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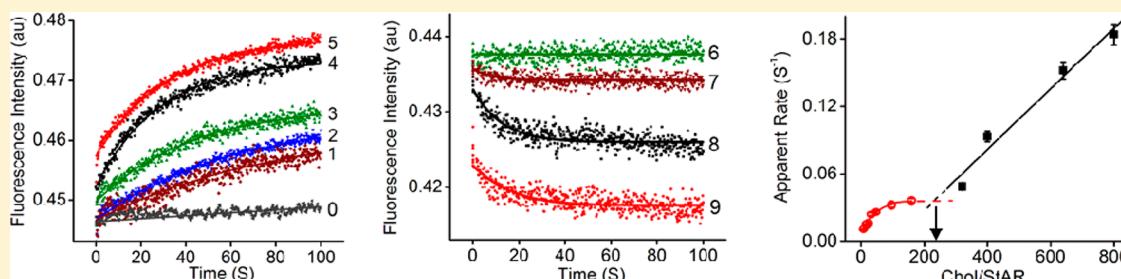
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ABSTRACT: Although the mechanism by which the steroidogenic acute regulatory protein (StAR) promotes steroidogenesis has been studied extensively, it remains incompletely characterized. Because structural analysis has revealed a hydrophobic sterol-binding pocket (SBP) within StAR, this study sought to examine the regulatory role of cholesterol concentrations on protein folding and mitochondrial import. Stopped-flow analyses revealed that at low concentrations, cholesterol promotes StAR folding. With increasing cholesterol concentrations, an intermediate state is reached followed by StAR unfolding. With 5 µg/mL cholesterol, the apparent binding was 0.011 s^{-1} , and the unfolding time ($t_{1/2}$) was 63 s. The apparent binding increased from 0.036 to 0.049 s^{-1} when the cholesterol concentration was increased from 50 µg/mL to 100 µg/mL while $t_{1/2}$ decreased from 19 to 14 s. These cholesterol-induced conformational changes were not mediated by chemical chaperones. Protein fingerprinting analysis of StAR in the absence and presence of cholesterol by mass spectrometry revealed that the cholesterol binding region, comprising amino acids 132–188, is protected from proteolysis. In the absence of cholesterol, a longer region of amino acids from position 62 to 188 was protected, which is suggestive of organization into smaller, tightly folded regions with cholesterol. In addition, rapid cholesterol metabolism was required for the import of StAR into the mitochondria, suggesting that the mitochondria have a limited capacity for import and processing of steroidogenic proteins, which is dependent on cholesterol storage. Thus, cholesterol regulates StAR conformation, activating it to an intermediate flexible state for mitochondrial import and its enhanced cholesterol transfer capacity.

Unlike cells that synthesize and secrete polypeptide hormones, steroidogenic cells do not store a significant reservoir of previously synthesized hormone that can be released on demand.^{1–6} Instead, acute steroidogenic responses, such as the rapid release of cortisol in response to stress, require rapid, *de novo* synthesis.⁷ When the acute steroidogenic response occurs, steroid hormones are synthesized and released within a few minutes of trophic hormonal stimulation, especially within the adrenal glands and gonads.⁷ The rate-limiting and regulated step in the acute steroidogenic response is the movement of cholesterol from the outer to the inner mitochondrial membrane; once it is across the matrix side of the inner membrane, cholesterol is converted to pregnenolone by cytochrome P450scc and its electron-donating partners, ferrodoxin and ferrodoxin reductase. The intracellular trafficking of cholesterol is regulated, at least in part, by the StART (StAR-related lipid transfer)⁸ family of proteins. Of these

proteins, steroidogenic acute regulatory protein (StAR) is the best known, playing a crucial role in facilitating the movement of cholesterol into mitochondria. Mutation of StAR results in an inborn disorder, known as congenital lipoid adrenal hyperplasia (lipoid CAH),^{9,10} which is characterized by premature death shortly after birth because of a salt-loss crisis.^{11,12} This cholesterol reservoir is maintained in the cytoplasm within vesicle membranes where it modulates the function of a variety of membrane proteins, including receptors and channels.¹³ In addition, the presence of high concentrations of cholesterol results in the deformation of cellular organelles.¹⁴

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All members of the StART family have lipid binding properties, because of their structural similarity with StAR.^{15–18} Full-length MLN64 has no steroidogenic activity, but a 50% deletion of the N-terminal amino acids of MLN64 (StARD3) creates a steroidogenically active protein similar to StARD1.^{19,20} Through the three-dimensional structure of StARD3 (N-216 MLN64), it can be seen that these proteins have a pocket to accommodate cholesterol.⁷ StAR and MLN64 bind cholesterol. StARDS binds cholesterol and 25-hydroxycholesterol. PCTP (StARD2) (phosphatidylcholine transfer protein) binds phosphatidylcholine. StARD10 binds phosphatidylcholine and phosphatidylethanolamine. CERT (ceramide transport protein) binds ceramides. StARD4 and StARD5 are widely expressed in steroidogenic cells, while StARD6 expression is restricted to testicular germ cells.¹⁷ In contrast to StARD1, StARD4, StARD5, StARD6, StARD10, and CERT lack N-terminal sequences, suggesting they are cytosolic proteins like StARD2 (PCTP).²¹ StARD4 and StARDS bind cholesterol with high affinity and specificity, suggesting that they are capable of facilitating the transport of cholesterol through an aqueous environment. All this suggests that StARD4 and StARDS play an important role in cellular cholesterol homeostasis.^{22,23} StARD4 and StARDS have negligible StAR-like activity to stimulate the movement of cholesterol from the OMM to the IMM. In contrast, StARD6 has robust StAR-like activity and physicochemical properties that closely resemble those of StAR (StARD1).¹⁷ StARD7 is a phosphatidyl choline binding protein²⁴ abundantly expressed in trophoblast cells.²⁵ StARD4 and StARDS resemble an N-62 StAR molecule without its mitochondrial leader peptide;^{26,27} it is possible that StARD4 or StARDS might function to “load” cholesterol into the OMM. StARD4 knockout mice had minimal changes in weight and serum lipids, and no changes in steroidogenesis; therefore, it can be concluded that other factors appear to compensate for any role played by StARD4.²⁸

Structural analyses have revealed a hydrophobic sterol-binding pocket (SBP) in StAR.^{29–31} To form its hydrophobic core,³² StAR must undergo a conformational change upon activation.^{17,32–36} Because cholesterol transport during acute steroidogenesis regulation occurs at a rapid rate, it is highly unlikely that StAR interacts with cholesterol molecules individually for fostering into the mitochondrial space (IMS). In that scenario, hundreds of StAR and cholesterol molecules should be available in the limited mitochondrial space, which is not realistic. N-62 StAR, StARD3, or StARD6 does not enter the mitochondrion to facilitate cholesterol transport,^{26,27,37} suggesting that a dynamic state of interaction exists prior to mitochondrial cholesterol transport. A folded protein is in an energetically stable state that is less likely to be able to foster the adequate amount of cholesterol required during acute physiological demand to initiate rapid synthesis of pregnenolone. We hypothesize that cholesterol functions as a facilitator, thereby concentrating the cytoplasmic StAR and altering its conformation. The concentration of cholesterol may also decrease the volume of the cell or slow its protein degradation. In this study, we have applied a combination of cell biology and protein biochemistry analyses to determine the effects of the environmental influence of cholesterol concentration on StAR conformation and how these changes might influence the import of cholesterol and StAR into the mitochondria. The results of this study indicate that the environmental influence, namely cholesterol concentration, is the most critical for its

transport, which is independent of the number of StARs present at the cytoplasm.

MATERIALS AND METHODS

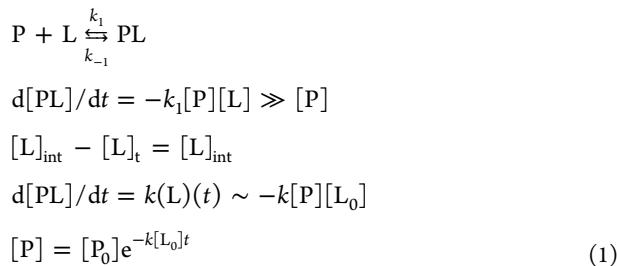
Protein Purification. Overexpression of both N-62 StAR and full-length StAR in nonsteroidogenic COS-1 cells showed 100% activity,²⁶ so truncated N-62 StAR was subcloned into the pQE-30 expression vector (Qiagen), which has a His tag coding region, as described in detail previously.³⁸ After transformed *Escherichia coli* subclones had grown with 0.05 mg/mL ampicillin and 0.01 mg/mL kanamycin at 37 °C to an optical density of 0.5, they were induced with 0.4–1 mM IPTG for 4 h. The bacteria were isolated by centrifugation at 5000 rpm for 15 min, resuspended in lysis buffer [50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole (pH 8.0)], and sonicated on ice with 10 s pulses and 30 s rests for 10 min. After the soluble fraction had been separated by centrifugation at 15000g for 30 min, it was then passed through a Ni-NTA-superflow (Qiagen) agarose column and washed first with lysis buffer and then with 50 mM NaH₂PO₄, 300 mM NaCl, and 25 mM imidazole (pH 8.0). The column was then eluted with 250 mM imidazole (pH 7.5). The eluates were dialyzed against a buffer consisting of 20 mM NaH₂PO₄ and 50 mM NaCl (pH 7.4), after which the proteins were purified using a gel-filtration column and fast protein liquid chromatography (FPLC) (AKTA, GE). The concentrations of the unfolded proteins were determined using the guanidinium hydrochloride denaturation method to avoid errors in extinction coefficients.³⁹ The molecular mass of N-62 StAR is 26931 Da; hence, 1.0 μg of StAR represents ~2.2 × 10¹³ molecules.

Western Blot Analysis. Unless otherwise mentioned, 12.5 μg of protein was separated by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Following the transfer, the membrane was blocked with 3% nonfat dry milk for 45 min, probed overnight at 4 °C with the indicated primary antibodies, and then incubated with the HRP-conjugated goat anti-rabbit or anti-mouse IgG (Pierce) for 45 min at room temperature. The signals were developed with chemiluminescent reagent (Pierce). All Western blot experiments were performed at least three times independently.

Fingerprinting of StAR Subdomains. StAR (10 μg) was mixed with 10 mg/mL cholesterol and incubated with varying amounts (1–100 units) of trypsin (Promega) in 20 mM Tris (pH 7.4) at room temperature for 30 min. Proteolysis was terminated by the addition of an equal volume of 2× SDS sample buffer containing 1 mM PMSF. The protected protein domains were separated on a 17 or 20% SDS–PAGE gel and stained with Coomassie blue or silver nitrate. Bands were excised, destained, reduced with DTT (Roche), alkylated with iodoacetamide (Sigma), and digested with trypsin (Promega) overnight as previously described.⁴⁰ The resultant peptide fragments were analyzed via liquid chromatography and tandem mass spectrometry (LC–MS/MS) using a nanoACQUITY UltraPerformance LC system (Waters) coupled with a Q-ToF Premier mass spectrometer (Waters). The smaller peptides were separated using a linear water/acetonitrile gradient (0.1% formic acid) on a nanoACQUITY column [3 μm Atlantis dC18, 100 Å pore, 75 μm inside diameter × 15 cm (Waters)] with an inline Symmetry column [5 μM C18, 180 μm inside diameter × 20 mm (Waters)] as a loading/desalting column.

Protein imported into mitochondria was first synthesized in a cell-free system, labeled with [³⁵S]methionine, and proteolyzed for 30 min in the presence of cholesterol, glycerol, and mitochondria. The reactions were stopped following the same protocol as described above. After SDS-PAGE, the protected bands were visualized by exposing the gel to X-ray film.

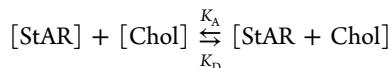
Stopped-Flow Spectrophotometry. The kinetics of protein unfolding were measured in a Jasco spectropolarimeter interfaced with an SF4 stopped-flow module (Biologic). A stock concentration of StAR (20 μM) was loaded into a syringe at a flow rate 14 mL/s. The source cholesterol concentration was 20% (Sigma), and working concentrations were developed by gradual dilution one time for all experiments. Cholesterol concentrations were used at a factor of 10⁻³. The fixed protein solution and varying concentrations of soluble cholesterol (2.5–250 μg/mL) were mixed with a “Delta” mixer with a dead time of 3.5 ms. For the unfolding experiments in which StAR was incubated with cholesterol in the presence of glycerol, 20 μM StAR was mixed with 5 or 10% glycerol, and the amount of cholesterol was varied as described above. For the unfolding experiments in which StAR was incubated with only cholesterol, the maximal concentration of cholesterol was maintained at 500 μg/mL in the reservoir. Fluorescence was measured by excitation at 280 nm, and the resulting emission was monitored at 330 nm with an optical cutoff filter at 305 nm. Data were collected in four independent segments in which each segment contained a minimum of 10 injections. Cholesterol concentrations from 5 to 100 μg/mL were mixed in a 1:1 ratio with protein to give final cholesterol concentrations of 2.5–50 μg/mL. Kinetics were analyzed using the following equations:



where P and L represent protein and ligand, respectively.

The observed rate constant (k_{obs}) was calculated by fitting the kinetic traces to a second-order rate equation: $yt = at + b + c/(1 + dt)$, where a is the slope, b is the offset, c is the amplitude, and d is the observed rate of the reaction with respect to time.

Each $k_{a(obs)}$ represents an average of at least four independent values that were plotted against cholesterol concentration to obtain the K_A of StAR in the presence of cholesterol following the relationship in eq 1.



$$k_{obs} = k_A[A_o] + C$$

The observed dissociation constant, $K_{d(obs)}$, was calculated by fitting each kinetic experiment to the following second-order rate equation:

$$Y = at + b + \sum_{i=1}^N c_i e^{-k_i t}$$

where c_i is the amplitude, k_i is the rate, and i is the order kinetics where 1 is the first order and 2 the second order. Next, the resulting apparent rate constants (k_{app}) were calculated from the kinetic analysis developed by Santolini.⁴¹ k_{app} is the observed change in rate of cholesterol association with the carrier, resulting in increased fluorescence, and is denoted by $k_{a(app)}$. Similarly, with a further increase in cholesterol concentration, the StAR's fluorescence decreases, which is denoted as $k_{d(app)}$.

Isolation and Purification of Mitochondria. Mitochondria were obtained from mouse Leydig MA-10 cell lines or, as a control, from rat adrenal glands. The adrenal glands were diced in mitochondrial isolation buffer [250 mM sucrose, 10 mM HEPES, and 1 mM EGTA (pH 7.4)]; cell lines were washed twice with PBS and then incubated with 1 mM HEPES (pH 7.4) for 30 min at 4 °C. Tissues and cells were homogenized in a hand-held, all-glass Dounce homogenizer with 40 gentle up and down strokes, and the cell debris was removed by centrifugation at 3500g for 10 min. The supernatant containing the mitochondrial fraction was purified by differential centrifugation following a previously reported procedure,^{42,43} and the pellet was washed and resuspended in an energy regeneration buffer [125 mM sucrose, 80 mM KCl, 5 mM MgCl₂, 10 mM NaH₂PO₄, 10 mM isocitrate, 1.0 mM ATP, 1.0 mM NADP, 0.1 mM ADP, and 25 mM HEPES (pH 7.4)] prior to storage either at −86 °C or in liquid nitrogen. The protein concentration was determined using the Bio-Rad Protein Assay following the manufacturer's instructions (Bio-Rad), and the mitochondrial protein concentration was used as an index of the amount of mitochondria.

Protein Import. Cell-free synthesis of [³⁵S]Met-labeled StAR was performed with a TNT rabbit reticulocyte system (Promega). Ribosomes and associated polypeptide chains (incompletely translated) were removed by centrifugation at 150000g for 15 min at 4 °C.⁴⁴ For all protein import experiments, 100 μg of isolated mitochondria isolated from MA-10 cells was incubated with [³⁵S]StAR in a final volume of 100 μL, and the mixtures were incubated in a 26 °C water bath. Import reactions were allowed to proceed for the indicated time and were terminated by the addition of 1 mM *m*-chlorophenylhydrazone (mCCCP) and an equal volume of boiling 2× SDS sample buffer. For proteolysis, 500 μg/mL proteinase K (PK) was added in the presence and absence of cholesterol. To understand the effect of cholesterol on protein import, the kinetic import analysis of the synthesized protein was performed with preimport of 0.01, 1.0, 10, 50, 100, and 200 μg/mL soluble cholesterol into the isolated mitochondria, keeping all the components equilibrated at 26 °C for 20 min. The reaction was stopped when the mixture was transferred into a boiling water bath and 2× SDS sample buffer pre-equilibrated at 80 °C was added. After the reaction mixtures had been subjected to SDS-PAGE analysis, the gels were fixed in a methanol/acetic acid mixture (40:10), dried, and exposed to a phosphorimager screen.

RESULTS

Real-Time Folding and Unfolding of StAR in Response to Cholesterol Concentrations Using Stopped-Flow Analysis. During steroidogenesis, cholesterol is converted to pregnenolone upon entering the mitochondria. StAR acts as a carrier for mitochondrial cholesterol transport. Because the mechanism by which this carrier protein rapidly transports cholesterol is not known, we hypothesized that an increased

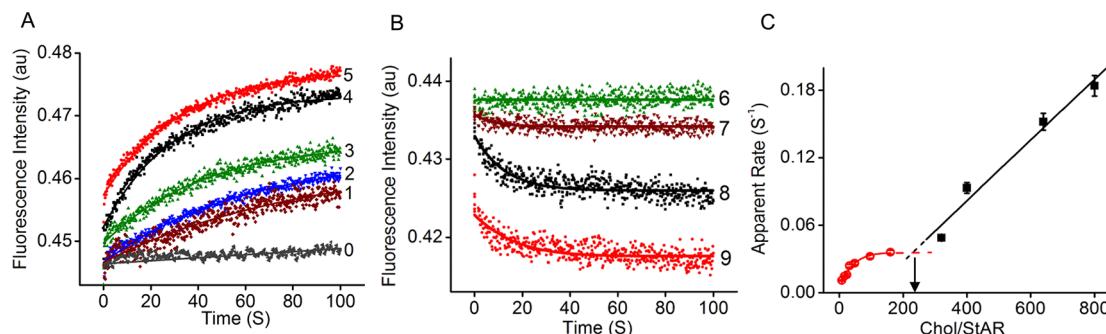


Figure 1. Effects of increasing cholesterol concentrations on StAR folding kinetics in real time as determined by stopped-flow fluorescence. (A) Refolding of 20 μM StAR by 5 $\mu\text{g}/\text{mL}$ (1, brown line), 10 $\mu\text{g}/\text{mL}$ (2, blue line), 20 $\mu\text{g}/\text{mL}$ (3, green line), 30 $\mu\text{g}/\text{mL}$ (4, black line), and 50 $\mu\text{g}/\text{mL}$ cholesterol (5, red line). StAR refolding was measured with a dead time of 3.5 ms. Increased fluorescence was observed with up to 50 $\mu\text{g}/\text{mL}$ cholesterol. (B) Unfolding of StAR with increased cholesterol concentrations: 75 $\mu\text{g}/\text{mL}$ (6, green line), 100 $\mu\text{g}/\text{mL}$ (7, brown line), 150 $\mu\text{g}/\text{mL}$ (8, black line), and 200 $\mu\text{g}/\text{mL}$ (9, red line). (C) Apparent binding of cholesterol, K_{app} , plotted with an increasing cholesterol:StAR ratio using the data obtained in panels A and B. The dashed lines are extrapolated pointing with an arrow, indicating an intermediate state in which changes in fluorescence were observed.

substrate concentration underlies its rapid transport into the mitochondria for catalysis. Therefore, StAR must change its conformation rapidly to adjust cholesterol concentrations. To determine the effects of the substrate on StAR conformation and cholesterol transfer, we conducted stopped-flow analysis using cholesterol (2.5–250 $\mu\text{g}/\text{mL}$) and 20 μM StAR to measure the rates of protein folding and unfolding (Figure 1A–C and Table 1). Whereas an increase in the magnitude of the

fluorescence intensity was observed with 100 $\mu\text{g}/\text{mL}$ cholesterol, which continued with up to 250 $\mu\text{g}/\text{mL}$ cholesterol, suggesting that increased cholesterol concentrations denature StAR (Figure 1B).

$k_{\text{d}(\text{app})}$ was also calculated by fitting the kinetic traces to a second-order rate equation. As shown in Table 1, $k_{\text{d}(\text{app})}$ increased from 0.049 to 0.093 s^{-1} from 100 to 125 $\mu\text{g}/\text{mL}$ cholesterol, respectively, confirming that increased cholesterol concentrations indeed unfolded StAR. The highest $k_{\text{d}(\text{app})}$ of 0.184 was observed in the presence of 250 $\mu\text{g}/\text{mL}$ cholesterol, a concentration was 3.7-fold higher than that observed with 100 $\mu\text{g}/\text{mL}$ cholesterol (Table 1).

To improve our understanding of how cholesterol increases the level of StAR folding at lower concentrations but induces unfolding at higher concentrations, the $t_{1/2}$ or the time required to initiate half of the folding or unfolding was next determined (Figure 1C and Table 1). Figure 1C shows StAR folding and unfolding in response to an increasing cholesterol concentration. StAR folding was observed with as few as eight molecules of cholesterol (Table 1) per molecule of StAR. As shown in Table 1, the $t_{1/2}$ of this reaction was 63 s, suggesting a slower rate of folding. Thus, further increasing cholesterol levels should result in a more tightly folded StAR and a reduced $t_{1/2}$. As expected, increasing cholesterol levels up to approximately 200 $\mu\text{g}/\text{mL}$ (800 molecules) per molecule of StAR continued to decrease $t_{1/2}$ (Table 1). These results suggest that cholesterol has a dual role with respect to StAR folding. Whereas it facilitates StAR folding at lower concentrations, at higher concentrations, StAR is unfolded, which may be due to the breaking of its hydrophobic core by cholesterol. These results also suggest a limit to the number of cholesterol molecules StAR can transport within the cell.

After plotting the apparent rate of binding of cholesterol to StAR, we observed a saturation in StAR folding after the addition of approximately ≥ 200 molecules of cholesterol (Figure 1C). After joining the two curves through extrapolation, we could predict the maximal number of molecules of cholesterol that StAR can transport before unfolding, which is shown by the black arrow line extrapolated from the midpoint of the folded and unfolded curves. For example, the maximal association between cholesterol and StAR was observed with a ratio of one molecule of StAR to 230 molecules of cholesterol (Figure 1C). StAR folding converted to unfolding after its

Table 1. Real-Time Unfolding of StAR by Cholesterol and Apparent Binding of Cholesterol with StAR Using Stopped-Flow Fluorescence Analysis

[cholesterol] ($\mu\text{g}/\text{mL}$)	cholesterol:StAR ratio	apparent rate (s^{-1})	$t_{1/2}$ (s)
2.5	8	0.011	63
5.0	16	0.014	49
10.0	32	0.0240	29
15	48	0.026	27
30	96	0.032	22
50	160	0.036	19
100	320	0.049	14
125	640	0.093	7
200	800	0.152	4
250	1300	0.184	4

fluorescence signal is attributed to a more organized and refolded protein structure, a decrease indicates protein unfolding. As shown in Figure 1A, protein folding was observed at cholesterol concentrations as low as 5 $\mu\text{g}/\text{mL}$, continuing until the concentration reached 50 $\mu\text{g}/\text{mL}$. The $k_{\text{a}(\text{app})}$ was calculated by fitting the kinetic traces to a second-order rate equation⁴¹ and was 0.011 s^{-1} in the presence of 2.5 $\mu\text{g}/\text{mL}$ cholesterol (Table 1). The apparent binding constant (K_{app}) is the observed change in rate of cholesterol association with the carrier, resulting in increased fluorescence, and is denoted $k_{\text{a}(\text{app})}$. Similarly, with a further increase in cholesterol concentration, the carrier protein's fluorescence decreases, which is denoted as $k_{\text{d}(\text{app})}$. After the cholesterol concentration had been increased by 20-fold from 2.5 to 50 $\mu\text{g}/\text{mL}$, the apparent rate of cholesterol-induced folding to StAR increased 3-fold from 0.011 to 0.036 s^{-1} (Table 1). Although further increasing the cholesterol concentration to 75 $\mu\text{g}/\text{mL}$ did not induce a conformational change (data not shown), a reduced

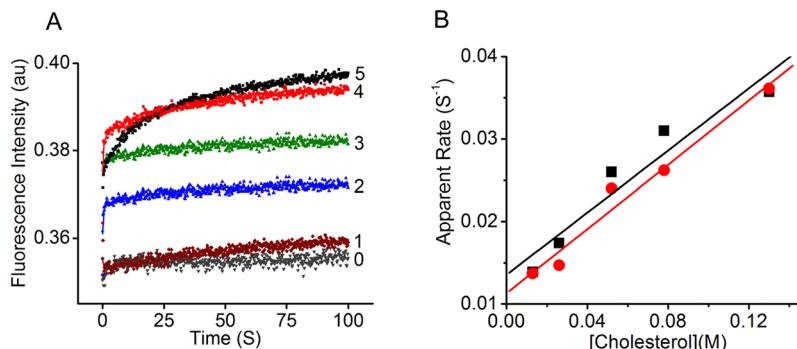


Figure 2. StAR folding kinetics induced by cholesterol after equilibrating with 5% glycerol. Cholesterol-induced StAR folding was assessed in real time by stopped-flow fluorescence as in Figure 1A. (A) Refolding of 20 μM StAR containing 5% glycerol by 5 $\mu\text{g}/\text{mL}$ (1, brown line), 10 $\mu\text{g}/\text{mL}$ (2, blue line), 20 $\mu\text{g}/\text{mL}$ (3, green line), 30 $\mu\text{g}/\text{mL}$ (4, black line), and 50 $\mu\text{g}/\text{mL}$ cholesterol (5, red line). Refolding was measured with a dead time of 3.5 ms. Increased fluorescence was observed up to 50 $\mu\text{g}/\text{mL}$ cholesterol. (B) The apparent binding (K_a) of cholesterol to StAR was determined in the presence (black squares and line) and absence (red circles and line) of glycerol.

association with approximately 300 molecules of cholesterol; with 800 molecules of cholesterol, StAR was unfolded. These results suggest that conformational changes in StAR mediate its translocation and turnover of a large number of cholesterol molecules in the acute phase of steroidogenesis. Specifically, in areas of high cholesterol concentrations, including cytoplasmic stores or in the mitochondria after cholesterol transfer, StAR is partially unfolded. Upon cholesterol transfer, StAR is once more refolded.

Viscosity Is Not Associated with StAR Folding.

Chaperones may participate in the maturation pathways of certain newly synthesized proteins. Although StAR is a mitochondrially targeted protein, its entry into the mitochondria is slow compared to the entry of other mitochondrial proteins.²⁷ Thus, it may be influenced by the density of other proteins within its microenvironment. To determine the effect of viscosity on StAR folding, StAR was first premixed with 5% glycerol, a hydrophilic compound that does not interfere with protein aggregation, and then incubated with increasing concentrations of cholesterol (Figure 2). Stopped-flow fluorescence analysis revealed StAR folding at cholesterol concentrations as low as 5 $\mu\text{g}/\text{mL}$ and up to 50 $\mu\text{g}/\text{mL}$ (Figure 2A). k_{app} was 0.01 s^{-1} in the presence of 5 $\mu\text{g}/\text{mL}$ cholesterol and glycerol. When the cholesterol concentration was increased by 10-fold (from 5 to 50 $\mu\text{g}/\text{mL}$), k_{app} increased from 0.01 to 0.018 s^{-1} , which was not altered by the addition of 5% glycerol. After plotting the K_{app} data as a function of time, we used the slope of the resulting line to calculate the K_a values of StAR binding with cholesterol in the presence and absence of glycerol (Figure 2B), which were 0.48 and 0.50 $\text{M}^{-1} \text{s}^{-1}$, respectively (Table 2). Thus, StAR associates with cholesterol regardless of the presence of molecular crowders, such as glycerol, without the need for chemical chaperones as observed with other steroidogenic proteins active at the intermembrane space.⁴⁵

StAR Is Reorganized into Smaller Domains in Association with Cholesterol. The N-terminal region of StAR is tightly folded compared to the C-terminal region, allowing it to interact with the outer mitochondrial membrane for a longer time, facilitating the transport of cholesterol into the mitochondrion.³³ To determine the changes in domain organization in the presence of cholesterol, we next conducted trypsin proteolysis of StAR in the presence and absence of cholesterol, and the digested bands were analyzed by mass spectrometry (Figure 3 and Table 3). As shown in panels A and B of Figure 3, bands of different sizes were produced when

Table 2. Glycerol-Induced Real-Time Unfolding of StAR by Cholesterol and Measurement of the Apparent Binding of Cholesterol with StAR by Stopped-Flow Fluorescence

[cholesterol] ($\mu\text{g}/\text{mL}$)	$K_{\text{app}} (\text{s}^{-1})$	
	StAR	StAR with glycerol
5	0.0140 \pm 0.001	0.0134 \pm 0.003
10	0.0240 \pm 0.002	0.0174 \pm 0.001
20	0.0260 \pm 0.001	0.026 \pm 0.005
30	0.032 \pm 0.004	0.031 \pm 0.002
50	0.036 \pm 0.002	0.0357 \pm 0.002
$K_a (\text{M}^{-1} \text{s}^{-1})$	$(4.8 \pm 0.1) \times 10^{-4}$	$(5.0 \pm 0.2) \times 10^{-4}$

StAR was digested in the absence and presence of cholesterol, respectively. Trypsin digestion of StAR in the presence of cholesterol produced six bands, including the first two with similar molecular masses (Figure 3A,B). After mass spectrometric analysis of the protected bands in the presence of cholesterol and after the actual and theoretical sizes of the bands had been matched, band 3 comprised amino acids 62–274 and included the 19-amino acid leader sequence, resulting in a molecular mass of 28073.01 Da (Figure 3C). However, band 4 comprised amino acids 62–264 in which 19 amino acids from the N-terminus and 10 amino acids from the C-terminus were proteolyzed, resulting in a band of 23935.58 Da. Band 5 comprised residues 62–213 and had a molecular mass of 17208.63 Da; band 6 comprised amino acids 88–213 and had a molecular mass of 14112.21 Da (Figure 3C).

As shown in panels D and E of Figure 3, ribbon (left) and surface (right) representations of StAR fragments of residues 133–213 (band 1) and 133–264 (band 4 in the gel) were produced, respectively. The trypsin-resistant region of residues 133–213 comprised four β -sheets (blue) and an α -helical region (pink) (Figure 3D). The surface representation of the binding of a single cholesterol molecule (in spheres) to the cholesterol binding groove of StAR highlighted the fact that the cholesterol binding region of StAR (from amino acids 88–213 in the crystal structure), which comprises α -helix 3 (residues 142–147) and β -sheets D (residues 151–160), E (residues 164–171), F (residues 182–192), and G (residues 198–203), is preserved in all the bands (Figure 3D,E). However, sheets A–C, H, and I, α -helices 1 and 2, and part of α -helix 4 underwent proteolysis (Figure 3D,E). As compared with our previous report, StAR proteolysis in the absence of cholesterol

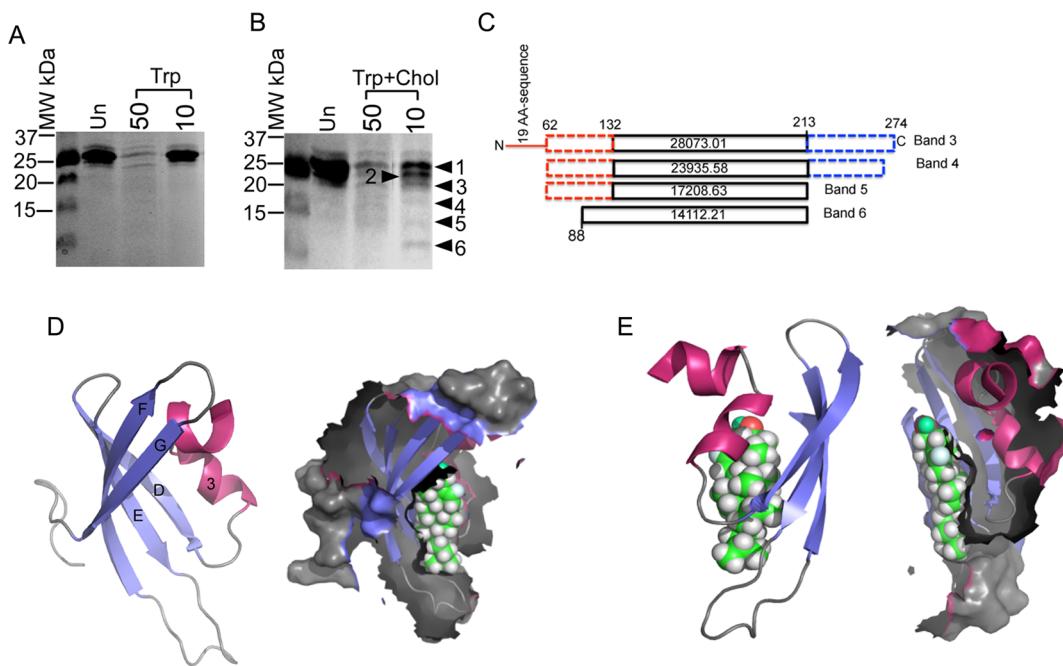


Figure 3. Trypsin proteolysis of StAR in the absence or presence of cholesterol. StAR (10 μ g) in the (A) absence or (B) presence of 10 μ g/mL cholesterol was trypsinized (10 or 50 units). The protected bands (1–6) were excised for subsequent mass spectrometric analysis. (C) Summary of the mass spectrometric analysis of the protected bands (bands 3–6). (D) Ribbon (left) and surface (right) representations of the StAR fragment, spanning amino acids 133–213 (band C0). The trypsin-resistant region that spanned amino acids 133–213 and comprised four β -sheets, namely, residues 151–160, 164–171, 182–192, and 198–203 (blue), as well as an α -helical region, residues 142–147 and 252–278 (pink). Random coil regions are colored gray. The surface representation contains a single cholesterol molecule (spheres) fitted into the cholesterol binding groove of StAR. This panel was generated using Protein Data Bank entry 2I93. (E) Ribbon (left) and surface (right) representation of the trypsin-resistant fragment of StAR that spanned amino acids 133–188 (band 4 in the gel). The protected region comprises two β -sheet regions of residues 151–160 and 164–171 (blue), a partially digested α -helix of residues 182–192 (blue), and α -helical regions of residues 142–147 and 129–139 (pink). The gray regions are random coils. The surface diagram shows partial digestion of the cholesterol binding domain.

resulted in one protected band of 16000 Da, which comprised amino acids 62–188/193 and retained the same leader sequence.³⁵

Mass spectrometric analysis revealed that amino acids 62–132 were digested into three discrete bands only in the presence of cholesterol. Sequence analysis of the specific region revealed areas likely to be trypsinized because of easy accessibility (e.g., amino acids 62–88, 88–108, 88–122, and 122–132) upon cholesterol binding. The Chou–Fasman and Robson Gariner methods individually or in combination indicated that the region spanning amino acids 62–132 is predominantly helical with a maximum of two sheets, indicating a rigid conformation. Thus, in the presence of cholesterol, StAR had a more open conformation, which facilitated digestion at the theoretically predicted positions. Alternatively, in the absence of cholesterol, amino acids 62–188/193 were completely protected. In summary, these data suggest that cholesterol facilitates the organization of StAR domains into smaller units, which may permit its simultaneous association with multiple cholesterol molecules.

Faster Clearance of Cholesterol from Mitochondria Is Mandatory for Further StAR and Cholesterol Import. Steroid secretion is constitutively linked to steroid synthesis, and steroidogenic cells store very little steroid hormone; therefore, a rapid steroidogenic response requires rapid synthesis of new steroid. After cholesterol is catalyzed to pregnenolone inside the mitochondrion, it then needs to be transported back quickly to the cytoplasm, so that the next pool of cholesterol can be transported. Thus slower transport back

from the mitochondrial matrix to the cytoplasm may increase the level of storage of cholesterol in the cytoplasm. Cholesterol stabilizes StAR structure by interacting with a specific site (Figures 1A and 3C). The binding of cholesterol to a membrane can be replaced by a receptor blocker,⁴⁶ because cholesterol decreases the partial free volume of the lipid bilayer, resulting in a rapid (millisecond) increase in protein volume.^{47,48} StAR transfers cholesterol to the mitochondrion, although at a rate slower than that observed for other mitochondrially targeted proteins.⁷ However, within the mitochondrial matrix, cholesterol is metabolized by P450ccc.⁷ To improve our understanding of the processing capacity of StAR through the outer mitochondrial membrane (OMM), we incubated mitochondria with water-soluble 22(R)-hydroxycholesterol (22R cholesterol), which is directly transported into mitochondria without StAR. Unimported 22R cholesterol was removed from the reaction mixture by centrifugation of the isolated mitochondria.⁴⁹ To understand the dependence of StAR import on cholesterol clearance through its metabolism, we preincubated StAR with 22R cholesterol in the presence and absence of glycerol and added [³⁵S]StAR for 2 h, which was followed by treatment with PK to distinguish the imported and unimported StAR fractions. As shown in Figure 4, mitochondrial import resulted in a 37 kDa unimported StAR fragment and a 30 kDa imported StAR fragment. PK proteolyzed unimported 37 kDa StAR regardless of the addition of glycerol (Figure 4A,B), but the presence of an increased level of cholesterol protected unimported StAR from proteolysis. However, StAR import efficiency was significantly decreased

Table 3. LC–MS/MS Analysis of the Trypsinized Bands Protected by the Presence of Cholesterol^a

sequence	amino acid range
	Band 1
VVPDVVK	112–118
LYEELVER	133–140
MEAMGEWNPNVK	141–152
DFVSVR	183–188
GSTCVLAGMATDFGNMPEQK	194–213
	Band 2
MEAMGEWNPNVK	141–152
DFVSVR	183–188
GSTCVLAGMATDFGNMPEQK	194–213
	Band 3
VVPDVVK	112–118
MEAMGEWNPNVK	141–152
DFVSVR	183–188
	Band 4
VVPDVVK	112–118
LYEELVER	133–140
DFVSVR	183–188
	Band 5
VVPDVVK	112–118
LYEELVER	133–140
MEAMGEWNPNVK	141–152
DFVSVR	183–188
	Band 6
MEAMGEWNPNVK	141–152
DFVSVR	183–188

^aParent mass and MS/MS match proposed sequence. Note that methionine was observed as oxidized methionine.

upon addition of 100 µg/mL cholesterol as observed by the 50% decreased intensity of the imported 30 kDa band (Figure 4A). After incubation with 200 µg/mL cholesterol, StAR import was completely inhibited possibly because the mitochondria could not metabolize cholesterol at 26 °C (Figure 4A); therefore, the mitochondria were filled with cholesterol, preventing further StAR import. As shown in Figure 5B (100 µg/mL cholesterol), semiquantitative analysis of StAR band intensity revealed that the presence of excess cholesterol prevented the import of [³⁵S]StAR into the mitochondria. Thus, the import of StAR into the mitochondria depends on local cholesterol concentrations.

Specificity of Mitochondrial Protein Import. Because clearance of cholesterol from the mitochondria may be essential for further StAR import, we hypothesized that the mechanism underlying StAR import was slower and thus differed from those of other mitochondrial-targeted proteins. Therefore, we next examined whether wild-type StAR and P450scc compete for import into the mitochondria. While [³⁵S]StAR import was blocked by previously imported unlabeled StAR, the import of [³⁵S]P450scc was not (Figure 6A; imported fraction was 54 kDa), which suggested differences in import mechanisms between StAR and P450scc. To examine the importance of the StAR leader sequence for its mitochondrial import, we replaced the 62-amino acid N-terminal sequence of StAR with the 39-amino acid leader sequence of P450scc to generate SCC/N-62 StAR. Prior to importing the ³⁵S-labeled proteins into isolated mitochondria, we imported unlabeled wild-type StAR for 2 h followed by incubation with either [³⁵S]StAR or [³⁵S]SCC/N-62 StAR for an additional 2 h. The import of [³⁵S]StAR but not [³⁵S]SCC/StAR was blocked by previously imported unlabeled StAR (Figure 6B). This finding suggests that StAR mitochondrial import can be saturated and that only a finite, limited number of StAR molecules can be imported per unit of time. In addition, the mitochondrial import process may exhibit differences based on the leader sequence.

To confirm the observation that StAR mitochondrial import is mediated through a unique mechanism as compared to other mitochondrially associated proteins, we performed competition experiments with P450scc and aldehyde dehydrogenase (ALDH) leader sequences (Figure 6C). P450scc is a 514-amino acid (61 kDa) protein that is imported into the mitochondrial matrix as a 54 kDa protein upon cleavage of its 39-amino acid N-terminal leader sequence.⁵⁰ ALDH is a 517-amino acid protein with a small 19-amino acid N-terminal leader sequence that is removed upon its import into mitochondria, resulting in a protein of approximately 57 kDa.⁵¹ Import of [³⁵S]ALDH was blocked by prior import of unlabeled P450scc (Figure 6C). Competition experiments with a shorter N-terminal sequence (34 amino acids) of StAR fused with P450scc, [³⁵S]StAR(1–34)/P450scc, were next undertaken; unlabeled StAR blocked import of [³⁵S]StAR(1–34)/P450scc (Figure 6D). To improve our understanding of the unique folding of StAR, we fused 12 amino acids of COX-IV to the substituted 62-amino acid leader sequence of StAR to generate a 12COX/N-62StAR fusion protein (COX/StAR).

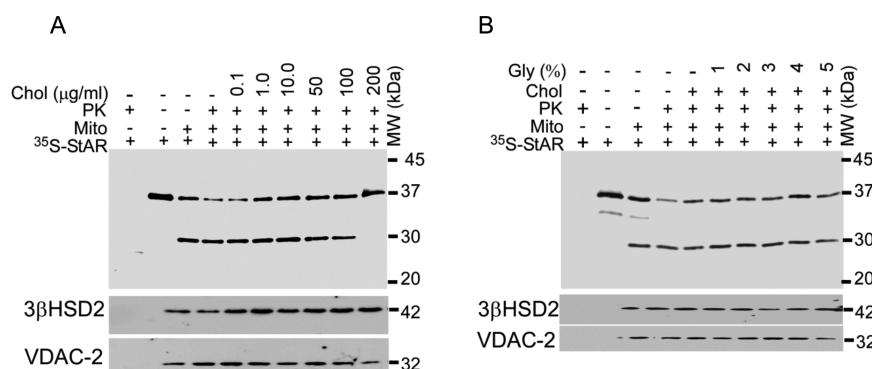


Figure 4. Effects of cholesterol on the mitochondrial import of StAR. ³⁵S-labeled StAR was incubated with mitochondria in the presence of the indicated concentration of cholesterol in the (A) absence and (B) presence of 1–5% glycerol. The imported fraction was identified after proteolysis with proteinase K. The levels of mitochondrial 3 β HSD2 and VDAC-2 were assessed using Western blot analysis as a control for the mitochondrial content.

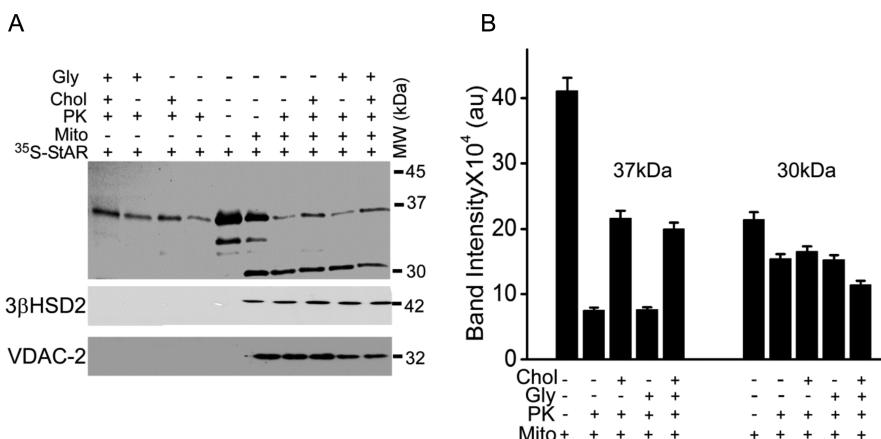


Figure 5. $[^{35}\text{S}]$ StAR mitochondrial import efficiency in the presence of cholesterol. (A) $[^{35}\text{S}]$ -labeled StAR was incubated with mitochondria in presence of 100 $\mu\text{g}/\text{mL}$ cholesterol and/or 5% glycerol. The imported fraction was identified after proteolysis with proteinase K. (B) Quantitative estimation of band intensity of the mitochondrial imported and unimported fractions after proteolysis, where the imported fraction was protected from PK by the inner mitochondrial environment. A large excess of cholesterol also protected unimported StAR from proteolysis.

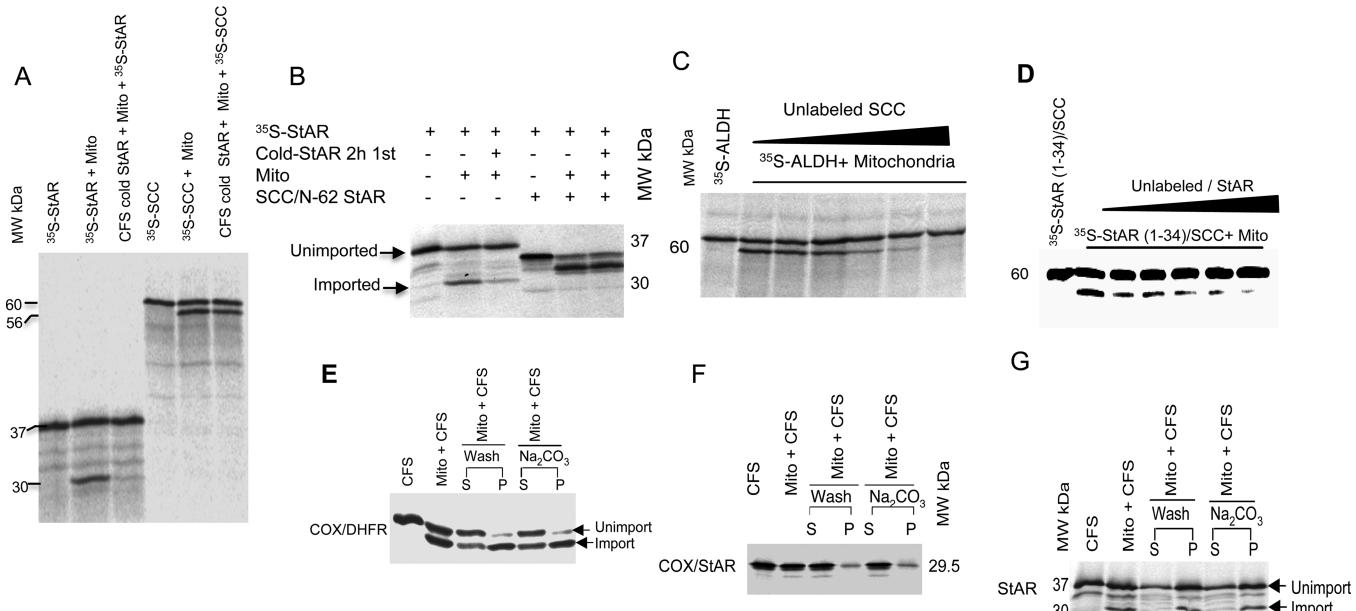


Figure 6. Mitochondrial import of StAR is signal sequence-dependent. (A) $[^{35}\text{S}]$ StAR and P450scC were imported into mitochondria preincubated with unlabeled StAR. (B) Import of $[^{35}\text{S}]$ StAR and $[^{35}\text{S}]$ SCC/N-62 StAR was blocked by the preincubation with unlabeled StAR. (C) Mitochondrial import of $[^{35}\text{S}]$ ALDH in the presence of increasing concentrations of unlabeled P450scC. (D) Competition between $[^{35}\text{S}]$ StAR/SCC and unlabeled StAR. (E–G) Import and extraction of COX-IV/DHFR, COX IV/StAR, and STAR by sodium carbonate following mitochondrial import, where the unimported fraction is in the supernatant (S) and the imported integrated fraction (P) is in the pellet.

The N-terminal leader sequence of COX-IV/StAR was not cleaved upon import (Figure 6F). Following import of COX/StAR into mitochondria, fractionation by washing with sodium carbonate, which interrupts the protein–protein interactions but not the lipid–protein interactions, was performed. Separation of these fractions indicated that COX/StAR remained in the supernatant (Figure 6F), suggesting that the COX-IV N-terminal leader sequence is not sufficient to transport cytoplasmic N-62 StAR into the mitochondria. Wild-type StAR, however, remained lipid-associated under these conditions, demonstrating that imported StAR was integrated into the mitochondrial membrane (Figure 6G). Thus, the dependence of StAR import on the specificity of its mitochondrial signal sequence indicates that the N-terminal domain, which was considered nonessential, is extremely

important for accurate folding of StAR, confirming that StAR import is different from that of other mitochondrially targeted proteins. It remains possible that StAR required interaction with VDAC⁴⁹ prior to processing through the Tom40 channel.

DISCUSSION

In this study, we have examined the effects of cholesterol on StAR conformation and mitochondrial import using reconstitution and functional complementation analyses. First and foremost, these studies have identified differences in the mechanisms by which mitochondrial proteins are imported. Prior to entering the mitochondrion, StAR must be activated by its substrate, cholesterol. Although a substrate-specific function was reported previously for proteins targeted to the endoplasmic reticulum,⁵² to the best of our knowledge this

report is the first to demonstrate activation of mitochondrial protein folding by cholesterol (Figure 1A). In the absence of significant cholesterol concentrations, the StAR conformation may not be flexible enough to permit mitochondrial import through a narrow protein channel. At the same time, faster cholesterol metabolism and therefore clearance from the mitochondria are necessary for StAR import. P450ccc mitochondrial import occurs more quickly as it is required for pregnenolone synthesis.²⁷ On the other hand, StAR expression is induced by hormonal stimulation; therefore, longer residency at the OMM^{27,49} is essential for the transfer of several cholesterol molecules into the mitochondrion.

The three-dimensional structure of StAR has been deduced from the crystal structure of the StART domain of StARD3 (MLN64 lacking its 218 amino-terminal residues). The crystal structure of StARD3 shows a globular protein with an α/β helix-grip fold and an elongated hydrophobic pocket measuring ~26 Å deep and 10 Å across at its widest diameter.²⁹ Computational modeling shows that StARD3 can accommodate a single molecule of cholesterol in this pocket, with the 3 β -OH group coordinated by the two polar residues facing the bottom of the pocket. This structure, the crystal structure of the closely related protein StARD4,³¹ and several computational models of StAR^{30,35} are all very similar. A recent, low-resolution (3.4 Å) structure of human StAR has confirmed the results of these models.⁵³ Interestingly, the StARD5 NMR structure was proposed to be preferentially involved in the transport of bile acids rather than cholesterol.⁵⁴ A comparative study with a similar family of proteins structure shows that even cholesterol ester transfer protein (CETP) has a very similar banana-like structure with elongated N- and C-termini.⁵⁵ It can then be concluded that cholesterol transport or cholesterol ester transport proteins have a similarity in their folding where entry and exit has a unique mechanism of release after transport from the cavity-forming proteins.⁵⁵

The mechanism by which StAR promotes steroidogenesis has been studied extensively but remains incompletely characterized. However, the R182L mutant, which is inactive *in vivo*¹¹ and *in vitro*, retains cholesterol binding capacity.⁵⁷ Many other mutants (e.g., splice variants) block cholesterol transfer without any hydrophobic interaction.^{58,59} Thus, while StAR may be a cholesterol transfer protein, its action is more complex. The presence of SBP is suggestive of its cholesterol transfer capacity, and computational modeling and related structural studies indicated that there was insufficient room for a cholesterol molecule to enter or exit the SBP of StAR.^{29–31,35,56} On the OMM, StAR undergoes a conformational change that is required for its activity.³³ Other studies suggested that StAR acts in the intramembranous space,²⁹ shuttling cholesterol from the OMM to the inner mitochondrial membrane (IMM). Thus, it is unclear whether StAR first binds cholesterol or associates with the OMM. An increase in ΔH occurred only in the presence of StAR, cholesterol, and mitochondria,³² suggesting that StAR picks up cholesterol from a source near the OMM, and not from cytoplasmic cholesterol. Because high cholesterol concentrations cannot remain at the OMM as they will result in the distortion of the mitochondrial architecture,⁶⁰ it is likely that cholesterol induces StAR folding. However, high cholesterol concentrations protected StAR from rapid proteolysis (Figure 4A). Thus, the mechanism of cholesterol transport depends on the environment, which includes the cholesterol molecules and StAR; formation of a molten globule conformation by StAR transports several

hundred molecules of cholesterol without distorting the mitochondrial architecture.

In this study, the level of StAR folding was increased upon interaction with as few as 10 molecules of cholesterol per molecule of StAR or up to 230 molecules (cross section after extrapolation of the folding and unfolding curve) before a breakpoint of unfolding with 300 molecules (Figure 1C and Table 1). The three-dimensional structure shows that the protein is mostly folded, and even under the most stable energetic condition, StAR required cholesterol to achieve the appropriate folding.²⁹ However, the solution structure had a cholesterol molecule inside it.³⁶ Thus, at lower cholesterol concentrations, StAR was refolded as seen by the increased fluorescence intensity, which decreased with a higher cholesterol concentration (Figure 1A). However, at cholesterol concentrations of $\geq 75 \mu\text{g/mL}$, StAR was unfolded and thus was easily proteolyzed. However, proteolysis was completely stopped with 200 $\mu\text{g/mL}$ cholesterol. It is unlikely that this will occur in living cells as unused cholesterol would be circulated in part by wild-type StAR, but not the R182L mutant of StAR.⁵⁷ Because both wild-type and mutant StAR interact with cholesterol with the same efficiency, differences in their folding may be responsible for the mutant's inability to transfer cholesterol to the mitochondria.⁶¹

During acute regulation of steroidogenesis, it is less likely that cholesterol enters the core of StAR and then is released for import into the mitochondrial matrix, especially if StAR has to transport a large number of cholesterol molecules. Once cholesterol is stored in sufficient quantities as a small pool at the OMM, it is more likely that cholesterol transfer by StAR is mediated by a push–pull mechanism in which changes in conformation will allow the transfer of several molecules of cholesterol into the mitochondrion. Cholesterol may possibly facilitate the necessary change from a loosely to tightly folded conformation of StAR. Once enough cholesterol is stored, the StAR conformation changes from a tightly folded conformation to a partially open molten globule-like conformation, resulting in the pushing of a large amount of cholesterol into the mitochondria.^{33,62}

In acute steroidogenesis, a substantial influx of cholesterol to the matrix side of mitochondria is essential for the synthesis of large amounts of steroid hormones in a short period of time. Therefore, a cholesterol pool must remain close to both the OMM and StAR. Mass spectrometric analysis of partially proteolyzed StAR in the presence of cholesterol revealed a protected region of amino acids 133–188, which spans the central cholesterol binding region that contains β -sheets of amino acids 151–160 and 164–171 as well as a complete and partial α -helix of residues 142–147 and 182–192, respectively. However, in the absence of cholesterol, the protected region spanned amino acids 62–188 only,³³ suggesting that the folding of StAR is different in the presence of cholesterol. Once StAR reaches the OMM, multiple cholesterol molecules, which are destined for transport into mitochondria, are transported by StAR. In mitochondrial isolation procedures, cholesterol is removed. As a result, the limited remaining endogenous cholesterol does not facilitate pregnenolone synthesis or alter the enthalpy (ΔH). Upon association with several molecules of cholesterol and mitochondria, the active conformation of StAR is achieved.³² Computer modeling of the StAR and StAR-related lipid transfer (StART) proteins predicted that StAR unfolds upon activation and import in association with the

membrane.³⁵ Thus, cholesterol assists in achieving the appropriate conformation of StAR at the OMM.

Mitochondrial competition experiments show that StAR's import into the mitochondria is unique in comparison to that of other mitochondrial targeted proteins (Figure 6). For example, import of StAR into the mitochondrion is blocked by its own N-terminal sequence, which does not occur with other mitochondrial targeted steroidogenic proteins (Figure 6B). However, the import of other steroidogenic proteins like P450scc into mitochondria is blocked by nonsteroidal mitochondrial targeted proteins like ALDH (Figure 6C,D). In addition, import of StAR into mitochondria is slower than that of other steroidogenic proteins imported into mitochondria,²⁷ as it requires interaction with the VDAC channel,^{49,63–65} suggesting that StAR requires a different folding process prior to import. The slower mitochondrial entry is facilitated by cholesterol-mediated unfolding of StAR. Therefore, we speculate that the transport of cholesterol occurs through the mitochondrial PTP channel. Proteins targeted to the mitochondrial matrix, such as StAR, must cross the OMM and IMM before reaching their final destination, where the protein may fold interdependently⁶⁶ or independently in a stepwise fashion.⁶⁷ StAR is active at the OMM prior to import;²⁶ thus, active StAR maintains an open conformation. Once StAR has associated with the mitochondrial membrane, its folding and activity are dictated by the domain organization and the presence of cholesterol.

In summary, we propose that cholesterol is the sole activator of StAR folding, and its signal sequence mediates the tight import regulation through the OMM. The slower unfolding of the StAR N-terminal sequence delays its import into mitochondria, and as a result, the cholesterol pool present at the OMM stabilizes StAR to an appropriate conformation for cholesterol delivery. The cholesterol binding region is located within the central core and remains intact. The loosely folded C-terminus of StAR interacts with cholesterol and fosters its transport through the PTP; this domain may be refolded into the active conformation and thus recycled multiple times. Thus, a large number of steroids can be synthesized via the delivery of cholesterol by a limited number of StAR molecules. Finally, StAR is imported relatively slowly through the mitochondrial import channel.²⁷

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ABBREVIATIONS

ALDH, aldehyde dehydrogenase; CD, circular dichroism; COX, cytochrome *c* oxidase; IMM, intermembrane space; OMM, outer mitochondrial membrane; PK, proteinase K; PTP, permeability transition pore; SCC, cytochrome P450 side chain cleavage enzyme; SBP, sterol binding protein; StAR, steroidogenic acute regulatory protein; StART, StAR-related lipid transport domain; VDAC, voltage-dependent anion channel.

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