Steroidogenic Acute Regulatory Protein Binds Cholesterol and Modulates Mitochondrial Membrane Sterol Domain Dynamics*

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The steroidogenic acute regulatory protein (StAR) mediates the rate-limiting step of steroidogenesis, delivery of cholesterol to the inner mitochondrial membrane. However, the mechanism whereby cholesterol translocation is accomplished has not been resolved. Recombinant StAR proteins lacking the first N-terminal 62 amino acids comprising the mitochondrial-targeting sequence were used to determine if StAR binds cholesterol and alters mitochondrial membrane cholesterol domains to enhance sterol transfer. First, a fluorescent NBD-cholesterol binding assay revealed 2 sterol binding sites (K_d values near 32 nM), whereas the inactive A218V N-62 StAR mutant had only a single binding site with 8-fold lower affinity. Second, NBD-cholesterol spectral shifts and fluorescence resonance energy transfer from StAR Trp residues to NBD-cholesterol showed (i) close molecular interaction between these molecules (R_{nm} = 33 Å) and (ii) sensitized NBD-cholesterol emission from only one of the two sterol binding sites. Third, circular dichroism showed that cholesterol binding induced a change in StAR secondary structure. Fourth, a fluorescent sterol transfer assay that did not require separation of donor and acceptor mitochondrial membranes demonstrated that StAR enhanced mitochondrial sterol transfer as much as 100-fold and induced/increased the formation of rapidly transferable cholesterol domains in isolated mitochondrial membranes. StAR was 67-fold more effective in transferring cholesterol from mitochondria of steroidogenic MA-10 cells than from human fibroblast mitochondria. In contrast, sterol carrier protein-2 (SCP-2) was only 2.2-fold more effective in mediating sterol transfer from steroidogenic cell mitochondria. Taken together these data showed that StAR is a cholesterol-binding protein, preferentially enhances sterol transfer from steroidogenic cell mitochondria, and interacts with mitochondrial membranes to alter their sterol domain structure and dynamics.

Research on the individual pathways for intracellular cholesterol trafficking has dramatically accelerated in recent years (for review, see Refs. 1–6). Among the more interesting pathways for intracellular cholesterol movement is the mechanism whereby cholesterol is transferred to (for review, see Refs. 7–10) and within (for review, see Refs. 11–14) mitochondria wherein the cholesterol side chain is cleaved to synthesize the first steroid of the steroidogenic pathway (15). Cholesterol delivery to mitochondria may be considered in two parts.

The first step is the transfer of cholesterol to the mitochondrial outer membrane from other intracellular sites, primarily lipid droplets and plasma membrane. This transfer of cholesterol to the mitochondria may be mediated by proteins such as sterol carrier protein-2 (SCP-2)1 (for review, see Refs. 7–10). The observation of normal phenotype and normal serum steroid (testosterone, progesterone, corticosteroids) levels in SCP-2 gene-ablated mice suggests that SCP-2 is not essential for gonadal and adrenal steroidogenesis (16, 17) and that additional/compensatory mechanism(s) must exist for cholesterol transfer to the mitochondria (11, 14).

The rate-limiting step in gonadal and adrenal steroidogenesis is the transfer of cholesterol from the relatively cholesterol-rich outer mitochondrial membrane to the cholesterol-poor inner mitochondrial membrane (for review, see Refs. 11–14). This regulated step in steroid production is catalyzed by the steroidogenic acute regulatory protein (StAR) (for review, see Refs. 11, 12, and 14). The importance of StAR in steroidogenesis is underscored by studies of mutations in humans and StAR gene ablation in mice. Humans carrying mutations that inactivate StAR exhibit markedly reduced gonadal and adrenal steroidogenesis, a condition that leads to accumulation of cholesterol in lipid droplets and a disease named congenital lipid adrenal hyperplasia (18, 19). Ablation of StAR gene in mice also results in a phenotype of impaired steroidogenesis and adrenal lipid accumulation (20).

The mechanism whereby StAR mediates cholesterol transfer from the outer to the inner mitochondrial membrane remains elusive. An early hypothesis favored stimulation of cholesterol movement as a result of StAR being physically transported through the outer to the inner mitochondrial membranes (for review, see Ref. 11). In this model the StAR translocation process was envisioned to promote formation of contact sites between outer and inner mitochondrial membranes, thereby allowing cholesterol to flow down a chemical gradient from the outer to the inner membranes. Consistent with this hypothesis,

1 The abbreviations used are: SCP-2, sterol carrier protein-2; StAR, steroidogenic acute regulatory protein; START, StAR-related lipid transfer; NBD-cholesterol, 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-chole-3β-ol; FRET, fluorescence resonance energy transfer; CMC, critical micellar concentration; PIPES, 1,4-piperazinediethanesulfonic acid.
immunoelectron microscopy localized StAR in the intermembranous space and the intermembranous face of the inner mitochondrial membrane (21), whereas immunoblotting of isolated mitochondrial membrane contact sites revealed the presence of StAR (22). However, recent studies from our laboratories favor another mechanism whereby StAR acts like most other typical lipid transfer proteins (23, 24). This model is based on observations that StAR is a cholesterol transfer protein (23) and acts on the outside of mitochondria (24). StAR molecules lacking a mitochondrial targeting sequence that were shown to be incapable of mitochondrial importation were able to stimulate conversion of cholesterol into steroid hormones in intact cells and isolated mitochondria (24). A third hypothesis has emerged from the characterization of a StAR-related lipid-transfer (START) domain found in a related protein, MLN64 (35% identity with the StAR START domain) (25–27). X-ray crystallography and molecular modeling revealed a hydrophobic tunnel in the MLN64 START domain capable of accommodating a cholesterol molecule (26). These data suggested that StAR could be a cholesterol carrier from the outer to the inner mitochondrial membrane.

To explore the properties of StAR and its action on mitochondria predicted by the existing models of StAR function, we performed experiments to address the following questions. Does StAR bind cholesterol? Do interactions with sterol affect StAR structure? Does StAR enhance mitochondrial sterol transfer by altering mitochondrial cholesterol domain dynamics? The data presented herein yielded the following new insights. (i) Fluorescence resonance energy transfer demonstrated the close molecular interaction between StAR and bound sterol. (ii) A fluorescent sterol binding assay established that StAR has two sterol binding sites with high affinity (ns Kd values). A functionally inactive StAR mutant had much lower affinity for cholesterol. (iii) By application of a fluorescent sterol transfer assay, it was shown that StAR dramatically enhanced mitochondrial sterol transfer and altered the sterol domain structure of the mitochondrial membrane.

**EXPERIMENTAL PROCEDURES**

**Materials**—NBD-cholesterol (22-N-(7-nitrobenz-2-oxa-1,3-diazol-4-ylamino)-23,24-bisnor-5-cholen-3α-hydroxy-5(25)-ene, 20% Tween 20, 2 mL) was obtained from Molecular Probes Inc. (Eugene, OR). Dehydroergosterol was prepared as reported previously (28, 29). GTP, isotocrit, cholesterol, N-acetyl-l-tryptophan amide, and guanidine-HCl were obtained from Sigma.

**Recombinant Proteins**—N-62 StAR containing a C-terminal 6-histidine tag (His6 tag) and C-terminal His6 tag N-62 StAR mutant with an A218V amino acid replacement were expressed in Escherichia coli and purified as described previously (23, 24). N-62 StAR concentration was 0.13 mg/ml phosphate-buffered saline (50 mM sodium phosphate, 75 mM NaCl, pH 7.4) containing 0.01% Tween 20. Since similar NBD-cholesterol binding and sterol transfer data were obtained with N-62 StAR in 50 mM sodium phosphate, 390 mM NaCl, pH 8.0, the presence of a small amount of Tween (diluted several orders of magnitude in the binding assay and sterol transfer assay) in the stock protein solution did not affect the results. The A218V mutant N-62 StAR was 4.58 mg/ml in 50 mM sodium phosphate, 300 mM NaCl, pH 8.0. Both proteins were stored at 4 °C for immediate use or stored frozen at −80 °C. The A218V mutation was chosen because it functionally inactivates the protein and causes congenital lipid adrenal hyperplasia in humans (19). The A218V mutation did not result in global destabilization of the StAR molecule but did induce a modest change in StAR secondary structure as indicated by analysis of circular dichroic spectra. N-62 StAR had 24 ± 1.4% α-helix, 20 ± 1.4% β-sheet, 23 ± 1.4% turn, and a Tm (mid-point of thermal denaturation) of 45 ± 0 °C (n = 2). A218V N-62 StAR had 23 ± 1.6% α-helix, 27 ± 5% β-sheet, 19 ± 2% turn, and a Tm of 50 ± 4.7 °C (n = 3). Human recombinant SCP-2, isolated as described previously (30), was stored at 1 mg/ml in 10 mM potassium phosphate, pH 6.8, 2.5 mM dithiothreitol at −80 °C. Both proteins were stable stored at −80 °C under these conditions (24, 30).

**Cell Culture**—MA-10 mouse Leydig tumor cells were generously provided by Dr. M. Ascoli (Dept. of Pharmacology, University of Iowa College of Medicine). Cells were cultured as described by Clark et al. (31). Normal human skin fibroblasts (CWN), generously provided by Drs. Edward Neufeld and Peter Pentchev (National Institutes of Health, Bethesda, MD), were cultured as described previously (32). For isolation of mitochondria to be used in fluorescent sterol transfer assays (see "Fluorescence Sterol Transfer Assays"), MA-10 cells or CWN fibroblasts were cultured as above with the following modifications. The cells were grown and incubated on 20 × 20-cm trays (Nalge-Nunc, Milwaukee, WI). To obtain mitochondrial sterol acceptor membranes, the cells were cultured in serum containing medium supplement with cholesterol (15 µg/ml) for a total of 4 days. To obtain mitochondrial sterol donor and acceptor membranes, cells were cultured in serum-containing medium supplemented with 15 µg/ml cholesterol (for acceptor mitochondria) or dehydroergosterol (for donor mitochondria) for a total of 4 days. Cells were then washed with phosphate-buffered saline followed by isolation of mitochondria.

**Isolation of Mitochondria for Sterol Transfer Assay**—MA-10 and CWN cell mitochondria were isolated as described earlier for fibroblasts (10). Purification of mitochondria was estimated by Western blotting (10). Isolated mitochondria were suspended in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4 buffer. The stock mitochondrial membrane donor and acceptor protein concentrations were 0.18–0.25 mg/ml and 0.3–0.5 mg/ml, respectively. Mitochondria stored at 4 °C were used immediately for sterol transfer assay or stored frozen at −80 °C. Freezing the mitochondria did not adversely affect StAR (24) or SCP-2 (10) activity in sterol transfer. Protein concentration was determined by Lowry (33). A218V mutant N-62 StAR was 4.58 mg/ml in 50 mM sodium phosphate, 300 mM NaCl, pH 7.4 buffer. The stock mitochondrial membrane donor and acceptor protein concentrations were 0.18–0.25 mg/ml and 0.3–0.5 mg/ml, respectively. Mitochondria stored at 4 °C were used immediately for sterol transfer assay or stored frozen at −80 °C. Freezing the mitochondria did not adversely affect StAR (24) or SCP-2 (10) activity in sterol transfer. Protein concentration was determined by Lowry (33).

**Fluorescence Assay for Sterol Binding to StAR**—NBD-cholesterol or dehydroergosterol binding to StAR was monitored by steady-state fluorescence intensity using an ISS photon-counting fluorimeter (ISS, Champaign, IL) in the L-format and ratio mode to correct for wavelength and lamp intensity variations in the excitation system. The light source was a 300 watt xenon arc lamp. The temperature was maintained with a 101ES cooling system (Fisher). NBD-cholesterol was excited at 473 nm; emission was scanned from 500–600 nm; excitation and emission bandpasses were 4 and 4 nm, respectively. Dehydroergosterol excitation was at 324 nm, and fluorescence emission was scanned from 200 to 500 nm using excitation and emission bandpasses of 4 and 4 nm, respectively. The sample absorbance for NBD-cholesterol and dehydroergosterol at 324 nm was kept below 0.15 to avoid the inner filter effect. Light scatter was reduced by the use of dilute samples and appropriate low fluorescence cutoff filters in the emission path.

StAR binding affinity for NBD-cholesterol was determined at 37 °C as previously described for SCP-2 binding of NBD-cholesterol (34), with the following modifications. A 40-µl aliquot of N-62 StAR protein stock solution (0.13 mg/ml in 50 mM sodium phosphate, 75 mM NaCl, 0.01% Tween 20, pH 7.4) was diluted to 2 ml with 25 mM potassium phosphate buffer, pH 7.4. At the final dilutions used for the NBD-cholesterol binding assay, the concentration of Tween 20 (1.5 × 10⁻⁶ M) had no effect on NBD-cholesterol fluorescence in the presence or absence of StAR and did not affect NBD-cholesterol binding to StAR. In contrast, 0.1% Tween 20 (10-fold higher than normally used in the binding assay) significantly altered NBD-cholesterol fluorescence and binding to StAR. The integrated NBD-cholesterol emission was measured after each addition of the ligand. Each emission spectrum, corrected for the background and blank, was used to construct the binding isotherm.

Sigmoid ligand binding curves were analyzed by the Lineweaver-Burk plot for allosteric enzymes (35), where enzyme activity, v, was substituted with binding, b, defined in Equation 1,

\[ b = \frac{(V_I/F_I)B_{max}}{(1 + \frac{V_I}{F_I})} \]  

in which \( V_I \) = fluorescence of bound NBD-cholesterol at concentration i of ligand, \( B_{max} \) = fluorescence of NBD-cholesterol bound at maximal binding (i.e. binding site saturation), \( B_{max} \) = maximum binding site concentration for the given amount of protein is given by Equation 2,

\[ B_{max} = nE_c \]  

where \( n \) is the number of binding sites on the protein, and \( E_c \) is the protein concentration in the assay.

\[ V_I/F_I = K[I]/[NBD-cholesterol]^{b+1} \]  

in which the values are defined as described earlier (36).
where $E$ is the fluorescence energy transfer efficiency, $R_o$ is the critical distance for 50% efficiency, and $R_{O/2}$ is the actual distance between donor and acceptor. The energy transfer efficiency was calculated from STAR-TRp-quenching as well as from sensitized emission of NBD-cholesterol.

$$E = R_o^6 / (R_o^6 + R_{O/2}^6)$$

where $F_{DA}$ and $F_D$ are the fluorescence intensities of STAR at 340 nm in the presence and in the absence of NBD-cholesterol, respectively; $F_{AD}$ and $F_A$ are fluorescent intensities of acceptor (NBD-cholesterol) at 530 nm in the presence and in the absence of donor (STAR), respectively; $\epsilon_{DA}$ and $\epsilon_D$ are the molar extinction coefficients of acceptor and donor, respectively, at the wavelength of excitation (280 nm) (40). $R_o$ was calculated from Equation 7,

$$R_o = 0.211 [k^2/n^2]^{3/4} Q_{av}(\lambda)^{1/6}$$

in angstroms (Å), with wavelength expressed in nm and $Q_{av}(\lambda)$, the overlap integral, expressed in m$^2$ cm$^{-1}$ s·molecule$^{-1}$, for STAR protein and J(λ) were calculated as previously described (41, 42). The orientation factor $k^2$ and refractive index $n$ were assumed to be 2/3 and 1.4, respectively, as for protein solutions (39).

**Stern-Volmer Fluorescence Quenching**—To examine the surface exposure of STAR Trp, increasing acrylamide (5 mM stock solution) was added as 20-μl aliquots to STAR (40 μM in 2 ml of phosphate-buffered saline). STAR Trp was excited at 295 nm, and decreasing emission with increasing acrylamide was measured at 333 nm. Parallel experiments were performed with N-acetyl-l-tryptophan amide to assess quenching of Trp when acrylamide was completely accessible to the quencher in aqueous solution. The exposure of STAR-bound NBD-cholesterol to the aqueous was examined with KI as a quencher. Successive 10-μl aliquots of 5 mM KI (made freshly with 0.1 mM sodium hydrosulfite) were added to 2 ml of phosphate-buffered saline containing 80 μM STAR preincubated with or without 50 mM NBD-cholesterol (from a 2 mM stock solution in dimethyformamide). Fluorescence emission of NBD-cholesterol was measured at 530 nm with excitation at 470 nm. Parallel experiments were performed with free NBD-cholesterol (from a 2 mM stock solution in dimethyformamide). Fluorescence intensities were corrected for dilution, scattering, and ion strength. Quenching data were analyzed by the Stern-Volmer equation, Equation 5 (43) as follows.

$$F/F = 1 + K_{SV}[Q]$$

where $F$ and $F'$ are the fluorescence intensities in the absence and the presence of quencher respectively. $Q$ and $K_{SV}$ are the quencher concentration and Stern-Volmer quenching constant, respectively.

**Circular Dichroism of STAR with and without Cholesterol**—Circular dichroic spectra of STAR (2.9 μM) in the presence of cholesterol (1 μM) were obtained basically as described earlier for SCP-2 (44) except that the buffer used was phosphate-buffered saline (147 mM, pH 7.4) containing 0.01% Tween. For guanidine-HCl-induced denaturation studies, STAR (2 μM) was preincubated with or without cholesterol (30 μM) for 15 min followed by denaturation in the presence of increasing (up to 4 M) guanidine-HCl in phosphate-buffered saline, 0.01% Tween. After denaturation for 12 h at room temperature, the circular dichroic spectra were measured with a J-710 spectropolarimeter (JASCO, Baltimore, MD) at 25°C. Spectra were recorded from 250 to 210 nm at 50 nm/min with a time constant of 1 s and a bandwidth of 2 nm. For each circular dichroic profile an average of four scans was obtained.

**Fluorescence Sterol Transfer Assays**—Sterol transfer between donor mitochondrial membranes and a 10-fold excess of acceptor mitochondrial membranes was determined exactly as described earlier for L-cell mitochondrial membranes (10, 46, 47). For all sterol transfer assays, the donor and acceptor mitochondrial membrane final concentrations were 7 and 70 μg/ml, respectively, in 10 mM PIPES, pH 7.4, buffer. Performing the sterol transfer assays in a buffer (123 mM KCl, 5 mM MgCl$_2$, 10 mM KH$_2$PO$_4$, 25 mM HEPES, 100 μM GTP, 10 mM isocitrate, pH 7.4) previously used to determine STAR-mediated cholesterol transfer and steroidogenesis in mitochondria (24) did not significantly affect the sterol transfer assay. The following control experiments were performed. (i) Stability of the mitochondrial membrane donor was determined by measuring dehydroergosterol polarization in donor mitochondrial membranes in the absence of acceptor mitochondrial membranes for 4 h at a temperature of 37°C. The dehydroergosterol fluorescence polarization was not significantly altered in donor mitochondria isolated from either normal (CWN) human fibroblasts or MA-10 cells. (ii) STAR or SCP-2 were added to donor (CWN or MA-10 cell) mitochondria and dehydroergosterol polarization in the mitochondrial membranes was measured. Neither STAR nor SCP-2 significantly altered the dehydroergosterol polarization in donor mitochondrial membranes in the absence of acceptor mitochondrial membranes. These observations were consistent with those previously determined with L-cell fibroblasts mitochondria (10, 46, 47).

Spontaneous sterol transfer from mitochondrial membrane donors was determined by measuring dehydroergosterol polarization in mitochondrial membrane donors for 10 min to obtain a base line, followed by the addition of a 10-fold excess of mitochondrial membrane acceptor and measurement of dehydroergosterol fluorescence polarization for 4 min.

The effect of STAR or SCP-2 (1.5 μM) on sterol transfer from mitochondrial donor membranes (contain dehydroergosterol) to mitochondrial acceptor membranes (initially containing only cholesterol, but no dehydroergosterol) was determined by measuring dehydroergosterol polarization in mitochondrial membrane donors for 10 min to obtain a base line followed by the addition of a 10-fold excess mitochondrial membrane acceptor plus 1.5 μM STAR or SCP-2.

**Fluorescence Sterol Transfer Assays**—Molecular sterol transfer was calculated from measurements of dehydroergosterol polarization change during the dehydroergosterol transfer between mitochondrial membrane donor/acceptor pairs exactly as described earlier (10, 46, 47). Establishment of standard curves and the mathematical basis for the
FIG. 1. Fluorescence emission spectra of NBD-cholesterol in the absence and presence of N-62 StAR or A218V N-62 StAR. Panel A, NBD-cholesterol emission spectra (excitation at 473 nm) in the absence (spectrum 1) and the presence of 6.35 nM StAR protein (spectra 2–8). NBD-cholesterol concentration was as follows. Spectrum 1, 10 nM; spectrum 2, 10 nM; spectrum 3, 15 nM; spectrum 4, 30 nM; spectrum 5, 40 nM; spectrum 6, 60 nM; spectrum 7, 80 nM; spectrum 8, 100 nM. Panel B shows the emission spectra of NBD-cholesterol (15 nM) in buffer alone (spectrum 1), in the presence of 6.35 nM mutant StAR (spectrum 2), and in the presence of 6.35 nM StAR (spectrum 3). The maximum emission wavelengths of the three spectra were 562, 545, and 530 nm, respectively. arbitrary units.

FIG. 2. Stern-Volmer quenching of N-62 StAR bound NBD-cholesterol and of N-62 StAR Trp residues. All conditions were as described under “Experimental Procedures.” Panel A, KI quenching of NBD-cholesterol in the absence (open circles) and presence (solid circles) of StAR. Panel B, acrylamide quenching of N-acetyl tryptophanamide (solid squares) as well as StAR Trp in the absence (filled diamonds) and presence (filled triangles) of cholesterol. Panel C, the inaccessible fraction was determined from extrapolation to high acrylamide for data from panel A plotted as F0/Fω = F0/Fω.

RESULTS

The Sterol Binding Site of StAR; NBD-Cholesterol Spectral Shifts and KI Quenching—The recombinant N-62 StAR protein employed herein contained the START- but not mitochondrial-targeting domain (24). As noted above, x-ray crystallography and molecular modeling of the MLN64 START domain revealed a hydrophobic tunnel capable of accommodating a cholesterol molecule (26). These data indicated that StAR might also have a relatively hydrophobic ligand binding site. To test this possibility, the spectral properties of NBD-cholesterol were examined in the absence and presence of StAR. NBD-cholesterol exhibited much lower fluorescence emission in the absence of StAR. NBD-cholesterol emission was tested in aqueous buffer 10 nM (Fig. 1A, spectrum 1) and 100 nM (Fig. 1B, spectrum 1). In both cases, NBD-cholesterol emission was at least 10-fold lower than in the presence of StAR. Furthermore, NBD-cholesterol in buffer had a maximal emission near 562 nm (Fig. 1B, spectrum 1), consistent with exposure of the NBD group of NBD-cholesterol to the aqueous environment. In contrast, in the presence of StAR (Fig. 1A, spectra 2–8, and B, spectrum 3), the NBD-cholesterol fluorescence intensity increased >10-fold. This increase was much smaller, <2-fold, for NBD-cholesterol in the presence of the A218V mutant N-62 StAR. Concomitant with increased NBD-cholesterol fluorescence emission on binding to StAR, the NBD-cholesterol emission maximum wavelength was significantly blue-shifted by 32 nm from 562 to 530 nm (Fig. 1A, spectrum 8 versus 1 and B, spectrum 3 versus 1).

To determine if binding to StAR protected NBD-cholesterol from an aqueous-quenching agent, KI, quenching of NBD-cholesterol by KI was examined. StAR significantly reduced the KI quenching of NBD-cholesterol fluorescence (Fig. 2A). The Stern-Volmer quenching constant, KSV, for NBD-cholesterol in the absence and presence of StAR was 5.7 and 2.4 M⁻¹, respectively. Thus, the NBD-group of NBD-cholesterol was 2.4-fold less accessible to the water-soluble quencher KI when the NBD-cholesterol was bound to StAR as compared with NBD-cholesterol in aqueous solution.

StAR Binding of NBD-Cholesterol; Affinity and Stoichiometry—Because of the low solubility of cholesterol in aqueous buffer, critical micellar concentrations (CMCs) of 25–40 nM (48), radioligand binding assays requiring the separation of bound from free radiolabeled cholesterol are complicated by the presence of micelles, aggregates, and/or proteinecholesterol co-aggregates that preclude accurate determination of StAR binding parameters (26). To circumvent these problems, a fluorescent sterol (NBD-cholesterol) binding assay was used to


**StAR Binds Cholesterol and Alters Cholesterol Domains**

**Experimental Procedures** revealed that StAR contained two "versus binding sites for NBD-cholesterol. A plot of the data as $F_{\text{max}}/F_i$ (50, 51). However, because of its higher quantum yield, NBD-occurring fluorescent sterol with similar CMCs as cholesterol was chosen for use in StAR sterol binding studies and determination of fluorescence resonance energy transfer from StAR Trp (see "Fluorescence Resonance Energy Transfer of StAR Trp to Bound NBD-Cholesterol").

**Binding of NBD-Cholesterol to the A218V Mutant Recombinant StAR**—The A218V mutation in StAR causes congenital lipid adrenal hyperplasia in humans. Likewise, the N-62 StAR mutant A218V has negligible steroidogenic activity in transfected COS-1 cells (24) and only weakly transferred cholesterol from liposomes to liver mitochondria (23). To determine if these differences in A218V StAR from StAR might also be associated with altered ligand binding ability, the interaction of A218V StAR with NBD-cholesterol was examined. The interaction of equivalent amounts of NBD-cholesterol interaction with A218V StAR differed significantly from that with native StAR in several respects, as follows.

First, A218V StAR only weakly shifted the emission maximum of NBD-cholesterol from 562 to 545 nm (Fig. 1B, *spectrum 1 versus spectrum 2*), as compared with the shift from 562 to 530 nm (Fig. 1B, *spectrum 1 versus spectrum 3*) exhibited by NBD-cholesterol binding to StAR. Thus, the A218V StAR mutation altered the polarity of the ligand binding site to make it less hydrophobic in the region sensed by the NBD-fluorophore of NBD-cholesterol.

Second, the maximal fluorescence intensity of NBD-cholesterol in A218V StAR was markedly reduced (Fig. 1B) as compared with NBD-cholesterol bound to StAR (Fig. 1, A and B). When this difference was evaluated over the concentration range from 5 to 100 nM NBD-cholesterol, the maximal fluorescence intensity of A218V StAR-bound NBD-cholesterol was up to 50-fold lower than that of NBD-cholesterol bound to StAR (Fig. 3B versus 3A).

Third, A218V StAR exhibited much lower affinity for NBD-cholesterol than StAR. Analysis of the NBD-cholesterol binding data in Fig. 3B as described under “Experimental Procedures” yielded a $K_d = 232$ nM for A218V StAR binding NBD-cholesterol. This $K_d$ was 7.2-fold lower than that obtained for StAR.

Fourth, the shape of the A218V StAR binding curve for NBD-cholesterol (Fig. 3B) was much less sigmoidal than that of StAR (Fig. 3A). The best linear regression analysis of the binding data as described under “Experimental Procedures” showed that A218V StAR bound NBD-cholesterol only at a single binding site.

In summary, the A218V mutant StAR maximally bound only half as much sterol as StAR and at 7.2-fold lower affinity than StAR. The observation that A218V StAR binds NBD-cholesterol less and more weakly may be rationalized on the basis of the structural information available. It is possible that the more bulky valine side chain provides steric hindrance to the cholesterol binding site in StAR based on comparison with the x-ray crystal structure of MLN64 (26).

**StAR Trp, Aqueous-accessible or Buried**—The N-62 StAR portion of complete StAR protein has 4 Trp and 3 Tyr residues (31). The fluorescence emission of StAR Trp can be selectively resolved from that of Tyr residues by excitation at 295 nm, at which wavelength Trp is preferentially excited. The localization of the StAR Trp residues as being buried or aqueous accessible was determined by Stern-Volmer quenching (see "Experimental Procedures"). StAR Trp residues were poorly accessible to acrylamide, an aqueous quenching molecule (Fig. 2B). Acrylamide quenching constants, $K_{SV}$, calculated from these data were 25.2 M$^{-1}$ for N-acytetyl tryptophanamide, consistent with earlier data (52), and 2.8 M$^{-1}$ for StAR Trp. Since the StAR Trp was nearly 10-fold less accessible to acrylamide quenching than N-acytetyl tryptophanamide, these data suggest that StAR Trp residues were buried in the protein and not

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**Fig. 3. Fluorescence ligand binding assay for N-62 StAR (panel A) and the A218V mutant StAR (panel B).** Panel A, StAR (4.2 nM) was titrated with 5–100 nM NBD-cholesterol in phosphate-buffered saline, pH 7.4, at 37 °C. The inset represents a linear plot of the sigmoid curve as $F_{\text{max}}/F_i$ versus 1/[NBD-cholesterol]$^2$. Best linear regression plots for the binding curve in panel A yielded $n = 2$ and an average $K_d$ of 32.4 ± 4.1 nM for the two sites. Panel B, the mutant StAR (126 nM) was titrated with 0.01 to 1 μM NBD-cholesterol under the same conditions as for the native StAR. The inset represents a linear plot of the hyperbolic curve as $F_{\text{max}}/F_i$ versus 1/[NBD-cholesterol]. Best linear regression plots of the binding curve in panel B yielded $n = 1$ and a $K_d$ of 231.2 nM.

determine the binding parameters of StAR. NBD-cholesterol was chosen for the following reasons. (i) The CMC of NBD-cholesterol is higher than that of cholesterol (49). This suggested that concentrations <CMC of NBD-cholesterol could be used to determine sterol binding affinity and stoichiometry of StAR. (ii) NBD-cholesterol emission maximum shifted in the presence of StAR, indicating close molecular interaction rather than coaggregation. (iii) NBD-cholesterol fluorescence intensity increased in the presence of StAR.

As shown in Fig. 3A, increasing the concentration of NBD-cholesterol (over the range 5–100 nM) while maintaining the concentration of StAR constant (4.2 nM) resulted in increased fluorescence emission of NBD-cholesterol. Analysis of the sigmoidal-shaped binding curve (Fig. 3A), as described under “Experimental Procedures,” revealed that StAR contained two binding sites for NBD-cholesterol. A plot of the data as $F_{\text{max}}/F_i$ versus 1/[NBD-cholesterol]$^2$ was nearly linear (Fig. 3A, inset), indicating that the affinities of the two binding sites for NBD-cholesterol were very similar. Fitting the data in Fig. 3A to a single exponential yielded an average $K_p = 35.8$ nM, whereas fitting the data to two exponentials yielded two very similar $K_d$ values of 32.5 and 47.4, respectively. When multiple binding curves were analyzed, the single exponential fit was better and yielded an average $K_d = 32.4 ± 4.1$ nM ($n = 3$). It should be noted that the high affinity of StAR for NBD-cholesterol was not due to the fact that NBD-cholesterol is a synthetic fluorescent sterol. Similar binding curves (not shown) yielded a $K_d = 76 ± 10$ nM for StAR binding dehydroergosterol, a naturally occurring fluorescent sterol with similar CMCs as cholesterol (50, 51). However, because of its higher quantum yield, NBD-cholesterol was chosen for use in StAR sterol binding studies and determination of fluorescence resonance energy transfer from StAR Trp (see "Fluorescence Resonance Energy Transfer of StAR Trp to Bound NBD-Cholesterol").
much accessible to the aqueous environment. Extrapolation of a plot of \(F_0/F = (1 - F)/F\) versus [acrylamide] \(^{-1}\) to high acrylamide concentration yields an intercept that reflects the inaccessible fraction (43). For StAR Trp quenching by acrylamide such a plot yielded an intercept near 1 (Fig. 2C), consistent with all the StAR trp residues being inaccessible to the aqueous. Thus, these data showed that StAR Trp residues were buried in the protein and not aqueous-accessible.

Fluorescence Resonance Energy Transfer of StAR Trp to Bound NBD-Cholesterol; Quenching of StAR Trp Fluorescence Emission—As indicated under “Experimental Procedures,” FRET is possible when the donor and acceptor exhibit significant spectral overlap between the emission of the donor and the excitation/absorbance of the acceptor. When the donor is excited, FRET is observed as (i) reduced emission of the donor and (ii) appearance of sensitized emission of the acceptor. Sensitized emission refers to emission of the FRET acceptor (NBD-cholesterol) due to energy transfer rather than direct excitation of the acceptor. The spectral properties of NBD-cholesterol and StAR Trp are well suited for Forster energy transfer. StAR Trp at 295 nm resulted in maximal emission near 352 nm (Fig. 4, spectrum 1), which overlaps well with the NBD-cholesterol absorbance maximum centered near 340 nm (Fig. 4, spectrum 2). These data suggest that the StAR Trp and NBD-group of NBD-cholesterol may be an excellent donor/acceptor pair for FRET.

The extent of FRET from StAR Trp to bound NBD-cholesterol was examined by incubating NBD-cholesterol in the presence of StAR, exciting StAR Trp at 295 nm, and measuring emission over the range 300–600 nm (Fig. 5A). The reduced StAR Trp emission near 352 nm, and the appearance of sensitized NBD-cholesterol emission at 530 nm clearly demonstrated efficient FRET. The lack of complete StAR quenching by NBD-cholesterol suggested that not all four of the Trp residues in StAR are located near and/or correctly oriented to the NBD-cholesterol for efficient quenching. Of the four Trp residues in StAR, three are conserved in StAR and MLN64, and one in β strand 9 (Trp-241) on MLN64 was shown to directly contribute to the hydrophobic tunnel (26). Because of the structural similarity between StAR and MLN64, these data suggest that the NBD-cholesterol energy transfer occurs to one StAR Trp located in a ligand binding tunnel (analogous to that for MLN64) wherein at one Trp residue was in close proximity to the NBD group.

To further refine the above qualitative observations, the above tryptophan fluorescence-quenching data were used to determine the intermolecular distance between StAR Trp and bound NBD-cholesterol as described under “Experimental Procedures.” The overlap integral \(J\) was determined experimentally to be 5.68 ± 0.45 × 10⁻¹⁴ m⁻¹ cm⁻¹ (Table I), basically similar to that obtained for other proteins (for review, see Ref. 42). The transfer efficiency was calculated from Trp quenching (Equation 5, see “Experimental Procedures”) to be 64%, and the \(R_{2/3}\) value obtained thereby (Equation 4, see “Experimental Procedures”) was 32.6 ± 1.3 Å (Table I). Based on comparisons with MLN64 (26), this distance was consistent with the putative location of the energy donor (NBD-cholesterol) located in StAR.

Sterol Binding Parameters of StAR Determined by FRET and Sensitized Emission of Bound NBD-Cholesterol—Excitation of StAR Trp at 295 nm resulted in sensitized fluorescence emission at 530 nm from StAR-bound NBD-cholesterol. This sensitized emission increased with increasing NBD-cholesterol concentration (Fig. 5A) and was plotted as a binding curve (Fig. 5B). Analysis of the latter binding curve as described under “Experimental Procedures” showed that StAR bound NBD-cholesterol with a \(K_D\) of 49 nM. A plot of the hyperbolic increase in sensitized NBD-cholesterol emission as \(1/(1 - F/F_{\text{max}})\) versus [NBD-cholesterol]/[F/F_{\text{max}}] as described earlier (36) was linear (Fig. 5B, inset), consistent with a single binding site containing a Trp interacting with cholesterol.

In addition to the above-described Trp residues, N-62 StAR has 3 Tyr residues (31). Concomitant excitation of StAR Trp as well as Tyr residues at 280 nm resulted in maximal emission near 350 nm that exhibited slightly, about 8%, higher intensity.
Experimental Procedures.

The transfer efficiency of FRET from N-62 StAR Trp to bound NBD-cholesterol was calculated from the quantum yield of N-62 StAR Trp (0.24), the refractive index (1.4), and the orientation factor (2/3) as described under “Experimental Procedures.” J is the overlap integral. R₀ is the critical distance at which 50% energy transfer occurs. Rₑ is calculated from the quantum yield of N-62 StAR Trp (0.24), the refractive index (1.4), and the orientation factor (2/3) as described under “Experimental Procedures.” E is the energy transfer efficiency.

| Energy donor   | Energy acceptor | J         | R₀    | E    | Rₑ    |
|----------------|-----------------|-----------|-------|------|-------|
| N-62 StAR      | NBD-cholesterol | 5.68 ± 0.45 | 36.0 ± 1.1 | 64   | 32.6 ± 1.3 |

(Fig. 6A versus Fig. 5A). These data were consistent with the severalfold lower quantum yield of Tyr versus Trp as well as with efficient internal transfer of excited Tyr energy to the Trp residues, again typical of most proteins.

In the presence of NBD-cholesterol, excitation of StAR Trp as well as Tyr residues at 280 nm also resulted in the appearance of sensitized NBD-cholesterol emission due to energy transfer from StAR bound NBD-cholesterol (Fig. 6A). The sensitized emission increased with increasing NBD-cholesterol concentration (Fig. 6A). These data were plotted as a binding curve (Fig. 6B) which showed that StAR bound NBD-cholesterol with K_d = 93 nM. Again, the plot of the hyperbolic increase in sensitized NBD-cholesterol emission as 1/(1-F/F_max) versus [NBD-cholesterol]/(F/F_max) (Fig. 6B, inset) was linear, consistent with a single binding site.

When the StAR Trp and Tyr were excited at 280 and emission was monitored at 350 nm in the presence of increasing NBD-cholesterol, a decrease in StAR aromatic amino acid residue emission was observed (Fig. 6A). This decrease was plotted as a function of increasing NBD-cholesterol (Fig. 6C). These data, plotted as a binding curve (Fig. 6C), were analyzed as described under “Experimental Procedures” and yielded a K_d = 17 nM for StAR binding NBD-cholesterol. The plot of the hyperbolic increase in sensitized NBD-cholesterol emission as 1/(1-F/F_max) versus [NBD-cholesterol]/(F/F_max) (Fig. 6C, inset) was linear, consistent with a single binding site.

The NBD-cholesterol-sensitized fluorescence emission data were used to determine the intermolecular distance between StAR Tyr/Trp (excitation at 280 nm) and bound NBD-cholesterol (see “Experimental Procedures”). The transfer efficiency was calculated from the sensitized emission of StAR-bound NBD-cholesterol (see “Experimental Procedures”) to be 61%, and the Rₑ value obtained therefrom was 33.4 Å, essentially the same as that obtained from quenching of StAR Trp (excitation at 295 nm) (Table I).

Together these data suggested that FRET from StAR to bound NBD-cholesterol occurred at a single binding site from the same Trp excited either at 295 or 280 nm. Interestingly, the mean K_d = 53 ± 22 nM determined by FRET (Figs. 5 and 6) was very similar to the K_d = 32 ± 4 nM determined from exciting NBD-cholesterol at 473 and plotting the increase in NBD-cholesterol fluorescence emission at 562 nm (Fig. 3). Since FRET did not reveal the presence of the second binding site, noted by plots of fluorescence emission of NBD from StAR-bound NBD-cholesterol (Fig. 3), the second-bound NBD-cholesterol was probably not located closely enough and/or oriented such for FRET to occur in StAR.

**Effect of Sterol Binding on StAR Conformation** —The effect of cholesterol binding on StAR conformation was examined by acrylamide quenching and by circular dichroism, a technique sensitive to alterations in protein secondary structure, as follows.

First, Stern-Volmer quenching studies with acrylamide showed that cholesterol binding did not alter structure of StAR to make Trp residues more aqueous-accessible. Examination of the effect of sterol binding on the emission maxima of StAR aromatic amino acids upon excitation of StAR Trp at 295 (Fig. 5A) or StAR Trp/Tyr at 280 nm (Fig. 6A) showed that in neither case was the wavelength of StAR maximal emission near 350 nm shifted. In addition, binding of cholesterol to StAR did not significantly alter the StAR Trp quenching by acrylamide (Fig. 2B). The acrylamide Stern-Volmer quenching constant, K_SV, of StAR Trp was 2.7 and 2.5 M⁻¹ in the absence and presence of cholesterol.
Second, circular dichroic spectra of StAR were significantly altered in the presence of cholesterol. Circular dichroic spectra of StAR exhibited minima at 208 and 222 nm (Fig. 7A, open circles), consistent with the presence of substantial α-helical structure (53). In the presence of cholesterol, the minima at 208 and 222 nm were both significantly reduced (Fig. 7A, closed circles). The effects of cholesterol (Fig. 7A) and NBD-cholesterol (not shown) on StAR spectra were similar.

Third, the cholesterol-induced conformational change in StAR secondary structure observed by circular dichroism involved a polar group(s) in StAR, since circular dichroism spectral changes were absent in the presence of high salt (50 mM NaH2PO4, 300 mM NaCl, pH 8). Nevertheless, StAR binding curves for NBD-cholesterol were the same in the absence (Fig. 7B) or presence (not shown) of high salt.

Fourth, since lower molar ellipticity of StAR containing bound cholesterol (Fig. 7A) indicated less structured StAR in the presence of ligand, the stability of the unliganded and liganded StAR were examined by guanidine-HCl-induced unfolding. Guanidine-HCl-induced unfolding curves of StAR in the presence and absence of cholesterol were very similar (Fig. 7B). The concentrations for 50% unfolding, C50%, calculated from these curves were 2.4 and 2.5 M, respectively. In summary, these data showed that cholesterol binding significantly altered the relative proportion of α-helical structure; however, this did not alter the exposure of StAR Trp residues to the aqueous solution.

Spontaneous Sterol Transfer from Mitochondrial Membranes—The basic characteristics of spontaneous sterol transfer between mitochondrial membrane donors and mitochondrial acceptors were obtained for normal (CWN) human fibroblast mitochondria (Fig. 8A, bottom curve) and MA-10 mitochondria (Fig. 9, bottom curve). In the absence of mitochondrial membrane acceptors, dehydroergosterol polarization in mitochondrial membrane donors was unaltered over the time period examined (not shown). However, addition of 10-fold excess acceptor mitochondrial membranes elicited a slow spontaneous sterol transfer. The initial rates of molecular sterol transfer from CWN fibroblast and MA-10 cell mitochondria were 0.40 ± 0.03 and 0.04 ± 0.01 pmol/min (Table II). Thus, spontaneous sterol transfer from mitochondria isolated from steroidogenic cells (MA-10 cells) was 10-fold slower than that from mitochondria isolated from normal (CWN) human fibroblasts.

StAR Preferentially Enhanced the Initial Rate of Molecular Sterol Transfer from Mitochondrial Membranes of Steroidogenic MA-10 Cells versus Fibroblasts—StAR significantly enhanced sterol transfer from mitochondrial membranes isolated from normal (CWN) human fibroblasts (Fig. 8A, middle curve), whereas the A218V mutant StAR did not enhance sterol transfer (Fig. 8B). StAR also significantly enhanced sterol transfer from mitochondrial membranes isolated from MA-10 cells (Fig. 8B).
Sterol transfer was measured as described under "Experimental Procedures." The curves show the change in fluorescence polarization as a function of time after the addition of a 10-fold excess of acceptor (dehydroergosterol-deficient) membranes to donor (dehydroergosterol-rich) membranes. Closed circles, donor + acceptor only; open circles, donor + acceptor + 1.5 μM StAR; closed triangles, donor + acceptor + 1.5 μM SCP-2.

### Table II

| Mitochondrial membrane source | Proteina | Initial rateb | pmol/min |
|------------------------------|----------|--------------|----------|
| CWN fibroblasts              | None     | 0.40 ± 0.03  |          |
| N-62 StAR                    | 0.60 ± 0.04<sup>c,d</sup> |                |
| SCP-2                        | 4.10 ± 0.15 |                |
| MA-10 cells                  | None     | 0.04 ± 0.01  |          |
| N-62 StAR                    | 3.97 ± 0.36<sup>d</sup> |                |
| SCP-2                        | 1.58 ± 0.19 |                |

- Protein concentration was 1.5 μM N-62 StAR or 1.5 μM SCP-2.
- Values indicate mean ± S.D. (n = 3 or 4).
- a <i>p</i> < 0.05 and <i>p</i> < 0.01, respectively, as compared to no protein added.
- b <i>p</i> < 0.01 versus SCP-2.

Comparison of the magnitude of STAR-induced polarization changes showed that STAR was much more effective in enhancing sterol transfer from mitochondria isolated from the steroidogenic MA-10 cells. The initial rate of STAR-mediated molecular sterol transfer from normal (CWN) fibroblast mitochondria, 0.60 ± 0.04 pmol/min, was only 1.5-fold faster than spontaneous sterol transfer from these mitochondria (Table II). In contrast, the initial rate of STAR-mediated molecular sterol transfer from the steroidogenic MA-10 cell mitochondria, 3.97 ± 0.36 pmol/min, was enhanced 100-fold when compared with spontaneous sterol transfer from these mitochondria (Table II). Even when the absolute initial rates of STAR-mediated molecular sterol transfer from these two types of mitochondria were compared, the STAR-mediated molecular sterol transfer from MA-10 cell mitochondria was 6.6-fold faster than the STAR-mediated molecular sterol transfer from normal (CWN) human fibroblast mitochondria (Table II).

In summary, SCP-2 was nearly 7-fold more effective than STAR in enhancing the initial rate of molecular sterol transfer from mitochondrial membranes isolated from normal (CWN) human fibroblasts. In contrast, both STAR and SCP-2 (2.2-fold less so) effectively enhanced the initial rate of molecular sterol transfer from steroidogenic MA-10 cell mitochondria.

Dynamic Analysis of Spontaneous Sterol Transfer from Mitochondrial Membranes—To determine if mitochondrial membrane cholesterol may be present in multiple kinetic pools, the dehydroergosterol polarization data in Figs. 8 and 9 were fit to one- and two-exponential equations, and kinetic parameters of the sterol transfer process were resolved as described under “Experimental Procedures.” Spontaneous transfer of sterol from mitochondrial membranes isolated from normal (CWN) human fibroblasts displayed two kinetic pools of cholesterol, a slowly exchangeable sterol pool with a <i>t</i><sub>1/2</sub> of 564 ± 80 min, comprising 11% of the total sterol, and an essentially non-exchangeable sterol pool (<i>t</i><sub>1/2</sub> was too slow to be measured) comprising 89% of the total sterol (Table III). In contrast, spontaneous transfer of sterol from mitochondrial membranes isolated from steroidogenic MA-10 cells displayed only a single kinetic pool of cholesterol that was essentially a non-exchangeable sterol pool (<i>t</i><sub>1/2</sub> was too slow to be measured) (Table III). Thus, the mitochondrial membrane sterols of steroidogenic MA-10 cells were significantly different from those of the fibroblast mitochondria. In contrast to the fibroblast mitochondria, the MA-10 cell mitochondria exhibited only a single domain of essentially non-transferable sterol.

**STAR Preferentially Altered the Sterol Kinetic Pools of Mitochondrial Membranes Isolated from Steroidogenic MA-10 Cells versus Fibroblasts**—STAR significantly altered mitochondrial membrane sterol domains resolved by kinetic analysis. STAR-mediated transfer of sterol from mitochondrial membranes isolated from normal (CWN) human fibroblasts best fit two kinetic pools of cholesterol. Although the <i>t</i><sub>1/2</sub> of the slowly exchangeable sterol pool, 462 ± 70 min, was not significantly different from that of spontaneous sterol transfer, its fractional contribution was increased 2-fold (Table III). Thus, STAR did not induce formation of additional kinetically resolvable sterol domains in mitochondria of normal human fibroblasts. Instead, STAR increased the size of a slowly exchangeable sterol domain.
StAR-mediated transfer of sterol from mitochondrial membranes isolated from steroidogenic MA-10 cells also fit two kinetic pools of cholesterol. The transferable sterol pool exhibited a very rapid \( t_{1/2} = 10 \pm 1 \text{ min} \) and accounted for \( 64 \% \) of MA-10 mitochondrial membrane sterol (Table III). These data indicated that StAR induced the formation of a large, rapidly transferable sterol domain in mitochondria from steroidogenic MA-10 cells. The size of the StAR-induced, transferable sterol domain was 3-fold larger than that of StAR-mediated sterol transfer from normal CWN human fibroblast mitochondria. Finally, the \( t_{1/2} \) of the transferable sterol domain induced by StAR in MA-10 cell mitochondria was 56-fold faster than that of StAR-mediated sterol transfer in normal CWN human fibroblast mitochondria (Table III).

**Effect of SCP-2 on Sterol Kinetic Pools of Mitochondrial Membranes Isolated from Steroidogenic MA-10 Cells versus Fibroblasts**—SCP-2 qualitatively exhibited the same effects on mitochondrial sterol domains as noted above for StAR. However, the effects of SCP-2 were quantitatively very different from those of StAR. SCP-2-mediated sterol transfer of sterol from mitochondrial membranes isolated from normal (CWN) human fibroblasts best fit two kinetic pools of cholesterol. SCP-2 accelerated the \( t_{1/2} \) of the slowly exchangeable sterol pool, \( 65 \pm 10 \text{ min} \), nearly 9-fold as compared with spontaneous sterol transfer (Table III). Furthermore, SCP-2 increased the size of the transferable sterol domain, \( f_1 = 0.67 \pm 0.03 \), nearly 6-fold as compared with spontaneous sterol transfer from normal CWN human fibroblast mitochondrial membranes (Table III). Therefore, although SCP-2 did not induce formation of additional kinetically resolvable sterol domains in mitochondria of normal human fibroblasts, SCP-2 dramatically increased the size and decreased the half-time of the slowly transferable sterol domain.

In contrast to spontaneous sterol transfer from MA-10 cell mitochondria, which fit only a single non-transferable sterol pool, SCP-2-mediated transfer of sterol from mitochondrial membranes isolated from steroidogenic MA-10 cells fit two kinetic pools of cholesterol. The transferable sterol pool had a very rapid \( t_{1/2} = 35 \pm 2 \text{ min} \) and accounted for \( 51 \% \) of MA-10 mitochondrial membrane sterol (Table III). Thus, SCP-2 induced the formation of a large, rapidly transferable sterol domain in mitochondria from steroidogenic MA-10 cells. The size of the SCP-2-induced, transferable sterol domain was 2-fold smaller than that of SCP-2-mediated sterol transfer from normal CWN human fibroblast mitochondria. Finally, the \( t_{1/2} \) of the transferable sterol domain induced by SCP-2 in MA-10 cell mitochondria was <2-fold faster than that of SCP-2-mediated sterol transfer from normal CWN human fibroblast mitochondria (Table III).

Taken together with the data in the previous section, these findings suggested that StAR was much less promiscuous than SCP-2 and preferentially induced the formation of rapidly transferable sterol domains in mitochondria isolated from the steroidogenic MA-10 cells.

**DISCUSSION**

Significant advances in the molecular biology of StAR have greatly contributed to our understanding of the role that this protein plays in acute regulation of gonadal and adrenal steroidogenesis. In contrast, much less is known regarding the nature of putative ligand binding sites and the mechanism(s) of action of this intriguing protein. The full-length StAR protein is comprised of at least two functional regions, an N-terminal mitochondrial-targeting sequence encompassed within the 62 N-terminal amino acid portion of the protein and an N-62 portion of StAR comprising the cholesterol transfer component. The data presented herein provide several novel insights concerning the ligand binding site of StAR and mechanism whereby StAR enhances sterol transfer from mitochondrial membranes.

First, the results showed for the first time the close molecular interaction of StAR with a fluorescent cholesterol derivative. This represents a major advance over the use of cholesterol for ligand binding assays. Cholesterol has a very low CMC, near 20–45 nm (48). This extremely poor aqueous solubility makes it difficult to use standard radioligand binding assays (requiring separation of bound from free cholesterol) to distinguish true cholesterol binding to a protein from cholesterol aggregation/coaggregation with the protein. In contrast, the NBD-cholesterol-based fluorescence binding assays used herein did not require separation of bound from free sterol and, because of the much greater sensitivity of the photon-counting based fluorescence assay, were performed at very low concentrations of NBD-cholesterol (below the CMC). Two types of experiments demonstrated a close molecular interaction between StAR and NBD-cholesterol. (i) The emission maximum of NBD-cholesterol was 32-nm blue-shifted in the presence of StAR but not in the presence of the inactive A218V mutant StAR. When compared with a standard curve of NBD-group emission wavelength versus solvent dielectric constant (54), the blue-shifted NBD spectrum in the presence of active StAR was consistent with a shift of the NBD group from an aqueous to a more hydrophobic environment with a dielectric constant near 2. Consistent with the relatively hydrophobic nature of the StAR ligand binding pocket, computer models of the x-ray crystal structure of the MLN64 START domain showed the presence of a hydrophobic tunnel thought to be the cholesterol binding site (26). Taken together, these data suggested that the NBD group of NBD-cholesterol was buried deep in a putative “hydrophobic tunnel,” assuming a similar structure of StAR as for MLN64. It should be noted that the proposed orientation of NBD-cholesterol or cholesterol in StAR or MLN64, respectively, differs significantly from that of sterol in the binding pocket of SCP-2, a totally unrelated cholesterol binding/transfer protein. NBD-cholesterol binding to SCP-2 did not result in significant shift of the NBD emission spectrum, thereby suggesting that the NBD group of NBD-cholesterol bound to SCP-2 was localized near the surface opening of the SCP-2 protein.
binding pocket (51). NMR data confirmed that SCP-2-bound 3-[13C]cholesterol was oriented with the polar 3β-OH of the sterol oriented near the surface opening of the SCP-2 binding pocket (34). (ii) FRET from StAR Trp to bound NBD-cholesterol was detected as decreased StAR Trp emission and appearance of sensitized NBD-cholesterol emission. Calculation of the energy transfer distances from either the decrease in StAR Trp emission or the appearance of sensitized NBD-cholesterol emission revealed that the StAR Trp and NBD-cholesterol were within 33 Å. This distance was in the range of those reported for other lipid-binding proteins (38, 55). These data could not be accounted for by coaggregation of NBD-cholesterol with StAR.

Second, the cholesterol binding affinity of StAR was calculated for the first time, establishing that StAR binds sterol with high affinity, $K_D$ near 32 nm. This conclusion was based on data obtained from direct binding of fluorescent sterols (NBD-cholesterol with $K_D$ near 32 nm; dehydroergosterol with $K_D$ near 76 nm), FRET from StAR Trp to bound NBD-cholesterol (decreased StAR Trp emission), and FRET-induced sensitized emission from StAR-bound NBD-cholesterol. Native N-62 StAR bound sterol with 7.3-fold higher affinity than did the functionally inactive A218V N-62 StAR mutant. It should be noted that earlier preliminary data from this laboratory suggested that the full-length StAR protein binds NBD-cholesterol, but solubility problems with this protein preparation precluded accurate determination of binding parameters (56). Likewise, the use of micromolar concentrations of radiolabeled cholesterol resulted in cholesterol aggregates that precluded determination of $K_D$ of StAR for cholesterol (26).

Third, it was shown for the first time that StAR has two ligand binding sites for cholesterol with similar low nanomolar $K_D$ values. A direct NBD-cholesterol binding assay determined both cholesterol binding sites, whereas FRET-based binding assays detected only one of these sites. The basis for the latter observation was probably due to only one of these sites containing a Trp residue. The structure of the MLN64 START domain (26) suggests that this site is likely to be adjacent to Trp-241, which directly contributes to the hydrophobic tunnel and is in close proximity/orientation within the binding site for efficient FRET to bound NBD-cholesterol. Computer modeling of the MLN64 START domain (analogous to N-62 StAR) revealed a hydrophobic tunnel whose volume was estimated near 1900 Å$^3$ (26). This tunnel was postulated to be the ligand binding site. Because the volume occupied by cholesterol is about 741 Å$^3$ (57), the 1900 Å$^3$ hydrophobic tunnel in the MLN64 START domain (26) is sufficient to accommodate at least one or easily two cholesterol molecules. In addition, the MLN64 START hydrophobic tunnel contains two buried polar residues, Asp and Arg, each of which could provide an interaction site for a 3β-OH of each of two bound cholesterol molecules. The close sequence homology (35% identity) of N-62 StAR to the START domain of MLN64 as well as similar function in mediating cholesterol transfer for steriodogenesis (albeit in different types of tissues) (26) suggests that N-62 StAR may structurally adopt a similar hydrophobic tunnel/ligand binding cavity that may accommodate up to two cholesterol molecules. It should be noted that these observations showing two sterol binding sites in N-62 StAR were at variance with an earlier report wherein StAR bound radiolabeled cholesterol at a single sterol binding site (26). It may be that problems with cholesterol solubility experienced in the latter work precluded accurate measurements of the number of binding sites therein.

What is the functional significance of high affinity, low affinity sterol binding to StAR? High affinity, low capacity sterol binding is a general property of proteins that are capable of transferring sterols, SCP-2 (1, 34, 44, 58) and liver fatty acid binding protein (1, 50, 59). This binding activity appears to be critical for StAR action on mitochondrial membranes because a point mutation (A218V) that dramatically reduces affinity and capacity for sterol binding ablates the steriodogenic activity of StAR. Likewise, abolition of ligand binding to SCP-2 and liver fatty acid-binding protein also inhibits their ability to enhance membrane sterol transfer (60, 61). Our findings indicate that the hydrophobic tunnel of the START domain can accommodate two cholesterol molecules and possibly other hydrophobic molecules in addition to sterols. This high affinity hydrophobic ligand binding domain may well be the catalyst for membrane perturbation, forcing sterol ejection from the outer mitochondrial membrane.

Fourth, cholesterol binding elicits a structural change in StAR. This observation was supported by circular dichroism and by the sigmoidal shape of the NBD-cholesterol binding curve (Fig. 3A). Circular dichroic data showed for the first time that cholesterol binding to StAR altered the secondary structure of StAR to reduce the proportion of α-helix. Furthermore, the sigmoidal shape of the NBD-cholesterol binding curve and the presence of two binding sites was consistent with a conformational/allosteric effect wherein binding of the first sterol altered the conformation of StAR to allow binding of a second sterol. The functional significance of these ligand-induced alterations in StAR secondary structure and conformation is not yet clear but may be related to the fact that sensitized emission of StAR-bound NBD-cholesterol occurred from only one ligand binding site. In addition, the ligand-induced change in StAR structure may play a role in altering the cholesterol domain structure of the mitochondrial membrane, thereby induce formation of rapidly transferable sterol domains as observed with mitochondria isolated from steroidogenic MA-10 cells.

Fifth, StAR dramatically enhanced the initial rate of sterol transfer from the outer mitochondrial membrane. Our assay for sterol transfer detects the movement of probe (dehydroergosterol) from donor mitochondria to acceptor mitochondria, presumably the transfer of dehydroergosterol from the outer membrane of the donor to the outer membrane of the acceptor mitochondria. This assumption is predicated upon the fact that StAR did not cause a change in dehydroergosterol polarization when added to donor mitochondria in the absence of acceptor mitochondria. A change in polarization under these circumstances might be anticipated if StAR enhanced dehydroergosterol transfer from the outer to the inner mitochondrial membrane. Because the mitochondria were not incubated under conditions (i.e. incubation with an energy source for generation of required reducing equivalents) that support steriodogenesis (MA-10 cell mitochondria) or cholesterol side chain 27-hydroxylation (human fibroblast mitochondria), it is likely that the inner membrane substrate pool of cholesterol was occupied, limiting flux of dehydroergosterol to the inner membrane. This would be expected given the observation that a “back-up” of cholesterol resulting in cholesterol loading of the endoplasmic reticulum and, subsequently, conversion of free cholesterol into cholesteryl esters occurs when steroidogenic cells are incubated with an inhibitor of the mitochondrial side-chain cleavage enzyme, aminoglutethimide (62).

Our findings using a recombinant protein lacking a mitochondrial-targeting sequence are consistent with the hypothesis that StAR acts on the outer mitochondrial membrane to cause a selective perturbation resulting in transfer of sterol to closely opposed inner membranes as long a cholesterol metabolism is taking place. Our findings are inconsistent with a model that requires mitochondrial importation of StAR to effect sterol translocation. With a stoichiometry of two sterols bound per StAR molecule, it is also difficult to imagine StAR acting as...
a carrier protein with respect to cholesterol transfer to the inner mitochondrial membrane in steroidogenic cells because it is a low abundance protein. For example, the 2-g (wet weight) human corpus luteum produces 80–160 μmol of progesterone/24 h. This requires the movement of an equivalent amount of cholesterol to the inner mitochondrial membrane. Approximately 1.3–2.6 g of STAR protein would be needed per 24 h to move this amount of substrate to the inner mitochondrial membrane cholesterol side-chain cleavage enzyme, assuming STAR makes a one-way (i.e. non-recycling) trip with its charge of cholesterol. The fact that import-incompetent STAR is capable of stimulating mitochondrial steroidogenesis (24) argues against any model based on an intermembranous shuttle (26).

Sixth, STAR differentially stimulated the initial rate of sterol transfer from mitochondrial membranes of steroidogenic MA-10 cells as compared with normal CWN human fibroblasts. In contrast, SCP-2 appeared nearly equally effective regardless of mitochondrial source. It is interesting to speculate that the STAR-mediated preferential transfer of sterol from MA-10 mitochondria might be due to a STAR-receptor (protein or lipid) in the donor and/or possibly the acceptor mitochondrial membrane. Consistent with a requirement for a STAR receptor in the donor membrane to reveal differential effects of STAR and SCP-2, earlier data from our laboratory showed that STAR and SCP-2 were equally effective in mediating cholesterol transfer from liposomal model membrane donors (comprised of phospholipid and cholesterol, but no protein) to rat liver mitochondria (23). Furthermore, since trypsin treatment of the acceptor mitochondria did not significantly affect STAR-mediated cholesterol transfer from the donor liposomes, this suggested that a STAR receptor in the acceptor membrane was not essential. Putative STAR receptors have been proposed, including the peripheral benzodiazepine receptor (63) among others (24). It should be noted, however, that since STAR overexpressed in COS-1 cells enhances steroidogenesis (24), there cannot be an absolute specificity of mitochondrial responsiveness to STAR (i.e. non-steroidogenic cell mitochondria can respond to high levels of STAR). These findings do not rule out differences in relative sensitivity of mitochondria of steroidogenic cells that could be critical, however, under physiological conditions where STAR is present in low levels.

Seventh, STAR is not only a cholesterol-binding protein but, equally importantly, dramatically altered the cholesterol domain structure of mitochondrial membranes. Spontaneous sterol transfer from mitochondrial membranes was very slow ($t_{1/2} >$ days). STAR induced formation of a rapidly ($t_{1/2}$ near 10 min) sterol pool that accounted for 64% of mitochondrial membrane sterol. This effect of STAR was relatively specific for mitochondrial membranes isolated from steroidogenic MA-10 cells as opposed to mitochondria isolated from normal (CWN) human fibroblasts. This specificity of STAR was not shared by another sterol carrier protein (SCP-2), which promiscuously enhanced sterol transfer from mitochondrial membranes of steroidogenic MA-10 cells as well as normal (CWN) human fibroblasts. The significance of the STAR-induced alteration in mitochondrial membrane cholesterol domain structure is 3-fold. (i) STAR elicits sterol transfer by acting on the outer mitochondrial membrane (23, 24). (ii) A STAR-mediated membrane fusion mechanism has been ruled out (23). (iii) STAR is not present in large quantities of sterol-containing cells to transfer large quantities of cholesterol to the inner mitochondrial membrane solely by a cholesterol carrier mechanism (23). However, by altering the domain structure of the outer mitochondrial membrane, the effect of low quantities of STAR may be magnified to induce a much larger flux of cholesterol to the inner mitochondrial membrane than could be accounted for by a simple carrier mechanism. A similar mechanism not requiring an aqueous carrier has been established for SCP-2 (60) and liver fatty acid binding protein (61)-mediated sterol transfer between membranes.

In summary, the data presented herein provided several new insights into the mechanism of action of STAR. The finding that the data presented herein provided several new insights into the mechanism of action of STAR. The finding that the STAR binds cholesterol is consistent with the proposal that the STAR may act as a cholesterol binding/transfer protein, an activity separate from mitochondrial import of STAR (23, 24). Likewise, the finding that STAR alters mitochondrial cholesterol domain structure to induce formation of a rapidly transferable sterol pool is also consistent with this hypothesis. However, the latter observation may also support the view that localization of STAR in mitochondrial intermembrane (outer-inner membrane) contact sites (21, 22) may disrupt cholesterol domain structure, therein allowing the flow of cholesterol from the outer to the inner mitochondrial membrane, wherein the first committed step of cholesterol oxidation for steroidogenesis occurs. The fact that the inactive A218V mutant N-62 STAR has only a single, 7-fold lower affinity site for sterol binding suggests that either mechanism must take into account the requirement for high affinity binding of at least two cholesterol molecules/STAR.

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