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MECHANISTIC SEQUENCE OF MITOCHONDRIAL CHOLESTEROL TRANSPORT BY StAR PROTEINS

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Abstract: The steroidogenic acute regulatory protein (StAR) presents across the species and are part of a family of StART (StAR-related lipid transport) domain-containing proteins. The first member of this family StAR is now called StARD1. StARD1 helps in the movement of cholesterol from the outer to the inner mitochondrial membrane (IMM), where cholesterol is the only substrate to synthesize the first steroid, pregnenolone. StARD1 is an essential protein and in its absence very little steroid synthesis occurs resulting premature death shortly after birth. The biochemical studies show that the flexible C-terminus is required for cholesterol-binding activity of all StART family proteins, and flexibility of this domain is directly proportional with StARD activity. As a result, a slower rate of unfolding for the C-terminal domain leads to higher activity, while faster unfolding leads to minimal activity. Thus, only wild-type StARD1 maintains a flexible hydrophobic core but not the inactive mutant proteins. Cholesterol associated with the outer mitochondrial membrane (OMM) facilitates the formation of the protein hydrophobic core and stabilizes the protein. This article presents a detailed mechanism of action for StARD1 proteins to transport cholesterol from the OMM into the mitochondria.

Keywords: Mitochondrial cholesterol transport; StAR protein; Steroidogemesis; Protein import into mitochondria; Congenital Adrenal Hyperplasia.

Introduction

Proteins fold into unique, functionally active forms, which are determined by the energetic information specified by their primary amino acid sequences (Anfinsen, 1972; Anfinsen 1973). Proper protein folding and the extent of functional activity are directly linked and proportional, though activity can be observed to differing extents in unfolded, intermediate, or fully folded states (Sohl *et al.*, 1998). Typically, folding proceeds through several intermediate states (Redfield *et al.*, 1994), each having a lower Gibbs free energy than the previous state (Brooks *et al.*, 1998). The intermediate states may comprise partially folded forms of individual domains or of the entire protein. Intermediates that have a high degree of secondary structure but lack defined tertiary structure are often called the "molten globule" state (Privalov, 1996).

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The steroidogenic acute regulatory protein (StAR) is a nuclear-encoded, mitochondrially-targeted protein, which is required for adrenal and gonadal steroidogenesis and for male sexual differentiation. StAR proteins belong to a family of proteins containing StART (StAR-related lipid transport) domains, which are found in most species (Alpy and Tomasetto, 2005). There are 15 human START-domain proteins, named StARD1 through StARD15 [Soccio and Breslow, 2003). StARD1 moves cholesterol from the outer mitochondrial membrane (OMM) to the IMM where it is converted to pregnenolone, the precursor of all steroid hormones. In the absence of StARD1 very little steroid synthesis occurs, and newborns die shortly after birth (Lin *et al.*, 1995; Bose *et al.*, 1996). Human StAR is a 285-amino-acid protein with a putative N-terminal mitochondrial targeting sequence, though a N-terminal 62-amino-acid deletion mutant of StARD1 (N-62 StAR) retains 100% activity in COS-1 cells (Arakane *et al.*, 1996) or when N-62 StAR is incubated with isolated mitochondria (Bose *et al.*,

2008). StARD1 acts exclusively on the OMM (Arakane *et al.*, 1996; Bose *et al.*, 2002), and undergoes several post-translational modifications to reach its fully active form. StARD1 is initially expressed as a 37-kDa cytoplasmic protein, where it is activated by phopsphorylation of Ser 195 (Arakane et al, 1997), processed to a 32-kDa intermediate possibly at the inner mitochondrial space (IMS), and imported into mitochondria to be cleaved to the final 30-kDa mature protein (Stocco and Clark, 1996). This article will focus on the StARD1 isoform in this article but also present relevant data from other StARD1 isoforms. In this article, we review current studies on StAR proteins and present a detailed mechanism for StAR cholesterol transport in to mitochondria.

1. Pausing at the OMM

StARD1 is synthesized as a 37-kDa protein and imported into mitochondria as a mature 30-kDa protein, and has no known function. It is widely accepted that matured 30 kDa StARD1 is an inactive protein (Stocco and Clark, 1996). StAR does not need to enter into the mitochondria to transport cholesterol, so the active form of StARD1 occurs at the outer mitochondrial membrane (OMM). Alternately, StARD1 may maintain an active form during its entry into the mitochondria. So, it is important to define the sequence of folding states during mitochondrial import to define the active state of StARD1. Several biophysical experiments investigating StAR folding dynamics have shown that: i) StAR undergoes a pH-dependent unfolding with an increase in a-helical content at low pH (pH 3.5) and retention of secondary structure as low as pH 2.0; ii) cooperative unfolding in urea at pH 4.0; iii) the presence of a tight N-terminal domain that partially unfolds at pH 4; and iv) the C-terminal region is more solvent-accessible and partially unfolded at pH 2.5. Thus, StARD1 appears to be a pseudostable protein that adopts a molten globule state at low pH, which may be similar to the unfolded form adopted during mitochondrial import (Bose *et al.*, 1999).

Mitochondrial import of StARD1 can be envisioned as a series of unfolding events. During mitochondrial import, the N-terminal mitochondrial leader sequence should be the first domain to enter the mitochondrial membrane. This compact N-terminal domain must unfold during mitochondrial entry (Gaume *et al.*, 1998), a rate-limiting step that causes StARD1 to pause during mitochondrial entry (Bose *et al.*, 1999) and provides more time for the biologically active C-terminus to interact with the OMM. The interaction between the StAR C-terminus and the OMM is facilitated by an abundance of positively charged residues, which likely form electrostatic interactions with the negatively charged membrane phospholipids (Christinsen *et al.*, 2001). The plausible pause of StAR mentioned here is expected to occur concomitantly to a shared binding of the N-terminal presequence plus a part of the mature domain that follows the presequence, to the so called trans-site of TOM (Translocases, outer mitochondrial membrane), located at the inter-membrane face of the outer membrane. Because StARD1 unfolds during mitochondrial entry and is active during mitochondrial entry, it is logical to speculate that the active form of StARD1 is a molten globule form that acts directly on the OMM (Bose *et al.*, 1999; Christinsen *et al.*, 2001).

2. The StARD1 Unfolded State is an Essential Minimal-energy Form

Mitochondrial proton pumps establish an electrochemical H^+ gradient leading to a local reduction of pH on the cytoplasmic side of the OMM and minor reduction of pH in the mitochondria matrix. The structure of StAR lends itself to partial unfolding, as the N-terminal and C-terminal domains behave differently, and unfolding would increase the length of the loop linking the protected N-terminus and the membrane-associated C-terminus. Thus, partial unfolding induced by the mitochondrial electrochemical gradient and mitochondrial insertion result in a transition from a fully folded state into a molten globule state (Ryan and Jensen, 1995). By preserving the majority of the secondary structure, the molten globule state is the most reasonable means to minimize the energetic cost of inserting a compact protein structure into a membrane, as this transition to a flexible conformation lowers the energy required to open the structure.

3. C-terminal of StARD1 is in a Dynamic State of Folding

Swapping the StARD1 N-terminal signal sequence with the signal sequence of different inner membrane, matrix, and outer mitochondrial proteins, and thus we placed StARD1 in a different mitochondrial compartment. These StARD1 fusion protein(s) was only active when it was placed at the OMM. These data confirm that StARD1 functions primarily at the OMM. The active unimported StAR is a 37 kDa protein, and thus it is obvious to consider that pausing at the OMM will be generated by the 37 kDa form of StARD1. However, the requirements for StARD1-like activity on the OMM and the precise mechanism of this activity remained unknown. Confusion has arisen because StARD1 can bind cholesterol and transfer it between membrane systems *in vitro*, suggesting StAR acts by transferring cholesterol from cytoplasmic sources to the OMM (Kallen *et al.*, 1998; Tuckey et al, 2002). Further, *in vitro* cholesterol-binding and transfer activity is retained by a StARD1 mutant (R182L) that cannot facilitate the synthesis of pregnenolone (Baker *et al.*, 2007). This suggests that StARD1 activity must be carefully distinguished between its enrichment of cholesterol into membranes and its ability to induce steroidogenesis by increasing the movement of cholesterol from the OMM to IMM.

An initial approach to understand StARD1 dynamics determined that the overall structure remains stable at low pH, but several segments of the protein undergo conformational changes (Baker *et al.*, 2005). The movement of the C-terminal helix is pH-induced, which opens and closes access to the sterol-binding pocket. Limiting the movement of the C-terminal helix by creating a disulfide bond linking it to an adjacent structure eliminated the molten globule form and also eliminated cholesterol binding and StAR-like activity. However, StARD1 activity could be restored by cleaving the disulfide bond, suggesting immobilization of the C-terminal helix, and not the amino acid substitution, ablated StAR-like activity (Baker *et al.*, 2005). Thus, the overall molten globule model of StARD1 action is that movement of the C-terminal helix, but not complete unfolding of the protein, is required for cholesterol-binding activity (Baker *et al.*, 2005; Baker *et al.*, 2007).

4. StARD1 is Not a Globular Protein

ANS (1, 8 Anilino Napthalene Sulfonic Acid) has been used for decades to demonstrate protein conformational changes *in vitro* (Cunningham and Agard, 2003). Conformational changes are shown by ANS binding to hydrophobic regions near planar aromatic rings that are newly exposed by the change in shape, leading to an increase in ANS fluorescence (Masui and Kuramitsu, 1998). Using ANS fluorescence, a difference between the wild-type StARD1 and mutant R182L StARD1 proteins was determined. Wild-type StARD1 had approximately twice as many ANS binding sites as mutant StARD1 (Bose *et al.*, 2009). Similar free energies (ΔG) for the wild-type and mutant proteins indicate a difference in the internal hydrophobic groove of the wild-type and mutant proteins. Further, the K_{app} of wild-type StARD1 was twice than that of the mutant protein, suggesting that the internal hydrophobic groove in wild-type StARD1 is not globular (Tsujishita and Hurley, 2000), as was originally hypothesized (Bose *et al.*, 1999), but the groove of the mutant StARD1 proteins may adopt a more globular form, resulting in limited space and less flexibility. It is likely that the organization of the wild-type StARD1 is more dependent on its specific amino acid sequence (Bose *et al.*, 2009).

Biochemical studies on StAR typically probe the context of tryptophan residues within the protein structure, and this method has been used to identify the exposed and buried domains of this protein. The tryptophan residues in StARD1 are randomly distributed, suggesting that some of these residues are more exposed than others. StARD1 requires an interaction with the OMM to adopt an open conformation (Bose *et al.*, 1999). This conformational change is essential not only for cholesterol binding, but for transport of cholesterol into the IMM (Bose *et al.*, 1999; Baker *et al.*, 2007). Real time assessments of wild-type and mutant StARD1 unfolding suggests that, for both, the fast unfolding phase is not associated with a rapid collapse of the polypeptide chain. Thus, wild-type and mutant StARD1 should be able to function in cholesterol binding in an intermediate folding state. The aromatic circular

dichroism (CD) showed that both the wild-type and mutant StAR are folded; however, a small difference in ellipticity at 275 nm, which corresponds to tryptophan absorption, was observed between these two proteins. Thus, while the core of wild-type and mutant StARD1 proteins have a similar hydrophobic nature, subtle differences may exist between the unfolding process of the wild-type and the mutant forms of StARD1 that are responsible for the inactivation of the mutants (Bose *et al.*, 1996; Christinsen *et al.*, 2001). Therefore, the faster unfolding of mutant StARD1 is not the sole reason for its inactivity, as was originally considered (Bose *et al.*, 2008). Instead, the core of the mutant proteins may be folded differently than the outer part of the protein.

5. Hydrophobic Core of StARD1

Early folding states of a protein often are based on the spectral changes during the dead time. These spectral changes can be attributed to the fast formation of an ensemble compact state and is defined as the burst phase of a protein (Houry *et al.*, 1998). The broad unfolding of the burst-phase intermediate, the rapidity of its transition state, and the presence of a hydrophobic core in the intermediate are all typical characteristics of a molten globule state (Bose *et al.*, 1999; Baker *et al.*, 2007). Thus, in these terms, the intermediate state of wild-type StAR is identical to the molten globule state. The secondary structures formed in the molten globule state are similar to the native, folded form of StAR. However, the 2-fold difference in K_{app} values between wild-type and mutant StAR suggests accessibility of ANS to the hydrophobic core differs. As a result, the ANS fluorescence emission when bound to wild-type StAR is 3-fold greater than ANS emission when bound to mutant StAR, indicating that a higher molar amount of ANS binds to wild-type StAR. Another tool used to probe StAR secondary structure is TFE (trifluoroethanol), which has been extensively used to increase the α-helical content of proteins. TFE induces a more structured fold by reinforcing hydrogen bonding between carbonyl and amide groups due to the removal of water molecules, and this results in a more stable conformation (Luo and Baldwin, 1997). Since StARD1 interacts at the OMM, and therefore liberates water molecules during its transient stay at the OMM, water molecules at the OMM may play a role in the folding and interaction of StAR with the mitochondrial membrane. The formation of hydrophobic core is facilitated only when the protein removes water molecules from its core. Thus, TFE induces a StAR conformation similar to that present when StAR is associated with the OMM by removing water from the core of StARD1 and increasing α-helical content (Roderick *et al.*, 2002). This flexibility of the C-terminal helix (Baker *et al.*, 2005) allows wild-type StARD1 to hold and release cholesterol during its interaction with the OMM protein(s) (Bose *et al.*, 2008). Thus, even minor differences in the hydrophobic core could affect the flexibility of the protein.

In addition, the core of wild-type StARD1 is likely more nucleated than mutant StARD1, which means the core residues establish contacts first during folding and directs the proper subsequent folding of the rest of the protein. The preferential folding of the core residues (also called nucleus) of a protein followed by compact folding rest of the protein is called nuclear condensation. In the nucleation-condensation protein folding model, the formation of a weak nucleus is associated with a compaction of the polypeptide chain such that the tertiary and secondary structures are formed at the same time (Fersht, 1999). However, the formation of a relatively small folding nucleus is not the only rate-determining step in global protein folding, since a significant fraction of the overall structure should be in the correct conformation to enable contact between nucleating resides to form and be maintained. This type of behavior implies that folding is driven by the formation of a weak nucleus, where most of the polypeptide chain is involved in stabilizing the transition state. Thus, a loose hydrophobic core might be necessary to impart flexibility to StAR and to give it the ability to bind and release cholesterol (Bose *et al.*, 2009).

6. Role of Cholesterol

To examine the unique mechanism of StAR-mediated cholesterol import into mitochondria, protein transport was combined with functional complementation of steroidogenic activity. These studies identified differences in the mitochondrial

import process. P450scc possibly resides at the IMS facing the mitochondrial matrix and occurs more quickly due to the need in the matrix prior to or at the same time as cholesterol transport for pregnenolone synthesis (Chung *et al.*, 1986). Substituting StARD1's N-terminal 62 amino acids with the 39 amino acids of P450scc resulted in the import of SCC-N-62 StAR fusion into isolated mitochondria four times faster than StARD1 (Bose *et al.*, 2002). StARD1 is expressed upon hormonal stimulation, and thus, longer residency within the OMM is essential for the transfer of several cholesterol molecules into the mitochondrion (Bose *et al.*, 2002). In acute steroidogenesis, substantial cholesterol influx to the matrix side of the mitochondrion is essential for the synthesis of large amounts of steroid hormones in a short period of time. Therefore, the cholesterol pool should remain spatially close to both the OMM and StARD1 molecules (Bose *et al.*, 2009).

Mass spectrometric analysis of proteolytically digested StARD1 domains showed that these domains are partially resistant to proteolysis, suggesting that the stability of StAR in solution differs from the stability of StARD1 associated with the mitochondrial membrane. The presence of the StARD1 N-terminal sequence is nonessential for activity (Arakane *et al.*, 1996) and stability; however, this sequence is critical for targeting StARD1 to the mitochondrial membrane. Additionally, computer modeling of StARD1 and START proteins predicted that StARD1 unfolds for activation and import in association with the membrane (Murcia *et al.*, 2006). In isolated mitochondria, cholesterol is removed during mitochondrial preparation, and as a result, the limited remaining endogenous cholesterol does not facilitate pregnenolone synthesis. However, *in vivo*, once StARD1 reaches the OMM, multiple cholesterol molecules are transported by StARD1 into the mitochondria. So, it is logical to consider that StAR is stabilized by an association with the OMM and cholesterol within this membrane (Bose *et al.*, 2009). Therefore, once StAR has associated with the mitochondrial membrane, the folding and activity of StARD1 are dictated by the protein's domain organization and the presence of cholesterol within the OMM.

7. Similarity in Folding is Essential for Activity

Studies have found that StARD3 has approximately half the cholesterol transport activity of StARD1 (Bose *et al.*, 2000), and StARD4 has less than 30% of the activity of StARD1 (Soccio *et al.*, 2005). Further, StARD5, StARD6, and disease-causing mutants of StARD1 have no cholesterol transport activity (Soccio *et al.*, 2005). One disease-causing mutant (R182L) binds cholesterol with an affinity similar to that of wild-type StARD1, but does not stimulate steroidogenesis. This suggests cholesterol binding alone is insufficient for a START domain protein to achieve StARD1-like activity (Baker *et al.*, 2007). The crystal structures of StARD3 (Tsujishita and Hurley, 2000) and StARD4 (Romanowski *et al.*, 2002) and the computational modeling of StARD1 (Mathieu *et al.*, 2002; Yaworsky *et al.*, 2005) resulted in similar three-dimensional structures, but the activity of these isoforms vary. Therefore, structure alone failed to explain the differential activity of these proteins (Bose *et al.*, 1998), indicating specific mechanisms regulating the biological activity of START proteins.

In addition, StARD6 showed biophysical behavior and a structure that is similar to that of StARD1, which indicates StARD6 undergoes a similar molten globule conformational change when it associates with the OMM. Both StARD1 and StARD6 have similar changes in free energy when undergoing transient partial unfolding: both unfold with similar kinetics, both show a dramatic increase in enthalpy when associating with the OMM, and both have similar predicted structures. Thus, it is likely that both StARD1 and StARD6 act as molten globules on the OMM to induce the movement of cholesterol from the OMM to the IMM 28. This hypothesis is supported by the observation that StARD6 cholesterol-binding activity was greater than that of StARD1 or StARD3, but transport activity was equivalent.

The unfolding and refolding of both StARD1 and StARD6 are slow and require an interaction with the OMM. Partial proteolysis data showed that StARD6 has a longer flexible C-terminus than StARD1 and StARD3. However, all three proteins showed similar binding capacity with cholesterol, suggesting that C-terminal flexibility is not the

sole determinant of the degree of StAR activity. Additionally, real time unfolding and refolding of StARD1, StARD3, and StARD6 showed that StARD6 had the slowest kinetics, with unfolding and folding on the order of minutes, compared to StARD1 and StARD3 with unfolding and folding within seconds. Slower unfolding led to a greater cholesterol binding activity for StARD6 over StARD1 or StARD3, indicating the rate of unfolding/folding is directly related to cholesterol binding, but not necessarily related to cholesterol transport.

8. StARD1 Requires Docking at the OMM

StARD's ability to transfer cholesterol between membranes is distinct from its ability to induce steroidogenesis (Baker *et al.*, 2007). Initially, it was proposed that StAR acted in conjunction with an OMM receptor, but this hypothesis was challenged by early studies showing StAR-like activity on protease-treated mitochondria, where putative receptors should have been removed (Kallen *et al.*, 1998). Nevertheless, substantial data suggest that cholesterol import into mitochondria requires the 18-kDa Peripheral Benzodiapine Receptor (PBR) protein on the OMM. PBR is a five transmembrane OMM protein, with an amino-terminus that binds benzodiazepines to stimulate steroidogenesis and a carboxyl-terminus that binds cholesterol (Lacapere and Papadopoulos, 2003). Steroidogenic activity was lost in cells with RNAi-mediated knockdown of PBR (Hauet *et al.*, 2005). Further, several proteins appear to interact with PBR, including VDAC1 (Voltage Dependent Anion Channel 1), ANT (Adenosine Nucleotide Translocator) (McEnery *et al.*, 1992), an unknown 10-kDa protein (Blahos *et al.*, 1995), PRAX-1 (Peripheral Benzodiazapine Receptor-Associated protein1) (Galiegue *et al.*, 1999), and PBR-associated protein 7 (PAP7) (Li *et al.*, 2001). However, StARD1 has not been shown to associate with PBR, which is expected since StARD1 is not detectable in unstimulated cells. Photoaffinity labeled PBR forms a complex of 170-210 kDa (Doble *et al.*, 1987) or 200-240 kDa (Papadopoulos et al, 2007), but StARD1 is not a component of these complexes. Thus, until recently a StAR receptor, other than the general components of the translocase complex in the mitochondrial (TOM) remained unidentified. In an attempt to identify this receptor, we used native gradient gel electrophoresis of digitonin-lysed StARD1 complexes identified VDAC1and PCP (Phosphate carrier protein) as putative receptors (Bose *et al.*, 2008a; Bose *et al.*, 2008b). RNAi-mediated knockdown of VDAC1 or blocking its interaction with StARD1 using Koenig's polyanion completely inhibited StAR-like activity and expression of StAR. Koenig's polyanion is an organic compound specifically blocks VDAC1 at the OMM (Shimizu *et al.*, 1999). Inhibition of VDAC1 resulted in activation of a cysteine protease in the OMM that proteolytically degraded misfolding and unimported StARD1, which resulted from the absence of VDAC1. Cells remained viable in the absence of VDAC1 and PCP, but in the absence of PCP circulation of ATP was completely ablated. Finally, specificity of StARD1 association with VDAC1 and PCP is supported by the inability to detect PBR in the isolated native StARD1-complexes from digatonin-lysed cells (Bose *et al.*, 2008a). Thus, we concluded that StARD1 directly interacts with VDAC1 and PCP on the OMM (Bose *et al.*, 2008a).

These observations are wholly consistent with the literature implicating PBR in StARD1-induced steroidogenesis. The association of StARD1 with PCP is needed to phosphorylate StARD1 for maximal activity. StARD1 appears to be phosphorylated by PKA, and PAP7 appears to act as an anchoring protein to promote the interaction of PBR and the PKA regulatory subunit 1a (PKAR1a) (Li *et al.*, 2001). PCP may play a role in delivering phosphate to PKAR1a (Zara *et al.*, 1996). Only the cytoplasmically exposed domains of VDAC1 interacted with StARD1, and the StARD1 C-terminus forms contact with VDAC1, consistent with data showing that this domain of StARD1 also interacts with synthetic membrane models of the OMM (Yaworsky *et al.*, 2005). Thus, based on these data, it is suggested that StARD1 acts by interacting with VDAC1, which is part of a larger OMM complex including StARD1, VDAC1, PCP, PBR, PAP7, PKAR1a, and possibly other proteins. It remains unclear whether VDAC1 forms the channel through which cholesterol passes or if this function is served by PBR. Since VDAC1 knockout mice are viable (Anflous *et al.*, 2001) and VDAC3 knockout mice becomes impotent (Sampson *et al.*, 2001), but

VDAC2 and PBR knockouts are embryonic lethal (Lacapere *et al.*, 2003; Cheng *et al.*, 2003), we suggest that the channel is formed by PBR in association with other is unlikely.

Conclusion

This review summarizes the current understanding of how coordinated StARD1 conformational changes facilitate its activity at the OMM, and the interaction of StARD1 with various proteins during its transport of cholesterol from the OMM to the IMM. Further, StARD1 requires an interaction with a pool of cholesterol, in a mechanism that remains to be described fully. Compelling evidence indicates the active conformation of StARD proteins is a molten globule form with a hydrophobic core, resulting in a flexible protein that can easily associate with the OMM environment. The complexity of conformational changes, protein-cholesterol, and protein-protein interactions that govern StARD1 activity requires further investigation to fully describe the sequential events involved with StAR-mediated mitochondrial cholesterol transport.

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