4 Data Analysis

Data analysis was performed in collaboration with Dr. Jan Kubelka of University of Wyoming using Matlab (Mathworks Inc.,

Nattick, MA). The following binding model was considered:



where E is the enzyme, S is substrate, f is an effective number of enzyme monomers binding the substrate and fE.S stands for the complex. The binding constant is expressed as follows:

$$K_d = [fE][S]/[fE.S] \quad (2)$$

Using the total concentration of the enzyme $[E]_0$ and substrate $[S]_0$:

$$[E]_0 = f([fE] + [fE.S]) \quad (3)$$

$$[S]_0 = [S] + [fE.S]$$

in (2), the concentration of the complex is:

$$[fE.S] = ([E]_0 + f[S]_0 + K_d - \sqrt{([E]_0 + f[S]_0 + K_d)^2 - 4f[E]_0[S]_0}) / 2f$$

The observed fluorescence intensity Φ is then:

$$\Phi = [fE] \Phi_{fE} + [fE.S] \Phi_{fE.S}$$

Where Φ is the fluorescence of the enzyme Φ_{fE} (f-mer), and $\Phi_{fE.S}$ is the fluorescence of the complex. It is assumed that the fluorescence of the f-mer in the absence of bound ligand, is simply f times that of the monomer.

Result and Discussion

3.1 Expression and purification of TSPO

We have successfully expressed and purified RsTSPO in *E.coli* in both DDM and DPC detergents.

3.2 Binding study

RsTSPO solubilized in the nonionic detergent (DDM) exhibited greater TSPO fluorescence quenching than RsTSPO solubilized in the zwitter ionic DPC, which is line with the previously reported results (Li, 2013). TSPO in DDM also exhibit higher affinity to bind hemin ($K_d = 0.35 \mu\text{M}$) compare to DPC ($K_d = 0.575 \mu\text{M}$)

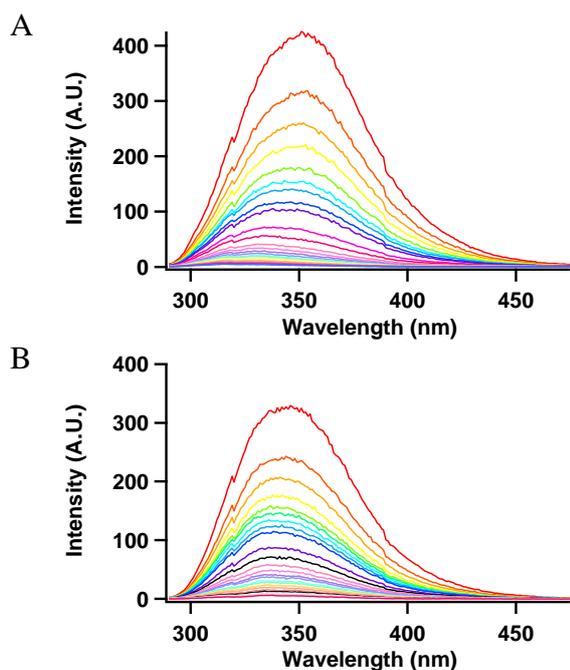


Figure 1. Fluorescence quenching of RsTSPO with hemin in DDM (A) and DPC (B)

A number of previous studies highlighted the critical role of lipids in bacterial and eukaryotic TSPO folding and function. For instance, it was shown in a study of mouse TSPO (Lacapere, 2001) that sodium dodecylsulfate (SDS) solubilized mouse TSPO was only able to bind steroid ligands, but not others. TSPO activity was recovered after reconstitution into lipid membranes. In binding studies of the bacterial RsTSPO, Li *et al.* (Li, 2013) compared the decylmaltoside (DM), a nonionic detergent, with DPC, a zwitterionic detergent. Their result showed greater TSPO fluorescence quenching in DM than in DPC. In our hands, efficient fluorescent quenching was observed for RsTSPO solubilized in either DDM or DCP, which indicates that RsTSPO was purified in a functional state in both detergents. However, RsTSPO solubilized in the nonionic detergent (DDM) exhibited greater TSPO fluorescence quenching than RsTSPO solubilized in DPC, which is line with the previously reported results (Li, 2013). Clearly, the nature of the detergent influenced the binding of ligands to TSPO, and the dissociation constants (K_d) observed for hemin was consistently lower in DDM,

which indicated higher ligand affinity. One possible explanation is that nonionic detergents such as DM and DDM can be less deactivating than Zwitterionic detergents such as DPC, as reported in the literature before (Seddon, 2004). However, the effect of the nature (i.e. nonionic vs. Zwitterionic) and concentration (i.e. 0.1 % DPC or 0.025 % DDM) of the detergent on the hydrophobic porphyrin ligands (e.g. ligand aggregation) cannot be excluded as a possible explanation for this observation, and more experiments are required to provide a workable theory.

Conclusion

RsTSPO in DDM has higher affinity to bind hemin compare to RsTSPO in DPC.

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