Steroidogenic acute regulatory protein (StAR) plays an essential role in steroidogenesis, facilitating delivery of cholesterol to cytochrome P450\textsubscript{sec} on the inner mitochondrial membrane. StAR is synthesized in the cytoplasm and is subsequently imported by mitochondria and processed to a mature form by cleavage of the NH\textsubscript{2}-terminal mitochondrial targeting sequence. To explore the mechanism of StAR action, we produced 6-histidine-tagged N-62 StAR (His-tag StAR) constructs lacking the NH\textsubscript{2}-terminal 62 amino acids that encode the mitochondrial targeting sequence and examined their steroidogenic activity in intact cells and on isolated mitochondria. His-tag STAR proteins stimulated pregnenolone synthesis to the same extent as wild-type StAR when expressed in COS-1 cells transfected with the cholesterol side-chain cleavage system. His-tag StAR was diffusely distributed in the cytoplasm of transfected COS-1 cells whereas wild-type StAR was localized to mitochondria. There was no evidence at the light or electron microscope levels for selective localization of His-tag StAR protein to mitochondrial membranes. In vitro import assays demonstrated that wild-type StAR preprotein was imported and processed to mature protein that was protected from subsequent trypsin treatment. In contrast, His-tag StAR was not imported and protein associated with mitochondria was sensitive to trypsin. Using metabolically labeled COS-1 cells transfected with wild-type or His-tagged StAR constructs, we confirmed that wild-type StAR preprotein was imported and processed by mitochondria, whereas His-tag StAR remained largely cytosolic and unprocessed. To determine whether cytosolic factors are required for StAR action, we developed an assay system using washed mitochondria isolated from bovine corpora lutea and purified recombinant His-tag StAR proteins expressed in Escherichia coli. Recombinant His-tag StAR stimulated pregnenolone production in a dose- and time-dependent manner, functioning at nanomolar concentrations. A point mutant of StAR (A218V) that causes lipoid adrenal hyperplasia was incorporated into the mitochondrial electrochemical gradient and protein import.

His-tag protein. This mutant was steroidogenically inactive in COS-1 cells and on isolated mitochondria. Our observations conclusively document that StAR acts on the outside of mitochondria, independent of mitochondrial import, and in the absence of cytosol. The ability to produce bioactive recombinant StAR protein paves the way for refined structural studies of StAR and StAR mutants.

Steroidogenic acute regulatory protein (StAR)\(^1\) is essential for efficient adrenal and gonadal steroidogenesis (1, 2). The evidence that StAR is critical for steroid hormone production has been derived, in part, from the demonstration that mutations in the StAR gene cause congenital lipid adrenal hyperplasia, a disease in which adrenal and gonadal steroid synthesis is severely impaired at the cholesterol side-chain cleavage step (1, 3, 4). Targeted disruption of the murine StAR gene results in a phenotype in the homozygous null mutants similar to that of congenital lipid adrenal hyperplasia in humans (5). Although these observations demonstrate that StAR plays a key role in steroidogenesis, the mechanism of the action of the protein remains obscure. The protein is believed to stimulate the movement of cholesterol from the mitochondrial outer membrane to the inner membrane where cytochrome P450\textsubscript{sec}, the enzyme that catalyzes the first step in steroid hormone synthesis, resides.

StAR expression is acutely regulated by trophic hormones (6, 7). Cyclic AMP influences StAR gene expression, like many proteins important for steroidogenesis (8–10), and enhances StAR activity by triggering posttranslational modifications (11–13).

The NH\textsubscript{2} terminus of StAR is characteristic of proteins destined to be imported into mitochondria (14–16). Radiolabeled pre-StAR is incorporated into isolated mitochondria and processed to the mature 30-kDa protein (15, 17). Immunoelectron microscopy localized StAR to the intermembranous face of cristae and the intermembranous space (17). Based on these observations it has been suggested that contact sites form between the outer and inner membranes during the import of StAR into mitochondria, permitting cholesterol to move to P450\textsubscript{sec} on the inner membranes. Recent reports identifying StAR in isolated mitochondrial membrane contact sites (18), and the inhibition of StAR action by compounds that disrupt the mitochondrial electrochemical gradient and protein import. These authors contributed equally to this work.

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\†† The abbreviations used are: StAR, steroidogenic acute regulatory protein; His-tag StAR, 6-histidine-tagged N-62 StAR; m-CCCP, carbonyl cyanide m-chlorophenylhydrazone; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis.
Mechanism of StAR Action

(19), supported the notion that StAR import is obligatorily linked to the stimulation of steroidogenesis (20). However, we previously reported that NH2-terminal deletion mutants of StAR had steroidogenic activity equivalent to wild-type StAR, despite the fact that they could not enter into mitochondria (20).

To shed light on the mechanism of StAR action and resolve apparent discrepancies in the models of how StAR works, we embarked upon experiments to produce biologically active recombinant StAR and study its activity on isolated mitochondria. We elected to produce human StAR proteins lacking the mitochondrial import sequence in bacteria, and to incorporate a 6-histidine-tagged N-62 StAR (His-tag StAR) to facilitate their purification. By using a construct from which the first 62-amino acid residues had been deleted (N-62), we could examine the action of StAR independent of the protein import process. Here we show that the His-tag StAR proteins have biological activity equivalent to wild-type StAR and that they act without being imported into mitochondria. We further demonstrate that purified His-tag recombinant protein acts directly on mitochondria to stimulate pregnenolone synthesis.

EXPERIMENTAL PROCEDURES

StAR cDNA Constructs for Transfection—To produce both NH2- and C-terminal His-tag StAR proteins lacking the first 62 amino acids, a cDNA encoding human StAR sequences from amino acid 63–285 was cloned into the pQE-30 (21) and pET24 vectors (see below), respectively. His-tag sequences were subcloned into pSV-SPORT-1. The A218V mutation, which causes congenital adrenal hyperplasia (4), was introduced by site-directed mutagenesis into the His-tag StAR construct (20). DNA sequences of all constructs were verified before use (20).

Cell Culture and Evaluation of Steroidogenic Activity—COS-1 cells were grown to 50–80% confluence and transfected using 10 µg of LipofectAMINE™ (Life Technologies, Inc.) with 1 µg of either an empty pSV-SPORT-1 plasmid, wild-type or His-tag StAR cDNAs in pSV-SPORT-1 and 1 µl of a plasmid-directing expression of a fusion protein consisting of human P450scc adrenodoxin and adrenodoxin reductase (1, 3, 22), kindly provided by Dr. Walter L. Miller, University of California, San Francisco. Culture media were changed after 24 h, and some cultures received 5 µg/ml 22R-hydroxycholesterol. COS-1 cells were scraped after 36 h, and were sonicated for 5 s. Disrupted cells were centrifuged at 600 × g for 10 min, and supernatants were spun at 13,000 × g for an additional 20 min to isolate the cytoplasm and the mitochondrial-enriched subcellular fraction. In some cases the mitochondrial fraction was treated with proteinase K (15 µg/ml) at 4 °C for 90 min. After protein assay (Pierce), equal aliquots of protein from each fraction were pre-cleared with 30 µl of protein A-agarose in a total volume of 1 ml of RIPA buffer (50 mM Tris-HCl, 1% Nonidet P-40, 0.1% deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 5 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) for 10 min, incubated with 10 µl anti-human StAR antibody and 30 µl protein A-agarose at 4 °C on a rocking platform overnight. Immunocomplexes were washed four times by resuspension in 500 µl of RIPA buffer and collected by centrifugation. Pellets were resuspended in 50 µl of 2 × SDS sample buffer and then subjected to SDS-PAGE and fluorography. In some experiments protein bands corresponding to wild-type StAR preprotein, wild-type mature protein, and C-His-tag StAR were excised from the gels, and radioactivity was quantified by scintillation counting.

For pulse-chase experiments, the COS-1 cells transfected with plasmids expressing expression of 22R-HIPPA, the C-His-tag StAR were incubated in methionine/cysteine-free Dulbecco’s minimal essential medium without serum for 30 min and then labeled with [35S]methionine/cysteine (200 µCi/ml) for 20 min. After labeling, cells were changed into Dulbecco’s minimal essential medium containing 4 mM methionine and 4 mM cysteine and were chased for the indicated times. Cells were then scraped into 500 µl of RIPA buffer. Equal aliquots of protein from each cell extract were subjected to immunoprecipitation as described above.

Immunocytochemistry—COS-1 cells grown on uncoated coverslips were transfected with expression plasmids for wild-type and His-tag StAR and processed for immunocytochemistry (21). Non-specific antibody binding was blocked by incubation with 1.5% normal goat serum and then cells were incubated with primary antibody (1:500 dilution). Controls consisted of substitution of the primary antibody with preimmune serum used at a dilution equal to that of the primary antibody.

Immunoelectron Microscopy—COS-1 cells were fixed in 0.2 M cacodylate buffer (pH 7.4) containing 2% parafomaldehyde and 0.2% glutaraldehyde for 1 h at 4 °C, dehydrated in an increasing series of ethanol treatments, and embedded in LR Gold resin (Polysciences, West Grove, PA). Grids containing sections were incubated in blocking buffer (Tris-buffered saline (20 mM Tris, pH 7.5, 0.5 mM NaCl) containing 10% normal goat serum) for 30 min at room temperature and were subsequently incubated in blocking buffer containing a 1:400 dilution of rabbit anti-StAR antiseraum or preimmune rabbit serum overnight (~16 h) at 4 °C. Following three washes in Tris-buffered saline, grids were incubated in blocking buffer containing 18 nm colloidal gold conjugated to a goat anti-rabbit IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA) diluted 1:50. After three more washes in Tris-buffered saline, grids were fixed and counterstained with 1% osmium tetroxide followed by 7% aqueous uranyl acetate and Reynolds’s lead citrate. Specimens were observed and photographed using a Phillips transmission electron microscope.

Mitochondrial Import Assays—Wild-type and mutant StAR proteins were synthesized using an SP6 TNT-coupled in vitro transcription/translation kit (Promega) following the manufacturer’s protocol for 2 h at 30 °C. Import assays of radiolabeled protein were performed as described previously (23). Selected reactions were performed in the presence of valinomycin (5 µg/ml), an electrochemical uncoupler that blunts mitochondrial import. Some mitochondria were treated with 1-mercaptoethanol (0.5 mM) in the absence or presence of 0.5% Triton X-100 following the import reaction. Trypsin was neutralized, using soybean trypsin inhibitor (5 mg/ml), and mitochondria were washed prior to sonication, followed by separation of proteins by SDS-PAGE and autoradiography.

Preparation of Purified StAR Protein—CDNA-encoding StAR lacking the amino-terminal 62-amino acid residues was amplified from a StAR cDNA clone using the following primers 5′-GGGAATTCCATGCGTGCAAGAGACTCTCTC-3′ and 5′-GCCCTGAGCCAGGGTTGCTCGAGG-GCCC-3′ and cloned into the pET-24 vector (Novagen) using XhoI and NdeI restriction sites, creating a C-His-tag StAR cDNA. The C-His-tag StAR A218V mutant was constructed using identical primers and mutant cDNA template. These constructs were expressed in BL-21(DE3) cells (Novagen) at 37 °C using 1 µm isopropyl-ß-D-galactoside for 4 h, bacterial cell pellets were sonicated in 300 mM NaCl, 50 mM Na2HPO4, 20 mM Tris, pH 7.4, 10 mM 2-mercaptoethanol, and 0.5 mM PMSF. Bacterial lysates were centrifuged at 13,000 × g for 30 min and incubated with nitritolactriacetic acid-cholate resin (Qiagen) for 30 min. The resin was washed with lysis buffer (minus PMSF) and then lysis buffer containing 20 mM imidazole, until washes demonstrated A260 < 0.01. His-tag StAR was eluted using 250 mM imidazole, dialyzed into a solution consisting of 50 mM KCl, 10 mM HEPES, 1.0 mM dithiothreitol, 0.1 mM PMSF, and stored frozen in this buffer.

Assay of Steroidogenic Activity Using Isolated Mitochondria and Purified StAR Proteins—Bovine corpora lutea were homogenized at 4 °C using a buffer consisting of 0.25 mM sucrose, 10 mM Tris, pH 7.4, 1.0 mM EDTA, 1.0 mM dithiothreitol, 0.1 mM PMSF, 1.0 mM/l aprotinin, and 0.5 µg/ml pepstatin A. Bovine plasma albumin. Homogenates were spun at 600 × g for 10 min, and supernatants were spun at 13,000 × g for an additional 20 min to obtain the mitochondrial-enriched subcellular fraction. The pellets were washed with homogenization buffer and subjected to the centrifugation steps described above a second time. The final mitochondrial pellets were resuspended in a modified homogenization buffer that contained only 1 mg/ml bovine serum albumin and were used immedi-
ately or stored at −80 °C. Preliminary experiments established that freezing of the mitochondria did not impair StAR protein import and processing or steroidogenic activity. Isolated mitochondria were incubated at a concentration of 0.75–2.0 mg of protein/ml in 123 mM KCl, 5 mM MgCl₂, 10 mM KH₂PO₄, 5 mM HEPES, 250 ng/ml of the 3b-hydroxysteroid dehydrogenase inhibitor trilostane, 100 μM GTP, 10 mM isocitrate, 200 μM cholesterol, and the indicated concentrations of purified StAR protein. Incubations were conducted at 37 °C for the indicated times. As a control, purified StAR protein was heat-denatured at 100 °C for 5 min. Incubations were terminated by flash freezing of samples. Pregnenolone was quantitated by radioimmunoassay (3, 20).

In some experiments, [3H]-cholesterol (0.005 μCi/μl) was included in the incubation reactions to assess the conversion of exogenous substrate into pregnenolone. Samples were then flash frozen and extracted with 1.5 ml petroleum ether. The organic phases were dried under nitrogen gas and resuspended in chloroform/methanol (2:1, v/v) and loaded onto silica gel G thin layer plates, which were developed in hexane/ethyl acetate (7:3, v/v). Pregnenolone bands detected with iodine vapor were collected, and radioactivity was quantitated using a scintillation counter (24).

RESULTS

His-tag StAR Proteins Are Biologically Active—Before producing recombinant protein for in vitro studies, we documented that the His-tag did not affect the activity of StAR in COS-1 cells transfected with an expression plasmid for the human cholesterol side-chain cleavage system (1, 20, 25). Both the NH₂- and the C-His-tag StAR proteins stimulated pregnenolone secretion by COS-1 cells to the same extent as wild-type StAR (Fig. 1A). Moreover, the His-tag StAR containing the A218V mutation, which inactivates full-length StAR, was devoid of steroidogenic activity. Expression of each of the His-tag StAR proteins was documented by Western blot analysis (Fig. 1B). Wild-type StAR preprotein and mature protein were identified in transfected COS-1 cells and only a single protein of 32 kDa was identified in COS-1 cells transfected with
the His-tag StAR constructs. The apparent molecular mass of the His-tag StAR in the SDS-PAGE system (32 kDa) is greater than the calculated molecular mass of approximately 26 kDa, which may reflect posttranslational modification of the protein. The level of expression of the NH$_2$-His-tag StAR A218V mutant was approximately one-third of that for the NH$_2$-His-tag “wild-type” protein, probably reflecting relative instability of this mutant.

**FIG. 4.** In vitro import assay of wild-type and C-His-tag StAR proteins. $^{35}$S-labeled wild-type StAR and C-His-tag StAR proteins produced by an in vitro transcription/translation system were incubated with isolated yeast mitochondria. Wild-type StAR is imported and processed yielding trypsin-resistant mature protein (A). Uncoupling of the mitochondrial electrochemical gradient with valinomycin blocks StAR import. The C-His-tag StAR protein is not imported and is digested by trypsin treatment of the mitochondria (B).

**FIG. 5.** The C-His-tag StAR is not imported into mitochondria or processed in COS-1 cells. Cytosol and mitochondria-enriched fractions of COS-1 cells transfected with expression plasmids for wild-type (A, B, and C) or the C-His-tag StAR (D) were prepared after metabolic labeling. Some cultures were treated with either m-CCCP (B), which blocks import, or 1,10-phenanthroline (C), which blocks processing but not import, as described under “Experimental Procedures.” Aliquots were incubated with or without proteinase K before immunoprecipitation and fluorography. Mito, mitochondria.

**FIG. 6.** Pulse-chase experiments carried out with COS-1 cells transfected with wild-type and C-His-tag StAR expression plasmids. After transfection, COS-1 cells were pulse-labeled with [35S]methionine/cysteine for 20 min and then chased with an excess amount of cold methionine and cysteine for indicated times followed by immunoprecipitation of the proteins and SDS-PAGE. Shown are autoradiograms of wild-type StAR (A) and C-His-tag StAR (B) and the plot of immunoprecipitated radioactivity in wild-type preprotein, mature protein, and C-His-tag StAR (C).
The His-tag StAR Proteins Are Not Imported into Mitochondria—At the light microscope level, wild-type StAR was located in vermiform organelles representing mitochondria (Fig. 2A). The C-His-tag StAR protein was diffusely distributed in the cytosol of the transfected COS-1 cells without selective mitochondrial localization (Fig. 2B). Cells transfected with empty plasmid did not stain for StAR (Fig. 2C). Immunoelectron microscopy confirmed that the C-His-tag StAR protein was distributed throughout the cytoplasm and excluded from mitochondria (Fig. 3B), whereas wild-type StAR protein accumulated inside the mitochondria (Fig. 3A). Preimmune serum demonstrated negligible staining (Fig. 3C).

Wild-type StAR preprotein was imported into isolated yeast mitochondria and processed to the mature 30-kDa form, becoming insensitive to trypsin (Fig. 4A). However, StAR protein was degraded by trypsin following Triton X-100 permeabilization of the mitochondria. Treatment with valinomycin, which uncouples the mitochondrial electrochemical gradient, blocked wild-type StAR import. Although some of the C-His-tag StAR protein was associated with mitochondria, it was completely sensitive to trypsin in the absence of detergent, indicating that C-His-tag StAR was not imported by the mitochondria (Fig. 4B). Similar results were obtained when bovine corpus luteum mitochondria were used in place of yeast mitochondria (data not shown).

Transfected cells were labeled with [35S]methionine/cysteine and cytosol and organelle-enriched fractions were separated by differential centrifugation. In some cases the organelle frac-

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**Fig. 7.** Preparation of purified His-tag StAR proteins and in vitro pregnenolone assay for StAR activity. A, the indicated His-tag StAR proteins were purified from *E. coli*, resolved by SDS-PAGE, and identified by Coomassie blue staining. Each lane contained 5 μg of His-tag StAR protein. The loading of the lane containing the C-His-tag StAR mutant was adjusted to compensate for purity of the preparation to give 5 μg of the 30-kDa mutant protein. B, time course and dose response of C-His-tag StAR-stimulated pregnenolone production from isolated bovine corpus luteum mitochondria, as measured by radioimmunoassay. C, heat-denatured C-His-tag StAR did not stimulate pregnenolone synthesis in isolated mitochondria. D, effects of C-His-tag StAR and the C-His-tag StAR A218V mutant on mitochondrial pregnenolone synthesis. The amount of C-His-tag StAR mutant added was adjusted for the contamination by other proteins such that the final mutant concentration was 5 μM. Results of representative experiments are presented. Each experiment was replicated on at least two separate occasions.
TABLE I
C-His-tag STAR stimulates the conversion of [3H]-cholesterol into [3H]-pregnenolone

| Treatment                  | [3H]-Pregnenolone formation | Exp. 1 | Exp. 2 | Exp. 3 |
|----------------------------|----------------------------|--------|--------|--------|
| Control                    | cpm/μg protein (90 min)    | 682    | 236    | 122    |
| C-His-tag STAR             |                            | 2676   | 1602   | 603    |
| C-His-tag STAR A218V mutant|                            | 172    |        |        |

Investigations into the mechanism of action of STAR have failed to reveal to date how the protein promotes steroidogenesis. Indeed, proof that STAR acts directly on mitochondria, rather than via an intermediary pathway, has not been forthcoming. King et al. (17) reported stimulation of pregnenolone synthesis by isolated mitochondria incubated with STAR-expressing COS cell lysates. Because these experiments did not employ purified protein, the possibility remained that the STAR activity in the cell lysates was dependent upon factors present in the COS cell cytosol. This potential explanation deserved serious consideration in view of the evidence that import of STAR is not required for its steroidogenic activity. Our data, using purified recombinant STAR and isolated mitochondria, strongly suggest a direct effect of STAR on the organelles. Furthermore, our studies provide a system and a rationale for a search for the mitochondrial outer membrane molecules through which STAR acts to increase cholesterol metabolism.

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ing a direct effect of the protein on mitochondria. Because we do not know whether all of the recombinant protein was bio-
logically active, it is possible that StAR functions at much lower concentrations. Moreover, the in vitro assay system we used may not be optimal for documenting the steriodogenic activity of StAR. Thus, the minimally effective concentration of StAR needed to stimulate mitochondrial pregnenolone synthesis in vivo cannot be estimated.

We recently presented evidence that phosphorylation of StAR at serine residue 195 by protein kinase A is essential for maximal steriodogenic activity (11), consistent with earlier studies demonstrating that StAR is a phosphoprotein (13, 26). The recombinant proteins we employed were not phosphorylated. However, treatment with protein kinase A catalytic sub-
unit and ATP did not increase the steriodogenic activity of recombinant His-tag StAR. This finding was not unexpected, because we have found that removal of the NH2-terminal 62 amino acids overcomes the negative impact of mutating serine residue 195 to a nonphosphorylatable alanine residue (27), indicating that phosphorylation either increases the activity of wild-type StAR by retarding mitochondrial import or overcomes a negative influence of the NH2 terminus.

In the absence of cytochemical and biochemical evidence for targeting of His-tag StAR to mitochondria, we speculate that StAR stimulates delivery of cholesterol to the mitochondrial inner membranes as a result of either a few high affinity stable interactions with the cytoplasmic face of the mitochondria or as a consequence of transient interactions. What could be the nature of these interactions? First, it is notable that mitochondria from nonsteroidogenic cells (e.g. COS-1 cells) respond to StAR. Therefore, StAR’s action presumably involves molecules that are not uniquely expressed in steriodogenic tissues. Recent data implicate the peripheral benzodiazepine receptor, located on the outer mitochondrial membrane, in the pathway of StAR-mediated cholesterol translocation (28). Although the peripheral benzodiazepine receptor remains an attractive candi-
date for the mitochondrial StAR-interacting protein, alternativa-
tives should be entertained. One interesting possibility is that StAR participates in a process involving GTP hydrolysis. GTP hydrolysis is known to be important for substrate delivery to cyochrome P450sec (25), and GTPases participate in mem-
brane trafficking and membrane fusion events (29, 30). Re-
markably, there is homology between StAR and members of the RhGAP family of GTPase activating proteins, although the homology does not encompass the catalytic domain. This homology raises the possibility that StAR triggers a change in the structure of mitochondrial membranes through an effect on a GTPase.

Pulse-chase studies suggest that the short functional life of wild-type StAR, predicted from studies demonstrating rapid inhibition of steriodogenesis by drugs that inhibit protein synthesis (e.g. cycloheximide), is not because of rapid destruction of the protein. Rather, the protein’s short functional half-life appears to result from its rapid import into mitochondria. Orme-Johnson et al. (26) reported that the half-life of the StAR preprotein in rat adrenal cortex cells is 3–4 min. Our studies, carried out in a system that floods COS-1 cells with StAR preprotein, arrived at a longer half-life (15 min) that may be ascribed to saturation of the import process or possibly to a more efficient import system operating in cells with endogenous steriodogenic activity. We propose that mitochondrial import terminates StAR action, an interpretation that is entirely consistent with the idea that StAR acts outside of the mitochondria.

The conclusion that StAR stimulates steriodogenesis by acting on the outside of the mitochondria conflicts with the original notion that StAR must be imported to exert its function. The more recent observation that StAR is localized to contact sites between outer and inner mitochondrial membranes (18) is likely to reflect a role of this machinery in StAR import. The relevance of this finding to the steriodogenic function of StAR is entirely speculative, although our data strongly supports the notion that StAR import and StAR-mediated cholesterol transport are distinct processes.

Our studies have several other important implications. First, we have demonstrated a biological activity of a recombinant StAR protein that can be produced in E. coli in large quantities and easily purified, paving the way for protein crystallization and refined structural studies. Second, the activity of recombinant StAR protein on isolated mitochondria will permit further elucidation of its mechanism of action, which has escaped elucidation in experiments using intact cells.

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