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Validation of the mechanism of cholesterol binding by StAR using short molecular dynamics simulations

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ABSTRACT

We previously proposed an original two-state cholesterol binding mechanism by StAR, in which the C-terminal α-helix of StAR gates the access of cholesterol to its binding site cavity. This cavity, which can accommodate one cholesterol molecule, was proposed to promote the reversible unfolding of the C-terminal α-helix and allow for the entry and dissociation of cholesterol. In our molecular model of the cholesterol–StAR complex, the hydrophobic moiety of cholesterol interacts with hydrophobic amino acid side-chains located in the C-terminal α-helix and at the bottom of the cavity. In this study, we present a structural *in silico* analysis of StAR. Molecular dynamics simulations showed that point mutations of Phe^{267}, Leu^{271} or Leu^{275} at the α-helix 4 increased the gyration radius (more flexibility) of the protein's structure, whereas the salt bridge double mutant E169M/R188M showed a decrease in flexibility (more compactness). Also, in the latter case, an interaction between Met^{169} and Phe^{267} disrupted the hydrophobic cavity, rendering it impervious to ligand binding. These obtained results are in agreement with previous *in vitro* experiments, and provide further validation of the two-state binding mode of action.

1. Introduction

The steroidogenic acute regulatory protein (StAR) is the rate-limiting factor during the synthesis of steroid hormones [1,2]. It allows for the transfer of cholesterol from cytoplasmic pools via the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) [3–5]. Under the influence of trophic hormones [6,7], StAR is acutely synthesized *de novo* in the cytoplasm as a 37-kDa protein. Due to its N-terminal import sequence [8,9], StAR is translocated into the mitochondria to be proteolytically cleaved to yield a 30-kDa protein [10]. The steroidogenic acute regulatory protein-related lipid transfer (START) domain is responsible for the binding and transfer of cholesterol [11,12]. START domains are 200–210 amino acid lipid/sterol binding motifs conserved in several living organisms [13], and exert their function in lipid transport, metabolism, signal transduction and transcriptional regulation [14,15]. All START-containing proteins include a binding pocket that determines ligand binding specificity and function [16,17], with high affinity for ligands, including cholesterol, phosphatidylcholine, phospholipids and sphingolipids [13].

Fifteen START domain proteins are known [18]. Two START protein structures were resolved by crystallography: the N-terminus 218 amino acids-deleted human metastatic lymph node 64 (MLN64/StARD3) [12] and the mouse cholesterol-regulated START protein 4 (StARD4) [19]. Given the functional similarities between StAR and MLN64 [20], a three-dimensional homology model of StAR in the apo and holo states was proposed based on MLN64's structure [21]. StAR's molecular model is composed of four α-helices and nine β-sheets. Also, two functional subdomains are crucial for StAR's function. First, the cholesterol binding site, a hydrophobic cavity with a putative salt bridge between Glu^{169} and Arg^{188}, is responsible for ligand specificity and orientation within the cavity. Second, the C-terminal α-helix 4 stabilizes the holo-complex and gates the transfer of cholesterol [22].

Mutations in the StAR gene cause lipoid congenital adrenal hyperplasia (LCAH), a severe autosomal recessive form of congenital adrenal hyperplasia [23,24]. Most of these mutations are located at the C-terminal α-helix 4 or at the cholesterol binding site of StAR [21,24]. For instance, the clinical mutation of Glu^{169} to glycine or lysine causes LCAH [23], nevertheless the folding of the E169G mutant is not disturbed as other severe mutants [25]. In transfected COS-1 cells, StAR mutations E169L or R188M showed a substantial loss in steroidogenic activity [21]. As a result, Glu^{169} was proposed to form a salt bridge with Arg^{188} that may interact with the 3β-OH group of cholesterol [21]. This assumption was also based on MLN64 which contains a Asp^{332}/Arg^{351} salt bridge within its cholesterol

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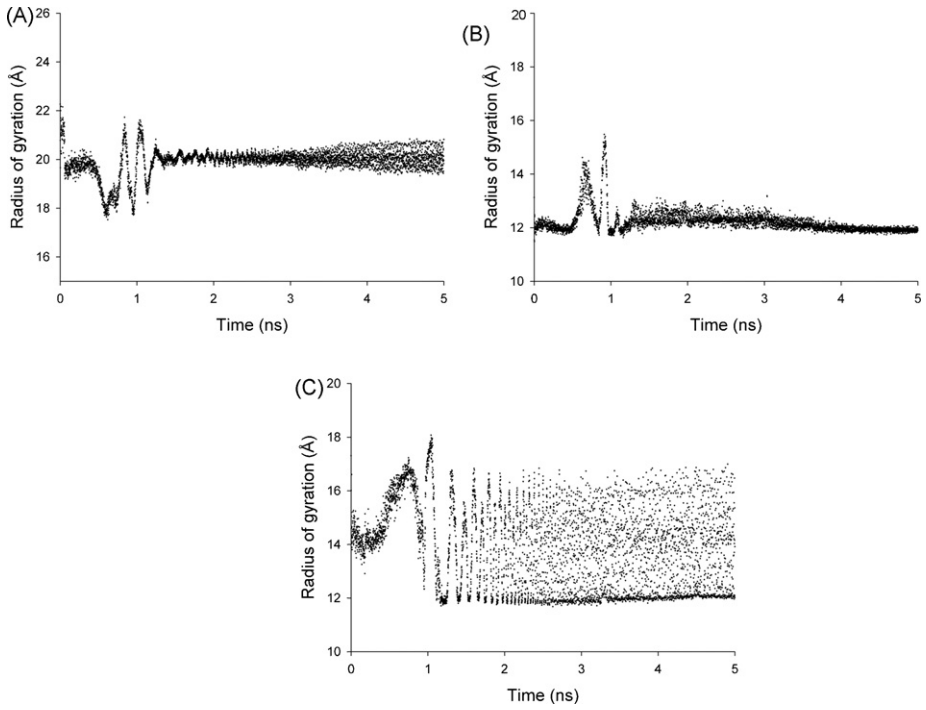

Fig. 1. MD trajectories of StAR WT. The radius of gyration of StAR WT is plotted for (A) the total protein structure, (B) α-helix 1 and (C) α-helix 4. The MD simulation was conducted at 300 K for 5 ns.

tunnel [12]. Such mutations indicate the importance of preserving the integrity of the putative salt bridge in StAR. Other clinical mutations affect the C-terminus of StAR, in particular its α-helix 4. For instance, the mutation of Leu^{275} to proline causes LCAH and exhibits only 13% of steroidogenesis activity compared to wild-type StAR, as analyzed in COS-1 cells [26]. Moreover, a truncation of ten C-terminal residues reduced StAR's activity by 70%, while a deletion of 28 residues abolished all activity in transfected cells [27]. Taken together, these observations indicate that StAR's α-helix 4 plays a critical role in the steroidogenic process.

We hypothesize that these clinical mutations alter the binding affinity of StAR for cholesterol and that this promotes its accumulation in LCAH [22]. Alteration of the ligand binding affinity of a protein requires an understanding of the effect of mutating individual residues in the ligand-binding site and the overall structure of the protein [28]. In this report, we sought to investigate the effect of mutating the salt bridge and residues 267, 271 and 275 in the α-helix 4 of StAR by molecular dynamics (MD) simulations. The MD simulations support the two-state model where α-helix 4 has a flexibility that permits it to gate the cholesterol binding.

2. Materials and methods

2.1. Molecular modeling

Initial coordinates for the molecular modeling of StAR were retrieved from the Protein Databank (code 1IMG). Site-directed mutagenesis *in silico* was done using the molecular modeling software SYBYL 8.0 (Tripos Inc., St. Louis, MO). Hydrogen atoms, not present in the PDB files, were added, then charges were assigned using the AMBER method. An energy minimization in the Tripos force field [29] was applied for the hydrogen atoms using the Powell algorithm with a convergence gradient of 0.05 kcal/mol for 1000 iterations. A second step of minimization was performed on the entire polypeptide to relieve bad van der Waals contacts by setting a Powell convergence gradient to 1 kcal/mol for 100 iterations, while applying a cutoff for non-bonded interactions (NB cutoff) of 8 Å and toggling minimum periodic boundary conditions. Connolly surfaces and cavities were rendered using the MOLCAD module in SYBYL.

2.2. Molecular dynamics (MD) simulations

All MD simulations were performed using the Tripos force field and AMBER charge assignments. Water molecules were not included in the simulations, therefore the dielectric constant was set equal to the distance between interacting atoms. The simulations trajectory was run for 5 ns with a 2 fs stepping at a constant temperature of 300 K, yielding a canonical ensemble (NVT). The NB cutoff was set to 8 Å. Bond length involving hydrogen atoms were constrained using the SHAKE algorithm [30], which permits longer time steps (1–2 fs), thus achieving extended MD runs for the same computational time. Dynamic analysis was done using the MSS module in SYBYL. Statistical analysis (one-way ANOVA) was performed using the SigmaStat software (Systat Software, San Jose, CA).

3. Results

3.1. MD simulations of WT StAR

According to the proposed 3D model of StAR [21], the C-terminal α-helix 4 contains several residues with hydrophobic side chains

Table 1
Summary of the mean radius of gyration of StAR WT and the mutants at 300 K.

Protein	Rg total	Rg α-helix 4 (Å)	Rg α-helix 1 (Å)
WT	19.9 ± 0.5	13.9 ± 1.6	12.2 ± 0.5
F267Q	20.6 ± 1.1	14.3 ± 1.6	12.7 ± 1.0
L271N	20.8 ± 1.6	16.1 ± 1.3	12.5 ± 0.8
L275P	23.3 ± 0.6	14.8 ± 1.8	15.8 ± 1.2
E169M/R188M	19.1 ± 1.1	14.8 ± 1.2	11.9 ± 0.1

The values of each MD trajectory (5000 points) were compiled and were significantly different ($p < 0.05$) when compared to the WT values, as analyzed by one-way ANOVA.

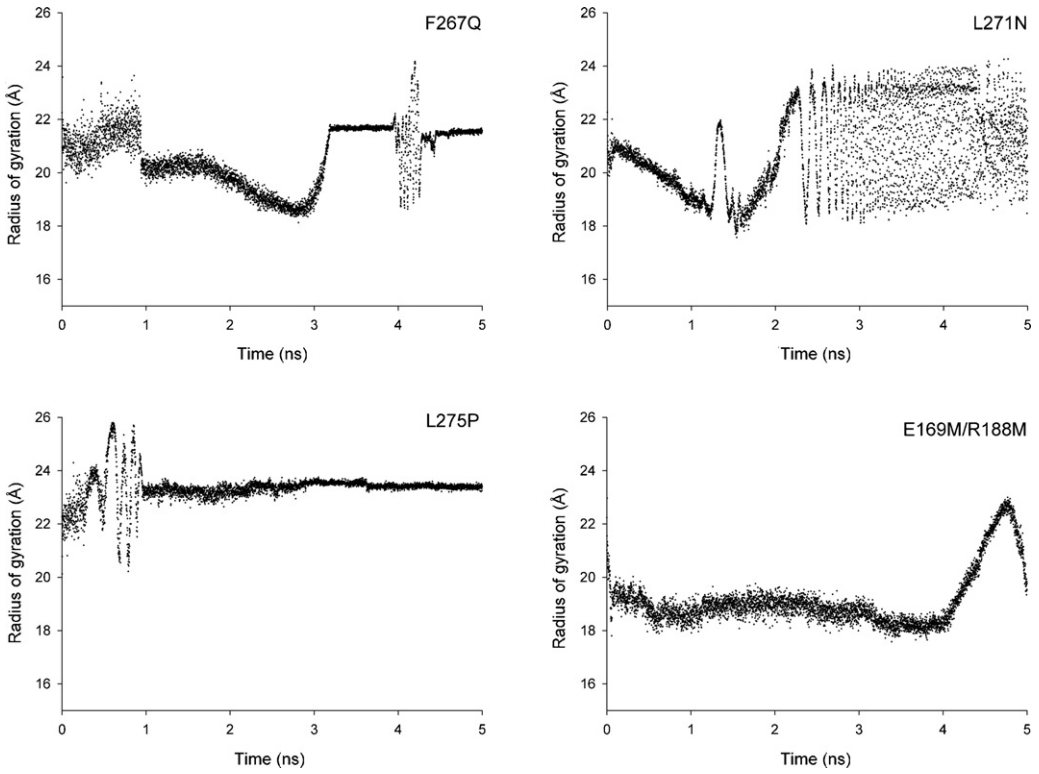

Fig. 2. Radius of gyration of the StAR mutants. The radius of gyration for the whole structure of StAR F267Q, L271N, L275P and E169M/R188M mutants are plotted for a 5 ns MD simulation at 300 K.

(Phe^{267}, Leu^{271}, and Leu^{275}) that form a tertiary hydrophobic core. These side chains create hydrophobic anchoring points favorable for a stabilizing clamp between α-helix 4 and the core of the protein. It is expected that upon binding of cholesterol to StAR, this clamping mechanism would help stabilize the complex by shielding it from the solvent exposure, until its later release during the steroidogenesis [22].

To gain insight into the molecular behavior of StAR at the atomic level, we conducted MD simulations on the WT protein, the α-helix 4 mutants (F267Q, L271N, and L275P) and the salt bridge (E169M/R188M) mutant and emphasized on the variation of their radius of gyration (Rg). Rg is a measure of unfolding and flexibility. The MD simulation of StAR WT showed a Rg that fluctuated between 17.5 and 22 Å for the first nanosecond, then stabilized with more discreet variations with an average of 19.9 ± 0.5 Å (Fig. 1A, Table 1). Interestingly, the C-terminal α-helix 4 had a high degree of Rg fluctuation (average 13.9 ± 1.6 Å), particularly an oscillation between 12 and 16.5 Å that started at 1.2 ns of the simulation (Fig. 1C). This variation of Rg as function of time suggests that the protein behaves with a contraction and expansion mobility, mainly at the α-helix 4 which exhibited a periodic fluctuation. In comparison, the other prominent α-helix of StAR, the N-terminal α-helix 1, remained stable throughout the simulation (Rg = 12.2 ± 0.5 Å) (Fig. 1B).

3.2. MD simulations of the mutants

To verify the importance of the hydrophobic interface, several α-helix 4 mutations were previously characterized [22,31], i.e. F267Q, L271N and L275P. All these mutations had a reduced binding of cholesterol, as well as a decreased steroidogenic activity. Mutating Phe^{267} to the polar glutamine is expected to weaken the tertiary structure of StAR. And, changing Leu^{271} to Asn (replacement of the leucine side chain with the isosteric polar asparagine side chain) would lead to an unfavorable desolvation of the asparagine side chain upon its burial at the hydrophobic interface; this can be expected to favor a local unfolding of the α-helix 4 [22]. Finally, concerning the clinical mutation L275P leading to LCAH [26], we hypothesized that the cyclization of the Pro^{275} side chain will reduce the extent of burial of the hydrophobic surface and weaken the strength of the hydrophobic interface.

The Rg of the F267Q mutant had an irregular pattern ranging between 18.5 and 24 Å (Fig. 2). Although the Rg of the L271N mutant also fluctuated between 18 and 24 Å, it showed an oscillation with an average of 20.8 ± 1.6 Å. The L275P mutation had a similar pattern as the WT protein, however it had the highest Rg average (23.3 ± 0.6 Å), indicating a greater flexibility for the overall structure. As for the salt bridge mutant, its Rg showed a slight and steady compactness when compared to the WT (Fig. 1A) that lasted for 4 ns (Rg = 19.1 ± 1.1 Å) (Fig. 2). These results suggest that mutations that favor the opening of the C-terminal α-helix 4 will lead to a more flexible StAR, whereas mutations increasing the hydrophobic core strength favor a more compact structural state.

3.3. Rg fluctuations of α-helix 1 and 4

The Rg of α-helix 4 was assessed for the mutants since it gates the binding of cholesterol [22]. Unlike WT StAR, the α-helix 4 of mutants showed intermittent Rg oscillation phases that varied between 12 and 20 Å (Fig. 3). The Rg maximum attained by the mutants (20 Å) suggests that α-helix 4 had more flexibility than the WT protein. The other major α-helix in StAR is α-helix 1, hence we assessed its Rg to verify if it had a flexibility during the simulations. The StAR mutants F267Q and L271N had a Rg varying between 12 and 14.5 Å (Fig. 4), a range that was less pronounced compared to the WT. However, StAR F267Q did not show an oscillation like the L271N mutant. On the other hand, L275P had a significant variation in its Rg that averaged 15.8 ± 1.2 Å. The Rg comparison of the StAR's main α-helices suggest that α-helix 4 undergoes a higher flexibility than α-helix 1, especially when point mutations are present at α-helix 4.

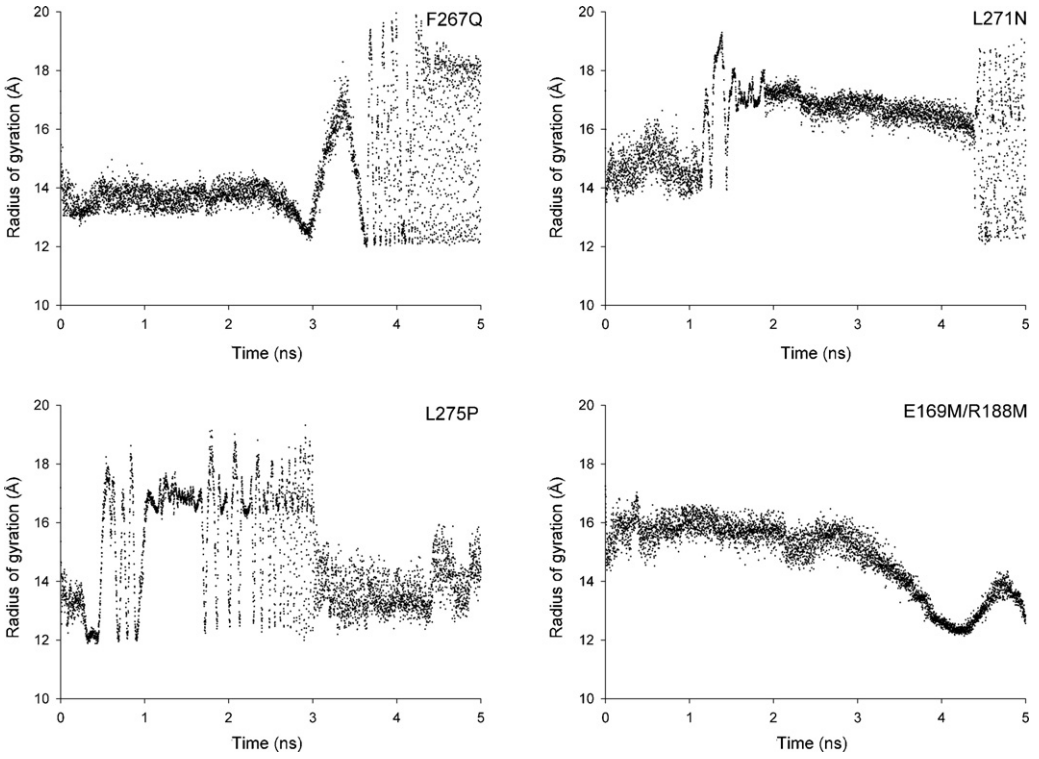

Fig. 3. Gyration radius of α-helix 4 of StAR WT and its mutants. The radius of gyration for StAR F267Q, L271N, L275P and E169M/R188M mutants are plotted for a 5 ns MD simulation at 300 K.

3.4. MD simulations of the salt bridge mutant

Some of the clinical LCAH mutations affect the putative salt bridge of StAR [21,32]. The double mutation of the salt bridge was previously found to abolish the specific cholesterol binding as well as the steroidogenic activity of StAR [22]. Here, the MD simulation of the E169M/R188M mutant showed a steady Rg (19.1 ± 1.1 Å) lasting 4 ns (Fig. 2) which was lesser than the WT (19.9 ± 0.5 Å). Also, in contrast with the α-helix 4 mutants and the WT StAR, α-helix 4 had a high mobility throughout the simulation (Rg ∼ 16 Å) that gradually

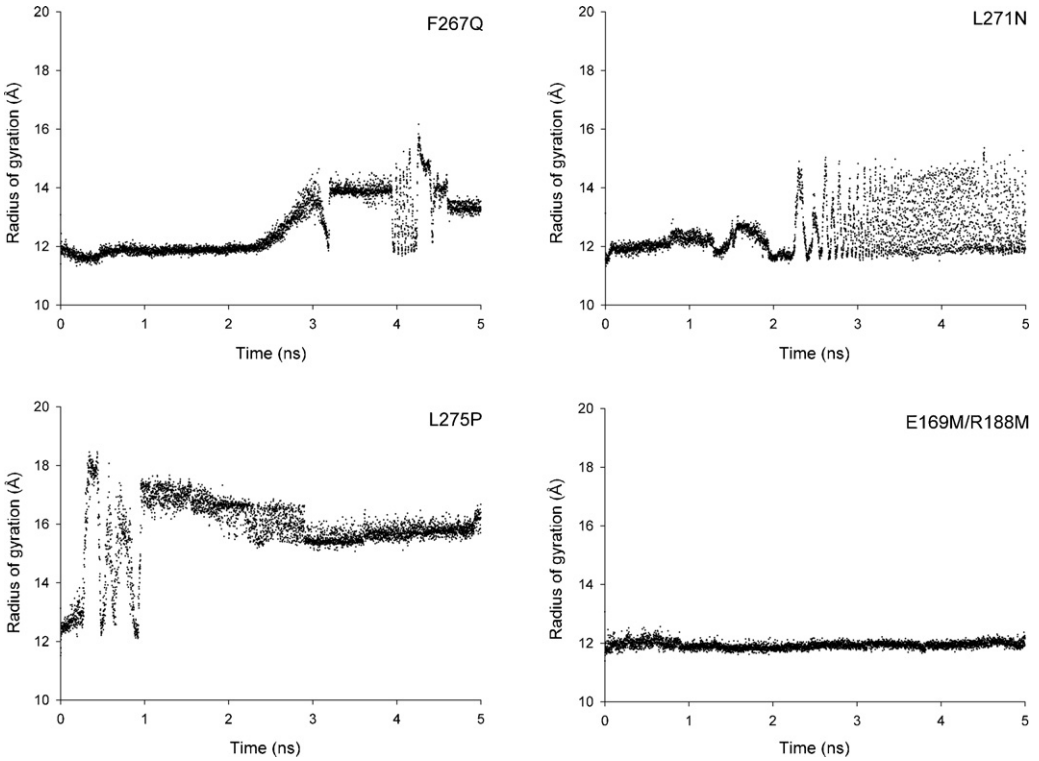

Fig. 4. Gyration radius of α-helix 1 of StAR WT and its mutants. The radius of gyration for StAR F267Q, L271N, L275P and E169M/R188M mutants are plotted for a 5 ns MD simulation at 300 K.

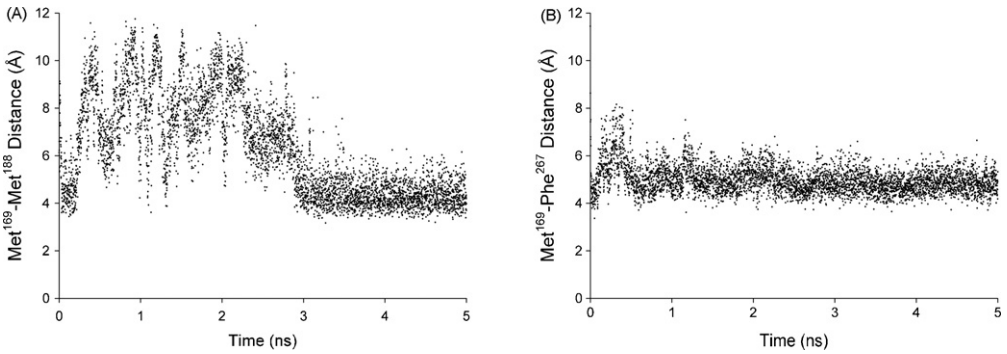

Fig. 5. Distance analysis between Met[169], Met[188] and Phe[267] of the StAR salt bridge mutant. The distance between (A) Met[169]-Met[188] and (B) Met[169]-Phe[267] is plotted for a 5 ns MD simulation at 300 K.

decreased to reach minimal levels around 12 Å (Fig. 3) indicating a non-dynamic and more compact structure (Fig. 3). On the other hand, α-helix 1 exhibited a sustained Rg of 11.9 ± 0.1 Å (Table 1), indicating a highly compact α-helical state (Fig. 4). These results are in agreement with the *in vitro* experiments where the double mutant displayed a higher α-helical content and thermodynamical stability [22].

Monitoring of the distance between the mutated residues revealed that the distance between Met[169] and Met[188] was very dynamic, fluctuated between 4 and 11 Å (average 6.3 ± 2.1 Å) before reaching a minimum at 4 Å (Fig. 5A). Further analysis of the MD trajectories revealed a dynamic interaction between Met[169] and Phe[267], the latter being located at the cholesterol gating domain (i.e. α-helix 4). The distance between the closest atoms of these side chains varied between 4 Å and 8 Å but had an average value of 5.0 ± 0.6 Å (Fig. 5B). This close distance Met[169]/Phe[267] is reminiscent of the formation of hydrophobic interactions that might explain the stabilizing effect on α-helix 4 as observed elsewhere [22] and depicted in Fig. 3. Surface analysis of the MD simulations revealed that StAR WT had some variations in its shape, however cholesterol could still dock into the hydrophobic pocket (not shown). Conversely, when mutating the salt bridge to methionine residues, the shape of the binding pocket experienced a gradual narrowing effect from the Met[169]/Phe[267] distance interval 8 Å to 4 Å (not shown), which led to a scission of the pocket when the distance between Met[169] and Phe[267] reached a minimum at 4 Å (Fig. 6). Furthermore, the severed binding pocket failed to accommodate a cholesterol molecule, as observed in docking experiments (not shown).

4. Discussion

To gain more structural insights into our previously described two-state model of StAR's binding mechanism [22], we studied the protein's behavior using MD simulations. The data indicated that any mutation within α-helix 4 conferred more flexibility to the protein, particularly at the α-helix 4 itself. The increased gyration radius may illustrate the first dynamic phase in the opening and closing of the gating domain of StAR, i.e. α-helix 4. In the case of the salt bridge mutant, the highly labile movement of the Met[169] and Met[188] is indicative of an increased entropy within the hydrophobic core, which translates into a compacting and stabilization effect on the overall protein structure, including the two terminal α-helices (1 and 4). Moreover, the decreased distance between Met[169] and Phe[267] enforces the hydrophobic interaction of α-helix 4 with the hydrophobic core, thereby keeping StAR in a closed state. Indeed, an increase in a protein's interior entropy does contribute to its stabilization [33]. Here, although the radius of gyration of the overall protein structure was reduced in the salt bridge mutant, the higher dynamics of the Met[168]/Phe[267] interaction is evocative of an increased stability to the protein as illustrated by the rise of the melting temperature [22]. In addition, this unexpected interaction caused a rift in the binding pocket which prevented the docking of cholesterol. This is in agreement with the *in vitro* experiments where salt bridge mutant had more helicity, an increased thermal stability and consequently no cholesterol binding [22]. Moreover, this corroborates with the covalent closing of α-helix 4 with cysteine disulfide bridges S100C/S261C or D106C/A268C, where StAR

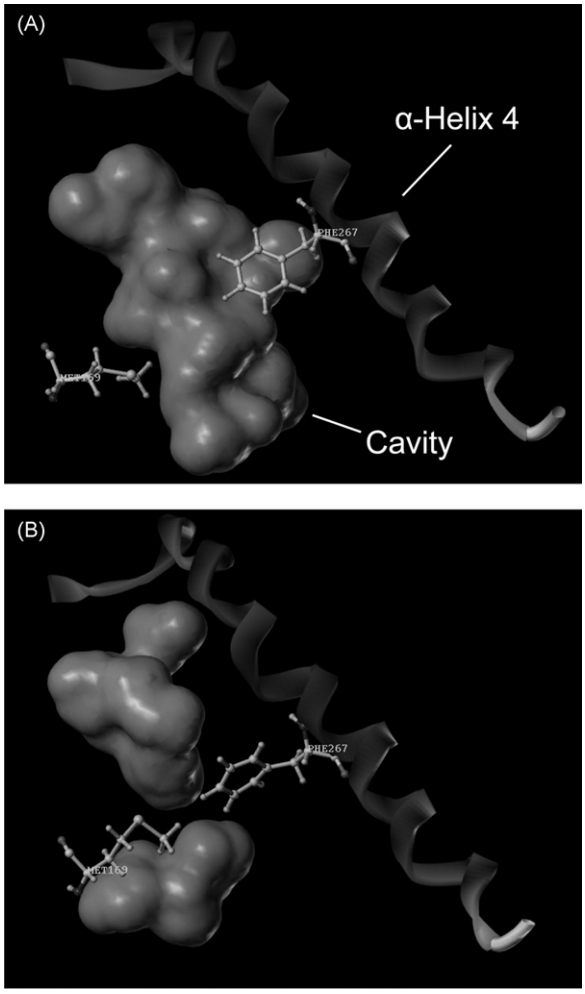

Fig. 6. Cavity analysis of the StAR salt bridge mutant E169M/R188M. Following MD simulations, the Connolly surface rendering of the hydrophobic cavity is depicted when the distance between Met[169]-Phe[167] was (A) 8 Å and (B) 4 Å. Phe[167] is present at α-helix 4.

lost half or completely its binding and steroidogenic activity, respectively, which were restored by the addition of a reducing agent [34].

According to steered MD simulations, it was proposed that a lid-like transient opening of loop Ω1 would provide sufficient conformational change to allow cholesterol egress from the START binding site, without further substantial rearrangement of the structure [35]. However, *in vitro* studies showed that upon binding of cholesterol, StAR lost helicity, then regained more helical content, compared to the apo-state [31]. Our MD simulations (α-helix 4 Rg oscillations) are in agreement with the model which suggests that α-helix 4 undergoes conformational unfolding and refolding upon binding to cholesterol. To observe the actual unfolding of the α-helix 4 would require a longer MD simulation on a microsecond scale. However, it is plausible to suggest that the Rg oscillations may reflect the equilibrium state between the opening and closing of α-helix 4 [22] on the nanosecond time scale. This micro-flexibility may therefore be a prelude to a large-scale movement, such as the unfolding/refolding of α-helix 4.

In conclusion, the MD simulations showed that point mutations of StAR, particularly at the binding pocket or the C-terminal α-helix 4, had a profound alteration in the variation of the gyration radius during the simulations. These two domains act in concert to provide StAR with its cholesterol transport activity. By losing one of the gating domain anchoring points, StAR is less able to bind cholesterol, and therefore its steroidogenic activity is reduced, whereas modifications in the salt bridge at the binding pocket abolishes the ligand binding as well as the steroidogenic activity of StAR. This expresses the intimate relationship between the structural integrity and the steroidogenesis, a crucial element for the proper functioning of StAR.

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