Steroidogenic acute regulatory (STAR) protein facilitates import of cholesterol into adrenal and gonadal mitochondria where cholesterol is converted to pregnenolone, initiating steroidogenesis. STAR acts exclusively on the outer mitochondrial membrane (OMM) by unknown mechanisms. To identify STAR domains involved in membrane association, we reacted N-62 STAR with small unilamellar vesicles (SUVs) composed of lipids resembling the OMM. Soluble 60-meric domains were digested with trypsin, Asp-N, or pepsin at different pH levels, and STAR peptides protected from proteolysis were identified by mass spectrometry. At pH 4 SUVs completely protected residues 259–282; at pH 6.5 this region was partially digested into 254–272, 254–273, and 254–274. Computer-graphic modeling of N-62 STAR indicated these peptides correspond to the C-terminal α-helix and that residues Leu275, Thr263, and Arg272 in α-form stabilizing interactions with Gln128, Asp150, and Asp100 in adjacent loops. CD spectroscopy of a 37-mer model of α4 (residues 247–287) indicated a random coil in aqueous buffer, but in 40% methanol the peptide was α-helical and achieved maximal α-helicity at pH 5.0 in the presence of SUVs. Reacting the 37-mer with diethyl pyrocarbamate incorporated into SUVs increased the number of modified residues. Thus the C-terminal α-helix is critically involved in the membrane association of STAR with OMM lipids. The membrane association and the probable three-dimensional structure of the C-terminal in the presence of OMM lipids are also pH-dependent. These results further support STAR undergoing a pH-dependent change in its conformation when interacting with the acidic phospholipid head groups of a membrane.

The steroidogenic acute regulatory (STAR) protein plays a critical role in steroidogenesis by facilitating the flow of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) where the cholesterol side-chain cleavage enzyme, P450acc, converts cholesterol to pregnenolone (1, 2). Full-length STAR is expressed as a 285-residue protein with a molecular mass of 37 kDa having a mitochondrial leader sequence that is cleaved to yield a 30-kDa protein upon entering the mitochondria (3, 4). Deletion of 62 N-terminal residues (N-62 STAR) yields a protein that remains in the cytoplasm yet retains full biologic activity (5). The mechanism by which STAR facilitates mitochondrial cholesterol import is not known. However, several lines of evidence indicate that STAR acts primarily and probably exclusively on or in the OMM (2). First, N-62 STAR remains in the cytoplasm, but is fully active (5). Second, bacterially expressed human N-62 STAR is active on isolated mitochondria in vitro (6, 7). Third, cytoplasmic N-62 STAR can transfer cholesterol to other membranes, such as the endoplasmic reticulum (8), and can transfer cholesterol between synthetic unilamellar vesicles in vitro (9). Fourth, STAR is inactive in the mitochondrial intramembranous space or when immobilized on the IMM, but manipulating the STAR leader to slow its mitochondrial entry or to immobilize STAR on the OMM shows that the activity of STAR is proportional to its residency time on the OMM (10). These data are consistent with data indicating that a tightly packed, protease-resistant N-terminal region (residues 63–188) of STAR slows its mitochondrial import to keep STAR on the IMM where it is biologically active (11).

Although a crystal structure of STAR has not been determined, the crystal structures of the STAR-related lipid transfer domains of two closely related proteins, N-216 MLN64 (12) and StarD4 (13), have been solved to 2.2-Å resolution. Both of these structures show an α/β-helix grip fold and an elongated hydrophobic pocket that can accommodate one molecule of cholesterol. N-216 MLN64 has about 50% of the activity of STAR to promote steroidogenesis in transfected cell systems (7, 14), and both spectroscopic data and the results of proteolytic cleavage indicate it is folded similarly to STAR (7). Both N-216 MLN64 and StarD4 have the same fold (12, 13); homology modeling indicates that hamster STAR also shares this fold (15). Thus there is substantial information about the probable three-dimensional structure of STAR.

Biophysical studies of N-62 STAR in solution and in association with membranes show that it exhibits pH-dependent molten globule properties (11, 16, 17). This might expose a cholesterol binding pocket, allowing this intermediate structure to bind EPC, egg phosphatidylcholine; CL, 1,2-dioleoylcardiolipin; SM, sphingomyelin; DEPC, diethyl pyrocarbamate; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; CD, circular dichroism; DHB, 2,5-dihydroxy benzoid acid; SUVs, small unilamellar vesicles.
deliver cholesterol from the OMM to the IMM (11). The C terminus appears to contain biologically relevant domains, because all missense mutations in StAR resulting in congenital adrenal hyperplasia are located in the C-terminal 40% of the protein (4, 18, 19). Furthermore, deletion of ten C-terminal residues ablates all activity (4, 5). Structural analysis indicates that the C-terminal 28 residues of StAR form an α-helix (Fig. 1). We hypothesize that this helix, which in StAR is essential to maintain steroidogenic activity, could hinge out to interact with lipid membranes. If inserted into the membrane, it might induce conformational changes to open the binding pocket and release cholesterol into the membrane.

Despite the available structural and biologic data, the orientation of StAR with respect to the OMM and the identities of the domains that interact with the OMM are unknown. Such information should facilitate understanding of the mechanism of action of StAR. We used mass spectrometric analysis of StAR peptides protected from proteolysis by synthetic lipid models of the OMM to show that the C-terminal α helix is the principal region of StAR that interacts with the OMM. Similar experiments plus spectroscopic studies using a 37-amino acid model of the C-terminal domain of StAR confirm pH-dependent interactions with lipid membranes.

**MATERIALS AND METHODS**

**Reagents**—Wild type His₆-N-62 StAR protein was expressed in *Escherichia coli* and purified as described previously (20). Egg phosphatidylcholine (EPC), tetraoleoylcardiolipin (CL), egg phosphatidylethanolamine, egg sphingomyelin (SM), and bovine brain phosphatidylinositol were obtained from Avanti Polar Lipids (Birmingham, AL). Pepsin (immobilized on agarose beads) was from Pierce, and trypsin (porcine,

---

**TABLE I**

| Molecular mass | Intensitya | Residues | Peptide sequence |
|----------------|------------|----------|-----------------|
| 669.36         | m³         | 179–184  | VGPRDF          |
| 814.49         | m³         | 248–254  | KGWLPKS         |
| 930.54         | m³         | 244–251  | SIDLKGWL        |
| 939.60         | w/m³       | 111–119  | KVVDPDGVKV      |
| 943.57         | s³, s⁶     | 82–90    | AMQKALGIL       |
| 1026.64        | m³         | 110–119  | SKVVDPDVGVKV    |
| 1043.63        | m³         | 243–251  | LSIDLKGWL       |
| 1154.70        | s², s⁶     | 248–257  | KGWLPKSIINQ     |
| 1155.70        | m²         | 245–254  | IDLKGWLPKS      |
| 1202.56        | m³         | 144–153  | MGEWNPNVKE      |
| 1242.73        | w/m³, m⁵   | 244–254  | SIDLKGWLPGKS    |
| 1282.77        | vs³, vs⁶   | 248–258  | KGWLPKSIINQ     |
| 1313.69        | m/s², m/⁶ | 123–133  | EVVVDQPMERL     |
| 1355.86        | s⁷, vs⁶    | 111–122  | KVVDPDVGVVFRL   |
| 1388.86        | vs⁶, s⁷    | 154–165  | IKVLQKIGKDTF    |
| 1446.89        | vs⁶        | 226–239  | VHLPLAGSPSSTKL  |
| 1577.93        | vs⁶        | 225–239  | MVLPLAGSPSSTKL  |
| 1624.00        | m³, s⁶     | 245–258  | IDLKGWLPKSIINQ  |
| 1686.04        | m³, m/s⁶   | 243–257  | LSIDLKGWLPGKSIN |
| 1711.00        | m³, s⁶     | 244–254  | SIDLKGWLPGKSIN |
| 1790.96        | s⁶         | 75–90    | YLQEGGAEAMQKALGIL |
| 1824.08        | s⁷, s⁶     | 243–258  | LSIDLKGWLPGKSIINQ |
| 1832.97        | m³         | 166–183  | ITHELAEEAGNLVGRPD |
| 1847.09        | vs³        | 225–242  | VHLPLAGSPSSTKL |
| 1862.00        | m³, s⁶     | 74–90    | AYLQEGGAEAMQKALGIL |
| 1869.12        | s³, m³     | 154–169  | IKVLQKIGKDFITHE |
| 1917.91        | m³/s³      | 138–153  | VERMAEGWPNVKE |
| 1978.14        | s³, m³/s³  | 225–242  | MVLPLAGSPSSTKL |
| 1980.06        | m³, w/m⁶   | 166–184  | ITHELAEEAGNLVGRPD |
| 2265.22        | m³, m³/s⁶  | 166–187  | ITHELAEEAGNLVGRPD |
| 2549.35        | w/m³, s⁶   | 261–282  | SQTQVDFAHNLRRLESHPASE |
| 2761.50        | s⁶         | 259–282  | VLSQTQVDFAHNLRRLESHPASE |

---

a MS/MS peak intensity: blank cell = not detected, w = weak, m = medium, and s = strong.

b Without lipids.

c OMM lipids.
sequencing grade-modified) was from Promega (Madison, WI). Sodium chloride, sodium phosphate, and diethyl pyrocarbamate (DEPC) were obtained from Sigma-Aldrich, and OPTIMA grade methanol was from Fisher (Fairlawn, NJ). All other reagents were from commercially available sources and of the highest purity.

Liposome Preparation—Lipid standards dissolved in chloroform were mixed to prepare vesicles composed of EPC, phosphatidylethanolamine, phosphatidylidyinositol, SM, and CL (51:26:11:4:3) to model the outer mitochondrial membrane (OMM) (21). Some samples also contained 5 parts of cholesterol. The chloroform was evaporated to dryness at ambient temperature using a probe sonicator pulsed three times for 3 min each (16).

Multilamellar vesicles composed of 82 mol% EPC and 18 mol% CL were prepared in pH 4.0, 20 mM sodium citrate buffer containing 150 mM KCl as above (16). SUVs were generated by equilibrating the lipid mixture with an extrusion apparatus (Avanti Polar Lipids, part number 610000) at 37 °C for 10 min and passing the solution through a 0.1-μm polycarbonate membrane 21 times (22, 23). An average particle size of 37-mer between residues 18 and 19, simplifying the MS analysis. The eluate was evaporated nearly to dryness, and the samples were dissolved to 50 μl with 0.1% formic acid. The peptide mixture from the final eluate was evaporated nearly to dryness and dissolved to 50 μl of 0.1% formic acid in water:acetonitrile (75:25). A 0.5-μl aliquot of the extract was manually injected with a Hamilton syringe, and C18 zip-tips were used to remove impurities. Each eluate was evaporated nearly to dryness, and the samples were prepared in water, and the entire sample was transferred onto a stain-free steel MALDI target for cocrystallization (ABI, Foster City, CA). The peptide was purified by HPLC, the molecular weight was confirmed by electrospray mass spectrometry, and the sample was lyophilized. The 37-mer (20 nmol/μl) was incubated at room temperature for 10 min in 200 μl of 20 mM phosphate buffer, pH 6.5, without or with SUVs composed of EPC:CL (82:12) at a total lipid concentration of 50 μM. The sample was de-salted using a Millipore 

Fig. 2. Mass spectrometric analysis. N-62 StAR was incubated with SUVs at pH 4.0, digested with pepsin for 30 min at 37 °C, subjected to HPLC, and analyzed by electrospray MS/MS. A, total ion chromatogram trace of the MS signal (upper panel), and zoom view of the retention window at 26.7 min (lower panel). B, MS ions detected at 26.7 min, with expanded views of the isotopic profiles of m/z 553.5 and 691.7 showing 5′ and 4′ ions, respectively. The calculated molecular masses of the 5′ and 4′ ions are 2761.48 and 2761.45. C, MS/MS spectra of the m/z 553.5 peak at 26.7 min corresponds to the peptide VLSQTVDFANHLKRLESHPASE. The top sequence shows ions from m/z 501 to 1300. The sequence corresponds to human StAR residues 259–282.

Peptide Limited Proteolysis—A synthetic 37-mer peptide corresponding to residues 247–284 of human StAR (excluding the C-terminal cysteine) was prepared by Dr. Hayden Ball (Protein Chemistry Technology Center, University of Texas Southwestern Medical Center, Dallas, TX). The peptide was purified by HPLC, the molecular weight was confirmed by electrospray mass spectrometry, and the sample was lyophilized. The 37-mer (20 nmol/μl) was incubated at room temperature for 10 min in 200 μl of 20 mM phosphate buffer, pH 6.5, without or with SUVs composed of EPC:CL (82:12) at a total lipid concentration of 50 μM. The sample was incubated with DEPC (0.1%, v/v) for 15 min at 25 °C. The peptides were isolated from the reaction mixture by zip-tip extraction/adsorption onto a pre-conditioned Millipore μC18 zip-tip (Millipore, Milford, MA) following the manufacturer’s instructions. The eluate was evaporated nearly to dryness in a SpeedVac; the peptide extracts were dissolved in 50 μl of 25 mM ammonium bicarbonate buffer, pH 8.5, and digested with 12 μl Asp-N for 3 h. This cleaves the 37-mer between residues 18 and 19, simplifying the MS analysis. The sample was de-salted using a Millipore μC18 zip-tip as above, and the final eluate was evaporated nearly to dryness and dissolved to 50 μl with 0.1% formic acid in water:acetonitrile (75:25). A 0.5-μl aliquot of the extract was mixed with 0.5 μl of saturated dihydroxy benzoic acid prepared in water, and the entire sample was transferred onto a stainless steel MALDI target for cocrystallization (ABI, Foster City, CA). The 37-mer peptide fragments were analyzed by MALDI MS. Analysis of N-62 STAR Plus Pepsin Digestion at pH 4.0—Prior to MS analysis, Millipore μC18 zip-tips were used to remove impurities. Each eluate was evaporated nearly to dryness, and the samples were dissolved to 10 μl with 0.1% formic acid. The peptide mixture from the pepsin digest (1 μl) was manually injected with a Hamilton syringe, and an LC-Packsings Ultimate HPLC (LC-Packsings, South San Francisco, CA) was used for solvent delivery. The samples were loaded onto a 75 μm x 15 cm, 5-μm particle size C-18 column (LC-Packsings) and eluted.
with 0.1% formic acid in 70% methanol and spotted for 30 s (150 nl of 15 cm, 5-β-cyano-4-hydroxycinnamic acid that was previously dissolved in 0.1% trifluoroacetic acid in 70% methanol and spotted for 30 s (~150 nl of sample/spot) onto a 100-well stainless steel MALDI target using a Probot (LC-Packings). MALDI-MS/MS analysis was performed on an ABI 4700 Proteomics Analyzer operated in the positive ion mode. MS/MS data were collected on all molecular ions having a signal-to-noise ratio of >15.

Homology Modeling of Human StAR—The sequence of human N-62 StAR (24) was aligned with the sequence of human N-216 MLN64 (12) using the program Swiss-PDB Deep View (version 3.7). The resulting alignment was examined manually and was submitted for automatic modeling using the human N-216 MLN64 template PDB ID: 1EM2 (12) on the Swiss-Model server (www.expasy.ch/spdbv/). The potential energy of the initial StAR model was minimized using the Amber7 program (force field ff99) at the Computer Graphics Laboratory at University of California at San Francisco. Energy minimization was performed with 250 steps of steepness plus 750 steps of conjugate gradient. The free energy of the structure reached the local minima after the run. The resulting model was checked using the program What If (www.cmbi.kun.nl.gv.servers/WIWWWI/), and the Ramachandran plots were performed in a 0.1-mm path length flat cuvette in a Jasco 720 spectrophotometer equipped with a Peltier temperature controller. The CD measurements were performed in a 0.1-mm path length flat cuvette in a Jasco 720 spectropolarimeter equipped with a Peltier temperature controller. The 37-mer (20 nmol) was mixed with varying concentrations of methanol at

**TABLE II**

| Molecular mass | Intensity* | Residues | Peptide sequence |
|----------------|-----------|----------|-----------------|
| 857.53         | s          | 153–159  | EIKVLQK         |
| 1049.53        | m/m/s, m/s, w/m/d | 133–140 | LYEEILVER       |
| 1095.53        | m/s, s, w/m/d  | 275–284 | LESHPASEAR      |
| 1114.60        | m/s, s, w/m  | 112–121 | VVPDVKVVF       |
| 1200.64        | m/s         | 239–248 | LTWLLSIDILK     |
| 1251.67        | s           | 274–284 | RLESHPASEAR     |
| 1313.66        | m/s, w/m/s, w/m/d | 122–132 | LEVVDQPMERc     |
| 1314.69        | m/s         | 86–97   | ALGILNSQEGWK     |
| 1379.71        | m/w         | 273–284 | KRLESHPASEAR     |
| 1404.61        | m/s, s, s, s | 141–152 | MEAMGEWNPNVIK  |
| 1429.79        | m           | 237–248 | TKTWLLSIDILK     |
| 1442.78        | m/s         | 86–98   | ALGILNSQEGWKK     |
| 1464.63        | m/s, w/mb   | 99–111  | ESSQDDNGKVMKSb  |
| 1716.00        | m/sb, m/sd  | 119–132 | VFRLLEVVDQMPERS  |
| 1774.83        | m/s, m     | 141–155 | MEAMGEWNPVNKEIK |
| 1500.93        | m/sb        | 218–236 | AEHPTCVMLHPLAEPK |
| 2081.24        | w/m, w/m, w/mf | 163–182 | DTFITHELAAEAAGNLVGP |
| 2112.93        | m           | 194–213 | GSTCVLAGMATDFGNMPEQKC |
| 2182.15        | w, m/sb, m/sf | 254–272 | SINQVLSTQVDLAFHNLR |
| 2217.11        | w/m         | 218–238 | AEEGPTCVMLHPLAEPK |
| 2243.15        | m/s, w/msf  | 141–159 | MEAMGEWNPVNKEIKVLQK |
| 2310.25        | w, m, wdf  | 254–273 | SINQVLSTQVDLAFHNLR |
| 2345.20        | w, w, wdf  | 122–140 | LEVVDQPMERLYEEILVER |
| 2379.26        | v, m, m     | 160–182 | IGKDTFTHELAAEAAGNLVGP |
| 2410.31        | w/m, w/mf  | 112–132 | VVPDVKVFRLEVVDQMPER |
| 2496.15        | w, wdf      | 153–152 | LEYELVERMEAMGEWNPV |
| 2466.35        | w           | 254–274 | SINQVLSTQVDLAFHNLRKR |
| 2642.38        | m           | 214–238 | GVRAEHEHPTCVMLHPLAEPK |
| 2847.56        | w/m, w/mf  | 156–182 | VLQKIGKDTFTHELAAEAAGNLVGP |
| 3082.62        | w           | 160–188 | IGKDTFTHELAAEAAGNLVGPDRFVSVR |

*a MS/MS peak intensity: blank = not detected, s = strong, m = medium, and w = weak.

*b No lipide.

c OMM lipids.

*d OMM lipids plus cholesterol.

e Peptide detected with oxidized Met.

**FIG. 3.** Summary of the N-62 StAR C-terminal peptides identified in experiments with SUVs. At pH 4.0, residues 259–283 were protected from digestion, and at pH 6.5 multiple fragments consisting of 254–272, 254–273, and 254–274 were detected. These data suggest complete protection at pH 4.0 and partial protection at pH 6.5.
pH 7.0, with buffers of varying pH with or without 50 μM lipid (82:18, EPC:CL). The pH 2 and pH 7 buffers were 4 mM phosphate, and pH 3–6 buffers were 4 mM citrate. Each preparation was incubated at room temperature for 5 min before the measurement, and the sample holder was maintained at 23 °C. Each spectrum represents the average of at least four accumulations with subtraction of the appropriate background. Each experiment was repeated three times, and the spectra from each experiment were averaged. The secondary structure analysis was performed using CDPro analysis tool SELCON3 (25).

Modification with Diethyl Pyrocarbamate—DEPC was prepared at 5% in 1:1 methanol:water and vortexed to dissolve the DEPC completely. The 37-mer (20 nmol) was mixed without or with 50 μM of 82:18 EPC:CL SUVs in 20 mM phosphate buffer, pH 6.5, incubated at 23 °C for 5 min, reacted twice with 5 μl of 5% DEPC (final concentration 0.1%) for 15 min at 25 °C, and the reaction was quenched with 1 μl of 1 mM imidazole. The peptide was extracted from the solution by adsorption onto a Millipore C18 zip-tip following the manufacturer’s instructions for conditioning and washing. The peptide was eluted with 20 μl of 70% acetonitrile and evaporated to ~2 μl in a SpeedVac. The peptide was reacted with 10 μl of 12 ng/ml endoproteinase Asp-N prepared in 25 mM ammonium bicarbonate buffer (pH 8) for 4 h, after which the peptide was extracted with a Millipore C18 zip-tip as described above. The sample was dissolved in 50 μl of 10% acetonitrile from which 0.5 μl was mixed with 0.5 μl of a saturated solution of 2,5-dihydroxy benzoic acid prepared in water, and the entire mixture was spotted on a MALDI target for MALDI-TOF analysis.

RESULTS

Interaction of N-62 StAR with Lipid Membranes—Although it is now clear that StAR acts on or in the OMM (10), it is not clear to what extent StAR becomes associated with the OMM, or which domains of the StAR protein interact with the OMM. To answer these questions, we designed “liposome protection” experiments. Purified, bacterially expressed human N-62 StAR was mixed with small unilamellar vesicles (SUVs) composed of 51% egg phosphatidylcholine (EPC), 26% phosphatidylethanolamine, 11% phosphatidylinositol, 4% sphingomyelin (SM), and 3% cardiolipin (CL), a lipid mixture designed to approximate the composition of liver OMM (21) or with SUVs of the same composition containing 5% cholesterol. The domains of StAR exposed to solvent were then digested with a proteolytic enzyme, and the membrane-associated, liposome-protected domains were identified by comparing the pattern of the resulting peptides with the pattern obtained under the same conditions, but in the absence of SUVs.

When N-62 StAR was digested with pepsin at pH 4.0, and the peptides identified in the presence and absence of lipids were similar except for a peptide of mass 2761.50, which was unique to the experiment conducted in the presence of lipids (Table I). The MS/MS spectrum of this peptide allowed us to decipher its sequence unambiguously, confirming that it comprises StAR residues 259–283 (Fig. 2). Similarly, when N-62 StAR was digested with trypsin at pH 6.5, equivalent peptides were identified in the presence and absence of lipids except for the C terminus of the protein (Table II). By contrast to the results at pH 4.0, the C terminus of the protein was not completely protected from enzymatic digestion but was partially protected at pH 6.5. The presence or absence of cholesterol in the SUVs had no effect on the protection at pH 6.5. The sequence RKR (residues 272–274 in the center of the C-terminal α helix) acted as an indicator for the efficiency of tryptic digestion. In the absence of lipids, proteolysis was complete, giving the tryp-
tic peptide 254–272, whereas the presence of lipids reduced the degree of proteolysis, resulting in multiple tryptic peptides comprising residues 254–272, 254–273, and 254–274. Although there may be differences in the efficiencies of the enzyme digestions under the two sets of conditions employed, these observations suggest complete protection at pH 4.0 and limited protection at pH 6.5. The clear reduction in the proteolysis of the C terminus of StAR at both pH 4.0 and 6.5 in the presence of lipids compared with the reactions without lipids suggests that the C-terminal helix is absorbed into the lipid membrane at the lower pH. These effects on the C terminus were the only marked difference between digestion in the presence and absence of lipids (Fig. 3).

**Modeling the Structure of Human StAR**—To understand how the domains protected from proteolysis relate to the overall structure of StAR, and to conceptualize how these data can provide a view of how StAR associates with a membrane, we constructed a model of human N-62 StAR using the human N-216 MLN64 crystal structure (12) as a template. The model was validated in the What If program. The root mean square for bond length relative to common refinement constraint values was 0.717 Å, and for the bond angles was 1.35°. The Ramachandran plots of all residues other than Gly and Pro show that all but one of the residues was in a favored or allowed region, and the counterpart of this residue in the N-216 MLN64 crystal structure (12) is also in the disallowed region. The free energy of the model was $-4.3 \times 10^3$ kcal/mol after energy minimization with the Amber7 program, whereas the free energy calculated for the N-216 MLN64 crystal structure was $-5.9 \times 10^3$ kcal/mole.

As expected, the overall predicted structure of N-62 StAR is remarkably similar to the crystal structures of both N-216 MLN64 (12) and StarD4 (13). In this model the barrel of the putative lipid-binding pocket is formed by nine twisted antiparallel $\beta$-sheets, stretched by the N-terminal $\alpha$-helix and parallel C-terminal $\alpha$-helix at each end (Fig. 4). Two flexible loop regions, $\Omega_1$ and $\Omega_3$, lie adjacent to the C-terminal $\alpha$-helix and may participate in opening and closing the lipid-binding pocket. The C-terminal $\alpha$-helix interacts with the main structure through interactions of the peptide-bond backbone of Leu$^{270}$ and Thr$^{263}$ with the side chains of Gln$^{128}$ and Asp$^{150}$ respectively. The side chains of Arg$^{272}$ (a4 helix) and Asp$^{106}$ (loop $\Omega_1$) are predicted to form two hydrogen bonds that result in a stronger association between the a4 helix and the $\Omega_1$ loop. The distance between the side-chain amine nitrogen of Arg$^{272}$ and the oxygen of the carboxylic acid side chain of Asp$^{106}$ is about 2.72–2.78 Å, allowing for an ionic association between these two groups. The presence of such an ionic association would reduce the flexibility of the C-terminal $\alpha$-helix, thus stabilizing the lipid-binding pocket. At pH 4.0, protonation of Asp$^{106}$ will result in the loss of such ionic association and allow the C-terminal $\alpha$-helix to interact with the lipid membrane. By contrast, at pH 6.5, the ionic association would remain intact, thereby reducing the flexibility of the C-terminal $\alpha$-helix and limiting the membrane interaction. Pairs of such residues are also conserved in the crystal structures of N-216 MLN64 (Asp$^{106}$-Arg$^{145}$) and StarD4 (Asp$^{106}$-Arg$^{218}$).

**Structure of the a4 Helix**—Biophysical studies of the interactions of N-62 StAR with SUVs suggest that StAR interacts with lipids in a pH-dependent manner and that these interactions are important in adopting a conformation that participates in cholesterol transport (9). Our mass spectrometric data indicate that the C-terminal $\alpha$-helix is the principal domain of StAR that associates with membranes, and our model of human N-62 StAR identified key interactions between the C-terminal $\alpha$-helix and the adjacent $\Omega_1$ and $\Omega_3$ loops. To characterize the behavior of this C-terminal helix in more detail, we synthesized a 37-amino acid peptide composed of human StAR residues 248–284 (the C-terminal cysteine residue was not included). The first residue is Lys, located at the 6 o’clock position, and the labeling proceeds clockwise with each sequential residue numbered with a subscript. Residues identified by the modeling to interact with atoms in adjacent structures are shown in bold, the acidic residues thought to interact with the surface of lipid membranes are underlined, and key hydrophobic residues are boxed. The sequence of the 37-mer is shown below, with key residues indicated.

![Figure 5](http://www.jbc.org/)
and can distinguish α-helix, β-sheet, and random coil structures (26). At pH 7.0, the 37-mer had a strong negative signal at 198 nm (which is characteristic of random coil) and very little signal at 208 nm (characteristic of α-helix) or at 218–220 nm (characteristic of β-sheet) (Fig. 6A). Computational analysis (25) indicates that these data correspond to a helical content of about 32%. As the pH is lowered, the CD spectrum and calculated α-helicity change minimally (Fig. 6A). Thus the 37-mer in aqueous solutions does not show a substantial conformational change. By contrast, intact N-62 StAR shows a substantial conformational change at about pH 3.5 in aqueous solutions, indicative of a transition to a molten globule structure (11). These results suggest that the α-helicity of the C terminus seen in the crystal structures of N-216 MLN64 and StarD4 requires stabilization by adjoining structural elements. Similarly, another fragment of STAR, 63–193, was relatively unstructured in aqueous media, but reverted to a folded structure in media containing a hydrophobic agent (17). Therefore, we examined the effects of a hydrophobic agent, methanol, on the CD spectrum of the 37-mer. At pH 7.0, addition of 10% or 20% methanol had no perceptible effect on the CD spectrum (Fig. 6B). As the methanol concentration was raised to 30%, the CD spectrum changed dramatically, with a diminution in the minimum at 198 nm and appearance of a minimum at 208 nm, indicating a transition from coil to α-helix. At 40% methanol the spectrum was typical of an α-helix, and computational analysis indicated about 90% helical content. The reduced polarity of the environment in 40% methanol at pH 7.0 reduces hydrogen bonding with solvent molecules and fosters the formation of intra-peptide hydrogen bonds, favoring the formation of an α-helix. Thus the 37-mer has an inherent tendency to form an α-helix, but the dielectric effect of water is sufficient to prevent the formation of internal hydrogen bonds needed to form an α-helix.

To model the behavior of the C terminus of STAR interacting with the OMM, we examined the CD spectrum of the 37-mer peptide in the presence of egg phosphatidylcholine:cardiolipin (EPC:CL) 82:18 SUVs, a lipid composition previously used to describe membrane association of N-62 StAR (16) (Fig. 7). At pH 7, the CD spectrum of the 37-mer in the presence of SUVs showed substantially more α-helical character that was seen in aqueous buffer at pH 7 (Fig. 7A). As the pH was decreased to 5, a sharp spectral minimum becomes apparent at 208 nm, indicating a strongly helical structure (Fig. 7B). Computational analysis of the predicted secondary structure using the program SELCON3 (25) indicated 84% helicity at pH 5 in the presence of SUVs. As the pH was lowered further, this structure was destabilized. When the pH was reduced to 4, there was a decrease in the signal at 208 nm and a slightly deeper minimum at 225 nm, indicating loss of helicity (Fig. 7B). The spectral data at pH 4 were highly variable, indicating this is a critical pH for this structural transition (Fig. 7C). This structural transition could be explained by decreased electrostatic interactions between negatively charged aspartic acid (Asp) and glutamic acids (Glu) and glutamic acids (Glu)2, both having pK values of about 4, with positively charged quaternary ammonium groups of phosphatidylcholine. At pH 3, helicity was virtually abolished, consistent with the view that increased protonation of the acidic amino acids reduces membrane association, leading in turn to reduced helical structure of the peptide.

Interaction of the 37-mer with SUVs—To model the interaction of the C terminal α-helix of N-62 StAR with SUVs we mixed the 37-mer with SUVs and performed proteolysis with trypsin at pH 6.5 and pepsin at pHe 4.0 as was done for N-62 StAR. In both reactions residues 247–254, which are not part of the C-terminal α-helix, are readily digested, consistent with a lack of membrane association. At pH 6.5, trypsin digestion yielded the same pattern of peptides as seen for this region with N-62 StAR (Fig. 3). At pH 4.0 in the absence of SUVs, 14 peptides were seen, whereas the presence of SUVs protected several of the pepsin cleavage sites so that only 10 peptides were seen (results not shown). By contrast, only 4 peptides were seen in the pepsin digest of N-62 StAR. The greater access of the 37-mer to proteolysis at pH 4.0 compared with N-62 StAR probably reflects its unstable conformation, as shown by the CD spectroscopy at pH 4.0 (Fig. 7C). Thus the interaction of the 37-mer with SUVs accurately reflects the spectroscopic data and shows that the isolated C-terminal helix does not behave in the same fashion as the C-terminal helix in the context of the intact protein. Thus other domains of StAR participate in fostering the interaction of the C-terminal helix with membranes as indicated by the computational model.

Limited Reactivity of αd Helix Histidines and Lysines in the C Terminus—The experiments examining the ability of liposomes to protect N-62 StAR or the 37-mer examined StAR from the perspective of a reagent (the proteolytic enzyme) in the aqueous phase. We wished to see if we could confirm these results using a reagent associated with the lipid phase of these systems. For this purpose we used diethyl pyrocarbamate (DEPC), which is only slightly soluble in water (to 40 mM) and will therefore preferentially associate with lipids. In a two-phase system, DEPC will associate with the lipid phase and will derivatize the histidine and lysine residues of accessible proteins to form carbethoxy modifications of the amine side.
chains, resulting in a net addition of 72 Da (27). The presence or absence of these modifications can be examined by proteolysis with Asp-N, which yields two peptides: peptide A, an 18-mer encompassing StAR residues 247–265, and peptide B, a 19-mer encompassing StAR residues 266–284 (Fig. 8A). MALDI-TOF spectra of the 37-mer peptide reacted with 0.1% DEPC in the presence and absence of SUVs composed of 82:18 EPC:CL suggest that more residues are modified in the presence of lipids. For example, the unmodified peptide A0 was prominent in the reaction without lipids but was not detected in the reaction with lipids. Similarly, the doubly modified A2 peptide was barely detectable in the reaction without lipids but was a major component after reaction in the presence of lipids. The B peptide yielded several products: in the absence of lipids the most intense species was the singly modified B1 peptide, whereas in the presence of lipids the triply modified B3 peptide was most abundant. These results indicate that the 37-mer is bound to or inserted into the lipid membrane, putting the reactive residues in close proximity to the lipid-soluble DEPC.

Thus reagents that probe the C terminus of StAR from both the aqueous phase (proteolytic enzymes) and from the lipid phase (DEPC) indicate that the C-terminal α-helix is associated with membranes.

**DISCUSSION**

Bacterially expressed N-62 StAR binds to SUVs comprised of lipids similar to those found in the OMM, and changes in pH yield membrane association of StAR as an active molten globule (16). Using tandem mass spectrometric analysis we identified the amino acid sequences of N-62 StAR that were protected from enzymatic digestion in the presence of SUVs. Modeling of these results by modeling StAR in silico and analyzing the behavior of a synthetic 37-mer in vitro indicated a pH-dependent nature of these membrane interactions. The interaction of N-62 StAR with lipids at both pH 4.0 and 6.5 protected peptide fragments that identify the C-terminal α-helix as the principal structure associating with lipid membranes. Modeling of human N-62 StAR identifies key interactions between residues in the C-terminal α-helix with the adjacent t11 and t13 loops. Hydrogen bonds are predicted to form between helix residues Leu275 and Gln128 (which lies between the β-sheet 3 and α-helix 2) and between Thr263 and Asn150 (between α-helix 3 and β-sheet 4). A closer ionic interaction appears to occur between the primary amine of the side chain of Arg272 and the side chain carboxylic acid of Asp106. This ionic association between Arg272 and Asp106 may contribute to the protection of the C-terminal α-helix at pH 4.0. Because the pKₐ of aspartic acid is about 4.0, pH 4.0 will disrupt the ionic association between Arg272 and Asp106. Loss of this association may allow the C-terminal α-helix to interact more avidly with the membrane, sequestering it from proteolysis. By contrast, at pH 6.5, Arg272 and Asp106 would form an ionic association and the C-terminal α-helix would be less free to interact with the lipid membrane. Therefore, membrane association would be governed both by the properties of C-terminal α-helix and the adjacent structural regions.

The synthetic 37-mer, a model of the C-terminal sequence of StAR, behaves like an α-helix in hydrophobic solutions and associates with lipid membranes. Although the 37-mer does not form an α-helix in aqueous solutions, CD spectroscopy showed it formed a helical structure in relatively hydrophobic solvents, which provide a better in vitro model of the environment of the C terminus in the intact protein. Similarly, the lipid environment of SUVs induced the 37-mer to assume a helical confor-
mation at pH 5.0. This is a higher pH than the pH 3.5 that was responsible for the molten globule transition of N-62 StAR in aqueous solution (11) but is consistent with the dramatic structural change and maximal binding of N-62 StAR seen at pH 5.0 in SUVs composed of 82:18 EPC:CL (16), i.e. the same composition of SUVs used in the present study. Similar behavior has been observed with other proteins: a peptide derived from CTP:phosphocholine cytidylyltransferase (28), which had a predicted α-helical structure, was largely random coil in aqueous buffer but acquired 54% helicity in the presence of 50% trifluoroethanol and 72% helicity in SUVs of 4 mM phosphatidylinositol (28). Derivation of His and Lys residues of StAR at pH 6.5 with DEPC sequestered in SUVs confirmed the observation that the C-terminal helix associates with membranes. Thus we have presented multiple lines of evidence showing that the C-terminal helix of StAR associates with lipid membranes and that this association is favored by hydrophobic, mildly acidic conditions that represent a reasonable model of the interaction of StAR with the zwitterionic phospholipid head groups of the cytoplasmic aspect of the OMM.

StAR’s formation of a pH-dependent molten globule in aqueous solution (11) and in the presence of lipids (16) suggests that the conformational changes we observed with the 37-mer could be necessary for the association of StAR with the OMM and delivery of cholesterol to the IMM. The association of N-62 StAR with SUVs composed of 82:18 EPC:CL (16) is maximal at pH 3.5–4.0. Mass spectrometric analysis of N-62 StAR proteolyzed in the presence of SUVs revealed the C terminus of the protein is well protected from enzymatic digestion. These data suggest that the orientation of N-62 StAR relative to the lipid membrane is such that the C-terminal α-helix of the protein may sit on or in the membrane, whereas the rest of the protein is exposed to solvent. Cholesterol release could result from a pH-dependent transition to a molten globule structure, involving the loss of association between the C-terminal α-helix and adjacent α1 and α3 loops. Interaction between the C-terminal α-helix and lipid molecules in the OMM could promote the hinging of the C-terminal α-helix facilitating the release of cholesterol.

Acknowledgments—We thank David Agard and the University of California at San Francisco (UCSF) Department of Biochemistry and Biophysics for use of the spectropolarimeter. Also, we thank Eric Pettersen and Dr. Elaine C. Meng at the Computer Graphics Laboratory, UCSF, for their assistance with the AMBER program.

REFERENCES

1. Stocco, D. M., and Clark, B. J. (1996) Endocr. Rev. 17, 221–244
2. Miller, W. L., and Strauss, J. F., 3rd (1999) J. Steroid Biochem. Mol. Biol. 69, 131–141
3. Clark, B. J., Wells, J., King, S. R., and Stocco, D. M. (1994) J. Biol. Chem. 269, 28314–28322
4. Lin, D., Sugawara, T., Strauss, J. F., 3rd, Clark, B. J., Stocco, D. M., Saenger, P., Rogol, A., and Miller, W. L. (1995) Science 267, 1828–1831
5. Arakane, F., Sugawara, T., Nishino, H., Liu, Z., Holt, J. A., Pain, D., Stocco, D. M., Miller, W. L., and Strauss, J. F., 3rd (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 16731–16736
6. Arakane, F., Kallen, C. B., Watari, H., Foster, J. A., Sepuri, N. B., Pain, D., Stayrook, S. E., Lewis, M., Gerton, G. L., and Strauss, J. F., 3rd (1998) J. Biol. Chem. 273, 16339–16345
7. Bose, H. S., Whittal, R. M., Huang, M. C., Baldwin, M. A., and Miller, W. L. (2000) Biochemistry 39, 11722–11731
8. Kallen, C. B., Billheimer, J. T., Summers, S. A., Stayrook, S. E., Lewis, M., and Strauss, J. F., 3rd (1998) J. Biol. Chem. 273, 26285–26290
9. Tuckey, R. C., Headlam, M. J., Bose, H. S., and Miller, W. L. (2002) J. Biol. Chem. 277, 47123–47128
10. Bose, H. S., Lingappa, V. R., and Miller, W. L. (2002) Nature 417, 87–91

FIG. 8. MALDI MS analysis of the unseparated mixture 37-mer reacted with 0.1% DEPC in the presence and absence of SUVs followed by Asp-N digestion. A, predicted Asp-N peptide fragments, A peptide: *KGWLPSIINVQVLSTQEV and B peptide: DFANHLRKLHSPASEAR with the m/z values of the modified peptides. The bold residues represent potential sites of modification with DEPC, and the asterisk represents a modification site on the lysine side chain and/or the free N terminus. B, B, 37-mer without SUVs yields abundant fragments that correspond to fewer A and B peptide modifications. C, 37-mer in the presence of SUVs yields a peptide that corresponds to a greater number of modifications.
11. Bose, H. S., Whittal, R. M., Baldwin, M. A., and Miller, W. L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7250–7255
12. Tsujishita, Y., and Hurley, J. H. (2000) Nat. Struct. Biol. 7, 408–414
13. Romanowski, M. J., Soccio, R. E., Breslow, J. L., and Burley, S. K. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 6949–6954
14. Watari, H., Arakane, F., Moog-Lutz, C., Kallen, C. B., Tomasetto, C., Gerton, G. L., Rio, M. C., Baker, M. E., and Strauss, J. F., 3rd (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8462–8467
15. Mathieu, A. P., Fleury, A., Ducharme, L., Lavigne, P., and LeHoux, J. G. (2002) J. Mol. Endocrinol. 29, 327–345
16. Christensen, K., Bose, H. S., Harris, F. M., Miller, W. L., and Bell, J. D. (2001) J. Biol. Chem. 276, 17044–17051
17. Song, M., Shaw, H., Mujeeb, A., James, T. L., and Miller, W. L. (2001) Biochem. J. 356, 151–158
18. Bose, H. S., Sugawara, T., Strauss, J. F., 3rd, and Miller, W. L. (1996) N. Engl. J. Med. 335, 1870–1878
19. Miller, W. L. (1997) J. Mol. Endocrinol. 19, 227–240
20. Bose, H. S., Baldwin, M. A., and Miller, W. L. (1998) Biochemistry 37, 9768–9775
21. de Kroon, A. I., Dolis, D., Mayer, A., Lill, R., and de Kruijff, B. (1997) Biochim. Biophys. Acta 1325, 108–116
22. Subbarao, N. K., MacDonald, R. I., Takeshita, K., and MacDonald, R. C. (1991) Biochim. Biophys. Acta 1063, 147–154
23. MacDonald, R. C., MacDonald, R. I., Menco, B. P., Takeshita, K., Subbarao, N. K., and Hu, L. R. (1991) Biochim. Biophys. Acta 1061, 297–303
24. Sugawara, T., Lin, D., Holt, J. A., Martin, K. O., Javitt, N. B., Miller, W. L., and Strauss, J. F., 3rd (1995) Biochemistry 34, 12506–12512
25. Seerarama, N., Venyaminov, S. Y., and Woody, R. W. (1999) Protein Sci. 8, 370–380
26. Johnson, W. C., Jr. (1988) Annu. Rev. Biophys. Biophys. Chem. 17, 145–166
27. Safarian, S., Moosavi-Movahedi, A. A., Hosseinkhani, S., Xia, Z., Habibi-Rezaei, M., Hosseini, G., Sorensen, C., and Sheibani, N. (2003) J. Protein Chem. 22, 643–654
28. Johnson, J. E., and Cornell, R. B. (1994) Biochemistry 33, 4327–4335
pH-dependent Interactions of the Carboxyl-terminal Helix of Steroidogenic Acute Regulatory Protein with Synthetic Membranes

Dustin C. Yaworsky, Bo Y. Baker, Himangshu S. Bose, Katrina B. Best, Lauren B. Jensen, John D. Bell, Michael A. Baldwin and Walter L. Miller

J. Biol. Chem. 2005, 280:2045-2054.
doi: 10.1074/jbc.M410937200 originally published online October 15, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M410937200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 28 references, 12 of which can be accessed free at http://www.jbc.org/content/280/3/2045.full.html#ref-list-1