Steroidogenic acute regulatory protein (StAR) stimulates adrenal and gonadal steroidogenesis by increasing the influx of cholesterol into mitochondria, where it is converted to pregnenolone to initiate steroidogenesis. StAR acts on the outer mitochondrial membrane where each molecule stimulates the mitochondrial import of several hundred molecules of cholesterol, but the precise mechanism of the action of StAR remains uncertain. StAR has a sterol-binding pocket that can accommodate one molecule of cholesterol. Direct assays show that StAR can bind cholesterol with stoichiometry approaching 1:1, and several disease-causing mutants with decreased or absent activity have correspondingly decreased cholesterol binding. We show that the StAR mutant R182L, which causes severe disease and is devoid of measurable activity in transfected cells or with isolated steroidogenic mitochondria, nevertheless, can bind as much [14C]- or NBD-cholesterol as wild-type StAR under equilibrium conditions and can transfer cholesterol between liposomes in vitro. Similarly, the artificial mutant S195A had 46.5% of the activity of wild-type StAR but bound cholesterol indistinguishably from wild-type. Competition assays showed that the rate of binding (t1/2on) for R182L was only 36% of the wild-type and the rate of dissociation (t1/2off) was 57% of wild-type, whereas the t1/2on and t1/2off for S195A and S195D were essentially the same for wild-type. These data indicate that cholesterol binding and transfer activities are distinct from its activity to induce steroidogenesis. StAR appears to act by other mechanisms in addition to cholesterol binding.

In the adrenal and gonad, the steroidogenic acute regulatory protein (StAR) facilitates the flow of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) where it is converted to pregnenolone by the cholesterol side chain cleavage enzyme, P450scC, thus initiating the synthesis of steroid hormones (1, 2). Loss of StAR activity ablates adrenal and gonadal steroidogenesis, causing lifethreatening congenital lipoid adrenal hyperplasia (3). Full-length StAR is expressed as a 37-kDa, 285-residue phosphoprotein having a mitochondrial leader sequence that is cleaved to a 30-kDa intramitochondrial form (4–7). Nevertheless, StAR acts on the OMM, and is inactive inside the mitochondrion: deletion of 62 NH2-terminal residues yields a protein (N-62 StAR) that remains in the cytoplasm, yet retains full biologic activity (8), and affixing StAR to the OMM yields constitutive activity, whereas localizing it to the intramembranous space or the matrix side of the IMM ablates activity (9). Only the COOH-terminal α-helix (C-helix) of StAR interacts with the OMM (10). Biophysical data show that StAR undergoes an acid-induced structural change to a molten globule state (11), and the association of StAR with the OMM is stronger under acidic conditions (10, 12–15).

The structure of StAR has not been determined experimentally, but the structures of three closely related proteins having “StAR-related lipid transfer” domains have been determined (16–18) permitting the construction of computational models of StAR (10, 15, 16). All of these structures are characterized by four α-helices and nine antiparallel β-sheets that define a hydrophobic sterol-binding pocket (SBP). Structural modeling indicates that the position of the C-helix is stabilized by a network of hydrogen bonds with the adjacent structures, especially by a salt bridge with Asp106 at the end of the loop between sheets β1 and β2 (residues 102–106) (10, 15). Molecular dynamics simulations show that the C-helix is closely associated with this loop, but opens widely under acidic conditions (14). Creation of disulfide bonds that prevent the movement of C-helix abolish StAR activity (14). Thus our current model is that the interaction of StAR with protonated phospholipid head groups on the outer mitochondrial membrane induces it to undergo a conformational change (molten globule transition) that permits the C-helix to swing open, permitting the binding and release of cholesterol from the SBP of StAR (19).

The mechanism by which StAR facilitates mitochondrial cholesterol import is not known. Multiple models of action for StAR have been proposed, but its precise molecular mechanism of action remains unclear (19). Phosphorylation of StAR at serine 195 by protein kinase A increases activity ~2-fold, as does the artificial StAR mutant S195D, but dephospho-StAR and the artificial mutant S195A retain substantial activity, indicating that phosphorylation is not required for activity (20, 21). StAR can bind cholesterol and transfer it from donor vesicles to an acceptor membrane (22–24). However, although StAR binds...
cholesterol with 1:1 stoichiometry (14, 16) estimates of StAR activity suggest each molecule of StAR is responsible for the mitochondrial import of up to 400 molecules of cholesterol per minute (25). Thus the correlation between cholesterol binding and StAR activity is not clear. Recent data indicate that StAR probably interacts with another OMM protein, the peripheral benzodiazepine receptor (26, 27). Our current model is that StAR mobilizes cholesterol previously bound to the so-called “cholesterol recognition amino acid consensus” domain of peripheral benzodiazepine receptor for import from OMM to IMM (19).

The cholesterol-binding activity of only a few functionally inactive StAR mutants has been examined, but to date all have had a degree of impaired cholesterol binding ability that correlated with activity (14, 16, 26, 28). We sought to examine this correlation more closely by focusing on mutations that affect either the geometry of the SBP or the phosphorylation of StAR. Based on structural modeling (10, 14), we have focused on mutations in the loop between sheets β5 and β6 (residues 172–182), which stabilizes the position of the C-helix. These include mutations R182L, commonly found among Palestinian Arabs (29, 30), and R182H, found in eastern Saudi Arabia (31), both of which cause severe disease, and R188C, which causes an attenuated form of lipoid CAH in Pakistan (28). We recently found that the R182C mutant, which causes a milder disease, retains activity (29, 30), and R182H, found in eastern Saudi Arabia (31), both of which also modulate activity (20, 21).

**MATERIALS AND METHODS**

**Preparation of Vectors and Proteins**—The amino-terminal ends of wild-type and mutant N-62 StAR cDNA constructs were fused to the Ssp Dna B intein domain and cloned into pTWIN (New England Biolabs) doubly digested with SapI and BamHI, as described (14). Each construct was prepared with an amino-terminal His$_6$ tag, which facilitated lipid-binding assays, and in each case the carboxyl-terminal cystine residue of StAR was changed to phenylalanine to reduce the reactivity of the protein; deletion or mutation of this residue does not affect StAR activity (8, 14). Bacterial lysates were cleared by centrifugation and loaded onto pre-equilibrated chitin-binding columns (New England Biolabs) according to the manufacturer’s recommendations. The columns were washed with 10 volumes of 300 mM NaCl, 25 mM Tris, pH 8.5, and eluted with 300 mM NaCl, 25 mM Tris, pH 7.0, at room temperature to induce intein self-splicing. The N-62 StAR was collected and dialyzed against 300 mM NaCl, 20 mM Tris, pH 7.5. For N-62 R188C, purification was performed in the presence of 1 mM phenethylsulfonyl fluoride in all the buffers, which was subsequently removed by dialysis against 300 mM NaCl, 20 mM Tris, pH 7.5. Far-UV CD spectroscopy was done at room temperature in a 2-mm path length cuvette in a Jasco spectropolarimeter with a Peltier temperature control.

**Protein Activity**—Mitochondria were prepared from mouse MA-10 Leydig cells as described (9), suspended in 0.25 M sucrose, 10 mM HEPES, pH 7.4, and stored at −80 °C. Purified N-62 StAR proteins (5 μM) were added to mitochondria (4 μg of Bradford protein) in 50 μl of 125 mM KCl, 5 mM MgCl$_2$, 10 mM KH$_2$PO$_4$, 25 mM HEPES, pH 7.4, 250 ng/ml trilostane, 100 μM GTP, and 10 mM isocitrate with 1 mM dithiothreitol (“import buffer”) (9), and conversion of mitochondrial cholesterol to pregnenolone was measured by radioimmunoassay. For kinetic experiments, 16 μg of mitochondrial protein was used in 200 μl of import buffer and a 20-μl aliquot was taken at each time point and assayed.

[^14C]Cholesterol Binding—Binding of [14C]cholesterol was done essentially as described (16). The purified His$_6$-tagged proteins were dialyzed into stock buffer (20 mM Tris, pH 7.5, 300 mM NaCl). Proteins (4 μM) were incubated with [14C]cholesterol (4 μM) (DuPont, 59 mCi/mmol) in 100 μl of binding buffer (50 mM phosphate, pH 8.0, 200 mM NaCl) containing 2% ethanol for 1 h at 37 °C. Proteins were then immobilized on the nickel-nitrioltriacetic acid resin, washed four times with binding buffer to remove unbound sterols, and eluted with binding buffer plus 0.5 M imidazole. The radioactivity of [14C]cholesterol bound to the eluted proteins was quantified by scintillation counting.

**Cholesterol Transfer Activity**—Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Lipid films containing palmitoyloleoyl phosphatidylcholine:palmitoyloleoyl phosphatidylethanolamine:cholesterol (1:1:1) were made by evaporation from a solution in chloroform:methanol (2:1) under a stream of nitrogen gas and then placed under strong vacuum for ~3 h. The dry films were hydrated with 10 mM HEPES, 0.14 M NaCl, 1 mM EDTA, adjusted to the appropriate pH. To make acceptor large unilamellar vesicles (concentration 1.2 mM), the lipid suspensions were freeze-thawed five times and then extruded through two 200-nm polycarbonate filters in a barrel extruder under pressure. Lipid films for donor vesicles were also composed of palmitoyloleoyl phosphatidylcholine:palmitoyloleoyl phosphatidylethanolamine:cholesterol (1:1:1) but also composed of palmitoyloleoyl phosphatidylcholine:palmityl oleoyl phosphatidylethanolamine:cholesterol (1:1:1) that contained 5 mol % BODIPY-cholesterol. The structure of BODIPY-cholesterol and its properties have been previously described (32, 33). The amount of probe was chosen from a separate experiment (not shown) where it was found that at 5 mol % BODIPY-cholesterol undergoes self-quenching. Lipid films containing BODIPY-cholesterol were hydrated to have one-tenth of the concentration of the acceptor vesicles and these suspensions were sonicated to clarity at room temperature in the dark, to make small unilamellar vesicles. N-62 StAR and R182L mutant stocks were centrifuged for 3 min at 13,000 × g and the supernatant used for the assays. An aliquot of the supernatant was used to determine the concentration of the proteins by the MicroBCA assay (Pierce). In a typical transfer assay, 10 μl of donor small unilamellar vesicles and 10 μl of acceptor large unilamellar vesicles were placed in an acryl 1-cm$^2$ fluorescence cuvette containing 2 ml of the buffer at the appropriate pH at 37 °C. After establishing a baseline, an aliquot of protein was added and the change in fluorescence at 515 nm followed as a function of time. The excitation wavelength was 500 nm and a 4-nm band path was used in excitation and emission with a 500-nm cut off filter in the emission monochromator. Values for 100% transfer were obtained by adding 20 μl of 20% Lubrol, vortexing, and brief sonication. The dependence of transfer on protein concentration is summarized using the...
**Cholesterol Binding and StAR Activity**

Extent of transfer obtained after 200 s of adding the protein. The pH curves were constructed by measuring transfer in a series of buffers at different pH values and taking the value at or close to the maximal extent of transfer measured after about 5 min.

**NBD-Cholesterol Binding**—Steady-state binding was measured by mixing N-62 StAR proteins (1 μM) in phosphate-buffered saline (containing 1 mM dithiothreitol) with various amounts of 22-(n-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino-23,24-bisnor-5-cholesten-3β-ol) (NBD-cholesterol) (Invitrogen) in 96-well plates (final volume, 200 μl), incubated 1 h at 37 °C and steady-state fluorescence intensity was monitored on a SpectraMax M2 microplate reader (Molecular Device Inc.). Fluorescence was always measured in the presence of protein. Excitation was set at 480 nm, emission was set at 540 nm, and cutoff was set at 515 nm. Various amounts of proteins in phosphate-buffered saline (containing 1 mM dithiothreitol) were mixed with 1 μM NBD-cholesterol and incubated at 37 °C for 2 h and steady-state fluorescence intensities were measured. Time course measurements were performed by mixing 1 μM wild-type or mutant N-62 StAR with NBD-cholesterol (1 μM). The fluorescence intensities were recorded for 2 h in 30-s intervals. Competitive binding assays were performed using cholesterol and NBD-cholesterol. Cholesterol (1 μM) or NBD-cholesterol (1 μM) taken from ethanol stocks (final ethanol concentration <2%) was first incubated with 1 μM wild-type or mutant N-62 StAR at 37 °C for 1 h, followed by the addition of 1 μM NBD-cholesterol or 1 μM cholesterol. Fluorescence intensities of bound NBD-cholesterol were then recorded at 37 °C at 30-s intervals for 2 h.

The buffer control was subtracted from the fluorescence data and analyzed by non-linear regression using GraphPad Prism version 3.0. For wild-type, R182L, S195A, and S195D, the steady-state NBD-cholesterol binding curves were analyzed as a sigmoid dose response with variable slope as a function of the logarithm of concentration.

\[
F_i = F_{\text{max}} / [1 + 10^{(\log K_m - \log C) \times \text{Hillslope}}] \quad (\text{Eq. 1})
\]

\(F_i\) is fluorescence of bound NBD-cholesterol at concentration \(i\) of ligand; \(F_{\text{max}}\) = fluorescence of NBD-cholesterol bound at maximal binding; \(C\) is the ligand concentration; and \(K_m\) is the ligand concentration when the fluorescence is half of \(F_{\text{max}}\) and the Hillslope is the steepness of the curve.

For R182H and R188C, the steady-state NBD-cholesterol binding curves were analyzed using a hyperbolic equation (one binding site) by plotting the fluorescence intensity versus the substrate (or protein) concentration.

\[
F_i = F_{\text{max}} \cdot C/(K_m + C) \quad (\text{Eq. 2})
\]

For the kinetic study, data were analyzed using a hyperbolic equation by plotting the fluorescence intensity versus time.

\[
F_i = F_{\text{max}} \cdot t/(t_i/2 + t) \quad (\text{Eq. 3})
\]

\(F_i\) is fluorescence of bound NBD-cholesterol at time \(i\); \(F_{\text{max}}\) is fluorescence of NBD-cholesterol bound at maximal binding; \(t\) is time; and \(t_i/2\) is the time when the fluorescence is half of \(F_{\text{max}}\).

For the competition binding kinetic studies, \(K_{\text{off}}\) values for cholesterol were determined using NBD-cholesterol to displace pre-equilibrated cholesterol. Data were analyzed using a hyperbolic equation by plotting the fluorescence intensity versus time as described above.

For the competition binding kinetic studies, \(K_{\text{on}}\) values of cholesterol were determined using cholesterol to displace pre-equilibrated NBD-cholesterol. Data were analyzed using exponential decay (one phase) by plotting the fluorescence intensity versus time.

\[
F_i = \text{Span} \cdot e^{-k_t} + \text{Plateau} \quad (\text{Eq. 4})
\]

\(F_i\) is initially equal to Span + Plateau and decreases to Plateau with a rate constant \(k\). The half-life \((K_{\text{on}})\) of the decay is 0.6931/\(k\).

**Structural Modeling and Molecular Dynamics**—Our model of human wild-type N-62 StAR (10) was modified by introducing missense mutations using the Swiss-Model program. Energy minimization and molecular dynamic simulations were done with Amber 7 (University of California, San Francisco Computer Graphics Laboratory) as described (14). Cholesterol was docked into the StAR model using the Dock 4 program (34) at the UCSF Computer Graphics Laboratory. The ligand conformation with lowest energy level was selected as a representative to visualize the substrate binding in StAR. Images were generated with Chimera (46).

**RESULTS**

**Protein Expression and Folding**—Wild-type and mutant StAR proteins were expressed as inteins in bacteria. Each was expressed as the fully active N-62 form with an NHi2-terminal His6 tag and purified to homogeneity. The wild-type, S195A, and S195D mutants were expressed at similar levels and were soluble up to about 4 mg/ml. The R182L and R182H mutants were expressed at a higher level than wild-type and remained in solution to over 20 mg/ml. Only small amounts of the R188C mutant could be obtained under the same conditions for expression and purification and this protein was not stable in solution. R188C precipitated after a few days at 4 °C, whereas wild-type remained in solution for more than 2 weeks at 4 °C.

Circular dichroism (CD) spectroscopy was used to assess the folding of the bacterially expressed N-62 StAR proteins (Fig. 1). The folding of the R182H, R182L, and R188C mutants was similar to that of wild-type, with the R188C mutant having a slight decrease in the signal at 208 nm, indicating a slightly altered conformation (Fig. 1A). The spectra of the S195A and S195D mutants were essentially the same as that of the wild-type, indicating very similar folding (Fig. 1B). Partial proteolysis of the wild-type and each of the mutants with either GluC or AspN yielded indistinguishable patterns on SDS-gel electrophoresis, indicating that the folding of all of these forms of N-62 StAR was quite similar (not shown).

**Activity of the Mutant Proteins**—The R182L mutant (29, 30) and the R182H mutant (31) were described in homozygously affected patients with severe congenital lipoid adrenal hyperplasia presenting in infancy, whereas the R188C mutant was recently described in a homozygote with an attenuated form of this disease called “non-classic congenital lipoid adrenal hyperplasia” (28). By contrast, the S195A and S195D mutants were
built to confirm that phosphorylation of Ser\(^{195}\) doubles the activity of StAR (20). The activities of these proteins were assessed in those previous publications by expression of vectors encoding the full-length StAR protein in COS-1 cells co-transfected with the F2 vector encoding a fusion protein of the cholesterol side chain cleavage enzyme system (H\(_{2}\)N-P450scc-ferredoxin reductase-ferredoxin-COOH) (35). Because our studies utilize the more soluble N-62 forms of these proteins, we first assessed the activity of each bacterially expressed purified N-62 protein to induce steroidogenesis by mitochondria isolated from steroidogenic mouse Leydig MA-10 cells. In this assay, StAR activity is measured as the amount of pregnenolone produced from endogenous mitochondrial cholesterol (9, 14, 28, 36). The proteins were quantitated by adsorption at 280 nm and by the Bradford method, and the accuracy of the measurements was confirmed by SDS-gel electrophoresis (not shown). In a standard 1-h incubation, wild-type N-62 StAR elicited 5 times more steroidogenesis than did buffer or the heat-denatured wild-type protein (Fig. 2A). After subtracting the buffer control, the R182H, R182L, and R188C mutants elicited 8.6, 5.6, and 20.5% of wild-type activity, whereas the S195A and S195D mutants elicited 46.5 and 102.5% of wild-type activity, respectively, consistent with previous results (20, 21). In comparison to the denatured wild-type control, neither R182L (\(p = 0.17\)) nor R182H (\(p = 0.12\)) elicited significantly more pregnenolone, whereas the increased activity of R188C (\(p = 0.043\)) was significant. As a percentage of the wild-type control, these activities are indistinguishable from those previously reported for the full-length proteins expressed in COS-1 cells (20, 28–31), demonstrating that the in vitro assay of bacterially expressed N-62 proteins is a reliable index of StAR activity in vivo.

To distinguish effects on net steroidogenic activity from effects on the kinetics of cholesterol import, the activities of the six StAR proteins were assayed as a function of time (Fig. 2B). The use of isolated steroidogenic mitochondria is ultimately limited by the pool of available cholesterol in their outer mitochondrial membrane. The wild-type and S195D mutant had indistinguishable kinetics and elicited the same level of activity, whereas the S195A mutant had similar kinetics but elicited a lower level of steroidogenesis, consistent with Fig. 2A. The R182H and R182L mutants had minimal activity above the

**FIGURE 1.** CD spectra of the N-62 StAR proteins. Protein concentrations were 0.15 mg/ml and the cuvette path length was 0.2 cm.

**FIGURE 2.** Activity of the purified proteins. A, purified proteins were added to mitochondria isolated from mouse MA-10 Leydig cells, incubated for 1 h, and pregnenolone produced from endogenous mitochondrial cholesterol was measured by immunoassay. The added StAR proteins are indicated; Denatured Designates wild-type StAR, heat denatured by boiling for 30 min. B, time course of steroidogenic activity in MA-10 mitochondria. Wild type, solid black line ( ); S195A, dot and dashed gray line ( ); S195D, dashed gray line ( ); R188C, dotted gray line ( ); R182H, solid gray line ( ); R182L, dashed black line ( ); denatured wild-type (WT), dashed black line ( ); buffer without protein, solid gray line ( ). In both panels, data are mean ± S.E. for three experiments, each performed in triplicate.
background seen with buffer alone or heat-denatured protein. However, the R188C mutant reached nearly the same level as the wild type, but did so much more slowly, reaching maximal activity at 6 h, whereas the wild type and Ser195 mutants reached maximal activity at about 3 h. These differences appear to be inherent to the specific forms of StAR tested, as they were seen in multiple assays with different mitochondrial preparations.

Cholesterol Binding at Equilibrium—The binding of cholesterol to StAR is readily assessed using fluorescent NBD-cholesterol (23). This assay is easier, faster, cheaper, and more sensitive than measuring the binding of \[^{14}\]C cholesterol. However, the addition of the NBD group to carbon 22 of cholesterol increases its molecular weight from 386 to 494; furthermore, the presence of the NBD group prohibits P450scC from using NBD-cholesterol as a substrate. Thus we first sought to validate the binding of NBD-cholesterol as an index of the binding of authentic cholesterol by comparing the binding of \[^{14}\]C cholesterol and NBD-cholesterol by selected StAR constructs. When assayed after 1 h of incubation, purified wild-type N-62 StAR bound \[^{14}\]C cholesterol well, whereas heat-denatured N-62 StAR did not (p = 0.0087) (Fig. 3A). The S195A and S195D mutants bound cholesterol equivalently to the wild-type. Surprisingly, the biologically inactive R182L mutant had 107% of the binding capacity compared with the wild-type (p = 0.072), and the R182H mutant had about half of this binding capacity (54%) (p = 0.013), whereas the R188C mutant retained only 10% of wild-type binding capacity, which was still significantly greater than the heat-denatured wild-type (p = 0.015).

We then used fluorescent NBD-cholesterol to measure binding (Fig. 3B) using the same conditions employed in Fig. 3A. NBD-cholesterol exhibits low fluorescence in aqueous buffers, but exhibits substantial fluorescence in a hydrophobic environment, such as when it is bound by StAR (23). As with \[^{14}\]C cholesterol, the wild-type bound NBD-cholesterol well, whereas the heat-denatured wild-type did not, and binding by the S195A and S195D mutants was indistinguishable from the wild-type. The binding activity of the R182H and R188C mutants was poor but was significantly greater than the heat-denatured wild-type control (R182H, p = 0.0060; R188C, p = 0.0048), whereas the binding activity of the R182L mutant was actually greater than that of the wild-type (p = 0.0033). Thus the binding of fluorescent NBD-cholesterol was very similar to that of \[^{14}\]C cholesterol, and thus is an appropriate substrate for studies of the cholesterol binding behavior of StAR.

Because the binding of the biologically inactive R182L mutant was equal to that of the wild-type protein, we examined this binding in more detail. Using a 1-h incubation time and a fixed 1 M concentration of each N-62 StAR protein, we first examined the binding of NBD-cholesterol as a function of its concentration (Fig. 4A). The wild-type, S195A, and S195D N-62 StAR bound NBD-cholesterol to precisely the same degree with almost identical dose-response curves, whereas the heat-denatured wild-type protein did not bind significantly. After subtracting the heat-denatured control, the binding capacity (B\(_{\text{max}}\)) of the R182H mutant was 29% of the wild-type value, and the B\(_{\text{max}}\) of the R188C mutant was 18% of wild-type, whereas the B\(_{\text{max}}\) of the R182L mutant reached 128% of wild-type. When the concentration of NBD-cholesterol was held constant at 1 M and the protein concentration was varied, the binding characteristics of the wild-type and S195A and S195D mutants were indistinguishable and the R182L mutant again behaved similarly to wild-type, whereas the R182H and R188C mutants had markedly attenuated binding, which was nevertheless greater than that of the heat-denatured wild-type protein (Fig. 4B). Thus, even though the R182L is inactive \(\textit{in vivo}\), its binding of cholesterol is very similar to that of wild-type StAR.

Cholesterol Transfer—Because R182L appeared to bind cholesterol normally, we determined whether it could transfer cholesterol between synthetic unilamellar vesicles as does wild-type N-62 StAR (24). This assay used fluorescent BODIPY-cholesterol (32, 33) in the donor liposome at 5%, where it is self-quenched; upon transfer to an acceptor liposome, fluorescence increases. Fluorescence was continuously monitored for 200 s, and the value at 200 s was plotted as a function of the concentration of the added StAR protein, showing that the R182L mutant and wild-type had very similar cholesterol-transfer capacities (Fig. 5A). When cholesterol transfer was similarly examined at pH values ranging from 3 to 7, both wild-type and mutant R182L StAR showed similar patterns, with maximal transfer below pH 4.5 (Fig. 5B). Thus the R182L mutant can transfer cholesterol \(\textit{in vitro}\) similarly to wild-type, even though it is inactive \(\textit{in vivo}\).

FIGURE 3. Binding of cholesterol probes. A, binding of 4 M \[^{14}\]C cholesterol to 4 M of the indicated bacterially expressed N-62 StAR proteins was measured by separating bound from free cholesterol after 1 h at 37 °C. B, binding of 1 M NBD-cholesterol incubated with 1 M of the indicated N-62 StAR proteins for 1 h at 37 °C. In both panels, data are mean ± S.E. for three experiments, each performed in triplicate. WT, wild type.
Kinetics of Cholesterol Binding—Because the R182L mutant bound cholesterol despite its inactivity in vivo, we examined the kinetics of this binding. The use of fluorescent NBD-cholesterol permitted continuous monitoring of binding. Wild-type N-62 StAR reached 50% saturation (t_{1/2} value) at 2.52 ± 0.06 min, whereas the R182L mutant had a t_{1/2} value of 5.66 ± 0.13 min (Fig. 6A). In this experiment, as in Fig. 4A, the saturation value of the R182L mutant was about 125% of that of the wild-type.

The amount of bound cholesterol must represent an equilibrium between its association and dissociation from StAR. We measured cholesterol association (t_{1/2on}) over 120 min by monitoring the fluorescence of NBD-cholesterol added to StAR previously saturated with cholesterol (Fig. 6B). The calculated t_{1/2on} for wild-type N-62 StAR was 10.2 ± 0.20 min and the calculated t_{1/2off} was 1.48 ± 0.03 min. For R182L, the calculated t_{1/2on} was 28.2 ± 0.54 min and the calculated t_{1/2off} was 2.59 ± 0.06 min, thus the association of cholesterol to R182L occurred at only 36% of the speed of its association with wild-type, and its dissociation from R182L occurred at about 57% of the speed of the wild-type. Thus net cholesterol turnover, estimated as the product of t_{1/2on} and t_{1/2off} for R182L was about 20% of wild-type. By contrast, the calculated t_{1/2on} for S195A was 11.52 ± 0.31 min and the calculated t_{1/2off} was 1.55 ± 0.02 min; for the S195D mutant the calculated t_{1/2on} was 10.86 ± 0.18 min and the calculated t_{1/2off} was 1.38 ± 0.06 min, values that were very similar to the wild-type (not shown).

Structural Analysis—Using our validated computational model of N-62 StAR (10), we explored the potential roles of Arg182 and Ser195 in StAR activity and cholesterol binding. Ser195 lies in a short loop (residues 171–182) connecting the β6 and β7 sheets (Fig. 7A). Based on the orientation of StAR in association with synthetic membranes having a lipid composition similar to the OMM, Ser195 is predicted to be exposed to the cytoplasm, on the opposite side of the protein from the membrane-associated C-helix (10). Mutagenesis of Ser195 would not be predicted to alter the mobility of the C-helix, which is essential for cholesterol binding (14), but might influ-
The modeling shows that Arg$^{182}$ normally forms a hydrogen bond with Leu$^{247}$, a residue on the short loop at the beginning of the COOH-terminal helix. Arg$^{182}$ appears to suppress the flexibility of the 171–182 loop, which, in turn, imposes spatial constraints on the shape and volume of the SBP. Neither the R182L nor R182H mutants can form these bonds. The lack of these hydrogen bonds is predicted to increase the flexibility of the loop region, opening the cholesterol binding pocket. Arg$^{188}$ and Glu$^{169}$ are the only charged residues inside the SBP; a salt bridge between Arg$^{188}$ and Glu$^{169}$ appears to play a crucial role in maintaining the conformation of the SBP. When cholesterol is modeled in the SBP, Arg$^{188}$ appears to be the primary binding site for the 3β-OH group of cholesterol (Fig. 7B). The R188C mutant would eliminate hydrogen bonding with Glu$^{169}$ and create a weak hydrogen bond with Thr$^{167}$; this would maintain the overall dimensions of the SBP, but would result in weak substrate binding.

To obtain a better understanding of how amino acid substitutions at Arg$^{182}$ may influence the conformation of StAR, we used molecular dynamic simulations to visualize the movements of the protein. The movement of the 171–182 loop, which includes Arg$^{182}$, is depicted by superimposing images of the molecule obtained every 10 ps (Fig. 8A). The wild-type and R182H mutant showed stable trajectories with conserved secondary and tertiary structure. By contrast, the 171–182 loop in the R182L mutant showed considerably greater mobility, resulting in a partially opened tertiary structure, which would open the SBP. The C-helix and 171–182 loop are closely associated and form a stable unit: the distance between the guanidino nitrogen of Arg$^{182}$ and the carboxylic oxygen of Leu$^{247}$ remained constant at 2.86 ± 0.11 Å throughout the 3 ns of molecular dynamics, favoring the formation of hydrogen bond (Fig. 8B). To compare the mobility of the 171–182 loop in the wild type and mutant proteins, we plotted the distances between the first side chain carbon (Cβ) of Arg$^{182}$ to the first side chain carbon (Cβ) of Leu$^{247}$ (Fig. 8C). For wild-type StAR, this distance remained fairly stable at 5.17 ± 0.32 Å and was only slightly greater for the R182H mutant (5.44 ± 0.44 Å). Replacing Arg$^{182}$ with His introduces a bulky, polar side chain projecting into the SBP, interfering with lipid binding. By contrast, the distance between the carboxyl carbons of Leu$^{247}$ and the R182L mutant fluctuated dramatically (7.40 ± 0.49 Å). This indicates that the 171–182 loop in R182L moves dramatically with respect to the COOH terminus, opening up the SBP; this may provide a more flexible hydrophobic environment for lipid binding, resulting in the reduced binding affinity of R182L. The enlarged SBP may also explain the increased lipid-binding capacity of the R182L mutant.

**DISCUSSION**

The mechanism of action for StAR remains unclear (19). It is now established that StAR acts on the outer mitochondrial membrane (9) where it undergoes a molten globule structural transition (11–14). It is also clear that StAR can act as a cholesterol transfer protein, able to transfer cholesterol between a variety of membrane systems (22–24), but it is not apparent that these two activities are correlated. The structures of proteins closely related to StAR (16–18) and computational mod-

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**FIGURE 6. Kinetics of cholesterol binding.** A, time course of binding of 1 μM NBD-cholesterol to 1 μM wild-type or mutant N-62 StAR. Wild-type, solid black line; R182H, solid gray line; R182L, dashed black line; heat-denatured wild-type, dashed gray line; buffer without protein, dot and dashed line. B, competition/displacement assays for binding and dissociation of cholesterol from wild-type (upper panel) and R182L N-62 StAR (lower panel). Solid lines, 1 μM cholesterol preincubated with 1 μM StAR protein was displaced by addition of 1 μM NBD-cholesterol. The data fit well to a hyperbolic curve (one binding site). Dashed lines, 1 μM NBD-cholesterol preincubated with 1 μM StAR protein was displaced by addition of 1 μM cholesterol. The data fit well to one-phase exponential decay. Gray lines, NBD-cholesterol in binding buffer.

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ence StAR activity by influencing its interactions with hypothetical partner proteins, such as PAP7, which appears to target protein kinase A to Ser$^{195}$ (37). This would be consistent with the Ser$^{195}$ mutants having CD spectra (Fig. 1B), kinetics of activity (Fig. 2B), and cholesterol-binding characteristics (Figs. 3 and 4) that are the same as wild-type despite the lower activity of the S195A mutant. Arg$^{182}$ lies at the beginning of β6-sheet, forming main chain interactions with the adjacent β5-strand.
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![Diagram of wild-type N-62 StAR](image)

FIGURE 7. Location of the mutants. A, ribbon diagram of wild-type N-62 StAR, showing the location of Ser\(^{195}\) in the cytoplasmically exposed loop joining sheets \(\beta_\alpha\) and \(\beta_\beta\), cholesterol, shown as a stick figure with a space-filling black wire-mesh surface, was docked into the model of wild-type N-62 StAR using Dock 4. Only a portion of the StAR molecule is shown as a ribbon diagram with the indicated residues, showing the locations of Arg\(^{188}\) and Arg\(^{188}\), represented as ball-and-stick diagrams.

eling of StAR (10, 14) indicate that StAR has an SBP that should bind cholesterol with 1:1 stoichiometry. Such binding has been demonstrated with bacterially expressed StAR (14, 16). StAR mutants that lack cholesterol-binding capacity also lack activity (14, 16, 23), and we have correlated the partial binding activity of the V187M and R188C mutants with clinical findings and partial steroidogenic activity measured in vitro (28). We now show that the StAR mutant R182L, which causes severe congenital lipoid adrenal hyperplasia and is devoid of measurable activity in a transfected cell system (29, 30), is able to bind cholesterol and transfer it in vitro. Thus cholesterol binding is not sufficient to ensure StAR function.

Modeling shows that there is not enough room for a cholesterol molecule to enter or exit the SBP of StAR without a conformational change, and CD data reveal such a conformational change when StAR binds cholesterol (12, 23). Our MD data show that the C-helix moves away from the loop between sheets \(\beta_1\) and \(\beta_2\) (residues 102–106) and opens the SBP when Asp, Glu, and His residues of the C-helix are protonated, thus modeling the interaction of StAR with the protonated phospholipid head groups of the OMM (14). Other MD simulations confirm the crucial role of the movement of the C-helix and its adjacent structures in cholesterol binding, but concluded that cholesterol entry was between the C-helix and the 171–182 loop (38). We tested our model by engineering disulfide bonds between the C-helix and the 102–106 loop. The SS mutant replaced Ser\(^{100}\) and Ser\(^{261}\) with cystines, and the DA mutant replaced Asp\(^{106}\) and Ala\(^{268}\) with cystines; each pair of cystines formed a disulfide bond. The SS mutant partially restricted the movement of the C-helix and reduced cholesterol binding and StAR activity by about half, whereas the DA mutant restricted movement further and eliminated measurable cholesterol binding and StAR activity (14). It has been suggested that these disulfide mutants introduced subtle but crucial structural changes that destroyed activity through means other than restricting the movement of the C-helix (38). However, the CD and fluorescence spectra of the wild-type and disulfide mutants were indistinguishable, and MD simulations show similar movements in the wild-type and disulfide mutants, except in the C-helix and adjacent loops (14). Compared with the starting structure, the Ca root mean square deviation values are 1.81 ± 0.11 Å for the wild-type, 1.64 ± 0.11 Å for the SS mutant, and 2.00 ± 0.16 Å for the DA mutant. The creation of the disulfides changes the Ser\(^{100}\)/Ser\(^{261}\) Ca distance from 8.44 ± 0.96 Å in the wild-type to 6.51 ± 0.19 Å in the SS mutant and changes the Asp\(^{106}\)/Ala\(^{268}\) Ca distance from 6.87 ± 0.33 Å in the wild-type to 6.96 ± 0.13 Å in the DA mutant. Thus the local effect was greater in the SS mutant, which retained partial activity, than it was in the DA mutant, which lost all cholesterol-binding and StAR-induced steroidogenic activity, consistent with the effects of these disulfide mutants arising from the restriction of the movement of the C-helix. Thus modeling and MD simulations using different computational approaches conclude that movement of the C-helix and adjacent structures is required for cholesterol binding but differ in emphasizing the 102–106 loop (14) versus the 171–180 loop (38); however, the former but not the latter is supported by direct experimental results.

Congenital lipoid adrenal hyperplasia can be viewed as resulting from StAR knock-out or site-directed mutagenesis experiments of nature (39). Patients have been described with two different mutations at Arg\(^{188}\): R182H and R182L. Seven individuals homozygous for R182H were described in five Saudi families; these individuals had unusual clinical courses, with the age of onset of symptoms ranging from 1 to 14 months of age (31). The reason for this clinical variability of the same genetic lesion on what appears to be the same genetic background is unclear. When tested in transfected cells (31) or when added to steroidogenic mitochondria in vitro (Fig. 2) this mutant lacked activity, but it retained partial capacity to bind cholesterol under steady-state conditions (Fig. 3). Similarly, three individuals homozygous for R182L all had onset of clinical symptoms of adrenal steroid deficiencies within the first week of life, and the two who were 46, XY genetic males had wholly normal female external genitalia, indicating a profound lesion in testicular steroidogenesis (29). Furthermore, this mutant had no activity in transfected cells (29) or when added to steroidogenic mitochondria in vitro (36) (Fig. 2). Thus this mutant is devoid of biological activity and achieved steady-state cholesterol binding equivalent to that of the wild-type protein (Fig. 3). By contrast, two siblings homozygous for the R188C mutant had the onset of clinically apparent adrenal insufficiency at about 2.5 years of age and are the only known 46, XY individuals with StAR mutations who had sufficient fetal testicular steroidogenesis to achieve male external genital development (28). Consistent with this attenuated phenotype, this mutant had partial activity with isolated mitochondria in vitro and had measurable but reduced steady-state cholesterol binding capacity (28) (Figs. 3 and 4). Thus there are major differences between R188C, where
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binding and activity correlate closely, and R182L, where they do not. A similar phenomenon has been described with the yeast oxysterol-binding protein termed Kes 1 or Osh 4, which has a structure that is distantly related to StAR (40). Targeting of this protein to the Golgi apparatus is required for activity, and that targeting requires phosphoinositide binding, but some inactive Kes 1 mutants retain phosphoinositide-binding capacity (41, 42).

To explore the role of cholesterol binding in StAR activity, we focused on R182L, where the discrepancy in these variables was profound. Kinetic analysis showed that both the rate of cholesterol binding ($t_{1/2on}$) and the rate of cholesterol dissociation ($t_{1/2off}$) were substantially slower for the R182L mutant, so that, even though net binding capacity at equilibrium was equivalent to wild-type, the rate of cholesterol transfer was reduced to about 20% of control. Whereas this slow cholesterol exchange could explain reduced activity, as discussed above, it remains clear that R182L is inactive in vivo and in vitro. Thus another explanation for the lost activity of R182L is needed.

It is apparent that StAR exerts two separate actions. First, StAR exerts a minor activity as a cholesterol transfer protein to deliver cholesterol to membranes, shown by the action of N-62 StAR to deliver cholesterol to non-mitochondrial membranes (22) and by its activity to transfer cholesterol between synthetic membranes in vitro (23, 24). Our data indicate that the biologically inactive R182L mutant retains this activity. This cholesterol-transfer activity probably represents a small portion of the cholesterol transferred to the OMM. Once StAR reaches the OMM its leader peptide will become associated with the mitochondrial protein-import machinery affixing StAR to the OMM, and preventing its re-cycling. Thus StAR is predicted to transfer stoichiometric amounts of cholesterol to the OMM.

Recent data suggest that the majority of cholesterol transferred to the OMM is delivered by StarD4, a leaderless protein having a structure very similar to StAR (17, 43–45). Second, once StAR becomes associated with the OMM, StAR exerts its major activity to foster the transfer of large amounts of cholesterol from the OMM to the IMM (9). This action requires its molten globule conformational change (11–14) and is predicted to move up to 400 molecules of cholesterol per molecule of StAR per minute (25). The loss of this activity causes congenital lipid adrenal hyperplasia, which can be caused by the StAR mutant R182L (29). As our present data show that R182L can bind and transfer cholesterol, it is now clear that cholesterol transfer does not explain the major activity of StAR. We speculate that this major activity, which requires the molten globule transition, is to mobilize cholesterol previously associated with the cholesterol recognition amino acid consensus domain of peripheral benzodiazepine receptor for mitochondrial import, possibly in association with the voltage-dependent anion channel protein, VDAC (19). Distinguishing these two activities of StAR should facilitate further studies of its mechanism(s) of action.

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