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Steroidogenic Acute Regulatory Protein Has a More Open Conformation Than the Independently Folded Smaller Subdomains†

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ABSTRACT: The acute steroidogenic response, which produces steroids in response to stress, requires the steroidogenic acute regulatory protein (StAR). StAR, a mitochondrial matrix protein, acts on the outer mitochondrial membrane (OMM) to facilitate the movement of cholesterol from the outer to inner mitochondrial membrane via an unknown mechanism. The N-terminal sequence was reported to be nonessential for activity. We show that alteration of the StAR amino-terminal sequence does not change the thermodynamic stability of StAR but offers protection from proteolytic degradation. A longer association between StAR and the OMM strengthens the interaction with cholesterol. Far-UV CD spectra showed that the smaller fragments of StAR domains were less α-helical compared to N-62 StAR but were structured as determined by limited proteolysis followed by mass spectrometry. The START domain consisting of amino acids 63−193 also exhibited protease protection for amino acids 84−193. The Stern−Volmer quenching constant (K_{SV}) of the N-62 StAR protein is 12.1×10^5 M^{-1}, with all other START fragments having significantly smaller K_{SV} values ranging from 6 to 10×10^5 M^{-1}, showing that N-62 StAR has a more open conformation. Only N-62 StAR protein is stabilized with cholesterol having an increased ΔH value of -5.6 ± 0.3 kcal/mol at 37 °C. These findings demonstrate a mechanism in which StAR is stabilized at the OMM by cholesterol to initiate its massive import into mitochondria.

Accurate protein folding is crucial for activity. Protein activity depends upon the final state of folding in a specific cellular compartment. Alternatively, a protein may be active at an intermediate state of folding prior to the final destination (*1*). As protein domains are independent evolutionary units and recombine with other domains to form a multidomain protein, any change in the folding of the domain organization will impact the activity of the protein. To understand the mechanism of physiological action, the relationship among thermodynamics, topology, and the various protein folding states must be elucidated. The process of protein folding to a native state or the active form following release from the ribosome remains one of the central unresolved issues in understanding the mechanism of action (*2*). Thermodynamically, protein folding is a spontaneous process; an unfolded protein refolds rapidly to the native structure, which represents the global free energy minimum (*3*). Understanding the rates and pathways involved in the proper folding of an active protein is a critical problem and requires a combination of cellular and thermodynamic approaches. Protein folding in vivo becomes more complex as additional factors such as ATP and chaperones are required for folding of the active protein.

The steroidogenic acute regulatory protein (StAR)[1] is a 285-amino acid cytoplasmic phosphoprotein that is synthesized upon hormonal stimulation as a 37 kDa protein and then transported to the matrix side of the inner mitochondrial membrane (IMM) as a 30 kDa protein (*4*). StAR is essential for the transport of cholesterol, which is required for the synthesis of steroid hormones, into mitochondria. Mutant StAR cannot transport cholesterol, and thus, these individuals have congenital lipoid adrenal hyperplasia (lipoid CAH), an inborn error of metabolism in which newborns die soon after birth due to a salt losing crisis (*5*, *6*). StAR is active before entering the mitochondria. Deletion of the signal sequence, the first 62 amino acids, resulted in a completely active protein as demonstrated by incubation of biosynthetic StAR with isolated steroidogenic mitochondria and by transfection of StAR cDNA in nonsteroidogenic COS-1 cells (*7*−*9*). These experiments indicated that the signal sequence was nonessential for StAR folding and activity. The 210 carboxy-terminal residues of StAR, called the START (StAR-related lipid transfer protein) domain, are conserved across species and serve as a versatile binding surface for lipids in many distinct processes

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[1]Abbreviations: StAR, steroidogenic acute regulatory protein; START, StAR-related lipid transport domain; Preg, pregnenolone; MS, mass spectrometry; CD, circular dichroism; Mito, mitochondria; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; Chol, cholesterol; 22-R, 22(*R*)-hydroxycholesterol; NATA, *N*-acetyl-L-tryptophanamide; SEM, standard error of the mean.

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in different tissues, including transfer of cholesterol, lipid transport and metabolism, and cell signaling (*10*, *11*). Thus, proteins with the START domain serve as a catalyst for the transport of substrate cholesterol into mitochondria.

Steroids are not stored but rather are synthesized as needed (*12*) by facilitation of cholesterol into the matrix side of the mitochondria by StAR. Thus, rapid folding into the active form is critical for StAR or other START domain proteins involved in regulation of steroid synthesis. Different hypothetical models have been proposed to explain this cholesterol transport mechanism: (1) the cholesterol desorption model (*13*), (2) the shuttle mechanism of StAR for cholesterol transport (*14*), (3) the StAR cocrystal structure that suggests the opening of the C-terminal domain for cholesterol release (*15*), and recently (4) the TSPO (peripheral benzodiazapine receptor or PBR)-specific protein import channel for cholesterol transport (*16*). We have proposed that StAR works as a molten globule at the outer mitochondrial membrane (OMM) (*1*) and interacts with the OMM resident VDAC1 (*17*), and thus, the cholesterol transport capacity of StAR is directly proportional to its residency time at the OMM (*18*). The rate of unfolding of StAR is slower than those of the inactive or mutant proteins (*19*), and this is due to the presence of a flexible hydrophobic core for N-62 StAR (*1*, *20*).

Despite extensive efforts, the mechanism of activation and mitochondrial import of the StAR protein has remained elusive. Here, we show that the StAR−mitochondrion association is stabilized by cholesterol, demonstrating that cholesterol has a significant role in achieving the active conformation of StAR.

EXPERIMENTAL PROCEDURES

Plasmid Expression System. For subcloning, human StAR cDNA (*21*) was used as the template for PCR with specific combinations of sense and antisense primers (Table S1 of the Supporting Information). The underlined nucleotides are restriction enzyme sites and are amplified following the previously described protocol (*17*). PCR-amplified DNA was purified by agarose gel electrophoresis, digested with *Kpn*I and *Bam*HI, ligated to predigested vector (pQE30), and transformed into competent *Escherichia coli* SG13009 (pREP 4) cells (Qiagen). For expression, at midlog phase, cultures were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 30 °C, and the incubation was continued for 4 h. In some cases, *E. coli* were grown at 37 °C and then induced at 15 °C from 6 h to overnight to enrich them with soluble protein (*22*). All StAR subdomains were expressed in *E. coli* and are herein termed biosynthetic proteins.

In Vitro Translation and Import of Protein into Isolated Mitochondria. Mitochondria from pig adrenal glands were isolated as described previously (*23*, *24*). The full-length StAR cDNA was translated in the presence of ^{35}S-labeled methionine in a TNT-rabbit reticulocyte system (Promega) with SP6 polymerase at 30 °C for 2 h. Ribosomes and the associated incomplete polypeptide chains were removed by ultracentrifugation at 148000*g* for 20 min at 4 °C. Partial proteolysis with proteinase K was terminated by the addition of PMSF to a final concentration of 2 mM and 2× SDS sample buffer (*18*), and then the mixture was transferred to a boiling water bath.

In Vitro and in Vivo Activity of StAR Subdomains and Cholesterol Binding. For expression of subdomains of StAR, we induced *E. coli* cells containing the expression constructs with 0.5 mM IPTG overnight. The bacterial pellet was resuspended in buffer [20% glycerol, 100 mM NaH_2PO_4, and 100 mM NaCl (pH 7.4)], sonicated, passed through the Ni^{2+} NTA-agarose column, eluted with the same buffer containing 250 mM imidazole, and then passed through a Pharmacia Superose 6/12 gel filtration column fitted to an FPLC system. These *E. coli*-expressed (Biosynthetic) StAR subdomains were added to 20 μg of pig adrenal mitochondria in a final volume of 100 μL of bioassay buffer (*18*). The mixture was then incubated for 1 h at 37 °C, and pregnenolone synthesis was assessed with a radioimmunoassay (*6*) (RIA, MP Biomedicals). For the determination of activity in transfected nonsteroidogenic COS-1 cells, all the constructs were subcloned in the pCMV vector cotransfected with F2 (*25*) following the previously described protocol (*17*, *18*). F2 is a fusion of cytochrome P450scc, adrenodocin, and adrenodoxin reductase. Media were collected from cultures 48 h after transfection and assayed by RIA (*6*).

Purified proteins in phosphate buffer were incubated with varying concentrations of NBD-cholesterol (Invitrogen) for 15 min at 37 °C, and fluorescence emission maxima were measured on a Shimadzu fluorescence spectrophotometer (Shimadzu, model 5301 PC). The NBD-cholesterol was excited at 460 nm, and the emission intensity was recorded at 534 nm. The relative fluorescence intensity was measured following the previously described procedure (*26*, *27*). The collected data points were processed using Kaleidagraph or Microcal Origin (Origin Lab).

Acrylamide Quenching. Fluorescence spectra of different proteins were recorded in a Shimadzu spectrofluorimeter (Shimadzu, model RF-5301PC) equipped with a variable emission and excitation band-pass. All the fluorescence experiments were conducted at room temperature and pH 7.5 using the appropriate buffer. Fluorescence quenching experiments were performed by the successive addition of 2 μL aliquots of 2 M acrylamide to the protein and by monitoring the change in Trp emission intensity at 340 nm. To correct for acrylamide absorption, the measured intensity was multiplied by the antilog of OD/2, where OD is the optical density at 295 nm and 0.5 cm is the effective path length of the cuvette. The Stern−Volmer quenching constant (K_{SV}) was calculated as the slope of the linear region of the F_0/F_{corr} versus [Q] plots, where F_0 and F_{corr} are the corrected fluorescence intensities in the absence and presence of different concentrations of quencher molecule, respectively (*28*, *29*).

Circular Dichroism. Far-UV (195−250 nm) circular dichroism (CD) measurements were taken using a 1.0 mm path length cuvette with 100−200 mg of protein/mL in 20 mM NaH_2PO_4 (pH 7.4) at 20 °C in an AVIV-215 spectropolarimeter (AVIV). Multiple scans were averaged to improve the signal-to-noise ratio. Appropriate buffer baselines were subtracted from the sample spectra and plotted as mean residue ellipticity $[\Theta]_r$. The protein concentration was determined using von Hippel's method (*30*).

Isothermal Titration Calorimetry (*ITC*). Mitochondria were prepared as described previously (*24*), and 0.3 mg of mitochondrial protein was suspended in 1.5 mL of buffer (*18*). The solution was titrated with increasing amounts of StAR protein in a MicroCal (Northampton, MA) VP-ITC calorimeter equipped with an automatic injector. All buffers were filtered and degassed before use. Data were collected by incubating mitochondria in the cell for 2 h prior to the injection of protein or cholesterol in 5 μL at a speed of 0.5 μL/s with a gap of 7 min for each injection for equilibration, or as described in the legend of Figure 6. Enthalpy (ΔH) was measured by integration of excess

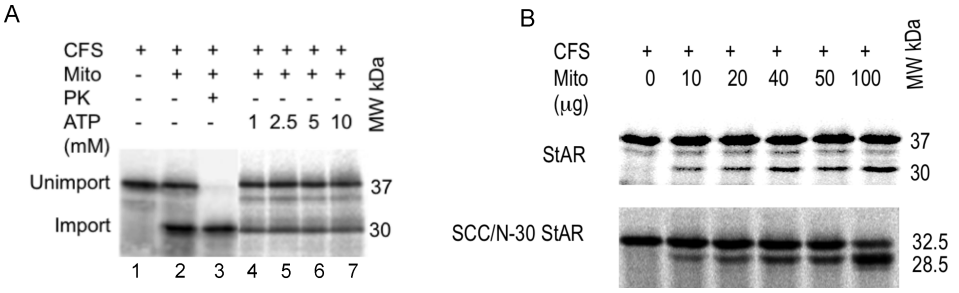

FIGURE 1: StAR does not aggregate at the mitochondrial membrane prior to import. Full-length [^{35}S]StAR and [^{35}S]SCC/N-30 StAR synthesized in a cell-free system (CFS) were imported into mitochondria with the following condition. (A) Import of 30 kDa StAR [lane 2 (after 2 h)] was protected from proteinase K (PK) digestion (30 min) (lane 3). Addition of 1, 2.5, 5, and 10 mM ATP did not affect StAR import (lanes 4−7, respectively). Panel A was generated from experiments performed at different times, and all lanes are numbered at the bottom. (B) Full-length ^{35}S-labeled StAR and SCC/N-30 StAR synthesized in a cell-free system were imported for 2 h at 26 °C with 10, 20, 40, 50, or 100 μg of mitochondria. The intensity of the mitochondrial imported fragments increased with the increase in mitochondrial protein concentration.

power generated by the reaction divided by the concentration of the injectant.

Fingerprinting of StAR Subdomains. Protein (20 μg) was incubated with 3 or 7 units of trypsin (Promega) in 20 mM Tris (pH 7.4) at 4 and 25 °C for 45 min. Proteolysis was terminated by addition of an equal volume of 2× SDS sample buffer containing 1 mM PMSF. The protected protein domains were separated on a 20 or 22% SDS−PAGE gel and stained with Coomassie blue. Bands were excised, destained, reduced with DTT (Roche), alkylated with iodoacetamide (Sigma), and digested with trypsin (Promega) overnight (*31*). The resulting peptide fragments were analyzed via liquid chromatography−tandem mass spectrometry (LC−MS/MS) on a nanoAcquity UPLC system (Waters) coupled with a Q-ToF-Premier mass spectrometer (Micromass/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 loading/desalting Symmetry column [5 mM C18, 180 μm (inside diameter) × 20 mm] (Waters).

RESULTS

StAR Requires a Longer Association with the Mitochondrial Membrane. Sensitivity to proteolysis can be used to assess the topology of a protein. Full protection from exogenous protease indicates complete translocation to the specific compartment of the mitochondrion. Conversely, digestion of certain domains to yield discrete protease-protected fragments indicates a membrane-spanning topology. The exact orientation of this topology can be identified via analysis of the protected fragments using biochemical methods. The use of proteases as a probe of topology is distinctly different from the use of proteases to determine protein conformation of purified proteins. Because the topology assay is performed in the absence of detergent, protection from protease is due to an intact membrane barrier (*18*). Import of the full-length ^{35}S-labeled StAR (37 kDa) into isolated mitochondria for 2 h followed by digestion with proteinase K for 30 min showed that the 30 kDa fragment was protected by the IMM, confirming StAR import (Figure 1A, lane 3). A longer association between StAR and the OMM strengthens the interaction with cholesterol. [^{35}S]StAR's slow import suggests a long residence time at the OMM. After 2 h, much the StAR protein remains unimported (Figure 1A, lane 2, and ref *18*).

Import of proteins into mitochondria is facilitated by the presence of ATP (*32*). ATP was not limiting for the import and activity of full-length [^{35}S]StAR as the addition of up to 10 mM ATP to the mitochondria did not show an increase in the 30 kDa mature (imported) form of StAR (*33*) (Figure 1A, lanes 4−7).

To determine whether mitochondria were a rate-limiting component for import, we measured the import of full-length [^{35}S]StAR and SCC/N-30 StAR in the presence of increasing amounts of mitochondria (10−100 μg) for 2 h (Figure 1B). Cytochrome P450scc is a mitochondrial matrix-targeted protein that has a 39-amino acid N-terminal leader sequence. Replacing full-length StAR's N-terminal matrix targeting 30 amino acids with the P450scc leader sequence (SCC/N-30 StAR) showed an increase in the rate at which StAR was imported (*18*). A 10-fold increase in the level of mitochondrial protein increased the import efficiency of StAR 4-fold, while the import efficiency of SCC/N-30 StAR increased 20-fold (Figure 1B, bottom panel). Thus, the availability of the mitochondrial import channels does not determine the import efficiency of StAR. This suggests that StAR's slower import efficiency depends on the N-terminal leader sequence and that the folding of StAR was altered upon substitution of the matrix targeting sequence.

StAR Subdomains Are Thermodynamically Stable. Folding of multidomain proteins depends on the relative rates of folding of the constituent domains (*34*). Specific domains may fold rapidly, both increasing the rate of folding and decreasing the rate of unfolding of its neighbors (*35*). As proposed by molecular dynamics studies, StAR uses an "in" (transient opening) and "out" (transient out) mechanism for transport of cholesterol (*36*) into mitochondria, and thus, StAR domains should be folding and unfolding in a dynamic process (*20*). All forms of StAR protein studied herein were overexpressed in *E. coli* and purified from the cytosol without any chaotropic reagent (Figure 2A). The N-terminal 62-amino acid truncated StAR retained the same activity as full-length StAR in transfected COS-1 cells; therefore, N-62 StAR is denoted here as StAR (*30*).

We next sought to determine the StAR domain containing catalytic activity as measured by pregnenolone synthesis. Addition of 63−193 StAR to cholesterol-containing isolated mitochondria showed an almost 2.5-fold increase in StAR activity compared to that of either the buffer or mitochondria alone (Figure 2B). The addition of 194−285 StAR in combination with 63−193 StAR did not show a further increase in activity. The activity remained similar to the level of mutant R182L StAR. Moreover, addition of N-terminal amino acids 1−193 together with the 194−285 StAR subdomain did not show an increase in steroidogenic activity, suggesting that the catalytic ability of the

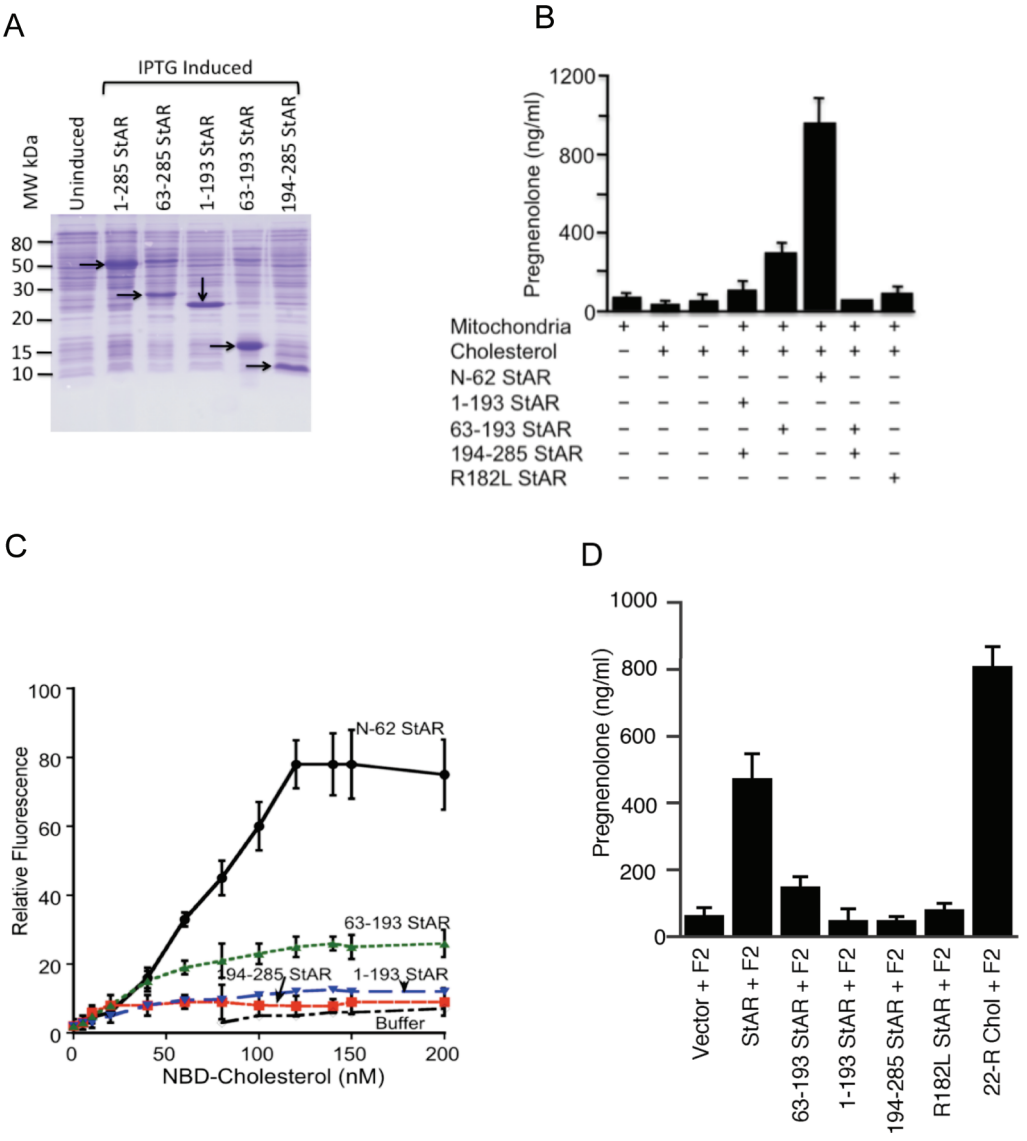

FIGURE 2: Determination of stabilities of different StAR subdomains. (A) Expression of smaller StAR subdomains before and after induction of IPTG as indicated in the figure. (B) Accumulated pregnenelone synthesis. StAR subdomain proteins were added to a fixed amount (20 μg) of isolated mitochondria and cholesterol. The extent of pregnenolone synthesis was 9-fold greater for N-62 StAR. (C) Binding of StAR and StAR sudomains with an increasing concentration of NBD-cholesterol. The relative intensity of the fluorescence maximum at 560 nm was plotted vs NBD-cholesterol concentration. In all panels, data are means ± SEM from three or four independent experiments, each performed in triplicate. (D) Measurement of accumulated pregnenolone synthesis by StAR and indicated StAR subdomains in COS-1 cells expressing the F2 fusion protein. COS-1 cells were transfected with F2 and cotransfected with StAR, 1−193 StAR, 63−193 StAR, and 194−285 StAR. The pregnenolone concentration was measured by RIA. As a control, cells containing F2 were also incubated with 22(*R*)-hydroxycholesterol, thus bypassing the need for StAR. Data are means ± SEM of three independent and separately performed experiments in triplicate.

63−193 subdomain is not sufficient to transport cholesterol at levels similar to that of N-62 StAR (Figure 2B).

The pregnenolone synthesizing capacity of StAR is 7−9-fold higher than that of mutant R182L StAR. Thus, we sought to determine if StAR and StAR subdomains bind cholesterol similarly. StAR binding of [^{14}C]cholesterol and StAR binding of the somewhat larger fluorescent compound NBD-cholesterol are equivalent (*37*), so assays were completed with NBD-cholesterol. As the size of the StAR subdomains is smaller, we used a proportionate amount of protein throughout the experiment; thus, the relative change in fluorescence should be proportional to the binding of NBD-cholesterol. As previously observed, the binding assays yielded sigmoidal curves for StAR (*26*), but the smaller subdomains, except 63−193 StAR, showed minimal binding (Figure 2C). StAR binds cholesterol with a nearly 1:1 stoichiometry (*14*), and mutant StAR binds cholesterol with the same efficiency (*19*); thus, it appears the subdomain of residues 194−285 cannot act in a manner independent of the subdomain of residues 63−193 for cholesterol binding.

To determine the activity (as measured by pregnenolone production) of smaller StAR subdomains, we cotransfected COS-1 cells with expression vectors for StAR and F2 (*25*). The activity of N-62 StAR was 510 ng/mL, whereas the activity of 63−193 StAR was 160 ng/mL, which is >3-fold lower than and comparable to the bioactivity observed with other methods (Figure 2D). Therefore, all three experiments confirmed the mild cholesterol fostering capacity of 63−193 StAR.

Equilibrium CD. As a general indicator of the similarities or differences in the three-dimensional structures of these bacterially expressed proteins, we performed far-UV CD spectroscopy (Figure 3) (*38*). A comparison is made among full-length StAR,

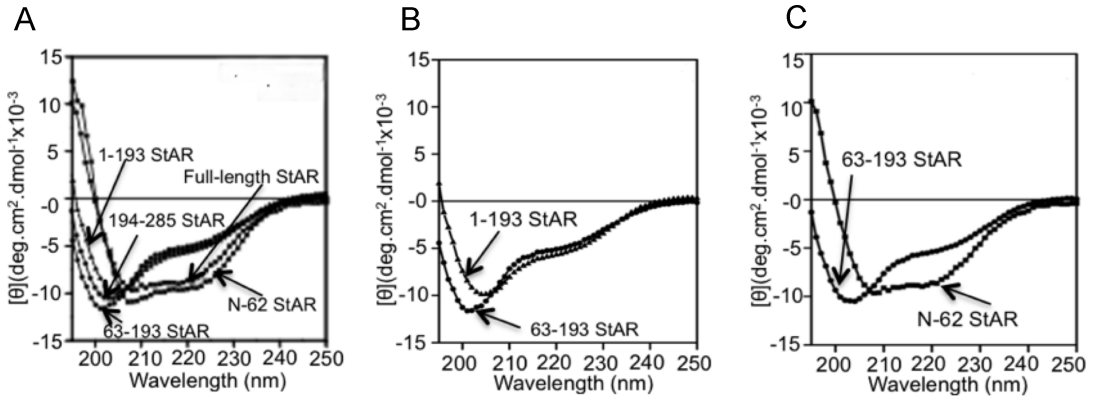

FIGURE 3: Equilibrium CD spectra of StAR and StAR subdomains. The far-UV CD spectra were recorded from 195 to 250 nm at 20 °C. Multiple scans were averaged to increase the signal-to-noise ratio. (A) Equilibrium CD spectra of N-62 StAR (■), full-length StAR (◆), 63−193 StAR (●), 1−193 StAR (▲), and 194−285 StAR (▼). (B) Equilibrium CD showing the similarity of the conformations of the 63−193 StAR (●) and 1−193 StAR (▲) subdomains. (C) Direct comparison of equilibrium CD between N-62 StAR (■) and the 63−193 StAR (▲) subdomain.

N-62 StAR, StAR subdomains 1−193 and 194−285, and the 63−193 subdomain reported by Song et al. (*39*). Subtraction of the spectrum of the buffer from the spectra of the protein samples showed that there was no absorption at 250 nm. The spectra of full-length StAR and N-62 StAR were superimposable (Figure 3A) and indistinguishable from our previously published spectra of this protein (*1*, *22*) but differed from the spectra of subdomains 63−193 and 1−193 of StAR. N-62 StAR and full-length StAR proteins had intensity minima at 208 and 222 nm, typical of an α-helical conformation; by contrast, the intensity minima of StAR subdomain 1−193, 63−193, or 194−285 shifted toward 204 nm (Figure 3A), suggesting a conformation change from predominantly α-helical (*1*) to coil transition (*38*, *39*). As purified full-length StAR was difficult to maintain in solution and could not be purified without urea denaturation and refolding, we did not continue with full-length StAR.

To show that the first 62 amino acids have a minimal contribution to the structural conformation of the 1−193 subdomain, we replotted the spectra of the 1−193 and 63−193 subdomains in Figure 3B which shows nearly superimposable spectra having no minima at 222 nm and strong minima close to 204 nm. A similar comparison of CD spectra of the 63−193 StAR subdomain with those of the N-62 StAR protein shows that a conformational change in N-62 StAR was possible when the 63−193 subdomain was fused with the 194−285 subdomain (Figure 3C). Thus, the fusion of 63−193 StAR with 194−285 StAR is essential for maintaining an α-helical conformation.

Fingerprinting of StAR Subdomains by Partial Proteolysis and Mass Spectrometry. The three-dimensional structures of the START domains of StARD3 (*14*) and StARD4 (*40*) are known by X-ray crystallography, and various modeling ventures show a very similar structure for StARD1 and StARD3 (*27*, *36*, *41*). The far-UV CD spectra of StAR subdomains show a shift from α-helix to a coil transition due to the loss of the StAR subdomain 63−193 (Figure 3C). As we have previously characterized StARD1 (*1*) and STARD3 (*42*) by partial proteolysis and mass spectrometric analysis of the resulting peptides, we applied the same approach to StAR subdomains 1−193, 63−193, and 194−285 (Figure 4). Thus, we performed limited proteolysis with trypsin on the independently folded subdomains of 1−193 StAR, 63−193 StAR, and 194−285 StAR (Figure 4A−C). The trypsin proteolysis of the 1−193 StAR subdomain resulted in two proteolysis resistant bands approximately 13 and 14 kDa in size (Figure 4B). Upon longer incubation with the protease of the 63−193 subdomain, only one trypsin resistant 10 kDa band was observed (Figure 4A). Mass spectrometric analysis indicated that amino acids 84−193 were protected from digestion (Figure 4A and Table S2 of the Supporting Information) and that the smaller and more sensitive band lacked 11 amino acids (112−122). The small (< 7 kDa) and more intense band resulted from the complete 1−193 domain and thus was a mixture of bands 1 and 2 (Table S3 of the Supporting Information). Surprisingly, the domain including only amino acids 63−193 also exhibited protease protection for amino acids 84−193, confirming that the signal sequence did not affect the conformation of StAR. Similarly, the 194−285 StAR subdomain was also resistant to proteolysis (Figure 4C) and resulted in a 6.5 kDa fragment containing all the amino acids from position 194 to 285 (Table S4 of the Supporting Information). Figure 4D shows a summary of the protease resistant bands found for each subdomain compared to N-62 StAR (*1*).

StAR Subdomains Are Tightly Folded. To further investigate the difference in microenvironment among these subdomains of the StAR protein, we measured acrylamide quenching of Trp residues. Acrylamide is an electron deficient compound that deactivates the excited fluorophores by collisional quenching (i.e., electrons are transferred from the excited indole group to acrylamide). This quenching effect allows the detection of minor structural differences in protein molecules, since proteins with greater Trp accessibility and stronger acrylamide binding undergo a greater decrease in fluorescence intensity. K_{SV}, which provides an index of quencher accessibility, was calculated for both N-62 StAR and smaller subdomains of StAR based on the fluorescence intensity of each. For reference, we employed the model Trp compound *N*-acetyl-L-tryptophanamide (NATA), which has a completely open chain conformation with a K_{SV} value of $16.5 \pm 0.3\ M^{-1}$. At an excitation of 295 nm, the K_{SV} of N-62 StAR (*19*) was $12.0 \pm 0.2\ M^{-1}$ (Figure 5). However, the K_{SV} values of subdomain 1−193 ($6.0 \pm 0.2\ M^{-1}$), subdomain 63−193 ($10 \pm 0.3\ M^{-1}$), and subdomain 194−285 ($7.8 \pm 0.1\ M^{-1}$) are lower (Figure 5). These results demonstrate that N-62 StAR has a more open conformation than the StAR subdomains.

Isothermal Titration Calorimetry. The result described above demonstrated that the StAR conformation is significantly open. However, because of the slow unfolding of StAR at the mitochondrial membrane, the mechanism by which StAR unfolds at the mitochondrial membrane and becomes activated for

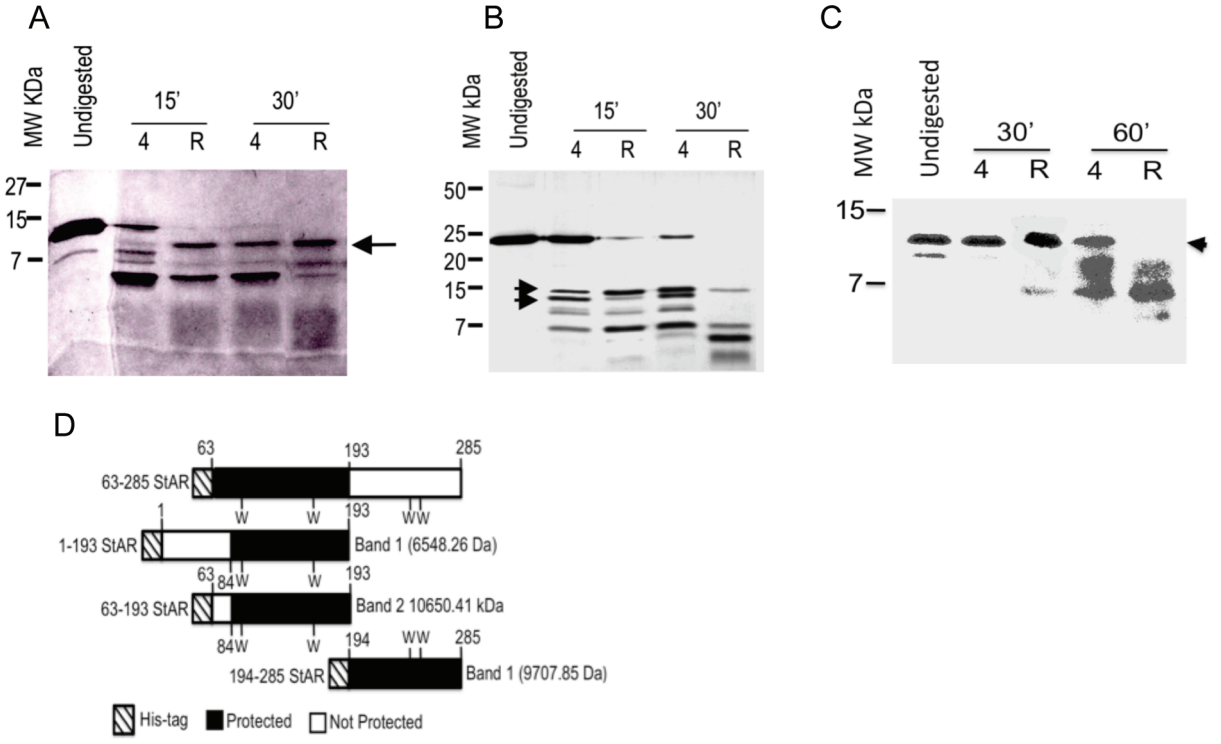

FIGURE 4: Proteolytic digestion of smaller subdomains of StAR. (A−C) Limited proteolysis with trypsin at two different temperatures. The proteins 1−193 StAR (A), 63−193 StAR (B), and 194−285 StAR (C) were digested at 4 °C and room temperature for 15 and 30 min (A and B) and for 30 and 60 min (C), electrophoresed via 22% SDS−PAGE, and stained with Coomassie blue. The trypsin resistant bands, indicated by the arrowhead, and the undigested bands were excised from the gel and subjected to mass spectrometric analysis. (D) Schematic presentation of the mass spectrometric analysis. The black regions depict the trypsin resistant amino acid sequence, and white regions depict the trypsin sensitive amino acid regions.

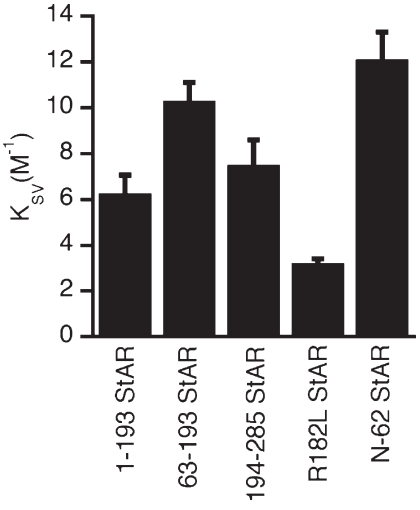

FIGURE 5: Stern−Volmer quenching constant (K_{SV}). Acrylamide quenching of Trp fluorescence (K_{SV}) for N-62 StAR, the 1−193, 63−193, and 194−285 subdomains, and mutant R182L StAR. All the data are means ± SEM from three independent experiments, each performed in triplicate.

facilitating cholesterol into mitochondria is perplexing. The human disease model of lipoid congenital adrenal hyperplasia (lipoid CAH) revealed that patients with a StAR mutation failed to metabolize cholesterol esters, causing severe changes in the mitochondrial architecture (*6*), and was confirmed by StAR knockout mice (*43*). We hypothesized that cholesterol plays an active role in the stabilization and activation of StAR. StAR is active prior to its import into mitochondria, and thus, the active conformation of StAR is likely attained at the mitochondrial membrane. The free energies (ΔG) of these START domains are very similar in isolation (*19*), but the ΔG of a complex three-component system (e.g., mitochondria, StAR, and cholesterol) cannot be measured directly; instead, the stoichiometry of complex systems is best measured by enthalpy (ΔH) (*44*). Therefore, we used ITC to measure the enthalpy of this three-component system. There was no change in ΔH when only two components were combined at a constant temperature of 37 °C; increasing amounts of StAR (Figure 6A) or cholesterol (Figure 6B) in the presence of a constant amount of mitochondria (20 μg of mitochondrial protein); increasing amounts of mitochondria in the presence of a constant amount (0.8 μg) of N-62 StAR (Figure 6C); and increasing amounts of cholesterol in the presence of constant amounts of N-62 StAR, R182L N-62 StAR, or BSA (Figure 6D) had no effect on ΔH. However, subjecting 20 μg of mitochondrial protein, 20 μg of cholesterol, and 0.8 μg of StAR to changing temperatures had a significant effect on ΔH for N-62 StAR, with ΔH increasing in a temperature-dependent fashion (Figure 6E). Incubation of mitochondria and cholesterol with BSA or the inactive mutant R182L N-62 StAR had no effect upon ΔH. When 0.8 μg of StAR and 20 μg of cholesterol were combined with increasing amounts of mitochondria, ΔH increased (Figure 6F). Thus, ΔH is directly related to the amount of titrant added, and the extent of cholesterol import increases at 37 °C.

New Paradigm for the Cholesterol-Induced Active StAR Conformation. To determine whether specific domains of StAR associate similarly with mitochondria and cholesterol, we incubated equal amounts of mitochondria and cholesterol with either the biosynthetic StAR N-terminal domain (amino acids 63−193) or the biosynthetic StAR C-terminal domain (amino acids 194−285)

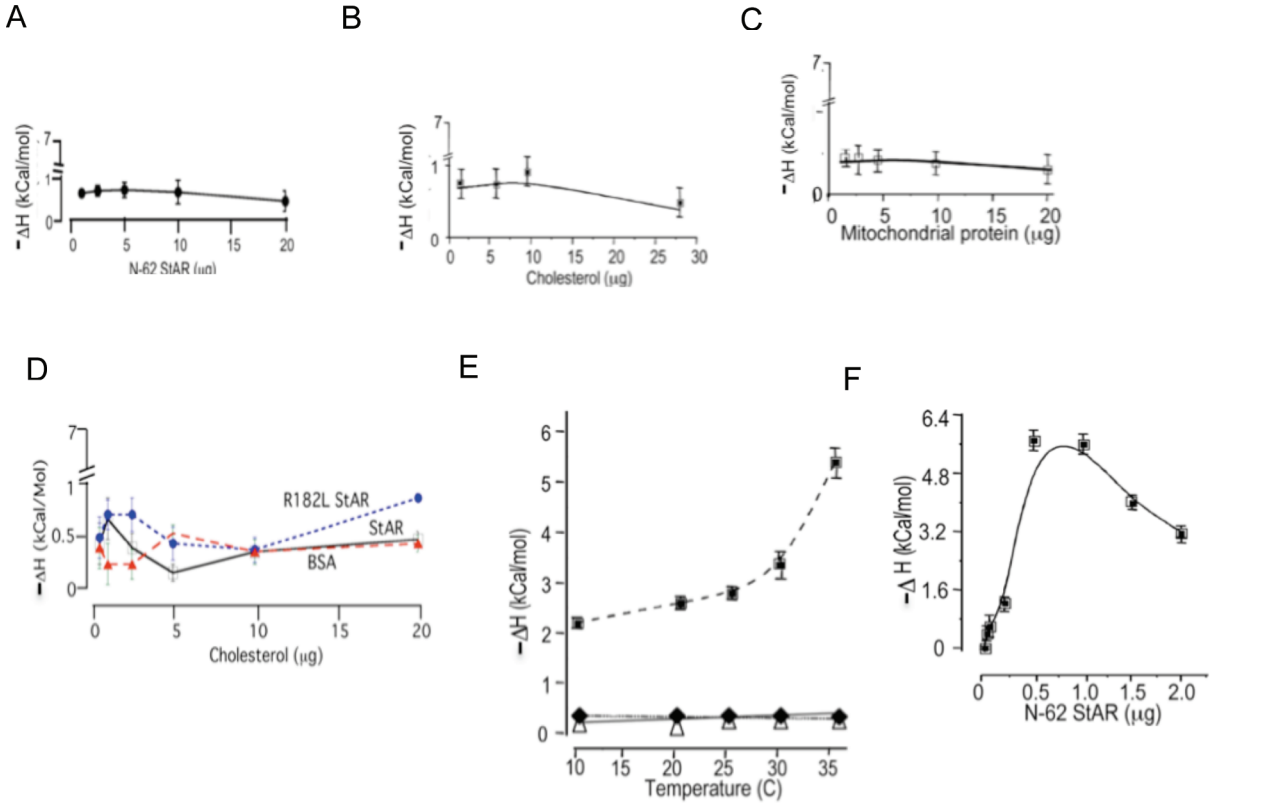

FIGURE 6: Isothermal titration calorimetry. Panels A−D represent control experiments in which only two of the three components of the system are combined. (A) ΔH for 20 μg of mitochondrial protein with increasing amounts of N-62 StAR. (B) ΔH for 20 μg of mitochondrial protein with increasing amounts of cholesterol. (C) ΔH for increasing amounts of mitochondrial protein and 0.8 μg of N-62StAR. (D) ΔH for 0.8 μg of N-62 StAR, R182L N-62 StAR, or BSA with increasing amounts of cholesterol (no mitochondria). (E) ΔH for 0.8 μg of N-62 StAR (■), R182L N-62 StAR (△), or BSA (●) added to 20 μg of mitochondrial protein and 20 μg of cholesterol with an increase in temperature. (F) ΔH for 20 μg of mitochondrial protein, 20 μg of cholesterol, and increasing amounts of N-62 StAR. All the data are means ± SEM from three independent experiments, each performed in triplicate.

(Figure 7A). There is an increase in enthalphy, only when StAR is incubated with mitochondria and cholesterol (Figure 7A); however, any combination of smaller subdomains under identical condition is similar to a negative control. These experiments clearly demonstrate that N-62 StAR is stabilized in the presence of cholesterol. The isolated subdomains either individually or in combination do not show the same effect.

As changes in ΔH correlated with the amount of added cholesterol, we used this measurement to determine the approximate stoichiometry of StAR-induced mitochondrial cholesterol import. With 20 μg of mitochondrial protein and 20 μg of cholesterol, ~0.8 μg of N-62 StAR elicited a maximal effect (Figure 6F). While technical limitations restrict the accurate determination of the protein concentration, we can conclude that 0.8 μg of N-62 StAR protein was responsible for the import of 20 μg of cholesterol. As the molecular weight of cholesterol is smaller than that of StAR, a large pool of cholesterol is transported by one molecule of StAR.

The kinetics of pregnenolone synthesis show that most activity is observed after 30 min of incubation (Figure 7B) and was completed in less than 1.5 h, consistent with previous observations (*18*). By subtracting the minimal import during this lag time, we can certainly conclude a large number of cholesterol molecules were imported per molecule of N-62 StAR; this time period correlates with the biological lifetime of a StAR molecule (*4*, *37*). Thus, both measurements of ΔH and direct measurements of pregnenolone synthesis indicate that each molecule of StAR is responsible for the import of a large number of cholesterol molecules.

A possible source of the increase in enthalpy is the catalytic conversion of cholesterol to pregnenolone by P450scc. To test this possibility, we repeated the experiments via replacement of cholesterol with an equal amount of 22(*R*)-hydroxycholesterol (22R-OH). This soluble oxysterol is a substrate for P450scc but does not require the action of StAR to enter the mitochondria (*25*); hence, 22R-OH will generate the heat of the cholesterol side chain cleavage reaction but will not generate any heat associated with StAR's action on the OMM. Combining 22R-OH with 20 μg of mitochondria and 0.8 μg of StAR produced pregnenolone but did not show a significant increase in ΔH (Figure 7C). Thus, the increase in ΔH could be attributed to the action of StAR rather than to the reaction converting cholesterol to pregnenolone.

DISCUSSION

In this study, we have combined mitochondrial protein transport and reconstitution with functional complementation of steroidogenic activity to examine the unique mechanism of StAR import required for transporting cholesterol into mitochondria. First and foremost, these studies have identified differences in the process of import into mitochondria. The import of P450scc, a protein that resides in the matrix, occurs more quickly because of the need for this protein to be present in the matrix prior to or at the same time as cholesterol to synthesize pregnenolone. On the other hand, StAR is expressed on hormonal stimulation, and thus, longer residency is essential for the transfer of several cholesterol molecules into the mitochondrion. In acute

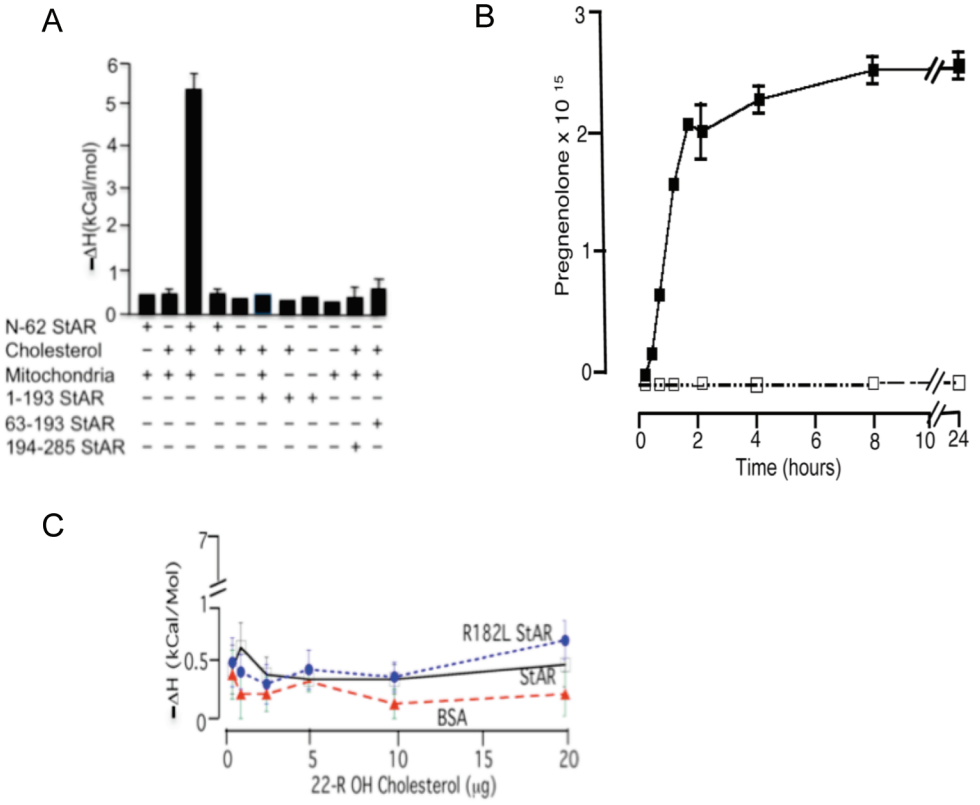

FIGURE 7: Stability and activity of the smaller StAR subdomains. (A) The change in enthalpy, ΔH, was measured following the addition of StAR subdomains in the presence of 20 μg of mitochondrial protein and cholesterol. The results showed no change in enthalpy with the addition of smaller StAR subdomains, and the enthalpy changed only when N-62 StAR was added to mitochondria in the presence of cholesterol. (B) Time course of pregnenolone synthesis with the ordinate representing the number of pregnenolone molecules produced vs time. Mitochondria were incubated with cholesterol and StAR (—) or without StAR (− · · −). Pregnenolone conversion starts after 30 min and is completed within 1.5 h. One molecule of N-62 StAR imported several molecules of cholesterol per hour. (C) There is no increase in ΔH when increasing amounts of 22R-OH are substituted for cholesterol in combination with N-62 StAR, R182L N-62 StAR, or BSA and mitochondria (20 μg of mitochondrial protein). All the data are means ± SEM from three independent experiments, each performed in triplicate.

steroidogenesis, substantial cholesterol influx to the matrix side of the mitochondrion is essential for the synthesis of a large amount of steroid hormone in a short period of time. Therefore, it is expected that the cholesterol pool should remain close to both the OMM and StAR.

Mass spectrometric analysis of partially proteolyzed domains showed that these domains are partially resistant to proteolysis, suggesting that the stability of StAR in solution differs from the stability of StAR in association with the mitochondrial membrane. The presence of the StAR signal sequence is nonessential for the activity and stability of StAR; however, this sequence was critical for targeting StAR to the mitochondrial membrane. Once StAR reaches the OMM, multiple cholesterol molecules, which are destined for transport into mitochondria, are transported by StAR. In isolated mitochondria, cholesterol is removed during the mitochondrial preparation, and as a result, the limited remaining endogenous cholesterol does not facilitate pregnenolone synthesis. Computer modeling of StAR and START proteins predicted that StAR unfolds for activation and import in association with the membrane (*36*). The stability of StAR protein, as shown by the increase in ΔH, was attained with an association with mitochondria and cholesterol (Figure 6). Thus, cholesterol assists in the stabilization of StAR at the OMM. At this time, however, we cannot explain the mechanism of StAR binding and release of cholesterol, and these questions will be addressed in future studies.

Most of the intracellular cholesterol is stored as cholesterol esters as this substance cannot remain in the cell as free cholesterol. We reasonably speculate that cholesterol is transported through the mitochondrial permeability transition pore (*45*). Translocated 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 interdependently (*46*) or independently in a stepwise fashion (*47*). StAR is active at the OMM prior to import. It is possible that StAR forms a complex with multiple molecules of cholesterol (*48*), as one molecule of StAR may transport 300−400 molecules of cholesterol (*49*). Therefore, the stability, kinetics, and folding pathways are influenced by environmental conditions, including cytoplasmic factors and chaperones. Once StAR has associated with the mitochondrial membrane, the folding and activity of StAR are dictated by the domain organization and the presence of cholesterol.

Both the structural studies (*14*, *40*) and computational models (*36*, *41*, *50*) indicated that there was insufficient room for a cholesterol molecule to enter or exit StAR's sterol binding pocket. Molecular dynamic studies and cross-linking experiments then showed that the carboxy-terminal C-helix of StAR acts as a door to the sterol binding pocket, and that disruption of hydrogen bonds between the C-helix and adjacent loops opens this door, permitting the entry and exit of cholesterol (*50*, *51*). We found no change in ΔH when cholesterol was combined with StAR; ΔH increased only when all three components were

combined. In summary, StAR picks up cholesterol from a source in the OMM, and not from cytoplasmic cholesterol. Substantial work will be needed to complete the molecular details of StAR's action and to delineate the precise itinerary of a cholesterol molecule on its way to the mitochondrial matrix.

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SUPPORTING INFORMATION AVAILABLE

Detailed information about the building of different constructs using a variety of combinations of oligonucleotides and the mass spectrometric analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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