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Structural integrity of the A147T polymorph of mammalian TSPO

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Abstract

Ligands of the transmembrane protein TSPO are used for imaging of brain inflammation, but a common polymorphism in TSPO complicates their application to humans. Here we determined the three-dimensional structure and side chain dynamics of the A147T polymorph of mammalian TSPO in complex with the first-generation ligand PK11195. We show that A147T TSPO is able to retain the same structural and dynamic profile of the wild-type protein and thus binds PK11195 with comparable affinity. Our study is important for the design of more potent diagnostic and therapeutic ligands of TSPO.

Keywords

NMR spectroscopy; membrane protein; polymorphism; structure; dynamics

Introduction

Translocator protein (TSPO) is a 169-residue protein, which is mainly found in the outer mitochondrial membrane of steroid synthesizing cells of the nervous system [1]. TSPO plays a role in mitochondrial respiration [2], cell proliferation [3], immunomodulation and apoptosis [4]. Expression of TSPO is upregulated in conditions of neuroinflammation as found in Alzheimer's and Parkinson's disease [5]. TSPO is a secondary binding site for benzodiazepines [6], and small molecules targeting TSPO might become useful in treating anxiety [7]. At the carboxylic terminus, TSPO contains a cholesterol recognition amino acid consensus (CRAC) sequence [8]. Cholesterol binds with nanomolar affinity to this sequence [9], but the direct role of TSPO in steroidogenesis is under debate [10].

Because TSPO is upregulated in activated microglia, small molecules, which bind to TSPO, are valuable tools to image inflammation in the brain [1c, 11]. The best characterized

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radioligand of TSPO is 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide, called PK11195 [12]. PK11195 binds with nanomolar affinity to mammalian TSPO [9], and is specific for the protein, as supported by TSPO knock-out studies in mice [10a]. PK11195 binds to a hydrophobic pocket on the cytosolic side of TSPO [13]. The binding pocket is formed by a tight bundle of five α -helices and closed by the loop region between the transmembrane domains one and two [14]. Within this loop region a short α -helix is found. TSPO residues, which mutational analysis showed to be important for cholesterol binding [13a], point away from the PK11195-binding pocket to the membrane environment [14].

To overcome limitations in the signal-to-background ratio of PK11195 in human positron emission tomography studies, a wide range of second-generation TSPO ligands were developed [11a, 15]. However, their application for medical purposes is complicated due to a polymorphism in TSPO, which results in the substitution of alanine by threonine at position 147 and decreases the affinity of many second-generation radioligands [16]. The change in binding affinity is most problematic for studies of Caucasians, where 30% of the population was reported to have T147 [17]. Besides its importance for diagnostic purposes, the A147T polymorphism was suggested to affect pregnenolone production [18], and to be associated with neurological diseases [19].

To understand the molecular basis of the A147T polymorphism on the function of mammalian TSPO and its interaction with ligands, we studied the structure and dynamics of the A147T variant of mouse TSPO (A147T-mTSPO) in the ligand-free state and in complex with (R)-PK11195 using NMR spectroscopy. We show that A147T-mTSPO is highly dynamic in the absence of a ligand, but folds into a defined three-dimensional (3D) structure upon binding to PK11195. We further show that the A147T substitution modulates the structure around the site of mutation, but does not affect the conformation of the TM1-TM2 loop and the cholesterol recognition sequence

Results

Three-dimensional structure of the A147T polymorph of mammalian TSPO in complex with PK11195

High-quality NMR spectra were obtained for the A147T-mTSPO-PK11195 complex reconstituted into fos-choline-12 micelles (Figure 1A and Supporting Information Figure S1). Triple-resonance experiments in combination with high-resolution side-chain NMR spectra enabled the sequence-specific assignment of 98% of the backbone and 95% of the side chain resonances of A147T-mTSPO. Subsequently, the 3D structure of the A147T-mTSPO-PK11195 complex was determined on the basis of a large number of NOE contacts (Table 1). The ensemble of 20 lowest-energy structures was well converged (Figure 1B). The transmembrane (TM) region of A147T-mTSPO is formed by five α -helices (Figure 1B). The five helices are organized in a tight bundle with the clockwise order TM1-TM2-TM5-TM4-TM3 (cytosolic view). The C-terminus of A147T-mTSPO is located on the cytosolic side [20], and is unstructured starting from R161. The five TM helices are connected by short loops (Figure 1B). The exception is the TM1-TM2 loop, which contains a 7-residue α -helix. The TM1-TM2 loop makes several contacts to the TM3-TM4 loop and the TM5 helix.

A147T-mTSPO binds one PK11195 molecule in the hydrophobic pocket, which is oriented toward the cytosol (Figure 1B).

A147T-mTSPO in complex with (R)-PK11195 has the same topology and distribution of secondary motifs as the wild-type protein [14]. The transmembrane region of the two structures can be overlapped with a backbone root-mean-square deviation of 0.95 Å (Figure 1C). The alanine to threonine substitution perturbed the local structure proportionally to the volume difference between the two types of side chain (Figure 1D). In addition, local structural rearrangements were found at the intermembrane space (IMS) side of TM5 (Figure 1C and S1), as evidenced by changes in cross peaks of L56 (TM2), V67 (TM2) and L137 (TM5) (Supporting Information Figure S2). In agreement with a conserved recognition mode and small side chain chemical shifts changes (Figure 1A and Supporting Information S3), the same mTSPO residues were found to make contacts with (R)-PK11195 in the A147T variant and the wild-type protein.

The chemical environment of TSPO's tryptophan residues is conserved

Chemical shifts are a valuable source of information on the local structure around a particular spin. To get further insight into the effect of the A147T mutation on the structure of mTSPO, we compared the chemical shifts of the variant and wild-type protein in complex with PK11195 (Figure 2). Backbone NH chemical shifts only changed in vicinity to the site of mutation including L144, which is one helical turn away from position 147 (Figure 2A,B). In addition, a 5-20% increase in helical content was observed for L137 and P138 in A147T-mTSPO. However, chemical shift-derived RCI-S² order parameters remained intact (Supporting Information Figure S4), demonstrating that the local mobility of N-H bonds in the main-chain was not perturbed. We also analysed the chemical shifts of tryptophan side chains, as TSPO is homologues to bacterial tryptophan-rich sensory proteins and tryptophan residues are believed to be functionally important [1a]. In agreement with similar 3D structures of A147T-mTSPO and wild-type mTSPO, only He1/Ne1 side chain resonances of W53 and W143, which are in spatial proximity to the site of mutation (Figure 2B), were perturbed by more than 0.2 units (Figure 2C,2D). W42 also showed slight changes in its He1/Ne1 chemical shifts due to the spatial proximity to T148 and L149. All other tryptophan side chains were not affected, consistent with a conserved environment.

Mobility of the side chains of TSPO in complex with PK11195

Ligand binding is driven by changes in enthalpy and entropy. In order to find out if the A147T substitution influences the motional properties of the side chains of mTSPO and thus modulates the entropic component of the interaction with PK11195, we probed the dynamics of the methyl groups of mTSPO in complex with (R)-PK11195. Cross-correlated relaxation rates (σ_{obs}) between dipolar couplings of two CH bonds were measured for well separated cross peaks in 2D ¹H-¹³C HSQC spectra [21]. 19 methyl groups distributed across the entire structure could be analyzed (Figure 3A and Supporting Information Figure S3). As more rigid methyl groups have larger σ_{obs} rates [21], the methyl groups of A50, I52 and L56 are most rigid. Moreover, comparison of σ_{obs} rates between wild-type protein and the A147 variant showed that the motional properties of the methyl groups were retained despite the A147 substitution (Figure 3A). This includes residues in the vicinity of PK11195 such as

L37 in the TM1-TM2 loop, as well as I66 and V67 on the IMS side. Notably, V26 is significantly more dynamic than the rest of the residues (Figure 3B), suggesting that the cytosolic end of TM1 might be important for the access of PK11195 to the binding pocket.

Structural integrity of the cholesterol recognition site

The cholesterol recognition sequence of mTSPO is located at the C-terminal end of TM5 and comprises residues L149-R156 [8]. The site of the A147T polymorphism is less than one helical turn away from it (Figure 4A). Substitution of alanine to threonine at the homologous position (A139T) in the tryptophan-rich sensory protein from *Rhodobacter sphaeroides* (RsTspO), a bacterial homologue of mammalian TSPO, decreased the cholesterol affinity by four-to-five fold [22]. However, the CRAC motif is not well conserved in the bacterial protein, resulting in a 10000-fold lower affinity of cholesterol to RsTSPO when compared to mammalian TSPO. We therefore asked if the A147T substitution modulates the structure of the CRAC motif. Figure 4A shows a superposition of the conformation of the CRAC motif as found in the 3D structures of wild-type mTSPO and the A147T variant both in complex with PK11195. Within the accuracy of the NMR ensemble the conformation of the main chain as well as the side chain orientations were identical. Identical conformations were also found for the side chains of Y152, Y153 and R156, residues essential for cholesterol binding [9, 13a]. In addition, backbone dynamics in the CRAC motif were not perturbed according to RCI-S² order parameters and the mobility of the methyl group at position 147 was unchanged (Figure 3A and Supporting Information Figure S4).

We then asked if the A147T substitution affects the structure of mTSPO when not bound to PK11195. Previous studies have shown that NMR resonances in ¹H-¹⁵N correlation spectra of ligand-free mTSPO cluster together [14, 23], suggesting that the mTSPO conformation is dynamic in the absence of a high-affinity ligand. For A147T-mTSPO, we also found narrow chemical shift dispersion (Figure 4B and Supporting Information Figure S5). Moreover, resonances of the wild-type protein and its A147T variant were superposable, suggesting that the polymorphism does not perturb the ligand-free state of mammalian TSPO.

Discussion

We determined the high-resolution three-dimensional structure of the A147T polymorph of mammalian TSPO in complex with the radioligand PK11195. Our study demonstrates that the A147T substitution, which is found in a large part of the Caucasian population, results in only local conformational changes in the 3D structure of mammalian TSPO in complex with PK11195. In addition, the mutation does not influence the structural integrity of the cholesterol recognition sequence, which is highly conserved in mammalian TSPO proteins (Figure 5). The structural integrity of the CRAC motif in mTSPO stands in contrast to structural changes observed between the crystal structures of wild-type RsTspO and its A139T variant [22]. In the crystal structure of the A139T variant of RsTspO the TM2 helix was tilted by 7.7° toward the TM5 helix. In addition, TM5 became less kinked resulting in a closer association of TM2 and TM5. These changes in the main chain were accompanied by the repositioning of the side chains of F144 and R148 on TM5 of RsTspO (Figure 4C) [22].

What is the reason for the structural differences between mTSPO and RsTspO, in particular in the region that is used by mammalian TSPO to bind cholesterol? RsTspO shares the same fold as mTSPO and preserves several interactions among conserved residues. However, the structures of the two proteins also differ in important aspects, and differ from the structure of the tryptophan-rich sensory protein from *Bacillus cereus* (BcTspO) [24]. The conformational differences are most pronounced in the packing of TM1 and the TM1-TM2 loop (Figure 4C). In addition, the end of TM1 and the connection to the short cytosolic α -helix is helical in the mTSPO-PK11195 complex, but has a loop conformation in RsTspO and BcTspO (Figure 4C). Because of these changes in the main chain, the side chains of the mTSPO residues A23 and V26, which show experimental NOE contacts to PK11195, are not pointing to the PK11195-binding pocket in the bacterial proteins, but are rotated toward the membrane. The structural differences, however, come with no surprise as bacterial tryptophan-rich sensory proteins bind PK11195 with 1000-fold lower affinity when compared to mammalian TSPO [22]. Moreover, the relative orientation of the TM2 and TM5 helices and the position of the TM1-TM2 loop is highly similar in wild-type BcTspO and the A138T variant of RsTspO as well as mTSPO and A147T-mTSPO, but different in wild-type RsTspO (Figure 4C), suggesting that the structural differences between wild-type and A139T RsTspO are caused by differences in crystal packing. Importantly, the orientation of the side chains of F144 and R148 differ in the structures of wild-type and A139T RsTspO, but the orientation of the homologous residues in wild-type BcTspO (W147 und S151), wild-type mTSPO and A147T mTSPO are very similar (Y152 and R156; Figure 4C), providing further support for the structural integrity of the cholesterol recognition sequence in the A147T polymorph of TSPO.

The structural integrity of the A147T variant of mTSPO in complex with PK11195 is in agreement with similar affinities of PK11195 to wild-type and A147T TSPO.[16a] In contrast to PK11195, which is called a first-generation ligand, the second-generation ligand PBR28 has a 50-fold reduced affinity to A147T TSPO [25]. This suggest that in case of PBR28 and other second generation ligands, A147T mTSPO is no longer able to retain the same structural and dynamic profile as the wild-type protein and thus binds these ligands with lower affinity. To probe this hypothesis, it will be important to probe the side chain dynamics of TSPO in complex with additional ligands. Characterization of side chain dynamics might also be useful for other membrane proteins. In addition, the structural integrity of the CRAC motif in mammalian TSPO highlights the limitations that are inherent to the transfer of findings from tryptophan-rich sensory proteins in bacteria, which do not contain cholesterol, to mammalian TSPO. Our study is thus important for the design of more potent diagnostic and therapeutic ligands of TSPO.

Experimental section

Protein and NMR sample preparation

^{15}N -, $^{13}\text{C}/^{15}\text{N}$ - and $^2\text{H}/^{13}\text{C}/^{15}\text{N}$ -labelled wild-type and A147T-mTSPO were expressed in *E. coli* using M9 minimal medium according to established protocols [14, 23]. NMR samples contained 0.5-0.9 mM protein solution in 10 mM sodium phosphate buffer, pH 6.0, in the

presence of approximately 2% (m/v) ^2H -DPC. For complex formation, 2.9 mM of (R)-PK11195 were added.

NMR spectroscopy

NMR measurements were performed on 900 and 800 MHz NMR spectrometers (Bruker) at 42 °C. Spectra were processed using TopSpin2.1 (Bruker) and analyzed using CARA [26]. To establish the sequence-specific backbone assignment of A147T-mTSPO in complex with (R)-PK11195, TROSY-based 3D HNCO, HNCA, HN(CO)CA, HNCACB, and HN(CA)CB experiments were recorded [27] on $^2\text{H}/^{13}\text{C}/^{15}\text{N}$ -labelled protein. Backbone resonance assignments were further supported by $\text{H}^{\text{N}}\text{-H}^{\text{N}}$ $i \pm 1, 2, 3$ connectivity, which was observed in a 3D ^{15}N -edited NOESY-HSQC spectrum (NOE mixing time of 200 ms) [28]. Analysis of experimental C^{β} and C^{γ} chemical shifts using PROMEGA [29] showed that all 14 proline residues of A147T-mTSPO were in *trans* conformation. Aliphatic side chain resonances were assigned using 3D (H)CCH-TOCSY, 3D H(C)CH-TOCSY (mixing times of 10 and 16 ms, respectively) [30] and 3D ^{13}C -edited NOESY-HSQC (100 ms mixing time) spectra recorded on $^{13}\text{C}/^{15}\text{N}$ -labelled A147T-mTSPO in complex with (R)-PK11195 (sample in D_2O), as well as a 3D ^{15}N -edited NOESY-HSQC spectrum (150 ms mixing time) measured in H_2O . NOE cross peaks between $^{13}\text{CH}_3$ methyl groups and H^{N} amides of the same spin system, which were observed in a 3D ^{13}C -edited NOESY-HSQC spectrum (150 ms mixing time) recorded on $^{13}\text{C}/^{15}\text{N}$ -labelled A147T-mTSPO in complex with (R)-PK11195 in H_2O , were used for verification of side chain assignments of valine, isoleucine, alanine and leucine residues.

RCI- S^2 order parameters were calculated from backbone chemical shifts collected at 42 °C using TALOS+ [31]. CCR rates in methyl groups were measured on $^{13}\text{C}/^{15}\text{N}$ -labelled protein in complex with (R)-PK11195 (samples in D_2O) using constant-time ^1H - ^{13}C HSQC experiments. Collected intensities were analyzed as described previously [21].

Structure calculation

The overall topology of A147T-mTSPO in complex with (R)-PK11195 was determined on the basis of 334 manually assigned medium-range $\text{H}^{\text{N}}\text{-H}^{\text{N}}$ contacts in the five transmembrane helices, 329 intramolecular long-range NOE contacts, which were observed among well-separated side chain methyl proton resonances, backbone amides and the tryptophan imidazole groups in 3D ^{13}C - and ^{15}N - edited NOESY-HSQC spectra (mixing times of 100 ms), as well as 55 ligand-to-protein contacts detected in 3D F1- $^{13}\text{C}/^{15}\text{N}$ -filtered/edited-NOESY- ^1H - ^{13}C -HSQC and 3D F1- $^{13}\text{C}/^{15}\text{N}$ -filtered/edited-NOESY- ^1H - ^{15}N -HSQC experiments (both 100 ms NOE mixing time). NOE cross peak intensities were calibrated and divided into four distance classes, as very weak 1.8-6.0 Å, weak 1.8-5.5 Å, medium 1.8-4.5 Å and strong 1.8-3.5 Å. The manually assigned medium- and long-range distance restraints were subsequently used together with a full set of cross-peaks from 3D ^{15}N - and ^{13}C -edited NOESY spectra (mixing times of 100 ms) for automatic NOE assignment and structure calculation in CYANA 3.0 [32]. The structure calculation was supplemented by dihedral angle restraints predicted from experimental chemical shifts using TALOS+ [31], as well as 98 hydrogen bond restraints for 63 $\text{H}^{\text{N}}\dots\text{O}=\text{C}$ hydrogen bonds in the transmembrane helices based on experimental hydrogen/deuterium exchange profiles

and supported by characteristic NOE contacts. Structure refinement was carried using a simulated annealing protocol implemented in XPLOR-NIH 2.33 [33].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

3D structure of A147T-mTSPO in complex with (R)-PK11195. (A) ^1H - ^{15}N HSQC spectra of A147T-mTSPO (orange) and wild-type mTSPO (silver) in complex with PK11195. Selected resonances are labelled. (B) NMR ensemble of the 20 lowest-energy structures of A147T-mTSPO (PDB id: 2N02). PK11195 is depicted by sticks. The cytosolic helix is labelled as α' . (C) Comparison of the 3D structures of A147T (orange) and wild-type mTSPO (silver; PDB id: 2MGY) in complex with PK11195. (D) Detailed view of the local

structure around position 147 in A147T-mTSPO (orange) and mTSPO (silver) both bound to PK11195.

Figure 2.

Chemical shift differences between A147T-mTSPO and wild-type mTSPO, both in complex with (R)-PK11195. **(A,B)** $^1\text{H}/^{15}\text{N}$ chemical shift perturbation (CSP) as a function of residue number (A) and mapped onto the 3D structure of A147T-mTSPO in complex with (R)-PK11195 (B). In (A), the location of helices is shown by black bars and the site of mutation is labelled. In (B), threonine at position 147 is colored black. **(C,D)** CSP of tryptophan imidazole protons upon substitution of alanine by threonine at position 147 as a function of residue number (C) and mapped onto the 3D structure of A147T-mTSPO in complex with (R)-PK11195 (D).

Figure 3.

Dynamics of methyl groups of A147T-mTSPO in complex with PK11195. **(A)** Cross-correlated relaxation rates (σ_{obs}) between dipolar couplings in methyl groups of A147T-mTSPO (orange) and wild-type mTSPO (silver). **(B)** Mapping of σ_{obs} values from (a; orange) onto the 3D structure of the A147T-mTSPO-CK11195 complex.

Figure 4.

Structural integrity of the cholesterol recognition sequence in mammalian TSPO. (A) Detailed view of the conformation of the cholesterol recognition sequence in A147T-mTSPO (orange) and wild-type mTSPO (silver; PDB id: 2MGY) both in complex with PK11195. Side chains of selected resonances are shown. (B) Superposition of ^1H - ^{15}N HSQC spectra of A147T-mTSPO (orange) and wild-type mTSPO (silver) in the absence of PK11195. (C) Comparison of the 3D structures of the A147T-mTSPO-PK11195 (orange) and mTSPO-PK11195 (silver; PDB id: 2MGY) complexes with those of the tryptophan-rich sensory proteins from *Bacillus cereus* (green; PDB id: 4RYQ) and from *Rhodobacter sphaeroides* (magenta; PDB id: 4UC1:A) and its A139T variant (light blue; PDB id: 4UC3:A). In wild-type RsTspO the TM1-TM2 loop was not observed and is therefore approximated by dots. The orientation of the side chains of Y152 and R156 in A147T-mTSPO, which are essential for cholesterol binding, is similar to the side chain orientation of the homologous residues in wild-type BcTspO and A139T RsTspO, but not in wild-type RsTspO.

Figure 5.

Alignment of mammalian TSPO sequences. A147 is marked in yellow. Residues in helical regions are colored orange.

Table 1

NMR constraints and structural statistics for the ensemble of 20 lowest-energy structures of A147T-mTSPO in complex with PK11195.

NMR distance & dihedral constraints	
NOE distance constraints ^{a)}	
Total NOE	3227
intraresidual ($ i-j = 0$)	724
sequential ($ i-j = 1$)	631
medium-range ($1 < i-j < 5$)	894
long-range ($ i-j \geq 5$)	923
protein to ligand	55
NOE restraints/residue	19.1
Hydrogen bonds restraints	63 (126)
Torsion angle constraints	264
Backbone (φ/ψ)	132/132
Structure Statistics	
Mean r.m.s.d. from exp. restraints ($\pm$ s.d.) ^{b)}	
NOE (Å)	0.0036 ± 0.0005
dihedral angles (deg)	0.3342 ± 0.089
Deviations from idealized geometry	
Bond lengths (Å) $\cdot 10^{-3}$	3.333 ± 0.122
Bond angles (deg)	0.6095 ± 0.01
Impropers (deg)	0.4242 ± 0.007
r.m.s.d. to the first structure	
backbone atoms (5...160) (Å)	0.70 ± 0.13
heavy atoms (5...160) (Å)	1.27 ± 0.12
Ramachandran plot (5...160)	
most favored region	94.9 %
additionally allowed region	4.5 %
generously allowed region	0.6 %

^{a)} 20 lowest-energy structures out of 105 submitted to structure calculation.

^{b)} None of the structures exhibited distance violations greater than 0.5 Å or dihedral angle violations $> 5^\circ$.