Structure of the mammalian TSPO/PBR protein

Mariusz Jaremko*[1], Łukasz Jaremko*†[1], Garima Jaipuria*, Stefan Becker* and Markus Zweckstetter*†‡[2]

*Max-Planck-Institut für Biophysikalische Chemie, Göttingen 37077, Germany

†Deutsches Zentrum für Neurodegenerative Erkrankungen (DZNE), Göttingen 37077, Germany

‡Center for Nanoscale Microscopy and Molecular Physiology of the Brain, University Medical Center, Göttingen 37077, Germany

Abstract

The 3D structure of the 18-kDa transmembrane (TM) protein TSPO (translocator protein)/PBR (peripheral benzodiazepine receptor), which contains a binding site for benzodiazepines, is important to better understand its function and regulation by endogenous and synthetic ligands. We have recently determined the structure of mammalian TSPO/PBR in complex with the diagnostic ligand PK11195 [1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide; Jaremko et al. (2014) Science 343, 1363–1366], providing for the first time atomic-level insight into the conformation of this protein, which is up-regulated in various pathological conditions including Alzheimer's disease and Parkinson's disease. Here, we review the studies which have probed the structural properties of mammalian TSPO/PBR as well as the homologues bacterial tryptophan-rich sensory proteins (TspOs) over the years and provide detailed insight into the 3D structure of mouse TSPO (mTSPO)/PBR in complex with PK11195.

The TSPO/PBR protein

The 18-kDa translocator protein (TSPO)/peripheral benzodiazepine receptor (PBR) is an evolutionarily conserved membrane protein found in most species including bacteria, plants and humans with some exceptions including *Escherichia coli* and *Saccharomyces cerevisiae* [1]. Although expressed throughout the body, human TSPO/PBR is present in high amounts in the outer mitochondrial membrane of steroid-synthesizing cells of the central and the peripheral nervous system [2]. TSPO/PBR was discovered in 1977, when Braestrup et al. [3] observed the existence of specific receptors on the membranes of rat brain cells, which specifically bind to an important group of psychoactive drugs, the benzodiazepines. TSPO/PBR was purified in complex with the voltage-dependent anion channel and the adenine nucleotide transporter from the mitochondrial membrane [4]. TSPO/PBR was referred to as PBR until 2006, when it was renamed due to its potential role in translocation of cholesterol from the outer to the inner mitochondrial membrane [5]. Recent studies, however, challenge the direct involvement of TSPO/PBR in cholesterol transport and steroidogenesis [6–8]. At the same time, TSPO/PBR has been implicated in several biological functions [9], including mitochondrial respiration [10], cell proliferation [11], immunomodulation and apoptosis [12,13]. In bacteria, tryptophan-rich sensory proteins (TspOs) are associated with the porphyrin biosynthesis pathway and consumption of reactive oxygen species [14].

TSPO/PBR is also important from a medical perspective as its expression is up-regulated in various pathological conditions including Alzheimer's disease, Parkinson's disease and multiple sclerosis [15]. In addition, the TSPO/PBR variant Ala[147]Thr is associated with human adult separation anxiety [16]. Increased expression levels of TSPO/PBR can be monitored by positron emission tomography/single photon emission computed tomography. These studies use TSPO/PBR-specific radiolabelled neuroimaging ligands such as [^{11}C](*R*)-PK11195 [1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide] to allow sensitive recognition of lesions and active disease processes in the brain [1,17–19]. TSPO/PBR ligands also have potential therapeutic applications such as attenuation of cancer cell proliferation [20] and neuro-protective effects [21]. Thus, many studies are pursued to develop synthetic ligands that specifically bind to TSPO/PBR and aid in the determination and restoration of active disease areas [15].

Structural properties of TSPO/PBR

A wide range of techniques have been used to probe the structural properties of TSPO/PBR including bioinformatics analysis, MD simulations, CD, IF spectroscopy, EM, NMR spectroscopy and biochemical assays. Analysis of the amino acid sequence of TSPO/PBR points to a high degree of homology between different species (Figure 1A) [22,23]. Hydropathy analysis of TSPO/PBR indicates five stretches of hydrophobic residues (Figure 1B), each long enough to

Key words: dynamics, nuclear magnetic resonance (NMR), peripheral benzodiazepine receptor, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK11195), structure, TSPO.

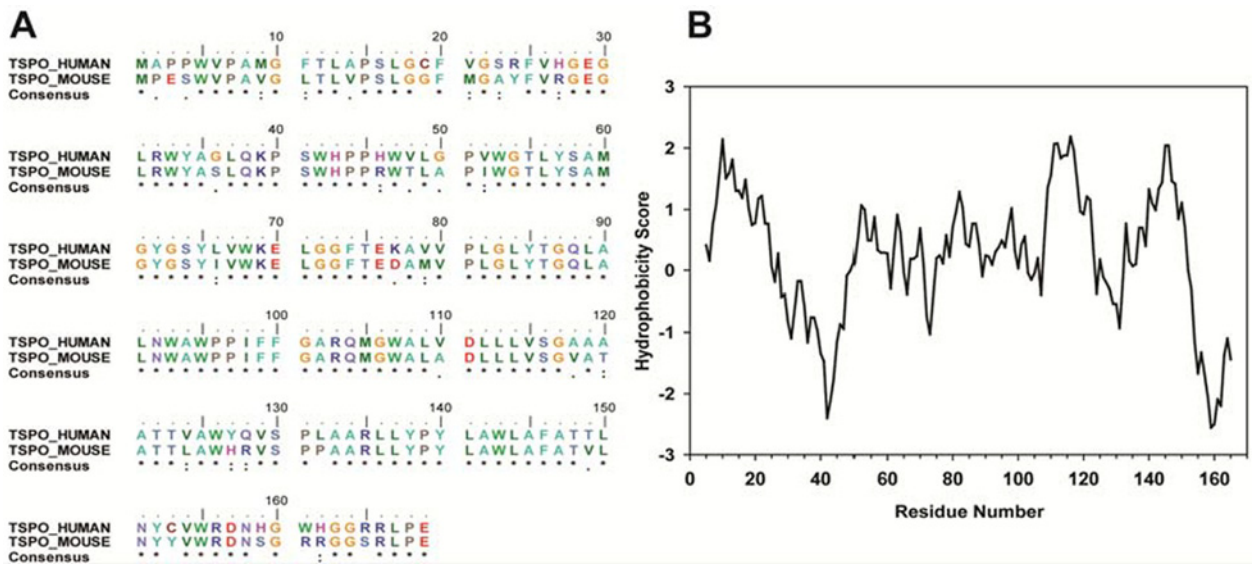
Abbreviations: CRAC, cholesterol recognition amino acid sequence; DPC, dodecylphosphocholine; PBR, peripheral benzodiazepine receptor; PK11195, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide; mTSPO, mouse TSPO; RsTspO, TspO from *Rhodobacter sphaeroides*; TM, transmembrane; TSPO, translocator protein; TspO, tryptophan-rich sensory protein.

[1]These authors contributed equally to the present work.

[2] **To whom correspondence should be addressed (email Markus.Zweckstetter@dzne.de).**

Figure 1 | **Sequence properties of mammalian TSPO/PBR**

(**A**) Alignment of the primary sequences of human and mTSPO/PBR. (**B**) Hydrophobicity of mTSPO/PBR as a function of residue number.



span the bilayer membrane. Early MD simulations predicted an energetically stable arrangement of the five transmembrane (TM) helices of TSPO/PBR with a short intramitochondrial N-terminus and a longer C-terminal tail pointing towards the extra-mitochondrial side [24]. These predictions were confirmed by immunodetection of epitope insertions made in the loop regions of TSPO/PBR and also by visualization of cysteine residues using thiol-labelling techniques [25]. Site-directed mutagenesis of specific residues on the C-terminal end led to the identification of a conserved cholesterol recognition amino acid sequence (CRAC) [26]. The far UV CD spectrum of mouse TSPO/PBR (mTSPO/PBR) solubilized in dodecylphosphocholine (DPC) micelles indicates a helical content of 45 %, which is further increased upon binding of PK11195 [27]. Changes in the secondary structure content upon ligand addition have also been confirmed by studying the hydrogen/deuterium exchange kinetics of amide protons by mid-IF spectroscopy. Increase in exchange rates and percentage of non-exchangeable amide protons upon binding of PK11195 to TSPO/PBR suggests that a more compact TSPO/PBR structure is generated on ligand addition [28].

The tendency of TSPO/PBR to form oligomeric complexes has been supported by biochemical experiments, as well as EM. While Lacapere and co-workers [29] observed association of at least four monomer units of mTSPO/PBR, Korkhov et al. [30] determined a low-resolution dimeric architecture of the homologues TspO from *Rhodobacter sphaeroides* (RsTspO) based on cryoEM of helical crystals. Consistent with the pronounced tendency of RsTspO to dimerize, two pentahelical RsTspO units were arranged as a dimer in the lipid bilayer [30]. Because TM helix 1 is the least conserved of the five TM regions and is more polar in RsTspO than in mTSPO/PBR, where the functional unit is a monomer, we suggested that TM helix 1 is involved in the dimer interface of RsTspO [22]. However, alternative models were also proposed [31,32].

NMR spectroscopy of membrane proteins

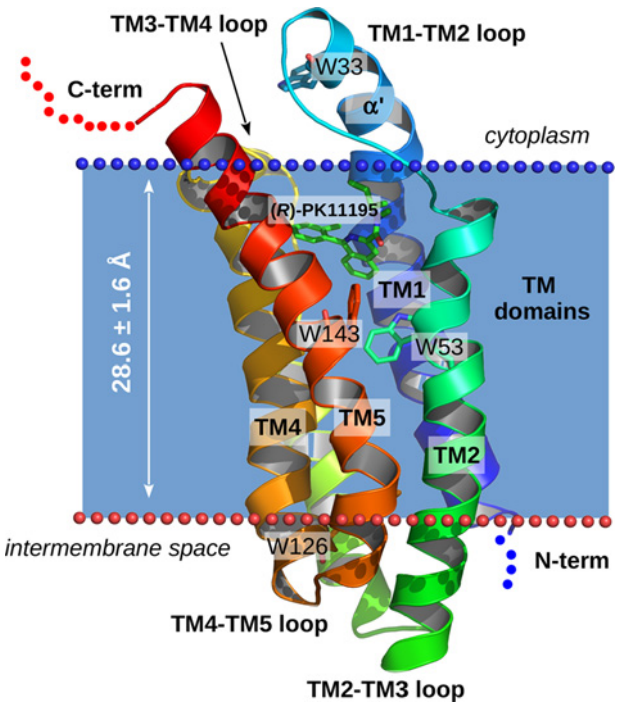
NMR spectroscopy and X-ray crystallography are two powerful techniques capable of determining the 3D structure of protein molecules at atomic resolution. While structure determination by X-ray crystallography requires the freezing of proteins at cryogenic temperatures in a crystal lattice, NMR spectroscopy is capable of determining the structure of both soluble and membrane proteins at near physiological temperatures. In addition, NMR spectroscopy can provide insight into time-dependent chemical phenomena, including reaction kinetics and dynamics in solution and the solid state at atomic resolution [33]. Although the cumulative molecular mass of a protein–detergent complex, the oligomerization state of the protein and a limited dispersion of an NMR spectrum may pose formidable hurdles for the structural study of membrane proteins by solution-state NMR, significant advances in sample preparation and experimental NMR methods have established NMR spectroscopy as a powerful method for studying membrane proteins both in the solution and in the solid state [34,35].

3D structure of mammalian TSPO/PBR in complex with PK11195

We recently determined the 3D structure of the 169-residue mTSPO/PBR in complex with (*R*)-PK11195 [36]. The structure was solved using solution NMR spectroscopy with mTSPO/PBR solubilized in DPC micelles. Consistent

Figure 2 | 3D structure of mTSPO/PBR in complex with (R)-PK11195

The backbone of mTSPO/PBR (PDB code: 2MGY; [36]) is shown in ribbon representation. The approximate positioning in the membrane is indicated in blue. The membrane thickness was estimated using the OPM server (http://opm.phar.umich.edu/server.php). Flexible C- and N-termini are marked by red and blue dots respectively. (*R*)-PK11195 is depicted in sticks. The side chains of four conserved tryptophan residues, out of a total of 12 tryptophan residues in mTSPO/PBR, are shown.



with the ability of mammalian TSPO/PBR to bind synthetic ligands and function as monomeric unit [37], mTSPO/PBR solubilized in DPC micelles is monomeric [36]. The mTSPO/PBR–PK11195 complex structure is characterized by a tight packing of its α-helical TMregions (Figure 2). When viewed from the extra-mitochondrial side, the clockwise order of the five TM helices is TM1–TM2–TM5–TM4–TM3. The same TM topology was recently observed for two bacterial tryptophan-rich sensory proteins [32,38]. mTSPO/PBR has a high content of proline residues in its TM region. TM1 is perturbed by Pro^{15}, whereas TM2 and TM3 contain the double-proline motifs Pro^{44}–Pro^{45} and Pro^{96}–Pro^{97} respectively. Proline residues are also present at positions 51 in TM2 and 81 in TM3. TM5 has a proline at position 139, which kinks this helix toward the intra-mitochondrial side. The most stable TM segments of the mTSPO/PBR structure are TM4 and TM5 according to hydrogen/deuterium exchange experiments [36]. TM4 and TM5 are the evolutionary most conserved regions of TSPO/PBR.

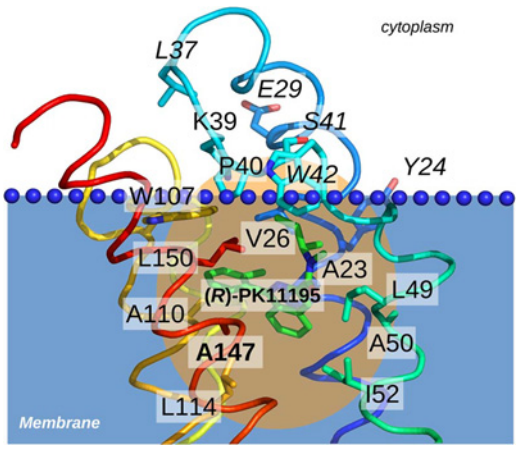
Mammalian TSPO/PBR is homologues to bacterial TspO proteins, which have a high content of tryptophan residues [39]. Accordingly, several tryptophan residues of mTSPO/PBR are conserved such as Trp^{33}, which is located in the short α'-helix in the TM1–TM2 loop, Trp^{53} in TM2, Trp^{126} at the end of TM4 and Trp^{143} in TM5 (Figure 2). Both Trp^{53} and Trp^{143} are in close contact to each other and contribute to the TM2/TM5 interface. Trp^{53} and Trp^{143} point toward the interior of the protein and make numerous contacts to neighbouring residues, consistent with the conservation of TM2, TM4 and TM5.

Topological analysis has suggested that the flexible C-terminus, the TM3–TM4 loop and the TM1–TM2 loop are located on the extra-mitochondrial side [25]. The intra-mitochondrial parts of TSPO/PBR are then formed by the N-terminus, the TM4–TM5 loop and the TM2–TM3 loop (Figure 2). Loop regions in mTSPO/PBR are short with the exception of the TM1–TM2 loop, which contains a seven-residue-long α-helical motif (Figure 2). Loops are mostly well-defined in the structure and have dynamic properties comparable to the TM α-helices [36]. The long TM1–TM2 loop forms a lid closing the top of the TM bundle and protects the PK11195 ligand from leaving the protein (Figure 2). On the intra-mitochondrial side, the TM2–TM3 loop is protruding from the membrane together with α-helical extensions of the C-terminal end of TM2 and the N-terminal end of TM3.

The C- and N-terminus of mTSPO/PBR in complex with PK11195 have no stable structure as evidenced by their close to random coil chemical shifts as well as

Figure 3 | The binding site for PK11195 and porphyrin

Selected view of the binding pocket of PK11195 as seen in the structure of mTSPO/PBR in complex with PK11195 (PDB code: 2MGY). Colour coding of helices is the same as in Figure 2. Residues, which are in direct contact with (*R*)-PK11195, are marked. Residues, which were additionally found to be important for ligand binding in mutagenesis studies [42,44,45], are labelled with italic characters. The orange ellipsoid highlights the region, which is important for binding to PK11195 and porphyrins. The polymorphic residue Ala^{147} is shown in bold.



negative steady-state Nuclear Overhauser Enhancement values [36]. The C-terminus is highly positively charged, whereas the regions exposed to the intra-mitochondrial side have an equal number of positive and negative charges [36]. The distribution of positively and negatively charged patches on the extra- and intra-mitochondrial side might be important for recognition of endogenous ligands and proteins interacting with TSPO/PBR, such as steroid acute regulatory protein and diazepam-binding inhibitor [40].

Interaction with small molecules

PK11195 binds with nanomolar affinity to TSPO/PBR from different eukaryotic species [41]. Residues, which are important for binding to PK11195, were identified by site-directed mutagenesis [42] and their importance is supported by the 3D structure of the mTSPO/PBR–PK11195 complex. In particular, residues Ala^{23}, Val^{26}, Leu^{49}, Ala^{50}, Ile^{52}, Trp^{107}, Ala^{110}, Leu^{114}, Ala^{147}, Leu^{150} of mTSPO/PBR directly contact (R)-PK11195 (Figure 3) [36]. On the other hand, PK11195 binds with an at least 1000-fold lower affinity to *Rs*TspO [43], suggesting possible structural differences between mammalian TSPO/PBR and bacterial TspO proteins (consistent with recent structural studies of two bacterial tryptophan-rich sensory proteins [32,38]). In case of mTSPO/PBR, Glu^{29} to Ala^{35} form a short α-helix in the extra-mitochondrial space, whereas the ^{27}RGE^{29} fragment is missing in the *Rs*TspO sequence. This might lead to a less stable lid on top of the PK11195-binding pocket and contribute to the lower PK11195 affinity of *Rs*TspO. Together with site-directed mutagenesis, the sequence differences support the importance of the TM1–TM2 loop for ligand binding [26,42]. Another region contributing to the functional and structural differences of mammalian TSPO/PBR and bacterial TspO proteins is probably the TM1 helix, as this is the least conserved region across different species. Indeed, two of the mTSPO/PBR residues, Ala^{23} and Val^{26}, which directly contact PK11195, are located in TM1 [36]. The binding cavity identified in the 3D structure of the mTSPO/PBR–PK11195 complex, is likely to be important for several other ligands. In line with this hypothesis, mutagenesis studies and computational analysis have suggested that residues Arg^{24}, Glu^{29}, Leu^{31}, Leu^{37}, Lys^{39}, Pro^{40}, Ser^{41}, Trp^{42}, Trp^{107} and Trp^{161} of TSPO/PBR (sequence numbering according to the human protein) are important for binding of the ligand Ro5-4864 [42,44,45]. Notably, although PK11195 binds with similar affinity to mutant TSPO/PBR, which carries either an alanine or a threonine at position 147, the rs6971 polymorphism strongly affects the binding of many second generation radioligands to TSPO/PBR [46,47]. This highlights the need to determine the 3D structure of both wild-type and the Ala^{147} variant of mammalian TSPO/PBR in complex with different small molecules.

Interaction with endogenous ligands

Numerous endogenous ligands are recognized and bound to TSPO/PBR including porphyrins, products of their degradation and cholesterol [15]. Cholesterol binds with nanomolar affinity to the CRAC motif (residues 147–159) close to the C-terminus of TSPO/PBR [26]. The CRAC motif L/V–X (1–5)–Y–X (1–5)–R/K has also been found in other TM proteins that interact with cholesterol [48]. An NMR analysis of a C-terminal TSPO/PBR peptide (residues 144–169) indicated a helical conformation for the Leu^{144} to Ser^{159} fragment of the peptide and a groove that could possibly

accommodate a cholesterol molecule [49]. Structural studies on full-length mTSPO/PBR revealed that in complex with PK11195 the CRAC motif forms a well-ordered α-helix [36]. The residues, which mutational analysis has shown to be important for cholesterol binding [26,50], point away from the protein interior towards the hydrophobic environment of the membrane. Together with neighbouring residues in TM4, the CRAC motif is the most stable part of the mTSPO/PBR-PK11195 structure [36].

Porphyrins bind to a different region of TSPO/PBR than cholesterol, such that substitution of Ala^{147} by threonine affects binding of porphyrins and second-generation radioligands [14,46,47]. In addition, PK11195 binding to TspO inhibits porphyrin degradation, suggesting that the two molecules compete for the same site (Figure 3), with PK11195 having higher affinity [14].

Concluding remarks

The 3D structure of mTSPO/PBR in complex with PK11195 has provided an important step towards a more detailed understanding of the molecular mechanism of mammalian TSPO/PBR and its interaction with small molecules [36]. Future structural studies will be required to provide insight into the influence of the $A^{147}T$ polymorphism on the recognition of second-generation radioligands, the analysis of interspecies differences, the structure and dynamics of TSPO/PBR in the absence of high-affinity ligands, the characterization of TSPO/PBR in complex with cholesterol and the binding of TSPO/PBR to other endogenous interaction partners.

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