

Chapter 15

Mammalian StAR-Related Lipid Transfer (START) Domains with Specificity for Cholesterol: Structural Conservation and Mechanism of Reversible Binding

Pierre Lavigne, Rafael Najmanivich, and Jean-Guy LeHoux

Abstract The StAR-related lipid transfer (START) domain is an evolutionary conserved protein module of approximately 210 amino acids. There are 15 mammalian proteins that possess a START domain. Whereas the functions and specific ligands are being elucidated, 5 of them have already been shown to bind specifically cholesterol. The most intensively studied member of this subclass is the steroidogenic acute regulatory protein (StAR) or STARD1. While its role in steroid hormone production has been demonstrated, much less is understood about how its START domain specifically recognizes cholesterol and how it releases it to be transferred inside the mitochondria of steroidogenic cell of the gonads and adrenal cortex. A major obstacle that is slowing down progress in this area is the lack of knowledge of the 3D structures of the START domain of StAR in both its free and complexed forms. However, 3D models of the START domain of StAR and mechanisms of binding have been proposed. In addition biophysical studies aimed at validating the models and mechanism have been published. What's more, the crystal structures of the free forms of 3 START domains (STARD3, STARD4 and STARD5) known to specifically bind cholesterol have been elucidated so far. In this chapter, we will review and critically summarize existing data in order to provide the most current view and status of our understanding of the structure and reversible cholesterol binding mechanism of START domains.

Keywords StAR, START domains · Cholesterol · Steroidogenesis · LCAH

15.1 Introduction

The steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) domain is a protein module of approximately 210 amino acids. This module

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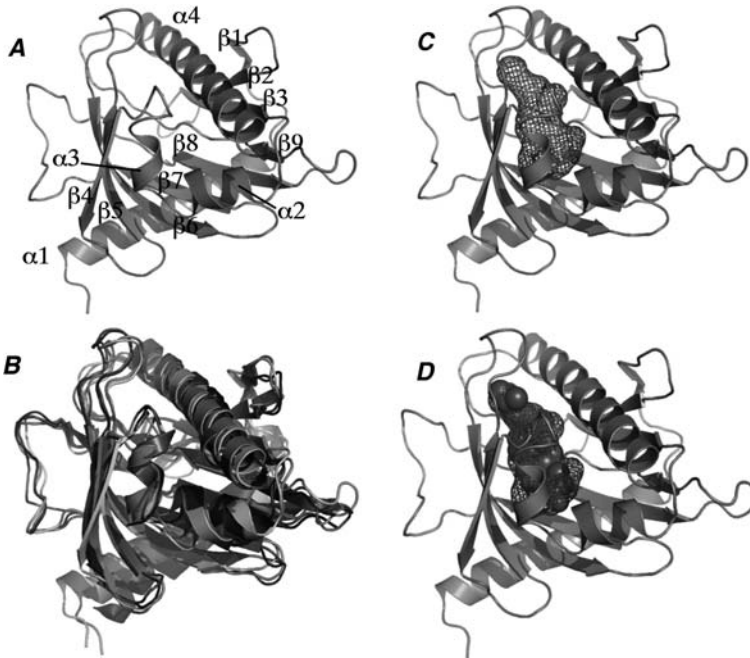


Fig. 15.1 **A.** The α/β helix grip fold. Note the presence of an N- and a C-Terminal helix gripping a twisted 9–10 stranded b-sheet. Depending on the algorithms used for secondary structure assignments, 9 or 10 β -strands can be identified. **B.** Superimposition of the backbone of the model of StAR (1IMG.pdb; Mathieu et al., 2002) and the crystal structure of MLN64 (1EM2.pdb, Tsujishita & Hurley, 2000), STARD4 (1JSS.pdb, Romonowski et al., 2002) and STARD5 (2R55.pdb, to be published). **C.** Molecular surface of the STARD1 internal cavity. **D.** One cholesterol molecule fits in the cavity. Figure made with Pymol (Delano, 2002)

has a α/β helix grip fold (Tsujishita and Hurley, 2000; Iyer et al., 2001 and Fig. 15.1A) and binds a wide variety of lipids, i.e. cholesterol, ceramides, Phosphatidylethanolamine (PE), Phosphatidylcholine (PC) (Schrick et al., 2004). Though not present in yeast, START domains are conserved through evolution and are found in bacteria, plants, flies, nematodes and mammals. In humans, 15 proteins possess a START domain (Alpy and Tomasetto, 2005). These proteins are involved in lipid metabolism, lipid transfer and cell signalling and have diverse expression patterns and cellular localizations. The diverse cellular functions and known ligands of the 15 mammalian START containing proteins were recently reviewed elsewhere (Alpy and Tomasetto, 2005). Here we will focus of the START domains that are known to bind cholesterol, i.e. StAR (STARD1), MLN-64 (STARD3), STARD4, STARD5 and STARD6 (Alpy and Tomasetto, 2005; Bose et al., 2008).

The discovery that StAR was responsible for the acute steroidogenesis has boosted much intense interest in an attempt to understand the molecular and structural biology of START domain containing proteins (Clark et al., 1994). In fact, StAR regulates the cholesterol mobilization to the mitochondria of steroidogenic

cells (gonads and adrenal cortex), following a ACTH stimulus. This mobilization is the rate-limiting step of steroidogenesis and the production of pregnenolone by the p450 cholesterol side chain cleavage complex located in the inner mitochondrial membrane (Arakane et al., 1998; Bose et al., 2002). StAR has a mitochondrial target sequence N-Terminal to its START domain (Arakane et al., 1998; Wang et al., 1998). However, this signal sequence is dispensable for cholesterol transport across the mitochondrial membrane. Indeed, a truncated mutant of StAR lacking its first 62 residue (N62-StAR) retains full activity in the stimulation of pregnenolone production by steroidogenic mitochondrial preparations (Bose et al. 2002). Mutations in the StAR gene cause lipoid congenital adrenal hyperplasia (LCAH), a severe autosomal recessive form of congenital adrenal hyperplasia (Bose et al., 1996; 2000). Most of these mutations are located at or near the C-terminal α -helix.

There are currently two active areas of research in the field of StAR. The first, which was recently reviewed in depth by Rone et al. (2009), focuses on the clarification of the elusive mechanism by which cholesterol enters the mitochondrial matrix. The other addresses the mechanism by which StAR reversibly binds and dissociates from cholesterol. This area will be updated and survey in detail in the present chapter.

MLN-64 is a member of the StAR group (Alpy and Tomasetto, 2005). Like StAR, MLN-64 possesses a sub-cellular localization domain that targets it to the late endosomes (Clark et al., 1994; Alpy et al., 2001) where it is thought to be involved in the mobilization of lysosomal cholesterol and its transfer to other organelles and membranes. Like StAR and in isolation, the START domain of MLN-64 can also stimulate steroidogenesis in steroidogenic mitochondrial preparations (Watari et al., 1997). In fact MLN-64 is thought to be involved in steroidogenesis in tissues that lack StAR expression such as the placenta (Watari et al., 1997). Conversely to StAR, the crystal structure of the START domain (without cholesterol) of MLN64 has been solved (Tsujishita and Hurley, 2000).

STARD4, STARD5 and STARD6 are members of the STARD4 group (Alpy and Tomasetto, 2005). They are the only START proteins with no subcellular localization domain and as such are thought to be cytosolic. Reports suggest that STARD4 and STARD5 are able to stimulate steroidogenesis. However, a recent study has shown that while STARD4, STARD5 and STARD6 can bind cholesterol, only STARD4 and STARD6 can stimulate pregnenolone production in vitro (Bose et al., 2008). By being able to diffuse through the cytoplasm, members of this family are thought to be able to provide multiple sources (membranes, lipid droplets and organelles such as the ER, endosomes and lysosomes) with cholesterol for steroidogenesis (Rone et al., 2009).

Despite all of the data discussed here and reviewed extensively elsewhere (Rone et al., 2009), we still do not know the molecular determinants responsible for the recognition of cholesterol by these START domains and the mechanism by which the START domains bind and release cholesterol. Although mundane at first glance, the mechanism of reversible binding is complicated by the fact that the binding site of cholesterol is buried inside the START domain and hence necessitates an opening reaction of some sort. Consequently, in this Chapter, we will review with in some detail structural and functional data from the literature in order to present an

up-to-date picture of the recent progress toward our understanding of the molecular recognition of cholesterol by START domains and how these protein modules reversibly bind cholesterol.

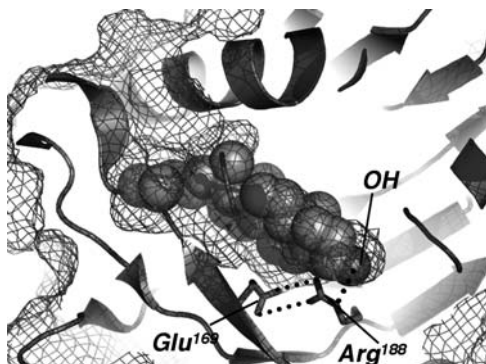
15.2 The START Domains That Specifically Bind Cholesterol Have a Highly Conserved α/β Helix Grip Fold

Our recent survey of the protein data bank has revealed that the crystal structures of 6 mammalian START domains have been solved so far. These START domains are those of STARD2 (Roderick et al., 2002), D3 (MLN64, Tsujishita and Hurley, 2000), D4 (Romanowski et al., 2002), D5 (2R55.pdb, to be published), D11 (Kudo et al., 2008) and D13 (2PSO.pdb, to be published). STARD3, D4 and D5 have specificity for cholesterol. STARD2 (PCPT) binds phosphatidylcholine (PC) and STARD11 (CERT) binds ceramides, (sphingolipid precursors). The lipid specificity of STAR13 (DCL-2) is not known as of yet.

Interestingly, apart from deletion and insertions in loops, the structures of all the START domains are very similar. Indeed, one can see in Fig. 15.1 that the α/β helix grip fold (Fig. 15.1A) of the START domains with affinity for cholesterol (Fig. 15.1B) and throughout the groups (not shown) is conserved. Another striking and common feature of these structures is the presence of an internal cavity large enough to fit one lipid molecule, i.e. cholesterol (Fig. 15.1C, D), ceramide (Kudo et al., 2008) or PC (Roderick et al., 2002).

While the structures of the START domains of STARD2 and STARD11 have been solved with their respective ligands, no structure of the START domain-cholesterol complex is currently available. Hence, the actual mode of binding and the determinants of the specificity of STARD1, 3, 4, 5 and 6 towards cholesterol remain to be understood and unravelled. In this regard, models for specific cholesterol complexes of MLN-64 (Murcia et al., 2006) and StAR (Mathieu et al., 2002; Yaworsky et al., 2005) have been proposed. In these models, the presence of conserved (between StAR and MLN-64) salt bridge between an acidic side-chain in β -strand 5 and an Arg in β -strand 6 at the bottom of the cavity was proposed to be a key determinant. More precisely, it was proposed that the cholesterol OH group forms a specific interaction with the guanidinium group of the conserved Arg (Fig. 15.2). On the other hand, it appears unlikely that molecular recognition and ligand selection rely only on one specific interaction (H-Bond). However, and as noted by others (Romanowski et al., 2002), it is quite possible that the actual shape of the cavity may play an important role in molecular recognition. To illustrate this, we present the molecular surface of the cavity of our 3D model of StAR with one cholesterol molecule located inside. As can be observed, the shape of the cavity matches almost perfectly that of the cholesterol molecule. Furthermore, by fitting the cholesterol molecule in this cavity, the OH group is perfectly positioned to interact with the guanidino group of the Arg side-chain of the salt-bridge (Fig. 15.2).

Fig. 15.2 Depiction of a putative specific interaction between cholesterol and a conserved salt-bridge in the model of StAR (1IMG.pdb, Mathieu et al., 2002). This salt-bridge is conserved in MLN64 and has been proposed to be involved in the specificity of interaction between its START domain and cholesterol (Murcia et al., 2006)



While surface complementarity is a hallmark of molecular recognition, it has to be emphasized that molecular recognition of small molecules by proteins usually involves more than one H-bond. In addition, it also has to be noted that the cholesterol binding site is totally buried inside the protein, which also a rather unusual feature. Hence, it is quite possible that the small number of H-bond may be balanced by perfect complementarity of non-polar and non-specific van der Waals interactions between cholesterol and the rest of the cavity. On the other hand, it appears as though these determinants are most likely different between the StAR (StAR, MLN64) and STARD4 (STARD4, 5 and 6) groups. Indeed, the salt bridge conserved in StAR and MLN-64 is not conserved in the STARD4 group. However, another salt bridge Asp-Arg in helix C is present in the structures of STARD4 and STARD5. This salt bridge is conserved in the primary structure of STARD6 (not shown) and could be involved, as proposed for MLN-64 and StAR, in the recognition of cholesterol. As one can appreciate, while we have a fair amount of structural data on the structure of START domains that specifically bind cholesterol, we are still awaiting the validation of our models in order to understand exactly what makes a START domain recognize cholesterol. This will come only with the crystal or NMR structures of START-cholesterol complexes from the StAR and STARD4 groups.

15.3 “To Be or Not to Be” a Molten Globule to Bind and Release Cholesterol Reversibly?

As one can appreciate, besides having a highly conserved fold, one peculiar feature of the structure of the START domains that bind cholesterol is the fact that their binding sites are shielded from the solvent and in the very core of the protein modules. Even though we are still trying to decipher the determinants of molecular recognition, this fact has prompted and stimulated many laboratories to search for the mechanism by which cholesterol and other lipids can reach the interior of

START domains (the binding site) in order to be transported to and then transferred inside the mitochondria.

In the late 1990's, the group of Walter Miller discovered that the N62-START domain behaved like a molten globule at acidic pH (from pH 4.5 to 3). Indeed, while the amount of secondary structure did not significantly changes from pH 4.5 to 3, the thermodynamic stability at 25°C (i.e. $\Delta G^{\circ}_u(25^{\circ})$) of the N62-START decreased significantly (Bose et al., 1999). Note that the stability of N62-StAR as measured by urea denaturation was observed to be constant from pH 8.3 to 4.5. This demonstrated that the while the secondary structure was maintained, the amount of stable and tertiary interaction was diminished at lower pH values. This is in complete agreement with the existence of a molten globule, i.e. a protein with native like secondary structure but loosely a packed native tertiary structure (Ptitsyn, 1995). In the same study, the Miller group found by proteolysis-MS analyses that the C-terminal region (193–285) was less folded at acidic pH than the N-terminus (63–188). Coupled to their hypothesis that the pH near the mitochondria OMM is acidic (~ 4.5), the Miller group put forth that a low pH induced molten globular state of StAR with the N-terminus more tightly folded than the C-terminus plays an important role in the cholesterol binding and transfer.

In 2002, we proposed an alternative mechanism for the reversible binding of cholesterol by N62-StAR (Mathieu et al., 2002). This mechanism is proposed to occur at neutral pH and was derived largely from the 3D model we had proposed for the N62-StAR, which was based on the crystal structure of STARD3. As initially uncovered in the crystal structures of STARD3 (Tsujiyama and Hurley, 2000) and STARD4 (Romanowski et al., 2002)) our model depicted a large internal cavity assigned to the cholesterol-binding site. In fact the volume of the cavity corresponded exactly to that of cholesterol (Mathieu et al., 2002 and Fig. 15.1C,D).

Based on the fact that such internal cavities destabilizes tertiary structures and that the C-Terminal helix could move independently from the rest of the molecule, we proposed the existence of an intermediate state with the N-terminus intact and the C-terminal helix undergoing a microscopic (independent of global unfolding) and reversible local unfolding (Mathieu et al., 2002; Roostaee et al., 2008; 2009). In this intermediate state (Fig. 15.3), the cholesterol-binding site would become accessible and explains cholesterol binding. In this mechanism, the C-terminal helix acts as a gate. Indeed, the refolding of the C-terminal helix when a cholesterol molecule is in the binding site it will lead to a more stable complex, with a lifetime long enough to carry and deliver cholesterol to its target organelle and/or transporter (e.g. TSPO (see Rone et al., 2009)).

15.4 Experimental Validation of the Two-State Model

We reasoned that if this mechanism is correct, the helical content of free N62-StAR should be less than optimal and that upon addition of cholesterol the helical content as well as the thermodynamic stability of N62-StAR should be increased at neutral

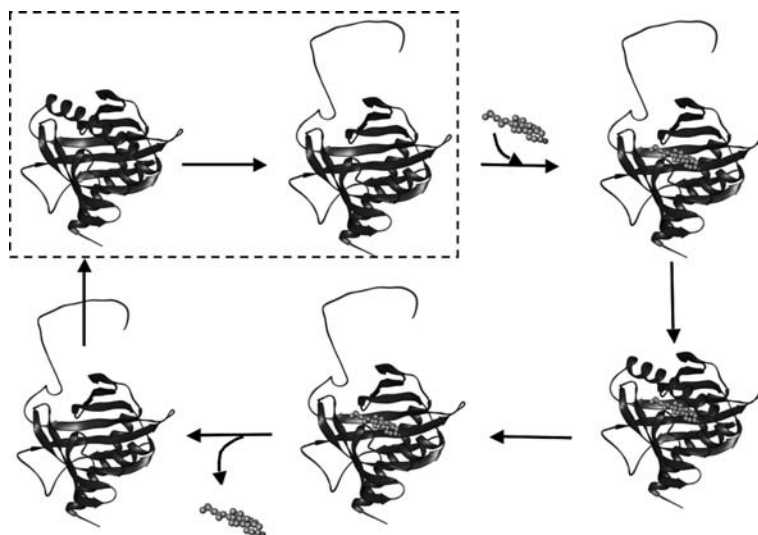


Fig. 15.3 The two-state model. Because of the presence of a cavity in the absence of cholesterol, the folded state of the closed and apo form of the START domain of StAR undergoes a local and reversible unfolding of its C-Terminal helix. *Boxed reaction*. The intermediate and partially unfolded state is proposed to initiate specific binding and allow for the dissociation of cholesterol

pH. As shown in Fig. 15.4, this is exactly what we have observed (Roostae et al., 2008; 2009). Indeed, as one can see, the far-UV CD spectrum (Fig. 15.4A) of N62-StAR with an equimolar concentration of cholesterol depicts more negative molar ellipticities than the free construct (of course the contribution of cholesterol has been subtracted). This is indicative of an increase in secondary structure (α and/or β). While there are many computational routines to determine the percentage of secondary structure from CD spectra, these have sizable uncertainties. On the other hand and more reliably, if the addition of cholesterol is accompanied by a transition from a mostly random coil C-terminus to the stabilization of this region into an α -helix, an isosbestic point is expected at 203 nm. Random coil and α -helix have the same molar ellipticity at 203 nm. However, the isosbestic point between the β structure and random coil is close to 208 nm. Therefore, if cholesterol had induced a random coil to β transition, an isosbestic point at ~ 208 nm would have been observed. As shown in Fig. 15.4A, an isosbestic point at 203 nm observed, hence demonstrating that the addition of cholesterol stabilizes the C-terminus into an α -helix from an otherwise mostly random coil configuration. Moreover, as expected, the melting temperature T° of the cholesterol-N62-StAR (1:1) complex is increase by almost 4° compared to the free construct (Fig. 15.4B). As described in detail elsewhere, the thermodynamic stability of the complex is also increased at all temperatures (Roostae et al., 2008). Finally, by monitoring the increase in α -helical content at 222 nm, we have titrated N62-StAR with cholesterol and confirmed, like MLN-64 (Tsuji-shita and Hurley, 2000) that the START domain of StAR binds

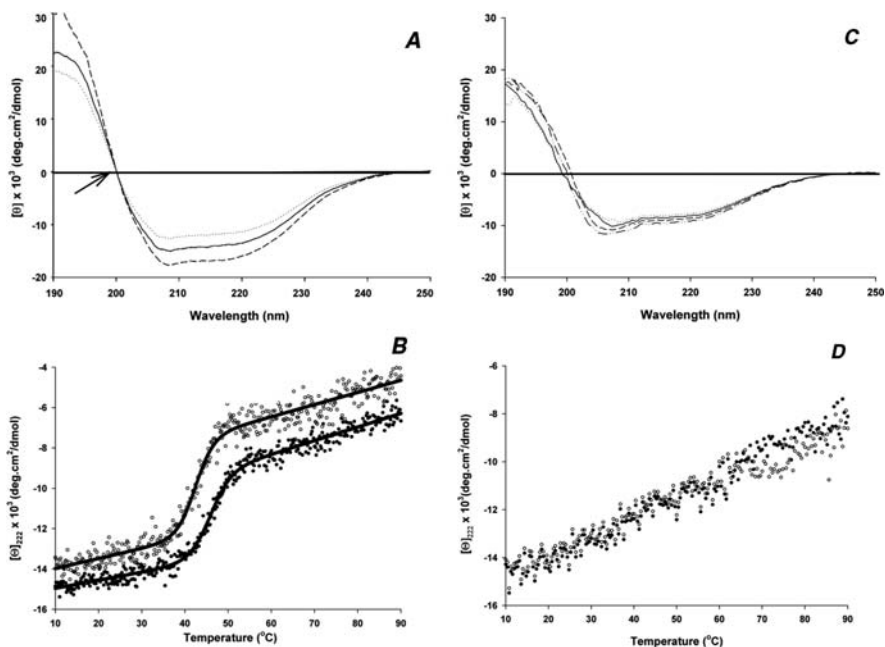


Fig. 15.4 The presence of stable tertiary structure in the START domain of StAR is a prerequisite for cholesterol binding. **A.** Far-UV CD spectra of the N62-StAR construct in absence (*solid line*), immediately (*dotted line*) and 20 minutes after addition of cholesterol at neutral pH (*dashed line*). Note the presence of an isosbestic point at 203 nm (*arrow*), which is indicative of the stabilization of α -helical structure from a random coil content upon addition of cholesterol and once equilibrium has been attained. **B.** Temperature-induced denaturation of the free (*open circles*) and 1:1 cholesterol complex (*solid circles*) of the N62-StAR construct at neutral pH. Note the cooperativity of the curves. This signifies that stable tertiary structure is present. **C.** Far-UV CD spectra of the N62-StAR construct in absence (*solid line*), immediately (*dotted line*), 20 min (*dashed line*) and an hour after addition of cholesterol at pH 3.5 (*dashed and dotted line*). **D.** Temperature-induced denaturation of the free N62-StAR construct (*open circles*) and a 1:1 N62-StAR:cholesterol mixture after 1 h of incubation (*solid circles*) at pH 3.5. Note that under these conditions, no cooperativity is observed, denoting the absence of stable tertiary structure even in presence of cholesterol

cholesterol in a 1:1 stoichiometry with apparent affinity of $\sim 3 \cdot 10^{-8}$ (Roostae et al., 2008).

For the sake of comparison and in order to evaluate the ability of the molten globular state of N62-StAR to bind cholesterol, we have repeated the same experiment at pH 3.5. As can be seen on Fig. 15.4C, the far-UV CD spectra of N62-StAR depicts a sizable content of secondary structure at acidic pH. However, N62-StAR is devoid of stable tertiary structure and does not bind cholesterol. Indeed, as one can see, the temperature denaturation curves monitored by CD of N62-StAR does not show any cooperativity and the presence of cholesterol does not alter the curve (Fig. 15.4D).

This data indicates that the molten globule state cannot provide the minimal tertiary structure necessary to allow for the specific binding of cholesterol. Hence, while a putative pH induction of a molten globular state of StAR near the mitochondrial membrane could be an effective way to release cholesterol, however it is not sure how such a state could reversibly refold into a structure with a stable tertiary structure and undergo such a global transition cyclically. In fact, once acidified N62-STARs solutions irreversible lose their capacity to bind cholesterol and induce steroidogenesis (Roostaei et al., 2008). Moreover, it is known that one StAR molecule can be responsible for the transfer of over 400 cholesterol molecules per minute (Artemenko et al., 2001). It appears to us that the local unfolding of the C-terminal is a much more efficient way to reversibly expose a well defined binding site to allow for a repetitive binding and release of many cholesterol molecule per STAR.

Furthermore, there are clear experimental evidences which show that restricting the movement of the C-terminal of N62-StAR hampers it's cholesterol-binding affinity and it's ability to induce pregnenolone production in vitro assays. By covalently attaching helix 4 to the loop between β -strands 1 and 2 with disulfide bridges S100C/S261C or D106C/A268C (Fig. 15.5), caused StAR to lose half or completely its binding and steroidogenic activity, respectively (Baker et al., 2005). However,

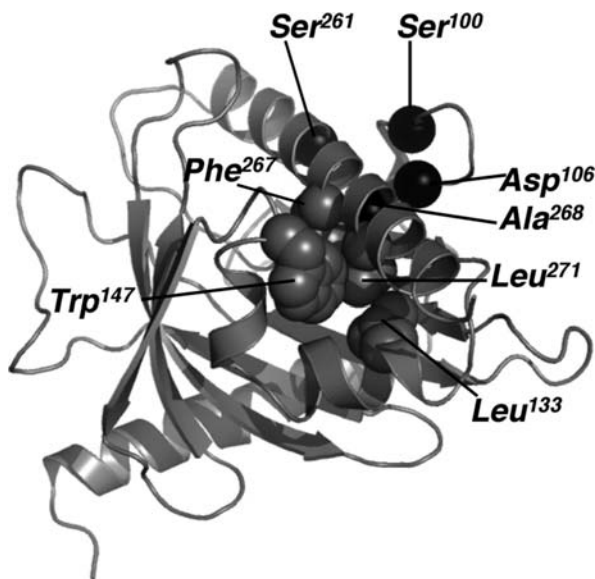


Fig. 15.5 A conserved hydrophobic core is present at the interface of helices 2, 3 and 4 (grey side chains). This hydrophobic cluster is present in the 3D model of StAR and the crystal structures of MLN64, STARD4 and STARD5 (not shown) and is proposed to stabilize the C-terminal helix in its folded state. Localization of the mutations made by Baker et al. (2005) in order to prevent movement of the C-terminal helix (black spheres)

adding a reducing agent restored the binding and activity. In addition, weakening hydrophobic and tertiary interactions at the interface of helices 2,3 and 4 (Fig. 15.5) in the fully folded state of N62-StAR in the bound and free forms, reduced the thermodynamic stability of both forms and abridged the steroidogenic activity of the mutants. This conserved hydrophobic cluster, involving Leu¹³³, Trp¹⁴⁷, Phe²⁶⁷ and Leu²⁷¹, is proposed to stabilize helix-4 in its closed form and provide the necessary stabilization free energy to generate a stable and functional complex (Roostae et al., 2000). While, mutating the conserved Phe²⁶⁷, Leu²⁷¹ to polar residues with similar respective volumes (i.e. Gln and Asn, respectively) promoted the opened form but prevented the formation of a stable and functional complex (Roostae et al., 2008; 2009).

15.5 Towards a Consensual Model for the Reversible and Specific Binding of Cholesterol by START Domain

As discussed, the START domains with affinity for cholesterol, have or can be predicted to have an internal cavity with a volume equal to that of cholesterol. Hence, in absence of cholesterol, the tertiary α/β helix grip fold will be destabilized. Since, helix-4 is free to move from the rest of the molecule, this excess in free energy should promote its unfolding (Fig. 15.3). In other words, that the lack of stabilization free energy created by the absence of a ligand should be naturally compensated by an increase in conformational entropy following the unfolding of the C-terminal helix, and hence promote the population of an intermediate state. This local unfolding (and intermediate state) can be seen as serving two purposes: **1** – stabilization of an otherwise unstable state and **2** – unveiling of the buried binding site. We propose that this is the state that recognizes cholesterol and from which cholesterol will dissociate in order to be delivered to organelles by processes still not fully understood (Rone et al., 2009). As discussed here, the discovery of the specific determinants or interactions responsible for the molecular recognition await the resolution of the 3D structure(s) of START-cholesterol complex(es). Nonetheless, it is increasingly evident that the species of START domains responsible for the formation of an initial complex should possess a minimal tertiary structure content capable to present (at least in part) the required structural determinants to allow for the formation of a specific complex. As discussed by others (Tsuji-shita and Hurley, 2000) and shown experimentally, a molten globular state with non-defined (specific) tertiary structure is a somewhat unsatisfactory purported entity to carry out such a function.

Interestingly, many of the experimental results that originally led authors to propose or validate the molten globule model, agree with and support the two-state model. Namely, the fact that the C-terminal helix is the most susceptible region of N62-StAR and that restricting the movement of the C-terminal helix by the engineering of disulfide bridges prevent binding and impede on the steroidogenic activity of N62-StAR.

At this stage, we believe that ambiguity or divergence in the interpretation of the results available in the literature stems from the hypothesis of a role of low pH. While, it is true that N62-StAR behaves like a bona fide molten globule at low pH, the existence of pH values in the 3.5–4.5 range near the mitochondrial membrane still needs to be demonstrated and the ability of such a state to specifically recognize any ligand or protein is contrary to the established understanding of biomolecular recognition.

15.6 Conclusions and Perspectives

Structural data on START domains that bind cholesterol and biophysical analysis of START domain-cholesterol interactions reviewed in here point to a common mechanism of molecular recognition and reversible binding. Indeed, many lines of evidence suggest the occurrence of a local and microscopic unfolding event of the α/β helix grip fold that leads to the population of key intermediate state with the C-terminal helix unfolded (Fig. 15.3). It is this intermediate state that is proposed to initiate the specific binding and release the cholesterol to its end point. The formation and the stability of the fully folded complex is also considered to be crucial. Indeed, the formation of an unstable complex would lead to a complex of short lifetime which would be unfit to deliver cholesterol to its final destination.

Finally, the definite validation of the two-state model awaits the characterization of the proposed movements or molecular unfolding events in the presence and absence of cholesterol. In this regard, we have recently published preliminary NMR data with and without cholesterol, which show that N62-StAR undergoes a slow exchange between two states with stable tertiary structure. The characterization of the structure and dynamics of both states is likely to shed the necessary light to definitely validate the two-state model.

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