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Mouse TSPO in a lipid environment interacting with a functionalized monolayer

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ABSTRACT

Translocator protein TSPO is a membrane protein highly conserved in evolution which does not belong to any structural known family. TSPO is involved in physiological functions among which transport of molecules such as cholesterol to form steroids and bile salts in mammalian cells. Membrane protein structure determination remains a difficult task and needs concomitant approaches (for instance X-ray- or Electron-crystallography and NMR). Electron microscopy and two-dimensional crystallization under functionalized monolayers have been successfully developed for recombinant tagged proteins. The difficulty comes from the detergent carried by membrane proteins that disrupt the lipid monolayer.

We identified the best conditions for injecting the histidine tagged recombinant TSPO in detergent in the subphase and to keep the protein stable. Reconstituted recombinant protein into a lipid bilayer favors its adsorption to functionalized monolayers and limits the disruption of the monolayer by reducing the amount of detergent. Finally, we obtained the first transmission electron microscopy images of recombinant mouse TSPO negatively stained bound to the lipid monolayer after injection into the subphase of pre-reconstituted TSPO in lipids. Image analysis reveals that circular objects could correspond to an association of at least four monomers of mouse TSPO.

The different amino acid compositions and the location of the polyhistidine tag between bacterial and mouse TSPO could account for the formation of dimer versus tetramer, respectively. The difference in the loop between the first and second putative transmembrane domain may contribute to distinct monomer interaction, this is supported by differences in ligand binding parameters and biological functions of both proteins.

1. Introduction

Translocator protein (18 kDa, TSPO), previously known as the peripheral-type benzodiazepine receptor [1] is a transmembrane protein that is evolutionarily conserved from bacteria to humans [2,3]. TSPO is expressed in almost all mammalian tissues and is mostly located in the outer mitochondrial membrane [3]. TSPO is involved in a wide range of physiological functions and the best established molecular one is the translocation of cholesterol from the cytosol to the mitochondrial matrix, the first step of steroids or bile salts formation in steroidogenic tissues and liver, respectively [4]. However, little is known about its three-dimensional atomic structure, oligomeric state, and its molecular mechanism. Initial hydropathy analysis suggested five transmembraneous domains for TSPO that were confirmed by topological studies [5]. Further, biophysical studies of peptides encompassing putative transmembrane domains and recombinant mouse TSPO demonstrated the five-helix fold of TSPO [6]. More recently, the first three-dimensional structure of TSPO from *R. sphaeroides* was obtained by electron cryomicroscopy of helical crystals [7]. From a functional point of view, the minimal unit for mammalian TSPO seems to be a monomer since recombinant mouse TSPO binds high affinity drug ligand PK 11195 and cholesterol [8]. However, several data suggest that TSPO is associated with other proteins either from the cytosol or from the mitochondrial membranes in the steroid biosynthesis for instance [9,10]. Moreover, it has been suggested that, in response to reactive

Abbreviations: PK 11195, N-butan-2-yl-1-(2-chlorophenyl)-N-methylisoquinoline-3-carboxamide; TSPO, 18 kDa translocator protein; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOGS-NTA-Ni, 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentanyl)iminodiacetic acid)succinyl] (Nickel salt); DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPE, 1,2-dimyristoyl-sn-glycero-3phosphoethanolamine; BAM, Brewster Angle Microscopy; TEM, Transmission Electron Microscopy; BB, BioBead; PPIX, Protoporphyrin IX; PET, Positron Emission Tomography

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oxygen species TSPO can form covalent polymers which can have functional properties [11]. Another possibility is the transient association of TSPO to form non-covalent polymers and this would be an explanation for the dimers observed in the tubular crystals of TSPO from bacteria [7]. Nevertheless, differences between species cannot be excluded. Thus, characterization of structural differences between mammalian and bacteria TSPO is an important issue.

Structural studies of membrane protein are intricate and several approaches are possible [12]. Among them, electron microscopy can be used to study membrane protein organization within the artificial membranes either by two-dimensional crystallization in solution or by reorganization of adsorbed proteins under a lipid monolayer at the air/water interface [13–15]. The latter has the advantage of concentrating tagged recombinant proteins under functionalized monolayers and in some case, allows to analyze isolated particles. The main difficulty to solve is the solubilizing effect of the detergent associated to the protein. Indeed, the detergent is necessary to stabilize the recombinant membrane protein, but it also solubilizes the lipid monolayer which is actually the matrix on which the protein in solution will interact. In this paper, we present a new strategy to overcome this problem by injecting pre-reconstituted membrane protein in lipid bilayer under the functionalized lipid monolayer. In this study, we show for the first time an image of a mammalian TSPO which suggests a different oligomeric state compared to bacterial orthologue described in a previous work using image analysis from an electron micrograph. Mammalian and bacterial TSPO have different number and composition of amino acids, their recombinant forms have a polyhistidine tag located in the C-terminus versus N-terminus, respectively. These differences may account for different polymeric associations. The loop between the first and the second putative transmembrane domains may be crucial in protein–protein association since it has been proposed to participate in bacterial TSPO dimer formation. In addition distinct ligand binding and transported molecules have been reported between mammalian and bacterial TSPO, giving different biological functions for these two proteins.

2. Material and methods

Lipids, *detergent and protein*: all lipids used in this study were purchased from Avanti Polar Lipids. Monolayer lipid ligand and diluting lipid are 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentanyl) iminodiacetic acid)succinyl] (Nickel salt) later named DOGS-NTA-Ni, and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC). These lipids were prepared in an organic solvent mixture chloroform/methanol (v/v) and stored at −20 °C. Lipids used for proteoliposomes were 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE) mixed in organic solvent at a DMPC/DMPE ratio of 9/1 (w/w), then solvent was removed and lipids resuspended in water and stored at −20 °C.

Detergent: sodium dodecylsulfate (SDS) was purchased from Sigma-Aldrich (France).

TSPO protein: His-tagged mouse recombinant TSPO was overexpressed in *E. coli* bacteria and purified in SDS as described [16,17].

TSPO reconstitution: DMPC/DMPE lipid mixture was added to mouse recombinant TSPO purified in SDS to full solubility and SDS was removed by incubation with Bio-beads SM2 (BioRad, France) as described [8,18].

SDS removal was followed using a homemade protocol previously described [18].

TSPO intrinsic fluorescence was performed using a PTI spectrophotometer (Serlabo Technology, France) equipped with a stirrer and a temperature-controlled cell. Excitation and emission wavelengths were set at 290 and 340 nm, respectively. The slits were set at 2 and 4 nm for excitation and emission, respectively. Experiments were performed at room temperature, 20 °C.

TSPO high affinity ligand binding was measured using proteoliposomes and radioactive [^{3}H] PK 11195 as described [18]. The Kd value for PK 11195 was determined by curve fitting using the following equation:

$$\text{PK 11195 bound to TSPO protein}(\%) = 100 * [\text{PK 11195}]/(Kd + [\text{PK 11195}])$$

In our data, 100% of binding was measured to be equal to 25 ± 2 nmol PK 11195 bound per milligram of TSPO.

Monolayers: monolayers were prepared in a 8 mL Teflon trough (S = 20 cm^2) filled with buffer (50 mM, Tris–HCl at pH 8). Surface pressure was measured using the Wilhelmy method (Nima Technology, Coventry, UK).

Ellipsometry measurements were carried out with an in-house automated ellipsometer [19,20] in a "null ellipsometer" configuration [21]. He–Ne laser beam (λ = 632.8 nm, Melles Griot) was polarized with a Glan–Thompson polarizer and reflected on the surface of the trough (incidence angle of 52.12°). After reflection on the liquid surface, the laser light passed through a λ/4 retardation plate, a Glan–Thompson analyzer and a photomultiplier. The analyzer angle, multiplied by two, yielded the value of the ellipsometric angle (Δ) i.e., the phase difference between parallel and perpendicular polarization of the reflected light. The laser beam probed a surface of 1 mm^2 and a depth in the order of 1 μm. Initial values of the ellipsometric angle (Δ_0) and surface tension of pure buffer solutions were recorded on the subphase for at least half an hour. These values have been subtracted from all data presented below. Values of Δ were stable and recorded every 4 s with a precision of ±0.5°.

Brewster Angle Microscopy (BAM) [22] observations were carried out with a device mounted on the ellipsometer apparatus, allowing the visualization of the surface during protein adsorption. The interface is illuminated at the air–water Brewster incidence (53.1°) with He\Ne laser (λ = 632.8 nm, Melles-Griot, France) and the reflected beam is captured by a CCD video camera giving an image of the monolayer. The contrast is due to a change in the reflexion coefficient of the polarized incident light on the surface due to a change in the refractive index of the interface upon adsorption of molecules. If the molecules are not adsorbed at the interface, the grey level is equal to the background level and the picture is dark. On contrary, when the molecules are adsorbed at the interface, the induced modification of the refractive index changes the reflexion coefficient of the incident polarized light and the gray level is higher than the background level in all places where molecules are present. Hence, domains appear as bright regions on a dark background while a homogeneous layer appears as a homogeneous bright picture. Our device does not allow to quantify the exact amount of molecules present at the interface but the comparison between gray levels gives an indication on the relative amount of molecules. The resolution of the system is a few μm.

Transmission Electron Microscopy (TEM): solution samples were deposited on carbon-coated grids (400 mesh, EMS); monolayers were picked up by placing a carbon-coated grid either on the surface of the monolayer through or on top of the well for several minutes. In all cases, grids were blotted with filter paper and negatively stained with 2% (w/w) uranyl acetate for 30 s. Images were recorded with a JEOL 1200EX operated at 80 kV or a FEI Philips CM120 operating at 100 kV.

Image analysis: selected electron micrographs were digitized with a Nikon film scanner (Super cool scan 9000 ED) at 2000 pixels/in. Digitized images were first treated to increase contrast and to perform object picking using IMOD program [23]. Then, subimages were aligned rotationally and translationally generating an averaged image. Briefly, all images were compared by cross correlation, rotated and translated to get the best match using classical protocol available in SPIDER program [24].

Finally, correspondence analysis was followed by hierarchical ascendant classification using SPIDER program [25] leading to homogeneous