A pH-dependent Molten Globule Transition Is Required for Activity of the Steroidogenic Acute Regulatory Protein, StAR*  

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The steroidogenic acute regulatory protein (StAR) stimulates steroid biosynthesis by increasing the flow of cholesterol from the outer mitochondrial membrane (OMM) to the inner membrane. StAR acts exclusively on the OMM, and only StAR’s carboxyl-terminal α-helix (C-helix) interacts with membranes. Biophysical studies have shown that StAR becomes a molten globule at acidic pH, but a physiologic role for this structural transition has been controversial. Molecular modeling shows that the C-helix, which forms the floor of the sterol-binding pocket, is stabilized by hydrogen bonding to adjacent loops. Molecular dynamics simulations show that protonation of the C-helix and adjacent loops facilitates opening and closing the sterol-binding pocket. Two disulfide mutants, S100C/S261C (SS) and D106C/A268C (DA), designed to limit the mobility of the C-helix but not disrupt overall conformation, were prepared in bacteria, and their correct folding and positioning of the disulfide bonds was confirmed. The SS mutant lost half, and the DA mutant lost all cholesterol binding capacity and steroidogenic activity with isolated mitochondria in vitro, but full binding and activity was restored to each mutant by disrupting the disulfide bonds with dithiothreitol. These data strongly support the model that StAR activity requires a pH-dependent molten globule transition on the OMM.

Molten globules are compact, partially unfolded proteins that retain their secondary structure but have lost some tertiary structure (1); such partially unfolded structures are typically inactive but may be intermediates in membrane insertion (2). Steroidogenic acute regulatory protein (StAR),3 which is essential for normal adrenal and gonadal steroidogenesis, facilitates the flow of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM), where cholesterol is converted to pregnenolone by the cholesterol side chain cleavage enzyme, P450sc (3, 4). Mutation of human StAR causes potentially lethal congenital adrenal hyperplasia (5, 6); all missense mutations that cause this disease are found in the carboxyl-terminal 50% of the protein, indicating that these sequences are required for StAR activity (7–9). StAR is synthesized as a 37-kDa phosphoprotein with an amino-terminal mitochondrial leader sequence that is cleaved during mitochondrial entry (3, 10, 11).

The mechanism by which StAR moves cholesterol from the OMM to IMM remains unclear. The x-ray crystal structures of two closely related proteins, N-216 MLN64 (12) and StarD4 (13), reveal a β-barrel structure with a hydrophobic sterol-binding pocket (SBP) that will accommodate one cholesterol molecule, although the apparent access channel to the SBP is too small to accommodate a cholesterol molecule. Models of StAR show the same fold (12, 14, 15), suggesting action as a transport protein. Recent data suggest that the StarD4 family of related proteins, which lack mitochondrial targeting sequences, serve as cytosolic transporters for insoluble lipid molecules (16, 17). Furthermore, one molecule of StAR apparently facilitates the mitochondrial import of about 400 molecules of cholesterol (18), suggesting a similar mechanism for StAR action. This would suggest that StAR acts in the intramembranous space (12), but multiple lines of evidence indicate that StAR exerts its action on or in the OMM. First, deletion of 62 amino-terminal residues, including the leader peptide, results in a protein (N-62 StAR) that is confined to the cytoplasm but retains full activity, both in transfected cell systems and in isolated mitochondria in vitro (19–21). Second, fluorescence resonance energy transfer experiments indicate that StAR directly interacts with synthetic membranes (22). Third, N-62 StAR promotes cholesterol transfer from synthetic vesicles lacking P450sc to those containing P450sc (23). Fourth, affixing N-62 StAR to the cytoplasmic aspect of the OMM constitutively activates StAR, whereas localizing N-62 StAR to the intramembranous space or matrix side of IMM ablated all activity (11). Fifth, in vitro mitochondrial protein import assays show that constructs that slow mitochondrial entry increase StAR activity and constructs that speed its entry decrease activity (11). Thus, all direct experimental evidence indicates that StAR’s site of action is the OMM. Finally, synthetic unilamellar vesicles having a lipid composition that models the OMM only protect StAR’s carboxyl-terminal α-helix (C-helix) from proteolysis, implying that when StAR interacts with the OMM, most of the protein remains exposed to the cytoplasm (15).

Spectral data and partial proteolysis indicate that StAR undergoes a structural transition to a molten globule at low pH in solution (24) and in association with synthetic membranes (22, 25). We have proposed that this conformational change is induced in vivo by StAR’s interaction with protonated phospholipid head groups of the OMM and is required for StAR activity (11, 22, 24). Consistent with this model, disrupting the mitochondrial proton pump with mCCCP (26) or with lipopolysaccharide (27) abolishes StAR activity, and liposome protection of the C-helix is more complete at pH 4.0 than at pH 6.5 (15). As the available structural (12, 13) and modeling (12, 14, 15) data indicate that no access route to the SBP of StAR is large enough to accommodate a cholesterol molecule, it is apparent that StAR must undergo a conformational change to permit cholesterol to reach the SBP. This conformational change is apparently associated with the spectroscopic changes interpreted as a
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FIGURE 1. Modeling and molecular dynamics of N-62 StAR. A, ribbon diagram of the model of N-62 StAR; the salt bridges between Asp-106 and Arg-272 and between Glu-136 and Arg-274 are shown as ball-and-stick representations (white, carbon; blue, nitrogen; red, oxygen). B, total energy levels of MD simulations as a function of time. Black, neutral; red, acidic; green, acidified C-helix. C, root mean square deviation (RMSD) of α-carbons during MD of the initial N-62 StAR structure. D, images of Asp-106–Arg-272 after 2800 ps of MD under neutral (top) and acidified C-helix conditions (bottom). The carboxyl carbon (C) of Arg-106 in the E11 loop (left) is bonded to two oxygen atoms, (O31 and O32), and the guanidino carbon (C) of Arg-272 in the C-helix (right) is covalently bonded to two nitrogen atoms (Nus2 and Nus1). Under neutral conditions, Arg-106 forms two hydrogen bonds to Arg-272 (O31-Nus2 and O62-Nus1), but under acidified C-helix conditions, the E11 loop and C-helix movement and bonding are disrupted.

molten globule (24). Nevertheless, the molten globule model of StAR’s action has been controversial. Therefore, we have sought direct evidence for the potential involvement of the molten globule structural transition in StAR activity. We now show that the conformational changes that have previously been characterized as a molten globule transition (24) are required for StAR activity.

MATERIALS AND METHODS

Molecular Dynamics (MD)—The parallel MD program AMBER7 using the amber force field ff94 (28) plus the modified ffmod

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MATERIALS AND METHODS

Molecular Dynamics (MD)—The parallel MD program AMBER7 using the amber force field ff94 (28) plus the modified ffmod. mod_phipsi.2 file (AMBER 8) were used at the University of California San Francisco Computer Graphics Laboratory. Programs were run on the socrates host of a Hewlett-Packard AlphaSever mainframe that included a 32-processor GS1280 and four 4-processor ES455s. The model of human N-62 StAR (15) was solvated using the pre-equilibrated TIP3P water model in a rectangular box with a minimum solute-wall distance of 10 Å. Images of the model were generated with the program Chimera (45). We used periodic boundary simulations based on the particle mesh Ewald method (AMBER 7), and the nonbonded cutoff, which is used to limit the direct space sum for particle mesh Ewald, was set at default value (8 Å). The system was subjected to a steepest descent energy minimization (1000 steps) with positional restraints on the protein and second minimization (1000 steps) on the whole system. At minimization convergence, the whole system was heated to 300 K over 100 ps, and another 100 ps equilibration was performed under constant pressure with the restraints switched off to allow the water box to relax. The whole system was then subjected to a 3-ns production MD run. (1.5 × 10^8 steps at 2 fs/step). The system energy, temperature, and pressure were checked before and after each MD run.

Protein Preparation—Wild-type full-length StAR DNA cloned into pTWIN1 (New England Biolabs Inc.) (a gift from X. Chen) was modified by deleting the region encoding the first 62 residues and adding an amino-terminal His6 tag fused to the Ssp DnaB intein domain at StAR’s amino terminus and then cloned into the SapI and BamHI sites. The carboxy-terminal Cys-285 residue was mutated to Phe, and the SapI site in the cDNA was mutated to facilitate cloning, yielding pTWIN-N-His6-C-N-62 StAR. Transformed Escherichia coli BL21(DE3) (New England Biolabs) grown in Luria broth were induced with 0.5 mM Tris, pH 8.5, 500 mM NaCl. Bacteria were lysed by sonication and cleared of debris, and the supernatant was filtered (0.45 μm) before loading onto chitin-binding columns (New England Biolabs). The columns were prepared, loaded, washed, and eluted according to the manufacturer’s suggestions. Protein was collected, dialyzed against 20 mM Tris, pH 7.5, 300 mM NaCl, and concentrated using 15 ml of ultrafiltration cells with YM10MWC membrane (Amicon, Inc.). Protein concentration was estimated at A\text{\text{\text{280}))/0.134} mg^{-1} cm^{-1} calculated from the amino acid sequence using the ProtParam program, ExPAsys. Free sulphydryl groups were titrated at 412 nm with 5,5'-dithiobis (2-nitrobenzoate) at pH 7.5 in the absence and presence of 2% SDS (Elman’s reagent instructions, Pierce). The N-62 StAR mutants S100C/S261C (SS) and D106C/A268C (DA) were constructed by site-directed mutagenesis, sequenced, and expressed as above.

Mass Spectrometry—Bands of purified wild-type, SS, and DA mutant N-62 StAR were excised from SDS-PAGE gels and digested into ~1 mm² pieces. Samples were destained twice with 100 μl of 25 mM NH₄HCO₃ in 50% acetonitrile to eliminate formation of spurious disulfides (29), evaporated to dryness in a SpeedVac centrifuge, and reacted with 50 μl of 55 mM iodoacetamide in 25 mM NH₄HCO₃ for 1 h at 20 °C in the dark. After removing the supernatant, the gel pieces were evaporated to dryness, digested with 10 μl of 12.5 ng/μl sequencing grade modified trypsin (Promega, Madison, WI), and extracted with 50 μl of 5% formic acid in 50% acetonitrile and then sonicated for 30 min. The supernatant was evaporated nearly to dryness and dissolved in 10 μl of 0.1% formic acid. A 1-μl aliquot was chromatographed on a 1100 nano-high pressure liquid chromatograph (Agilent Technologies, Santa Clara, CA) using a 75 μm × 15 cm, 5-μm particle size C-18 column (Phenomenex, Torrence, CA) and eluted at 0.1% formic acid with a 30-min linear gradient of 5–60% acetonitrile flowing at 5 μl/min. The effluent was routed into a QSTAR (o)-TOF MS (ABI, Foster City, CA) operated in electrospray positive mode. Data were acquired in IDA-mode scanning from 305 to 1400 m/z, low Q1 resolution, 25-V collision voltage, with a time-of-flight m/z range of 50–2000 for the MS/MS acquisition.

Spectroscopy—Protein samples were diluted to 0.3 mg/ml in 20 mM Tris-HCl, pH 7.5, and CD spectroscopy was performed at room tem-
temperature in a 1-mm path length cuvette in a Jasco 720 spectropolarimeter equipped with a Peltier temperature controller. For pH studies, samples were dialyzed against 20 mM phosphate buffer at the desired pH and clarified at 10,000 \( \times \) g for 10 min to eliminate aggregates, and the protein concentrations were determined following the CD spectroscopy. Each spectrum represents the average of at least five accumulations with subtraction of the appropriate background. Each experiment was repeated three times, and the spectra from each experiment were averaged and converted to mean residue ellipticity (\( \Theta \)). Secondary structure compositions were estimated using the three computational tools in CDPro (30). Analysis with CONTIN (31) yielded the lowest normalized root mean square deviation score; thus, data are portrayed
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FIGURE 4. Mass spectrometric analysis. A, analysis of the tryptic peptides of wild-type N-62 StAR. Left, the m/z 728.3 (3+) ion corresponds to the sequence SIINQVLSQTQVDFANHLR with an error of 9.1 ppm. Right, the m/z 510.7 (2+) ion corresponds to the sequence ESQDNGDK with an error of 19.6 ppm. In each panel, the inset shows an expanded view of the isotopic profile. B, mass spectrometric analysis of the SS mutant M5 ions, with expanded views of the isotopic profiles of the m/z 647.3 (5+) and the 808.8 (4+) ions, which correspond to the disulfide-linked peptides ECQQDNGCK-SIINQVLSQTQVDFANHRLR. These ions, plus the m/z 1074.1 (3+) ion, yield the shown disulfide-linked species of monoisotopic ion with an error of 21.6 ppm. C, mass spectrometric analysis of the DA mutant M5 ions, with expanded views of the isotopic profiles of the m/z 644.9 (5+) and 805.8 (4+) ions, which correspond to the disulfide-linked peptides ESQDNGCK-SIINQVLSQTQVDFANHRLR. These ions, plus the m/z 1074.1 (3+) ion, yield the shown disulfide-linked species with an error of 12.4 ppm.

with this program. Protein samples (0.5 mg/ml) in phosphate-buffered saline were excited at 280 nm, and emission spectra were recorded from 360 to 500 nm on a Spectra Max M2 microplate reader (Molecular Devices). Each experiment was performed three times, and the fluorescence spectra were averaged.

Cholesterol Binding—N-62 StAR (0.5 µM) in phosphate-buffered saline was mixed with various amounts of NBD-cholesterol 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino-23,24-bisnor-5-cholen-3α-ol) (Molecular Probes Inc.) in 96-well plates (final volume 200 µl) incubated 15 min at 37 °C, and steady state fluorescence was monitored on a SpectraMax M2 microplate reader. NBD-cholesterol was excited at 460 nm, and emission was recorded at 534 nm. Data were analyzed by non-linear regression using GraphPad Prism version 3.00 plotting NBD-cholesterol concentration versus the relative fluorescence.

Steroidogenic Activity—MA-10 cell mitochondria were isolated (11), suspended in 0.25 M sucrose, 10 mM HEPES, pH 7.4, and stored at −80 °C. About 1 µg of mitochondrial protein (Bradford method) was incubated with wild-type or mutant N-62 StAR at 37 °C for 60 min in 125 mM KCl, 5 mM MgCl₂, 10 mM KH₂PO₄, 25 mM HEPES, 250 ng/ml trilostane, 100 µM GTP, 10 mM isocitrate with or without 1 mM DTT. Conversion of cholesterol to pregnenolone was determined by radioimmunoassay.

RESULTS

Molecular Dynamics Simulations—We previously reported a computational model of human N-62 StAR (15) based largely on the x-ray crystal structure of N-216 MLN64 (12). N-216 MLN64 has 36% sequence identity with StAR (32), 50–60% of StAR’s activity in vitro (33, 34), and a tightly folded amino-terminal domain and a more loosely folded carboxyl-terminal domain (34), similar to StAR (24). The model closely resembles the structures of N-216 MLN64 (12) and StarD4 (13), scores favorably in the programs PROCHECK and WHATIF, and has a calculated free energy of −4.3 × 10³ kcal/mol, which is similar to the value of −5.9 × 10³ kcal/mol calculated based on the coordinates of the N-216 MLN64 crystal structure (Fig. 1A).

Models and crystal structures yield rigid representations, analogous to visualizing the protein at 0 K. To visualize the movement of the protein in solution at 300 K, we “hydrated” the model of human N-62 StAR in silico by adding 9631 water molecules as a 73.6 kcal/mol calculated based on the coordinates of the N-216 MLN64 crystal structure (Fig. 1A).

MD runs were performed encompassing 1.5 × 10⁶ molecular steps at 2 fs/step. The output files were written every 500 steps (1 ps) and the trajectory files were saved every 5000 steps, yielding 300 structures for each simulation.

MD runs were done under three computational conditions. First, all residues were left in their charged states (the “default” settings of the program) to model pH 7 (“neutral” conditions). Second, all Asp, Glu, and His residues were protonated to model acidic conditions near pH 4 (“acidic” conditions). Third, only the Asp, Glu, and His residues in the C-helix and in the adjacent Ω1 and Ω3 loops were protonated to model a neutral StAR molecule interacting with an acidified membrane (“acidified C-helix” conditions). The total energy under each condition remained stable during the MD runs (Fig. 1B). At neutral pH, the model showed stable trajectories with conserved secondary and tertiary struc-
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StAR had a partially opened tertiary structure. The distal end of the carboxyl-terminal helix became unfolded further, and the /H9024 loop and C-helix moved away from each other, opening and closing the SBP. Measurements of the C root mean square deviation showed that StAR is more labile under the two acidic conditions (Fig. 1C). The C-helix and /H9024 loop are closely associated; the distances between either of the resonating guanidino nitrogens of Arg-272 and either of the carboxylic oxygens of Asp-106 are 2.8 ± 0.1 Å under neutral conditions, favoring the formation of two hydrogen bonds (Fig. 1D).

To visualize the movement of the C-helix, we superimposed images of the molecule obtained every 100 ps, and we plotted the distances between key pairs of atoms during MD (Fig. 2). The distance between the carboxyl carbon (C) of Asp-106 in the /H1 loop to the guanidino carbon (C) of Arg-272 remained stable at 4 Å under neutral conditions, whereas under either acidic or acidified C-helix conditions, this distance fluctuated dramatically, reaching >10 Å (Fig. 2C, top). This indicates that the C-helix and /H1 loop move dramatically with respect to one another, opening and closing the SBP. Similar hydrogen bonding was seen between Glu-136 and Arg-274 in the static model at neutral pH. This distance varied from 4 to 6 Å during MD at neutral pH but was relatively more stable under acidic conditions, particularly when only the C-helix was acidified (Fig. 2C, bottom). Thus, acidic pH had little effect (or even a stabilizing effect) on the distance between Arg-274 in
the C-helix and Glu-136 in the short α2 helix that contributes to the roof of the SRP. Therefore, cholesterol is more likely to reach the SBP via a space between the Ω1 loop and the C-helix than between the Ω3 loop and the C-helix.

Design of Immobilizing Mutants—MD simulations based on different mathematical parameters might have yielded somewhat different results; therefore, we sought experimental confirmation of our computational results. To test the hypothesis that movement of the C-helix is required for StAR’s activity, we sought to immobilize this helix in the position identified by the modeling and by the MD under neutral conditions. We systematically modeled the effects of changing residues in the C-helix and adjacent loops to Cys, seeking pairs in which the side chains were ~4 Å apart, and then mutated them to Cys in silico. We identified two pairs of residues, S100C/S261C (SS) and D106C/A268C (DA), which, when changed to Cys, positioned their sulfhydryl groups to form disulfide bonds without altering the position of the Ca backbone of the C-helix or Ω1 loop (Fig. 3A). The model of each disulfide mutant had the same minimum energy as the wild type, suggesting that such mutants did not strain or alter the protein fold.

Structure of Immobilizing Mutants—The SS and DA mutants were created by site-directed mutagenesis, expressed as intein fusion proteins, purified, and analyzed by SDS-PAGE (Fig. 3B). The carboxyl-terminal residue of StAR, Cys-285, was changed to Phe to reduce reactivity; deletion of Cys-285 has no effect on StAR’s activity (19). Each mutant migrated more rapidly than the wild type, but when reduced with DTT, each had the same migration as the wild type, suggesting that disulfide bonds had been formed. Our wild-type N-62 StAR (containing the carboxyl-terminal C285F mutation) has three cysteines; modeling indicated that none forms a disulfide bond. Titrating the free sulfhydryls showed that the wild type, SS, and DA forms had two accessible sulfhydryls under native conditions and three titratable sulfhydryls when the proteins were denatured with SDS, indicating that the SS and DA mutants (which contain 5 Cys residues) each had one disulfide bond, presumably formed between S100C and S261C in SS and between D106C and A268C in DA.

To confirm the location of these disulfides, each mutant was purified on SDS-PAGE and subjected to mass spectrometric analysis. Protein bands excised from SDS gels were treated with iodoacetamide to prevent formation of spurious disulfide bonds and then digested with trypsin and analyzed by LC/MS and MS/MS. Comparison with the Swiss Protein Database (SwissProt.2005.01.06 entry number P49675) permitted identification of all peptides in the wild-type sample, including residues 99–107 with a predicted M+H monoisotopic mass (MI M+H) of 1020.4 and residues 254–272 (MI M+H = 2183.1) (Fig. 4A). The SS mutant contained an M+H ion of 3232.5 detected as 3+ (m/z 1078.2), 4+ (m/z 808.8), and 5+ (m/z 647.3) ions (Fig. 4B), and the DA mutant contained an M+H ion of 3220.52, detected as the 3+ (m/z 1074.1), 4+ (m/z 805.8), and 5+ (m/z 644.9) ions (Fig. 4C). Both of these M+H ions correspond to disulfide-linked peptides 99–107 and 254–272, and MS/MS spectra of the SS m/z 808.8 ion and the DA m/z 805.8 ion generated a nearly complete y-ion series, resulting in unambiguous peptide assignments (Fig. 5). Thus, the disulfide bonds in the SS and DA mutants were in the correct locations. Finally, far-UV CD and fluorescence spectroscopy, which are sensitive to the pH-induced structural changes in StAR (24), indicating that the folding of these mutants was indistinguishable from wild type (Fig. 6). Thus, the SS and DA mutants accurately model the conformation of wild-type StAR.

Immobilizing Mutants Lose Some Molten Globule Behavior—To test the effects of immobilizing the C-helix on the pH-dependent molten globule properties of StAR, we measured the CD spectrum of the wild type, SS mutant, and DA mutant at pH 2–7.5 (Fig. 7A). Analysis of this data by several algorithms showed that acidification induced a substantial increase in the α-helical content of wild-type StAR, with maximal helicity at pH 3.5, as reported previously (24) (Fig. 7B). Consistent with the overlapping spectra in Fig. 6, the calculated α-helical content of the
FIGURE 8. Activity of the disulfide mutants. A, binding of fluorescent NBD-cholesterol by wild-type (●), SS ( ), and DA N-62 StAR ( ), under native conditions (left) and following reduction with DTT (right). Data for the inactive StAR mutant M144R (×) and for the buffer without protein (+) are shown in gray. B, capacity of the wild type and SS mutant and DA mutant of N-62 StAR to induce steroidogenesis, as assessed by the capacity of each protein to promote steroidogenesis in mitochondria isolated from mouse Leydig MA-10 cells. Gray bars, native protein; black bars, protein reduced with DTT.

wild type and mutants was the same at pH 7.5, but the mutants showed substantially less change in α-helical content at acidic pH, with no peak at pH 3.5 (Fig. 7B). Thus, the disulfide mutants prevented the generation of the principal spectroscopic hallmark of the pH-induced molten globule transition described previously (24).

Activity of Immobilizing Mutants—StAR can bind cholesterol with 1:1 stoichiometry (12, 35). Wild-type N-62 StAR bound 14C cholesterol (not shown) and fluorescent NBD-cholesterol (Fig. 8A). Cholesterol binding by the SS mutant was reduced by about 50%. The engineered disulfide bond in the SS mutant is in the middle of the C-helix (S261C), which only partially restricted movement of this helix. Binding by DA was similar to the buffer background or binding by the inactive StAR mutant M144R (9). The DA mutant contains a disulfide closer to the carboxyl terminus (A268C), which more completely immobilized the C-helix and eliminated all measurable cholesterol binding. The cholesterol binding activity of both mutants was fully restored by treating the disulfides with DTT (Fig. 8A). Similarly, wild-type N-62 StAR elicited five times more steroidogenesis from mitochondria isolated from mouse Leydig MA-10 cells than did buffer. However, the activity of the SS mutant was reduced by about half and the activity of the DA mutant was similar to the buffer background. But these two mutants recovered full activity when the disulfides were disrupted with DTT (Fig. 8A). Thus, the SS and DA mutants were fully capable of normal function when their capacity to form a molten globule was restored by cleaving their disulfide bonds.

DISCUSSION

Although the mechanism of StAR’s action remains unknown, models of its action must account for the following facts. First, StAR works exclusively on the OMM. Deleting the StAR mitochondrial leader peptide prohibits StAR’s entry into mitochondria, yet such constructs are fully active in transfected systems (19–21). Immobilizing StAR on the OMM renders it constitutively active, whereas it is inactive in the intramembranous space or matrix; also, manipulations of the leader that slow StAR’s mitochondrial entry increase activity, whereas those that speed entry decrease activity (11). Thus, the role of StAR’s mitochondrial leader is to target its action to the mitochondrion (4) as lead-

we suggest that StAR is both a cytoplasmic cholesterol transport protein and the mitochondrial trigger of steroidogenesis. The action of StAR to stimulate steroidogenesis at the OMM is distinct from its cholesterol-transport activity as StAR bound to the OMM is active (11). Newly synthesized cytoplasmic 37-kDa StAR may bind cholesterol and transport it to a mitochondrion, in the fashion envisioned for members of the StarD4 family (17). However, once StAR’s mitochondrial leader peptide interacts with the outer membrane translocase machinery, StAR becomes associated with the OMM, and all further activity is confined to the OMM. In this location, StAR appears to act by mobilizing cholesterol that was previously loaded into the OMM. Although cholesterol is a normal component of the OMM and other membranes, the cholesterol in steroidogenic mitochondria behaves as two kinetically distinct pools: a stable pool, presumably the structural cholesterol, and a labile pool, which is imported and becomes the substrate for steroidogenesis (40). The chemical form of these two pools of cholesterol is the same; their difference lies in the physical nature of their association with the OMM. BPB, which participates in an as yet undefined fashion in StAR-induced steroidogenesis (39), consists of five transmembrane domains with a 26-amino acid carboxyl terminus exposed to the cytoplasm (41). This carboxyl terminus constitutes a “cholesterol recognition/interaction amino acid consensus sequence” or “CRAC domain” (42) having a helical structure with a groove that accommodates one molecule of cholesterol (43). We propose that cholesterol bound to the CRAC domain of BPB comprises the labile pool of cholesterol. StAR would thus act by associating with the OMM and interacting with protonated phospholipid head groups to disrupt the Asp-106/Arg-272 hydrogen bonds to initiate the mito-
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mitochondria to initiate steroidogenesis, our data indicate that StAR’s action is confined to the OMM and requires a proton-induced transition to a molten globule structure.

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