Molten-globule structure and membrane binding of the N-terminal protease-resistant domain (63-193) of the steroidogenic acute regulatory protein (StAR)

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The first step in steroidogenesis is the movement of cholesterol from the outer to inner mitochondrial membrane; this movement is facilitated by the steroidogenic acute regulatory protein (StAR). StAR has molten-globule properties at low pH and a protease-resistant N-terminal domain at pH 4 and pH 8 comprising residues 63–193. To explore the mechanism of action of StAR we investigated the structural properties of the bacterially expressed N-terminal domain (63-193 StAR) using CD, limited proteolysis and NMR. Far- and near-UV CD showed that the amount of secondary structure was greater at acidic than at neutral pH, but there was little tertiary structure at any pH. Unlike 63-193 StAR liberated from N-62 StAR by proteolysis, biosynthetic 63-193 StAR was no longer resistant to trypsin or proteinase K at pH 7, or to pepsin at pH 4. Addition of trifluoroethanol and SDS increased secondary structure at pH 7, and dodecylphosphocholine and CHAPS increased secondary structure at pH 2, pH 4 and pH 7. However, none of these conditions induced tertiary structure, as monitored by near-UV CD or NMR. Liposomes of phosphatidylcholine, phosphatidylserine and their mixture increased secondary structure of 63-193 StAR at pH 7, as monitored by far-UV CD, and stable protein–liposome complexes were identified by gel-permeation chromatography. These results provide further evidence that the N-terminal domain of StAR is a molten globule, and provide evidence that this conformation facilitates the interaction of the N-terminal domain of StAR with membranes. We suggest that this interaction is the key to understanding the mechanism of StAR's action.

Key words: CD, detergent, NMR, phospholipid, proteolysis.

INTRODUCTION

The steroidogenic acute regulatory protein (StAR) is synthesized as a 37 kDa protein, with an N-terminal mitochondrial signal sequence which is cleaved during entry into mitochondria to yield the 30 kDa intramitochondrial protein found in adrenal and gonadal steroidogenic cells [1]. StAR facilitates the movement of cholesterol from the outer to inner mitochondrial membrane, where it becomes the substrate for the cholesterol side-chain cleavage enzyme (cytochrome P450scc), which converts cholesterol to pregnenolone to comprise the first step of steroidogenesis [2]. Mutations in the human gene for StAR cause potentially lethal congenital lipoid adrenal hyperplasia [3–5]. Although StAR is translocated into the mitochondrial matrix, deletion of 62 amino acids from the N-terminus results in a cytoplasmic form of StAR (N-62 StAR) that retains complete activity and appears to interact with the outer mitochondrial membrane [6–8]. In contrast, deletion of only 28 amino acids from the C-terminus renders StAR inactive, and all known amino acid replacement (missense) mutations causing congenital lipoid adrenal hyperplasia are in the C-terminal region [4,5,9]. Thus it is the extramitochondrial precursor form that is active in steroidogenesis, and the C-terminal region that is especially important for biological activity. Although N-62 StAR does not enter the mitochondria, it associates with the outer mitochondrial membrane, since bacterially expressed N-62 StAR stimulates steroidogenesis in purified mitochondria *in vitro* [7,8,10], and even transfers cholesterol from phosphatidylcholine (PC) liposomes to isolated mitochondria [11].

We have reported [12] that the C-terminal domain (residues 194–285) of N-62 StAR is a molten globule, and that its N-terminal domain (residues 63–193) is tightly folded and protease-resistant (Figure 1). Molten globules are protein struc-

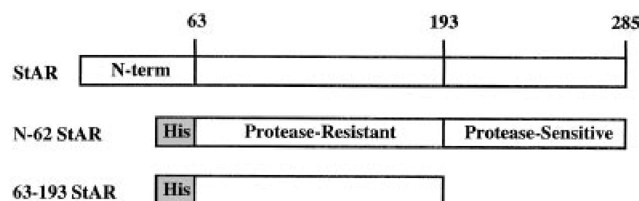

Figure 1 Primary structure of StAR

Upper structure: the full length StAR (37 kDa, 285 residues). The 62 N-terminal residues, including the mitochondrial leader peptide, can be deleted without loss of activity. Middle structure: the N-62 StAR studied previously [8,12], wherein the N-terminal 62 residues are deleted and replaced with a His_6 tag and an enterokinase site (18 residues). Partial proteolysis of this protein shows that residues 63–193 are protease-resistant, whereas residues 194–285 are digested quickly [12]. Lower structure: the biosynthetic 63-193 StAR studied in the present paper.

Abbreviations used: DPC, dodecylphosphocholine; HSQC, heteronuclear single-quantum coherence; NaPi, Na_2HPO_4/NaH_2PO_4 buffer system; PC, L-α-phosphatidylcholine; PS, L-α-phosphatidyl-L-serine; StAR, steroidogenic acute regulatory protein; 63-193 StAR, residues 63–193 of StAR; TFE, 2,2,2-trifluoroethanol.

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tures that retain their native secondary structure but have lost some or all of their tertiary structure; molten globules may be folding intermediates that retain their normal capacity for intracellular translocation [13]. The molten-globule state can expose the protein's interior, which is usually hydrophobic, and lose interactions between secondary structures; these changes can be monitored as a decrease in the near-UV CD signal, even though the far-UV CD signal remains. The molten-globule state can be induced by acid, heat, denaturants and other factors [14]. Molten globules are thought to play a number of key roles in biological systems; for example, transition to a molten globule permits water-soluble proteins to bind to phospholipid bilayers. Some water-soluble proteins have native molten-globule structures that facilitate binding to bilayer membranes at neutral pH [15,16], and a molten-globule structure of some toxins is induced by the acidic head groups of the phospholipid bilayer [17,18]. Thus the molten-globule state is essential for membrane binding of many water-soluble proteins. We have now investigated the structural and spectroscopic properties of this protease-resistant N-terminal domain, and have investigated the structural changes it undergoes when interacting with components of a membrane.

EXPERIMENTAL

Materials

L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, proteinase K, pepsin, dodecylphosphocholine (DPC), CHAPS, SDS, 2,2,2-trifluoroethanol (TFE), bovine brain PC, bovine brain L-α-phosphatidyl-L-serine (PS) and deuterated acetic acid (sodium salt; NaAc-d_3, 99 % ^{2}H) were all purchased from Sigma. $^{15}NH_4Cl$ (98 % ^{15}N) and deuterated SDS (SDS-d_{25}, 98 % ^{2}H) were purchased from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). All solutions for CD and HPLC were filtered through 0.2 μm cut-off filters.

Plasmid construction and expression

A DNA segment encoding the protease-resistant N-terminal domain (63–193) of N-62 StAR was constructed by PCR amplification of the cloned human cDNA [19] using the primers 5′-TCACGGATCCGATGACGATGACAAAATGGAAGA-GACTCTCTA-3′ and 5′-TACGTCAAGCTTTCATCATCGG-CGCTTGGCACAGCGCAC-3′.

The PCR product was digested with *Bam*HI and *Hin*dIII, ligated into pQE30 vector (Qiagen), predigested with the same enzymes and transformed into *Escherichia coli* SURE2 (Stratagene). The accuracy and orientation of the construct was confirmed by DNA sequencing. The resulting protein product has a His$_6$ N-terminal tag, followed by an enterokinase cleavage site, followed by the StAR sequence, as described previously [8,12,20].

Protein purification

E. coli M15 (pREP4) (Qiagen) were transfected with the plasmid expressing 63-193 StAR, grown at 37 °C in Luria–Bertani medium containing kanamycin and ampicillin, and induced with 1 mM isopropyl β-D-thiogalactopyranside for 4 h. ^{15}N-protein was produced in M9 minimal medium containing $^{15}NH_4Cl$. The bacteria were harvested at $D = 1.8$–2.0, resuspended in 20 mM sodium phosphate (Na_2HPO_4/NaH_2PO_4 buffer system; NaPi), pH 7.5, containing 1 mM 2-mercaptoethanol and 3 mM PMSF, treated with lysozyme (50 μg/ml), and incubated for 1 h at 4 °C. After sonication, the bacterial lysate was centrifuged for 90 min at 100000 $\boldsymbol{g}$. The supernatant was loaded on a DEAE-Superose column (Amersham Pharmacia Biotech) pre-equilibrated with 20 mM NaPi buffer, pH 7.5, containing 1 mM 2-mercapto-ethanol and 50 mM NaCl. The column was washed with 10 bed volumes of 50 mM NaCl in the same buffer, and the bound protein was eluted with a linear 50–350 mM NaCl gradient. The protein fractions were analysed by electrophoresis, and selected fractions were dialysed against 50 mM NaPi buffer, pH 8.2, containing 1 mM 2-mercaptoethanol, 300 mM NaCl and 20 mM imidazole (buffer N). The protein solution was loaded on to a Ni^{2+}-nitrilotriacetate column (Qiagen) pre-equilibrated with buffer N, washed with buffer N, and eluted with 50 mM imidazole followed by 150 mM imidazole. The fractions containing the protein were dialysed against 20 mM NaPi, pH 7.0, and stored at −70 °C for further use. Protein concentration was determined from the calculated molar absorbance coefficient (15340 M^{-1} cm^{-1} at 280 nm) [21]. The protein solutions were dialysed against 20 mM NaPi buffers at pH 2, pH 4 and pH 7, 20 mM sodium acetate (NaAc) buffer at pH 4 and pH 6, and 20 mM Tris/HCl at pH 8.3 overnight at 4 °C.

Conformational changes in hydrophobic conditions

Different amounts of TFE were added to biosynthetic 63-193 StAR to monitor its effects on protein conformation at pH 7.0. Different amounts of SDS were added to the protein solutions to titrate the conformational changes at various detergent/protein molar ratios at pH 7.0. DPC and CHAPS were added to protein solutions dialysed to pH 2.0, 4.0 and 7.0 at detergent/protein molar ratios of 10, 100 and 1000.

CD

Far-UV CD spectra were recorded on a Jasco J-710 spectropolarimeter at 190–260 nm using a 0.1 mm or 1 mm pathlength cell purged with a continuous flow of nitrogen gas. Protein concentrations were 0.01–0.5 mM. The scan speed was 50 nm/min, and 3–5 scans of each sample were averaged. Near-UV CD spectra were measured on the same instrument at 250–320 nm using a 10 mm pathlength cell, with a protein concentration of 1 mg/ml; 20 scans of each sample were averaged.

Limited proteolysis

The purified biosynthetic 63-193 StAR was dialysed against 20 mM NaPi (pH 7.0) or 20 mM NaAc (pH 4.0), and 60 μl aliquots were prepared at 1 mg/ml, at pH 7 and at pH 4 respectively. Two samples were incubated on ice or at room temperature for 30 min, then treated with trypsin [(63-193 StAR/trypsin, 100:1 (w/w)] or proteinase K [(63-193 StAR/proteinase K, 50:1 (w/w)] at pH 7, or with pepsin [(63-193 StAR/pepsin, 50:1 (w/w)] at pH 4. After incubation for 0, 10, 30, 45, 60 and 120 min, 5 μl samples were taken and protease activity was terminated by adding 5 × SDS gel sample buffer, containing 1 mM PMSF, to the samples digested with trypsin and proteinase K, or 1 M Tris for the sample digested with pepsin. After denaturation for 5 min at 95 °C, these samples were analysed by electrophoresis through an SDS/14 % acrylamide gel.

Preparation of phospholipid liposomes

Liposomes of PC, PS, or their 1:1 molar mixture were prepared by sonication using a probe-type sonicator [22]. Phospholipid solutions in chloroform/methanol were dried under nitrogen, the phospholipid films were prepared in a glass vial and residual organic solvent was removed under vacuum. NaPi buffer

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(20 mM), pH 7, containing 100 mM NaCl, was added to the lipid film and incubated for 1 h at room temperature with stirring, and the suspension was sonicated for 5×30 s on the ice bath. The concentration of phospholipid in the liposome solution was calculated using an average phospholipid molecular mass of 700 Da.

Gel-permeation chromatography

Phospholipid liposomes (1 mM) and biosynthetic 63-193 StAR (10 μM) were mixed in 20 mM sodium phosphate buffer (pH 7) containing 100 mM NaCl, incubated at 25 °C for 1 h, and 100 μl of this mixture was injected onto a Superose® 12 HR 10/30 column (Amersham Pharmacia Biotech), connected directly to a Hewlett Packard 1100 Series HPLC and pre-equilibrated with the same buffer. The eluate at 1 ml/min was monitored at 280 nm. Each peak was collected and the protein concentration was measured by the bicinchoninic acid method (Sigma) with BSA as a control [23].

NMR spectroscopy

All NMR spectra were acquired at 5–25 °C at 600 MHz using a Varian Inova-600 spectrometer equipped with a pulsed-field gradient unit and an actively shielded z-gradient triple resonance probe (5 mm or 8 mm). All samples (0.15–1.7 mM protein) contained $^2H_2O/H_2O$ (1:9) and 3-(trimethylsilyl)-tetradeutero sodium propionate (0.04 mM) that served as an internal chemical-shift reference at 0 p.p.m. The NMR data were processed by NMRPipe [24] and analysed with the SPARKY program (http://picasso.nmr.ucsf.edu) [25]. The one-dimensional NMR spectra were acquired with a presaturation pulse that was applied during the recycle delay (1.5 s) to suppress the H_2O signal. The one-dimensional spectra were each acquired with 8000 Hz spectral widths and 32K complex points. Before Fourier transformation, the data were zero-filled once to enhance the apparent digital resolution further. For samples uniformly labelled with ^{15}N, gradient-enhanced heteronuclear single-quantum coherence (HSQC) spectra [26] were acquired with 2048 complex points in *t*2 and 64 points in *t*1. Linear prediction and zero-filling were used for *t*1 to improve the sensitivity and resolution. The two-dimensional NOESY [27] (mixing time 100 ms) and TOCSY [28] (mixing time 50 ms) spectra were acquired in the States mode for the samples without ^{15}N label. Similarly, there were 4096 complex points in *t*2 and 512 points in *t*1. Zero-filling was applied to the *t*1 dimension. Apodization for two-dimensional data consisting of a cosine function was applied in both dimensions. Baseline correction in *t*1 was applied with a sixth-order polynomial function, but without correcting the region of the water signal, in cases where it was necessary.

RESULTS

pH-dependent conformation of 63-193 StAR

The N-terminal protease-resistant domain (63-193) of StAR was expressed as a soluble form in *E. coli* with an N-terminal His_6 tag and an enterokinase site, and was purified through DEAE-Superose and Ni^{2+}-nitrilotriacetate columns at pH 7. This is termed 'biosynthetic' 63-193 StAR to distinguish it from 63-193 StAR liberated from N-62 StAR by partial proteolysis; biosynthetic 63-193 StAR was used for the experiments described in the present paper. The presence of either an N-terminal or C-terminal extension containing a His_6 tag has no effect on N-62 StAR's activity *in vitro* [7,8,10], and hence presumably has no significant effect on protein folding. The calculated molecular

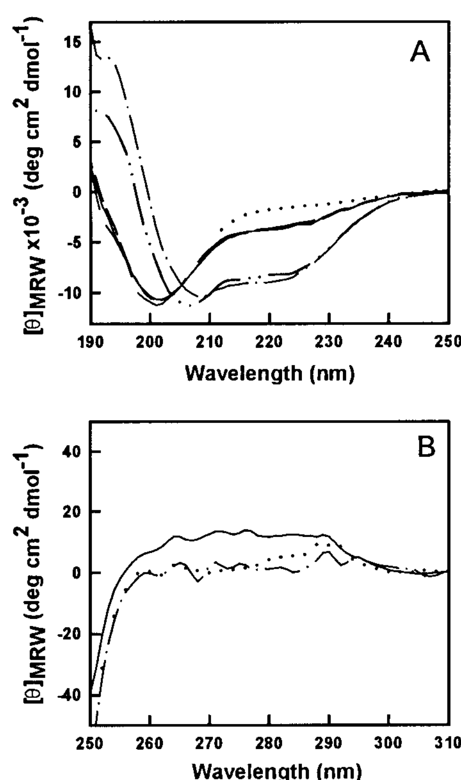

Figure 2 CD spectra of biosynthetic 63-193 StAR protein

(**A**) Far-UV CD spectra of 0.1 mg/ml biosynthetic 63-193 StAR measured at pH 8.3 (solid line), pH 7.0 (broken line), pH 6.0 (dotted line), pH 4.0 (single dot-and-dashed line) and 2.0 (double dot-and-dashed line). The buffer used for pH 8.3 was 20 mM Tris/HCl and 20 mM NaPi was used for the other pH values. The spectra were scanned five times using a 1 mm pathlength cell at 25 °C and smoothed. The far-UV CD spectrum of the unfolded protein in 6 M guanidinium chloride in 20 mM NaPi, pH 7, is shown as a dotted line. (**B**) Near-UV CD spectra of 63-193 StAR at 1 mg/ml measured at pH 7 with SDS (broken line), at pH 7 without SDS (solid line) and at pH 4 without SDS (dotted line), all in 20 mM NaPi. The SDS/protein molar ratio was 100. The spectra were scanned 20 times in a 10 mm pathlength cell at 25 °C and smoothed.

mass of this protein is 16.9 kDa, and its pI based on amino acid composition is 5.4. This fragment of StAR does not have biological activity *in vivo* as the translation-termination mutation R193X StAR causes congenital lipoid adrenal hyperplasia [3]. When tested with our isolated mitochondrial system *in vitro* [8], the biosynthetic 63-193 StAR was inactive as expected (results not shown).

We previously showed that N-62 StAR that contains the identical N-terminal extension (His_6 tag and enterokinase site) is a well-structured protein with a strong near-UV CD signal at pH 8.3 [20], and that it has full biological activity [8]. Such N-62 StAR undergoes very little structural change when the pH is reduced to 4.3, as shown by urea denaturation curves and far-UV CD spectroscopy [12]. Partial proteolysis of N-62 StAR protein showed that the 63-193 domain was quite resistant to the action of trypsin at pH 8 for up to 45 min at 20 °C and pepsin at pH 4 for up to 60 min at 20 °C [12], hence we expected that bacterially expressed 63-193 StAR should exhibit similar properties. The far-UV CD spectra of biosynthetic 63-193 StAR were essentially identical at pH 8.3, pH 7 and pH 6, but showed relatively little structure, as shown by the minimum at 200–204 nm and the relatively weak signals at other wavelengths (Figure 2A). In contrast, when biosynthetic 63-193 StAR was

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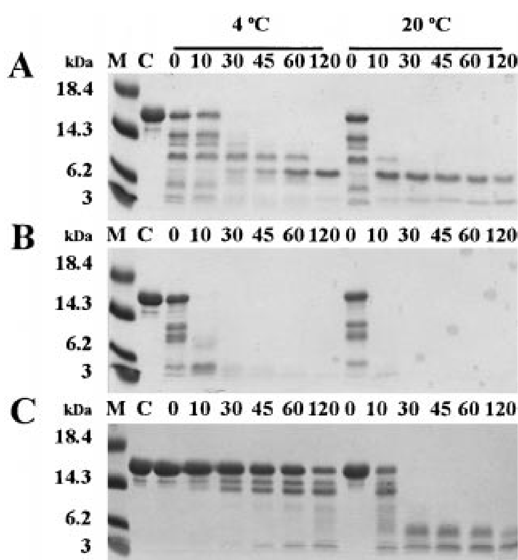

Figure 3 Limited proteolysis at pH 7 and pH 4

Bacterially expressed 63-193 StAR was digested with trypsin at pH 7 (**A**), proteinase K at pH 7 (**B**) and pepsin at pH 4 (**C**), at either 4 °C or 20 °C for the times shown in minutes above the gels, and the products were analysed by electrophoresis through SDS/14% acrylamide gel and stained with Coomassie Blue. The sample at 0 min was taken 10 s after the protease was added to the protein solution. M, prestained molecular-mass-markers; C, 5 μg of biosynthetic 63-193 StAR loaded as a control.

prepared at pH 4, there was a substantial increase in both its α-helical structure, as indicated by the shift of the minimum to 208 nm (π–π^* transition) and the increased signal at 222 nm (n–π^* transition), and also an increase in β-sheet structure, as indicated by the increased signal at 218 nm with a corresponding decrease in the contribution of random coils, shown by the decreased signal at 198 nm and below [29]. When the pH was reduced further to 2.0 some of this secondary structure was lost, consistent with acid-induced denaturation. The near-UV CD spectra of N-62 StAR showed substantial tertiary structure [20], but the near-UV CD spectrum of biosynthetic 63-193 StAR at pH 7 showed very little tertiary structure, which could be melted out by adding SDS (Figure 2B). Similar results were obtained when biosynthetic 63-193 StAR was refolded by step dialysis and by direct dialysis (results not shown). Thus biosynthetic 63-193 StAR has substantially less structure than N-62 StAR, but acquires structure and is stabilized near pH 4, indicating typical molten-globule characteristics. We suggest that this property facilitates the interaction of StAR or N-62 StAR with the outer mitochondrial membrane. The negatively charged head groups of phospholipids on the outer mitochondrial membrane favour an acidic environment near the outer mitochondrial membrane [30,31], and acidification is fostered by the mitochondrial proton pump, without which StAR is inactive [32]. Thus the peri-mitochondrial environment favours the formation of a pH-dependent StAR molten globule, which may expose hydrophobic domains of StAR that facilitate its interaction with the outer mitochondrial membrane.

Limited proteolysis

We previously showed [12] that 63-193 StAR was resistant to both trypsin (at pH 8) and pepsin (at pH 4) when this domain was proteolytically released from folded N-62 StAR. To determine if the biosynthetically produced 63-193 StAR was folded in the same fashion, we first examined the protease sensitivity of the biosynthetic 63-193 StAR. When digested with trypsin, biosynthetic 63-193 StAR was readily digested at 20 °C within 10 min and at 4 °C within 30 min (Figure 3A); in contrast, the 63-193 domain liberated from N-62 StAR was stable for up to

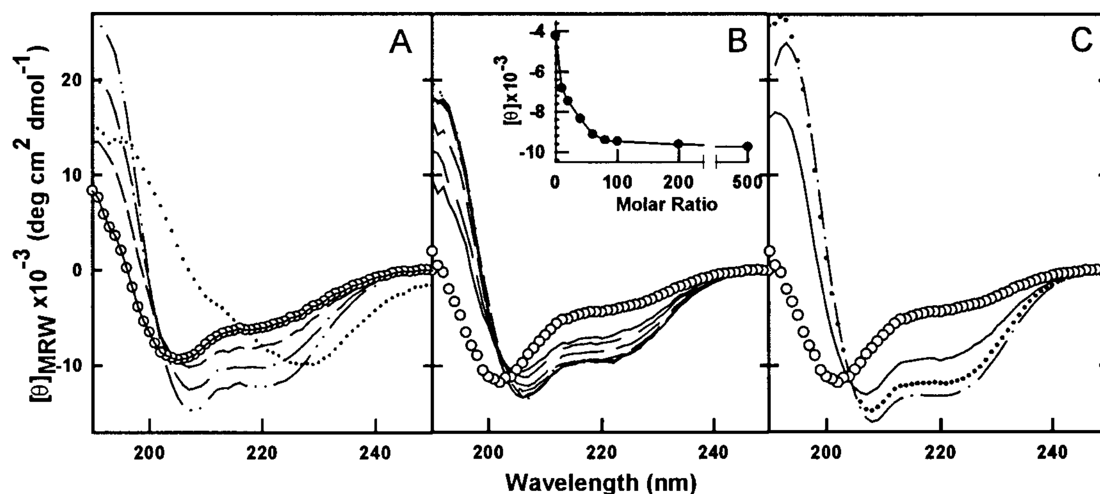

Figure 4 Effects of hydrophobic agents

(**A**) Effects of TFE. The far-UV CD spectra of 0.1 mg/ml biosynthetic 63-193 StAR in 20 mM NaPi, pH 7, were monitored in a 1 mm pathlength cell without TFE (○) and with TFE at 5% (solid line), 10% (broken line), 15% (dot-and-dash line), and 20% (double dot-and-dash line). The dotted line is the spectrum in 20% TFE at pH 4. (**B**) Effects of SDS. Far-UV CD spectra of 1.7 mg/ml (0.1 mM) biosynthetic 63-193 StAR in 20 mM NaPi, pH 7, with increasing molar ratios of SDS/protein from 10 to 500, monitored in a 0.1 mm pathlength cell at 25 °C. All SDS curves showed an isodichroic point at 203 nm. Inset shows the mean residue ellipticity at 222 nm as a function of the SDS/protein molar ratio. (**C**) Effects of DPC. Far-UV CD spectra were averaged for three scans in a 0.1 mm pathlength cell at 25 °C at pH 7 without DPC (○), or with a 100:1 molar ratio of DPC/biosynthetic 63-193 StAR at pH 7 (solid line), pH 4 (dotted line) and pH 2 (dot-and-dash line).

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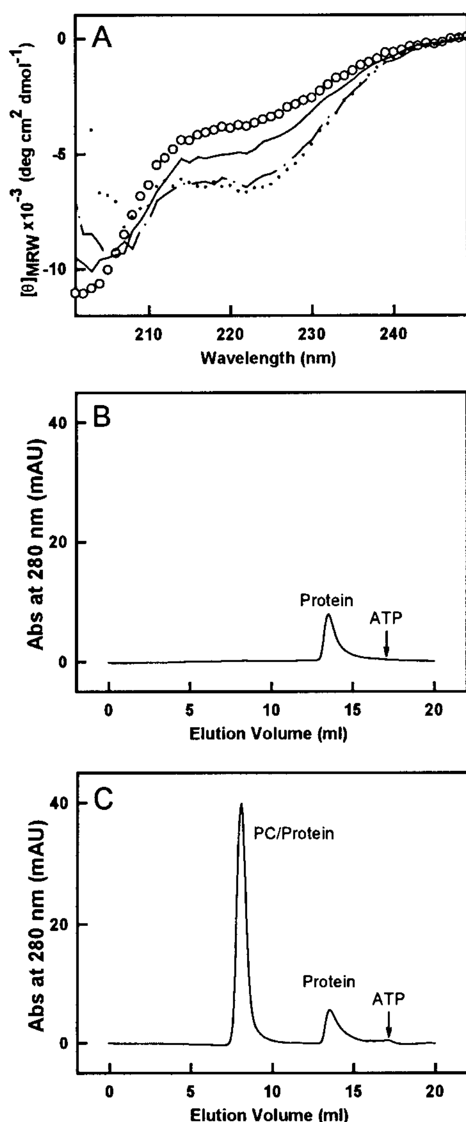

Figure 5 Binding of biosynthetic 63-193 StAR to phospholipid liposomes

(**A**) Far-UV CD spectra of 10 μM biosynthetic 63-193 StAR incubated in 20 mM NaPi, pH 7, for 1 h at 20 °C without phospholipids (○) or with 1 mM phospholipid liposomes composed of PC (solid line), PS (dotted line), or a 1:1 mixture of PC and PS (dot-and-dash line). (**B**) Gel-permeation chromatography of 10 μM biosynthetic 63-193 StAR. A 100 μl aliquot of protein in 20 mM NaPi, pH 7, 100 mM NaCl was injected onto a Superose-12 column connected to an HPLC and eluted with the same buffer at 1 ml/min. The total elution volume was measured with ATP. (**C**) Gel-permeation chromatography of 10 μM biosynthetic 63-193 StAR incubated with 1 mM PC for 1 h at 25 °C. Buffers and HPLC conditions were as in (**B**). The void volume was measured with liposomes.

45 min at either temperature [12]. Digestion with proteinase K at pH 7 was even more dramatic (Figure 3B). At pH 4.0, the biosynthetic 63-193 StAR was resistant to pepsin for 10 min at 20 °C and for up to 120 min at 4 °C (Figure 3C), consistent with the increased secondary structure at pH 4 shown by the far-UV CD spectroscopy (Figure 2). Thus biosynthetic 63-193 StAR is more sensitive to proteases than the 63–193 domain cleaved from N-62 StAR at both pH 4 and pH 8, indicating that the 194–285 domain is needed for the 63–193 domain to achieve its proper tertiary structure.

Secondary structure in hydrophobic agents

To determine whether the missing 194–285 domain of N-62 StAR stabilizes the folding of 63-193 StAR by providing a hydrophobic environment, we examined the effect of TFE on the far-UV CD spectrum of biosynthetic 63-193 StAR. TFE stabilizes α-helices, mimics the hydrophobic environment of protein cores and is widely used in NMR studies of membrane-associated proteins (Figure 4A) [33,34]. Increasing concentrations of TFE from 5 % to 20 % increased the secondary structure of biosynthetic 63-193 StAR at pH 7 with an isodichroic point at 203 nm, indicating a transition from random coil to α-helix. Aggregation was seen in 20 % TFE at pH 4, and also when the protein was dialysed against 20 % TFE in 20 mM acetate buffer (pH 4) at 4 °C (results not shown). To overcome aggregation, provide a hydrophobic environment and increase solubility, we added SDS. At an SDS/protein molar ratio of only 10:1, there was a substantial increase in α-helical structure at pH 7 (Figure 4B). Increasing the SDS/protein ratio increased the α-helical content further, with an isodichroic point of 203 nm. The maximal effect was seen at an SDS/protein ratio of 50:75 (Figure 4B, inset), as measured by the mean residue ellipticity at 222 nm, and might be correlated with micelle formation by SDS (critical micellar concentration = 8.1 mM) [35].

To control for the possible influence of the negative charge of SDS, we examined the effects of neutral detergents. At a detergent/protein molar ratio of 100:1, DPC induced a spectral shift at pH 7 that was essentially the same as the spectral shift seen with 100:1 SDS (Figure 4C). As the pH was decreased at 100:1 DPC, the secondary structure increased with an isodichroic point of 203 nm, and with a shape typical of globular, α-helical proteins, as seen with TFE and SDS. Addition of the detergent CHAPS at a detergent/protein ratio of 100:1 at various pH values was somewhat less effective in inducing secondary structure (results not shown). Ultracentrifugation for 1 h at 100000 g at 25 °C demonstrated that, with the exception of 20 % TFE at pH 4, the conditions used for CD measurements did not cause aggregation (results not shown).

Binding to phospholipid bilayer

The biosynthetic 63-193 StAR has molten-globule properties under several conditions, suggesting that it should bind to membranes. As a model for membrane binding, we used liposomes of PC, which has a net zero charge, and PS, which has a net negative charge at neutral pH. The interaction between biosynthetic 63-193 StAR and phospholipid vesicles was first monitored by far-UV CD (Figure 5A). PS liposomes increased the far-UV CD spectrum of biosynthetic 63-193 StAR to a greater extent than liposomes of PC or a 1:1 mixture of PC and PS. As expected from the acid-induction of the molten-globule conformation, liposomes of PS (which provides an acidic environment for the protein in a pH 7 buffer) induces the molten-globule conformation and increases membrane binding.

Gel-permeation chromatography showed that the protein does not aggregate at pH 7 and is eluted as a single peak (Figure 5B). To assess the quantitative interaction of 63-193 StAR with phospholipid vesicles, we mixed identical amounts (10 μM) of biosynthetic 63-193 StAR with liposomes composed of PC, PS or a 1:1 mixture of these two phospholipids. When mixed with liposomes, the protein formed a complex that eluted with the void volume (Figure 5C, results with PC; results with other liposomes not shown). Because the liposomes contribute to light scattering, the A_{280} measurements do not indicate the amount of protein associated with the liposomes. Direct protein measurements by the bicinchoninic acid method showed that the PC–

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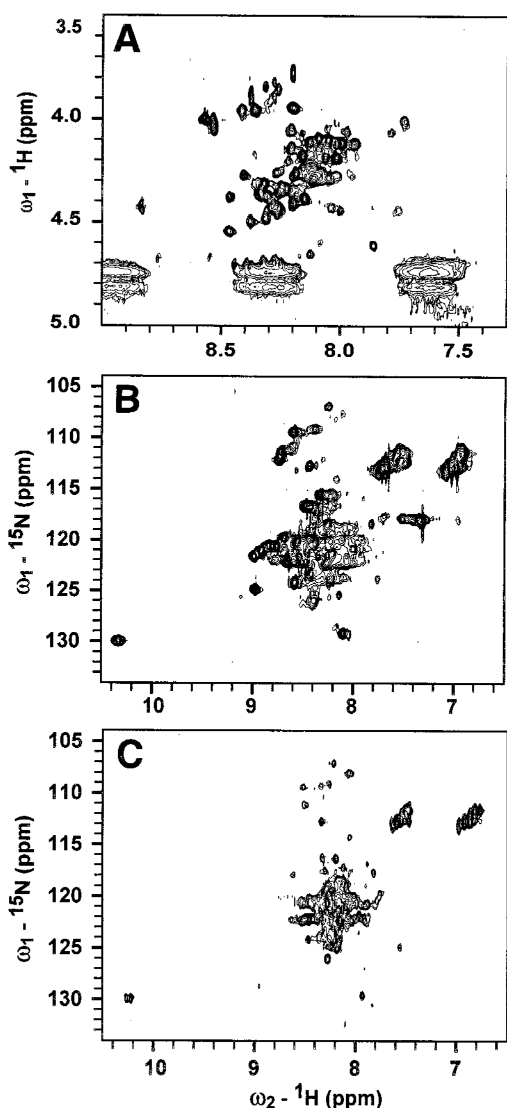

Figure 6 NMR spectroscopy

(**A**) Expanded αH–NH region of two-dimensional TOCSY with 50 ms mixing time at pH 2 (0.5 mM biosynthetic 63-193 StAR, 20 mM NaPi at 25 °C). There are approx. 60 peaks out of 135 amino acid residues counted without considering the possibility of two αH–NH cross-peaks from glycine, βH–NH cross-peaks from serine and threonine, or potential overlap. (**B**) Expanded HSQC at pH 4 with 100 mM CHAPS (0.5 mM biosynthetic 63-193 StAR, 20 mM sodium acetate at 25 °C). There are approx. 70 backbone amide peaks, but the cross-peaks for the side chain amides of asparagine and glutamine are amassed into one ill-defined signal. (**C**) Expanded HSQC at pH 7 with 100 mM CHAPS (0.5 mM biosynthetic 63-193 StAR, 20 mM NaPi at 25 °C). There are less than 70 backbone cross-peaks, and the side chain amides of asparagine and glutamine exhibit amassed cross-peaks similar to that at pH 4.

StAR complex contained 31 % of the added protein, the PS–StAR complex contained 45 % of the added protein and the complex with the 1:1 phospholipid mixture contained 47 % of the added protein. Thus different phospholipids have different affinities for biosynthetic 63-193 StAR. The increased association of biosynthetic 63-193 StAR with PS and with the 1:1 phospholipid mixture, and the nearly identical percentage of protein incorporated into the liposomes, are consistent with the stronger and nearly identical CD spectra with PS or the 1:1 mixture seen in Figure 5(A). It is not clear whether the biosynthetic 63-193 StAR associated with the liposomes by surface binding or by integration.

NMR

To examine the structure of biosynthetic 63-193 StAR in more detail we used NMR spectroscopy. In all cases at pH 7, with protein concentrations as low as 0.5 mM (20 mM NaPi, at 5, 10, 15, 20 or 25 °C), the NMR signals were broad without the dispersion that is characteristic of a folded protein. Thus the NMR data are consistent with the CD data showing that biosynthetic 63-193 StAR is largely in an unfolded random coil conformation at neutral pH. Even though CD spectra showed that biosynthetic 63-193 StAR adopts an α-helical conformation at pH 4, the one-dimensional NMR spectral peaks were very broad and indiscernible at pH 4 (20 mM NaAc-d_3, 5 °C), indicating that biosynthetic 63-193 StAR aggregated at a concentration just above 150 μM. At pH 2 (20 mM NaPi, 25 °C), resonances in the one-dimensional spectrum were only modestly better than at pH 4. The two-dimensional NOESY data were very poor, with only a few broad cross-peaks. In the two-dimensional TOCSY, about 60 distinct peaks could be observed, but nearly half of the residues failed to show αH-NH cross-peaks (Figure 6A). These observations are consistent with a molten-globular conformation, with defined secondary structure but no tertiary structure. The transiently structured region may sample multiple conformations on the NMR timescale. The resultant conformational flexibility results in exchange-broadened signals, which may become indistinguishable. In contrast, the flexible regions manifest sharp signals in TOCSY spectra.

Detergent molecules can prevent aggregation and enhance protein folding [36,37]. The use of uniformly ^{15}N-labelled protein expands the potential choices of detergents to include those not available in deuterated form. In HSQC spectra, cross-peaks correlate the ^{15}N-enriched nitrogen in the protein to its covalently bonded proton; therefore there is no signal from detergent. CHAPS prevented aggregation at pH 4, but the HSQC spectrum indicated that the protein did not fold into a tertiary structure, as there are some distinct sharp peaks and some weak broad peaks (Figure 6B). The cross-peaks for the side chain amides of asparagine and glutamine were amassed into one ill-defined signal with very broad linewidth. There are approx. 70 backbone amide peaks, only about half of the total residues. This suggests conformational averaging in certain regions of the molecule resulting in broadening of the signals for residues in these regions. Using SDS-d_{25}, homonuclear two-dimensional NOESY and TOCSY were acquired at pH 7 (results not shown). Although the one-dimensional spectrum had a narrow range of backbone amide and aliphatic methyl proton chemical shifts, the two-dimensional TOCSY showed approx. 90 distinctive αH–NH cross-peaks. There were also many peaks in the two-dimensional NOESY spectrum, especially in the NH–NH region, which is characteristic of α-helices. However, studies with the milder zwitterionic detergent, CHAPS, did not yield spectra as distinctive as that with SDS (Figure 6C). The HSQC spectrum showed peaks corresponding to less than half of the total residues, each with fairly broad linewidth. These data suggest that biosynthetic 63-193 StAR protein may be in a molten-globular form, even in the presence of mild detergents. With SDS, the conformation may be locked into an α-helix but there is no tertiary structure, which explains the narrow range of aliphatic methyl and backbone amide peaks. With the mild detergent CHAPS, conformational averaging occurs, as shown by the HSQC exhibiting certain distinctive peaks together with some broad and even missing peaks.

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DISCUSSION

When N-62 StAR is subjected to proteolysis at either pH 8 or pH 4, the 63–193 domain is protease-resistant, indicating it is tightly folded [12]. However, when the 63-193 domain is expressed in bacteria, it is less structured and more protease-sensitive at both pH values. Thus although our previous data show that the C-terminal 194-285 domain of StAR is a protease-sensitive molten globule [12], it exerts a dramatic effect on the folding of the 63-193 domain.

StAR is highly homologous to a protein termed MLN64 [38,39]. The StAR-like domain of MLN64 has recently been crystallized and solved at 2.2 Å (1Å = 0.1 nm) resolution [40]. By analogy with this structure, we can infer that the proteolytic cleavage sites in StAR between residues 188 and 193 are in the β-turn between the β6 and β7 sheets. Furthermore, the MLN64 structure implies that there are no distinct structural domains corresponding to 63-193 and to 194-285, and that α-helices and β-sheets from both of these regions contribute to the hydrophobic cholesterol-binding pocket.

Most water-soluble proteins have hydrophilic, polar residues outside and hydrophobic residues inside, so that they cannot incorporate into a lipid bilayer without dramatic structural changes. Numerous water-soluble proteins are able to penetrate membranes under certain conditions that induce molten-globule structures. For example, α-lactalbumin and lysozyme are tightly folded soluble proteins that bind strongly to phospholipid bilayers when they assume molten-globule conformations at low pH [41–45]. Colicin A can also convert to a molten globule at low pH to expose its hydrophobic α-helices and interact with a phospholipid bilayer, even one containing acidic phospholipids [17,46]. Sec A, a key component of *E. coli* protein translocase, is partially unfolded at neutral pH and incorporates into membranes by itself [16,47]. Apocytochrome *c*, the precursor of cytochrome *c*, is less structured but also penetrates lipid bilayers [48]. Both Sec A and apocytochrome *c* traverse the phospholipid bilayer and expose hydrophobic domains to the other side of the bilayers [47,48].

Acidic pH induces the formation of the molten-globule forms of the N-62 StAR and 63-193 StAR. StAR apparently experiences acidic conditions both at the outer mitochondrial membrane and in the intermembrane space, as disruption of the mitochondrial proton pump terminates StAR activity [32]. N-62 StAR transfers cholesterol from cholesterol-containing PC liposome to mitochondria *in vitro* [11]. N-62 StAR is not translocated into mitochondria but has complete activity, and immuno-gold electron microscopy shows that N-62 StAR associates with the outer mitochondrial membrane [6]. Thus it appears that StAR must interact with a phospholipid bilayer to transfer cholesterol. We have shown direct evidence for an interaction between StAR and a phospholipid bilayer by far-UV CD and gel-permeation chromatography. The lipid bilayer increased the secondary structure of biosynthetic 63-193 StAR and stable liposome–protein complexes were formed at neutral pH. Thus the available physical evidence all points to a mechanism for StAR action, where StAR interacts with the outer mitochondrial membrane, apparently forming a molten globule to take up or discharge cholesterol.

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