N-218 MLN64, a Protein with StAR-like Steroidogenic Activity, Is Folded and Cleaved Similarly to StAR†

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ABSTRACT: The steroidogenic acute regulatory protein (StAR) facilitates the movement of cholesterol from the outer to inner mitochondrial membrane in adrenal and gonadal cells, fostering steroid biosynthesis. MLN64 is a 445-amino acid protein of unknown function. When 218 amino-terminal residues of MLN-64 are deleted, the resulting N-218 MLN64 has 37% amino acid identity with StAR and 50% of StAR's steroidogenic activity in transfected cells. Antiserum to StAR cross-reacts with N-218 MLN64, indicating the presence of similar epitopes in both proteins. Western blotting shows that MLN64 is proteolytically cleaved in the placenta to a size indistinguishable from N-218 MLN64. Bacterially expressed N-218 MLN64 exerts StAR-like activity to promote the transfer of cholesterol from the outer to inner mitochondrial membrane in vitro. CD spectroscopy indicates that N-218 MLN64 is largely α-helical and minimally affected by changes in ionic strength or the hydrophobic character of the solvent, although glycerol increases the β-sheet content. However, decreasing pH diminishes structure, causing aggregation. Limited proteolysis at pH 8.0 shows that the C-terminal domain of N-218 MLN64 is accessible to proteolysis whereas the 244−414 domain is resistant, suggesting it is more compactly folded. The presence of a protease-resistant domain and a protease-sensitive carboxy-terminal domain in N-218 MLN64 is similar to the organization of StAR. However, as MLN64 never enters the mitochondria, the protease-resistant domain of MLN64 cannot be a mitochondrial pause-transfer sequence, as has been proposed for StAR. Thus the protease-resistant domain of N-218 MLN64, and by inference the corresponding domain of StAR, may have direct roles in their action to foster the flux of cholesterol from the outer to the inner mitochondrial membrane.

The rate-limiting step in steroidogenesis is the conversion of cholesterol to pregnenolone by cytochrome P450scc,[1] the cholesterol side chain cleavage enzyme system localized on the inner mitochondrial membrane (for review, see ref *1*). Movement of cholesterol from the outer to inner mitochondrial membrane is facilitated by the steroidogenic acute regulatory protein (StAR) (*2*). StAR is synthesized as a 37-kDa pre-protein and is processed to a 30-kDa intramitochondrial form, but the pre-protein appears to be the active moiety as StAR acts on the outer mitochondrial membrane (*3*−*5*). Mutations in human StAR cause congenital lipoid adrenal hyperplasia, a potentially lethal disease in which adrenal and gonadal (but not placental) steroidogenesis is severely impaired (*6*−*11*). All amino acid replacement mutations that cause lipoid CAH are in the carboxy-terminal 40% of StAR protein (*9*−*11*). Deletion of 62 amino acids from the amino-terminus of StAR (N-62 StAR) has no effect on biological activity, but deletion of 10 carboxy-terminal amino acids reduces activity by 70% and deletion of 28 C-terminal residues destroys all biological activity (*4*). Following the nonessential amino-terminal 62 residues is a tightly folded domain comprising residues 63−188, which appears to act as a "pause-transfer" sequence, permitting the loosely folded, biologically active carboxy-terminal domain to have increased interactions with the outer mitochondrial membrane (*12*).

MLN64 is 445-amino acid protein of unknown function that was cloned from metastatic breast carcinoma cells (*13*). The amino acid sequence of the carboxyl half of MLN64 is similar to StAR (*13*), and while full-length MLN64 has minimal StAR-like activity, deletion of 234 amino-terminal residues results in a protein (N-234 MLN64) that has substantial StAR-like activity in transfected cells (*14*). Like

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[1] Abbreviations: BSA, bovine serum albumin; CD, circular dichroism; DTT, dithiothreitol; EGTA, ethylene glycol-bis(β-aminoethyl ether)*N*,*N*,*N*,*N*-tetraacetic acid; ESI-TOF, electrospray ionization-time-of-flight; F2, fusion protein of P450scc−adrenodoxin reductase−adrenodoxin; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; LC-ESI MS, liquid chromatography-electrospray ionization mass spectrometry; MLN64, protein of unknown function cloned from human breast cancer cells; NADPH, reduced nicotinamide adenine dinucleotide phosphate; P450scc, mitochondrial cytochrome P450 specific for cholesterol side chain cleavage; PCR, polymerase chain reaction; PVDF, polyvinylidine difluoride; SDS, sodium dodecyl sulfate; StAR, steroidogenic acute regulatory protein; TFE, trifluoroethanol.

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N-62 StAR, N-234 MLN64 has no mitochondrial leader sequence and does not enter the mitochondrion but instead appears to act on the outer mitochondrial membrane. To study the StAR-like activity of MLN64, we prepared N-218 MLN64 in bacteria. We now show that, like N-62 StAR, N-218 or N-234 MLN64 has a two-domain structure including a protease-resistant domain comprising residues 229−414. N-218 MLN64 appears to undergo pH-dependent partial unfolding to resemble the molten globule configuration achieved by StAR. We have hypothesized that such a transition to molten globule configuration is a requisite component for StAR-like activity (*12*).

MATERIALS AND METHODS

Assessment of Steroidogenic Activity. The steroidogenicaly active N-218 MLN64 and N-234 MLN64 sequences were constructed by amplification using the human MLN64 cDNA sequence as a template. The oligonucleotides 5′AGCTGGATCCATGGAGCGGGAGTACATC CGCCAGGGGA 3′-(MLN64 S1) and 5′ AGCTAGGATCCATGAATGAATCAGATGAAGAA GTTGCT3′ (MLN64 S2) were used as sense primers and 5′AGCTAGAATTC TCACGCCCGGGCCCCCAGCTCGCT3′ (MLN64 AS1) as antisense primer following PCR program 4 (*15*). The amplified products were purified by gel electrophoresis using QIAquick (QIAGEN) columns, digested with *Bam*HI and *Eco*RI and repurified through the column. The digested N-218 MLN64 and N-234 MLN64 inserts were ligated into pCMV-Flag2 (Stratagene, San Diego, CA) that had been predigested with *Bam*HI and *Eco*RI and transformed into *Escherichia coli* DH5α. The accuracy of the constructs was determined by sequencing both strands of four clones. COS-1 cells were transfected with a plasmid expressing the catalytically active fusion protein termed F2 that consists of the cholesterol side chain enzyme, P450scc, and its electron-transport proteins, adrenodoxin reductase and adrenodoxin (*16*, *17*). Cells were cotransfected with F2 and plasmids expressing N-218 MLN64, N-234 MLN64, N-62 StAR, or empty vectors using lipofectamine, as described (*9*). Cultures were incubated in the presence or absence of 22R-hydroxycholesterol (5 μg/mL) and 5 pmol of trilostane for 40 h. Pregnenolone was determined by radioimmunoassay using reagents and protocols from ICN Pharmaceuticals (Costa Mesa, CA).

Construction of N-218 MLN64 Bacterial Expression Plasmid. The N-218 MLN64 sequence was constructed by amplification of human MLN64 cDNA using 5′ATCGGATCC GATGACGATGACAAAATGAATGAATCAGATGAAGAAGTT3′ (HBM1S) and 5′ AGC TAACTAAGCTTCACAGTCACG3′ (HBM2AS) as sense and antisense primers following PCR program 4 (*15*). The PCR product was purified by gel electrophoresis using QIAquick (QIAGEN) columns, digested with *Bam*HI and *Hind*III and ligated to pQE30 (QIAGEN), which had been digested to *Bam*HI and *Hind*III and amplified by transformation into *E. coli* DH5α. Plasmid DNA was then transformed into *E. coli* M15 (pREP4) cells, grown in LB medium containing 30 μg/mL kanamycin and 100 μg/mL ampicilin and induced with isopropyl-β-D-thio galacto-pyroniside (IPTG) when the turbidity reached 0.6 O. D. units. The culture was spun down at 37000*g* and resuspended in *E. coli* lysis buffer containing 1 mM PMSF (*18*).

Expression of Soluble Bacterial N-218 MLN64 Protein. *E. coli* cultures were grown in LB medium at 37 °C with shaking at 40−50 rpm. When the turbidity of the culture reached 0.2−0.25, the culture was transferred to a shaking incubator at 15−18 °C and induced with 0.4 mM IPTG overnight. The bacterial culture was harvested at 37000*g*, washed with resuspension buffer (20 mM Tris, 50 mM NaH_2PO_4, pH 8.0, containing 40% glycerol), lysed by addition of 100 μL of DNase I (10 mg/mL), RNase I (10 mg/mL), and lysozyme (1 mg/mL) per liter of culture, and sonicated 3 times for 30 s at 4 °C. Following sonication, the bacterial pellet was spun down at 37000*g* for 30 min at 4 °C; the supernatant was saved and the bacterial pellet was reprocessed as described for the original pellet. The supernatant was loaded onto a 10-mL bed volume Ni-NTA superflow (QIAGEN) column precharged with $NiSO_4$ and equilibrated with resuspension buffer. The Ni^{2+} column was washed with 20 bed volumes of resuspension buffer containing 500 mM NaCl, then rewashed with 10 bed volumes of resuspension buffer containing 20 mM imidazole; finally, N-218 MLN64 was eluted with 300 mM imidazole, 20 mM Tris, 50 mM NaH_2PO_4, pH 7.2 or 4.5, containing 40% glycerol and dialyzed through a Centriprep-10 (Amicon) column. N-218 MLN64 was injected onto a Biosil-125 Gel filtration column (Bio-Rad) connected to an HPLC system (Varian, CA) with a flow rate of 0.5 mL/min.

In Vitro Bioassay of MLN64 and StAR Proteins. Mouse Leydig MA-10 cells (*19*) were grown at 37 °C in Waymoth's medium supplemented with 15% horse serum, 25 mM HEPES, 5% FBS, and gentamycin in a humidified atmosphere containing 5% CO_2. Mitochondria were isolated from MA-10 cells by a modification of a previously described procedure (*20*). MA-10 cells were harvested at 500*g*, and the pellet was washed with 5 mL of 0.25 M sucrose, 1 mM EGTA, 10 mM Hepes, pH 7.4, 0.1% BSA, 1 mM DTT, and 1 mM PMSF and resuspended in the same buffer. Debris were removed from the homogenate by centrifugation at 1500*g* for 10 min at 4 °C, and then the mitochondria were pelleted from the supernatant at 10000*g* for 10 min. The crude mitochondria were washed in the same buffer (*21*), and the mitochondrial protein concentration was determined by the Bradford method (sigma); mitochondria were either used immediately or stored at −70 °C. Isolated MA-10 cell mitochondria (2 μg of mitochondrial protein) were incubated with purified, bacterially expressed N-62 StAR or N-218 MLN64 at 37 °C for 60 min (*3*, *22*, *23*) in the presence of 1 mM NADPH, and the conversion of cholesterol to pregnenolone was determined by radioimmunoassay of pregnenolone.

Western Blotting. Different amounts (300, 100, 30, and 3 ng) of purified N-218 MLN64 protein, N-62 StAR, and human placental homogenate (a generous gift of Dr. Susan Fisher, UCSF) were electrophoresed on an SDS 12.5% polyacrylamide gel and electroblotted to a PVDF (polyvinylidine difluoride) membrane. The membrane was blocked with 5% nonfat dry milk for 45 min followed by overnight probing with antibodies and incubation with our anti-human N-62 StAR antiserum (*12*). The color was developed with ECL reagent (Amersham).

Protein Concentration. Protein concentrations for all spectroscopy experiments were determined spectroscopically using an absorption coefficient (3.0620×10^4) calculated

from the amino acid composition of the N-218 MLN64 protein: $A_{280} = (5690W + 1280Y + 12S\text{-}S)/0.1M_r$, where W, Y, and S-S represent the number of tryptophan, tyrosine residues, and cysteine disulfide bonds (*24*). The molecular mass (M_r) of the recombinant protein was calculated from the amino acid composition of N-218 MLN64, which contains 4W, 6Y, and 3C.

Enterokinase Digestion. A total of 1.0 mg of Ni-affinity purified N-218 MLN64 protein was incubated with 1U of enterokinase (Novagen, Madison, WI) at 21 °C in the presence of 1.0 M urea overnight, and the digestion was terminated with 1× sample buffer containing 2 mM PMSF. The control and enterokinase-digested N-218 MLN64 was electrophoresed on an SDS 12.5% polyacrylamide gel and electroblotted to a PVDF membrane and processed for Western blotting with our antiserum to human StAR.

Gel Filtration. N-218 MLN64 purified through the one-step Ni^{2+} affinity chromatography was subjected to one-step purification through an HPLC gel filtration column. A total of 100 μL of Ni^{2+}-column purified protein was injected onto a Biosil-125 analytical gel filtration column (Bio-Rad, CA) and an isocratic buffer gradient containing 10 mM Tris, 25 mM NaH_2PO_4, pH 7.0, or 25 mM NaH_2PO_4, pH 4.5, containing 20% glycerol was run at 0.5 mL/min.

Circular Dichroism. N-218 MLN64 was equilibrated by dialysis with appropriate buffers from pH 2.0−7.0 in the absence of glycerol. The concentration-dependent far-UV CD spectra of N-218 MLN64 were recorded in a JASCO-720 spectropolarimeter in 50 mM NaOAc or 25 mM NaH_2PO_4 buffer or 10 mM Tris and 25 mM NaH_2PO_4 buffer at 190−250 nm. Protein concentrations ranging from 16 μg/mL to 5.5 mg/mL at pH values ranging from 2 to 7.0 were analyzed in 0.1-mm or 1.0-mm path length quartz cuvettes at 20 °C. The higher concentrations were studied in the presence of 40% glycerol. Temperature-dependent far-UV CD spectra were also recorded.

Preparative Ultracentrifugation. N-218 MLN64 at 1.0 mg/mL was equilibrated at pH 4.0, 4.5, and 7.0 in 25 mM NaH_2PO_4 containing 40% glycerol, gradually heated to 95 °C for 2 min, and cooled to 4 °C for 10 min in a PCR thermocycler for 8 cycles. After thermal cycling, the protein samples were spun at 100000*g* for 60 min at 20 °C in a Beckman TL 100 ultracentrifuge fitted with a TLV rotor. The absorbance of the protein samples at 280 nm was recorded before and after heat denaturation after diluting the samples 10 times with appropriate buffer.

Mass Spectrometry. A total of 250 ng of N-218 MLN64 was incubated with 1.0, 4, or 10 U of trypsin (Promega, sequencing grade) in 50 mM Tris buffer, pH 8.0, for 5, 15, 30, 45, and 60 min at 4 and 20 °C. The digestion reactions were terminated with 1× SDS sample buffer containing 1 mM PMSF, boiled for 5 min, electrophoresed on an SDS 20% polyacrylamide gel, and stained with silver nitrate. For preparative purposes, 35 μg of N-218 MLN64 was digested either at 4 or 20 °C and stained with Coomassie blue. The predominant bands were excised, cut into 1-mm pieces, and extracted three times with 50% CH_3CN/25 mM aqueous NH_4HCO_3 in silanized Eppendorf tubes for in-gel digestion (*25*). Following lyophilization, the gel pieces were rehydrated in 50 μL of aqueous 10 mM dithiothreitol/25 mM NH_4HCO_3. After incubation at 56 °C for 1 h, the supernatant was removed and 50 μL of aqueous 55 mM iodoacetamide/25 mM NH_4HCO_3 buffer was added and incubated for an additional 45 min at room temperature. The supernatant was then removed and 100 μL of 25 mM NH_4HCO_3 buffer was added; the sample was then vortexed for 10 min, and the supernatant was discarded. The gel pieces were again rinsed in 100 μL of 25 mM NH_4HCO_3 and vortexed for 10 min, and the supernatant was discarded. The gel pieces were dehydrated with 50 μL of 50% CH_3CN/25 mM aqueous NH_4HCO_3 followed by vortexing for 5 min. The gel pieces were lyophilized and then rehydrated with ∼50 μL of 10 ng/μL trypsin (Promega; sequencing grade), digested overnight at 37 °C, and sonicated in 100 μL of water for 5 min; the supernatant was saved in a silanized Eppendorf tube. The gel pieces were then re-extracted three times with 50% CH_3CN/5% aqueous trifluoroacetic acid (TFA), and the extracts were pooled together; the volume was reduced to 10 μL by vacuum centrifugation and 10 μL of 0.1% TFA was added. A 1-μL aliquot of each sample was diluted to 20 μL with water and injected onto a capillary HPLC system interfaced to a PE Biosystems Mariner electrospray ionization mass spectrometer (LC-ESI MS). Solvent A (0.1% formic acid) and solvent B (5:2 ethanol/*n*-propanol, 0.05% formic acid) were used to develop a 180 μm × 15 cm C-18 capillary column packed with 3-μm particles (LC Packings, CA) with a gradient from 5 to 60% solvent B in 60 min. The molecular masses of the peptides were determined by mass spectrometry and compared with calculated masses for peptides anticipated for the cleavage of the MLN64 protein by trypsin, assuming cleavages at lysine and arginine residues. For comparison, a band for undigested N-218 MLN64 was excised from the SDS−polyacrylamide gel and digested with trypsin, and the peptides were identified by LC-ESI MS.

RESULTS

N-218 MLN64 Stimulates Steroidogenesis. To test the steroidogenesis-enhancing activity of MLN64 and its N-terminal deletions, we cotransfected monkey kidney COS-1 cells with a vector expressing the F2 fusion of the human cholesterol side chain cleavage system (*16*) and with vectors expressing various StAR and MLN64 constructs and measured the resulting pregnenolone synthesis (Figure 1). Cells expressing the F2 fusion protein cotransfected with an empty vector had a low level of steroidogenesis using their endogenous cholesterol or that supplied by LDL in the serum of the culture medium as substrate for the F2 fusion enzyme. These same cells were also incubated with 22R-hydroxycholesterol, which is freely equilibrated in both the cytoplasm and the mitochondria thus bypassing the action of StAR-like proteins and thus providing an index of the cell's maximum steroidogenic capacity (*8*, *26*, *27*); using 22R-hydroxycholesterol as substrate increased pregnenolone production 10-fold. Cotransfection with vectors expressing either full-length (1−285) StAR or N-62 StAR increased steroidogenesis nearly 6-fold over the level seen with the empty vector to a level about half that seen with 22R-hydroxycholesterol, consistent with previous reports (*4*, *8*, *28*). Cotransfection with the vector expressing full-length (1−445) MLN64 had no effect as compared to the vector control, but cotransfection with either the N-218 or N-234 forms of MLN64 increased steroidogenesis about 3.5-fold over the level seen with the vector control. Thus the N-terminally deleted MLN64 constructs had about 60% of the activity

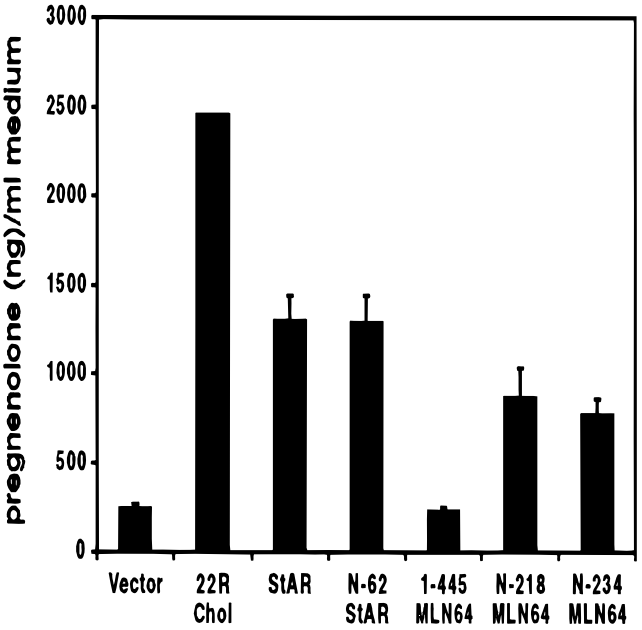

FIGURE 1: Relative stimulation of pregnenolone synthesis by MLN64 and StAR proteins in COS-1 cells expressing the F2 fusion protein of the human cholesterol side-chain cleavage system. COS-1 cells were transfected with a plasmid expressing F2 and cotransfected with a plasmid expressing N-218 MLN64, N-234 MLN64, full-length MLN64, N-62 StAR, or full length StAR, and the amount of pregnenolone produced was measured by radioimmunoassay. As a control, cells containing F2 were also incubated with water-soluble 22R-hydroxycholesterol, which bypasses the need for StAR. Data are the means (±SEM) of three separate experiments, each performed in triplicate.

seen with either full-length or N-62 StAR; this is somewhat greater than the 37% level of StAR's activity reported previously for N-234 MLN64 (*14*). Therefore, because N-218 MLN64 corresponds more closely than N-234 MLN64 to the size and sequence of N-62 StAR, and because the N-218 construct consistently appeared to elicit a slightly greater steroidogenic response, we used the longer N-218 MLN64 construct for all further studies of the folding similarities between truncated MLN64 and StAR proteins.

Expression and Purification of Recombinant N-218 MLN64. To study the physical characteristics of the N-218 MLN64 protein, we built a vector for expression of N-218 MLN64 in bacteria using the same strategy we had used previously for bacterial expression of N-62 StAR (*18*). The vector contains a *lac*-inducible T5 promoter upstream from a bacterial leader sequence followed by a 6-His sequence followed by N-218 MLN64; treatment of bacteria with IPTG induced expression of the expected 28-kDa protein (Figure 2A). The N-218 MLN64 protein was purified by nickel affinity chromatography, digested with enterokinase, and purified to homogeneity by HPLC (Figure 2B). The calculated molecular mass of the final N-218 MLN64 protein, including the leader sequence, is 27883.6 and the calculated isoelectric point (p*I*) is 7.92; the molecular mass obtained from the mass spectrometric analysis (ESI-TOF) was 27883.6.

Expression of MLN64 in Vivo. We recently raised a high-affinity rabbit antiserum to bacterially expressed human N-62 StAR (*12*). When log dilutions of bacterially expressed N-218 MLN64 and N-62 StAR were analyzed by Western blotting, both the purified N-218 MLN64 and the N-62 StAR could be detected in samples containing only 0.3 ng of protein

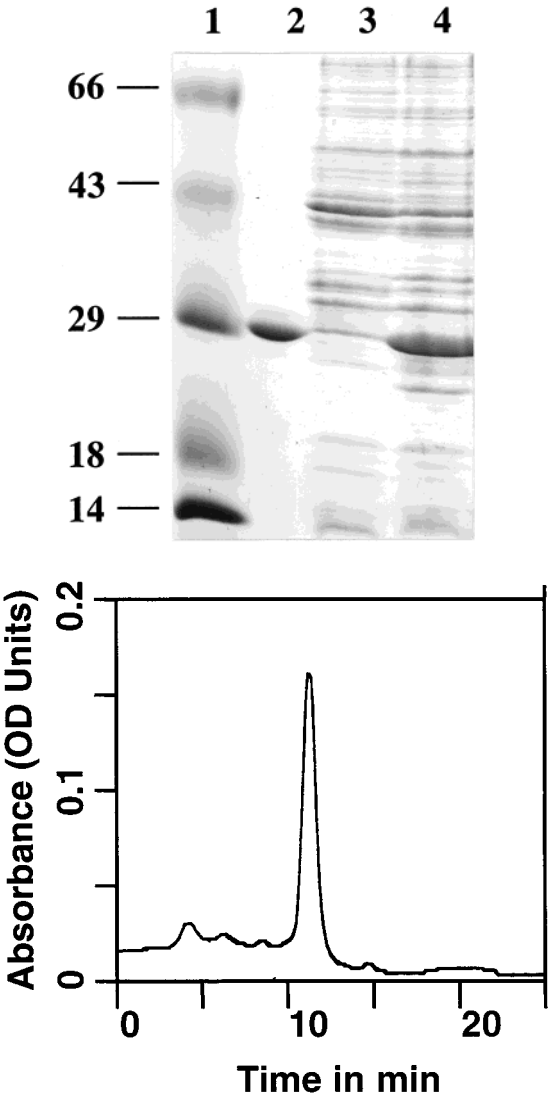

FIGURE 2: *E. coli* expression and purification of N-218 MLN64 and N-62 StAR. Upper panel: SDS-polyacrylamide gel. Lane 1, prestained molecular weight markers; lane 2, purified N-62 StAR; lane 3, total cytoplasmic protein from uninduced bacteria expressing N-218 MLN64; lane 4, total cytoplasmic protein from IPTG-induced bacteria expressing N-218 MLN64. Note the appearance of the prominent 28-kDa band of N-218 MLN64. Lower panel: Gel filtration chromatogram of N-218 MLN64 purified by Ni−NTA chromatography. Approximately 100 ng of Ni-NTA column-purified material was injected onto an analytical HPLC−Gel filtration column (Biosil-125, Bio-Rad, CA) fitted with a Rainin HPLC (Varian, CA) system. The isocratic gradient was run with a flow rate 500 μL/min, and the protein was eluted at retention time of 11 min.

(Figure 3A). Thus both N-62 StAR and N-218 MLN64 have similar antigenic epitopes, suggesting that the proteins are folded similarly. Analysis of subcellular fractions from transfected *E. coli* (not shown) indicated that most of the N-218 MLN64 remains in a soluble cytoplasmic form rather than in inclusion bodies, in contrast to the opposite finding with N-62 StAR (*18*).

The placenta is the largest steroidogenic organ and converts large amounts of cholesterol to pregnenolone and thence to progesterone to maintain human pregnancy (*29*). However the placenta does not express StAR (*28*), and StAR mutations that cause congenital lipoid adrenal hyperplasia do not disrupt placental biosynthesis of progesterone (*10*).

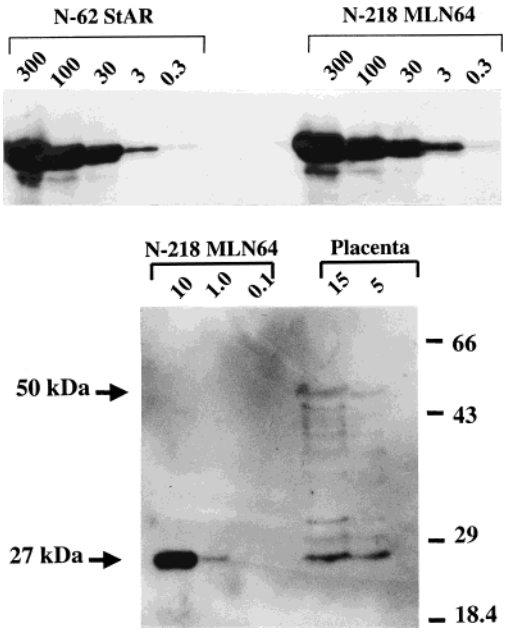

FIGURE 3: Western blots. Upper panel: Blots of bacterially produced N-62 StAR and N-218 MLN64; samples of 300, 100, 30, 3, and 0.3 ng of each protein were analyzed on the same gel. Lower panel: Expression of MLN64 in the human placenta. Left: samples of 10, 1.0, and 0.1 ng of bacterially expressed N-218 MLN64; right: samples of total protein homogenate from human placenta at 17 weeks' gestation; the two lanes contain 15 and 5 μg of total protein by Bradford determination.

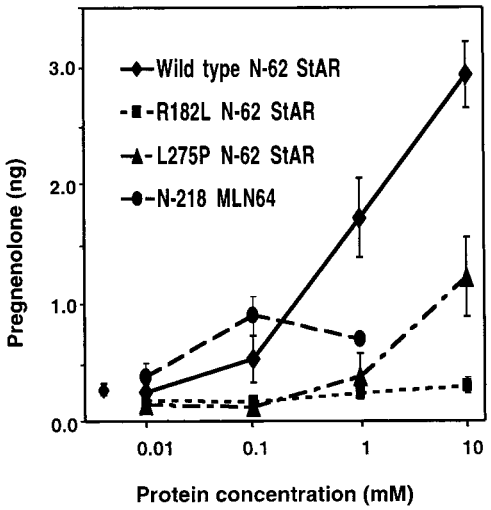

FIGURE 4: Biological activities of the bacterially expressed N-218 MLN64 and N-62 StAR proteins. Purified proteins at different concentrations were incubated with isolated MA-10 mitochondria at 37 °C for 1 h, and pregnenolone production was measured by radioimmunoassay. Note that the value for a buffer blanks (no added protein) is shown at the left. The proteins analyzed are wild-type N-62 StAR (closed symbols), R182L N-62 StAR (■), L275P N-62 StAR (▲), and wild-type N-218 MLN64 (closed symbols). Data are the mean ±SEM of four experiments, each performed in triplicate.

It has been suggested that MLN64 might substitute for StAR in the placenta (*14*), but this has not been examined directly. Western blotting of a total protein homogenate from midterm (17-week) human placenta showed the presence of apparently full-length (50 kDa) MLN64 and various proteolytic products (Figure 3B). The most prominent band had migration indistinguishable from N-218 MLN64, and no smaller fragments were seen. Thus the placenta cleaves the 445-amino acid MLN64 protein to a small fragment of about the same size as N-218 MLN64; as shown in Figure 1 such a fragment would be expected to have StAR-like activity.

In Vitro Assay of Bacterially Expressed Protein. To determine if the bacterially expressed N-218 MLN64 was biologically active, we established an in vitro assay of StAR activity using mitochondria isolated from steroidogenic mouse Leydig MA-10 cells and measured the production of pregnenolone from the cholesterol endogenously present in the outer mitochondrial membrane. This system is similar to previously described assays (*3*, *22*, *23*). The system was first validated by testing wild-type N-62 StAR and N-62 StAR containing the mutations R182L or L275P. The R182L mutant is wholly inactive in vivo, whereas the L275P mutant retains about 15−20% of activity (*9*); furthermore, spectroscopic analysis shows that the R182L mutant is grossly misfolded, whereas the folding of L275P is only slightly different from the wild-type N-62 StAR (*18*). At a protein concentration of 280 μg/mL (10 μM), the activity of N-62 StAR was about 5−6-fold higher than that of the R182L mutant or buffer control, indicating that the bacterially expressed N-62 StAR maintains its bioactivity, as previously suggested by spectroscopic analysis (*18*) (Figure 4). Similarly, the L275P mutant had a modest degree of activity, consistent with the low level of activity of this mutant in full length StAR expressed in whole cells (*9*). Bacterially expressed N-218 MLN64 was also biologically active but elicited its maximal, 2-fold response at a very low concentration of 0.1 μM. Its activity at 1 μM was no greater than at 0.1 μM, and at concentrations higher than 1.0 μM, the activity of N-218 MLN64 decreased, apparently due to aggregation; aggregation at high concentrations was confirmed by preparative ultracentrifugation. The aggregation of N-218 MLN64 at high concentrations could be eliminated by the addition of 20−40% glycerol, but the steroidogenic activity of the mitochondria was severely impaired by glycerol concentrations above 10% (results not shown), and hence the steroidogenic activity of N-218 MLN64 in 20−40% glycerol could not be assessed. Thus, the protein employed in our experiments is biologically active and hence correctly folded, but unlike N-62 StAR it was not soluble at moderate to high concentrations, probably due to the removal of a domain that would normally protect against hydrophobic protein−protein interactions.

Influence of pH, Ionic strength, and Solvent Polarity. Protein conformation and folding are often sensitive to pH. As pH decreases, most native proteins first lose tertiary structure, and then, at lower pH, their secondary structure collapses to an unstructured random coil. To test the effects of protein concentration and pH on the folding of N-218 MLN64, we used far-UV CD spectroscopy (Figure 5). For some proteins, lowering the pH induces abrupt changes in the near-UV CD region (260−300 nm) but induces little change at the far-UV CD region (*30*); however, for most proteins far-UV CD spectroscopy can distinguish secondary structural transitions by changes in the spectral minima near 198 nm for random coil at 208 and 222 nm for the α-helices and at 218 nm for β-sheets. Reducing the pH to 3.5−4.0 increased the α-helical structure of N-62 StAR, indicating the formation of a molten globule, i.e., the reduced pH decreased tertiary structure but increased secondary structure (*12*). Consistent with the data in Figure 4, CD data in aqueous

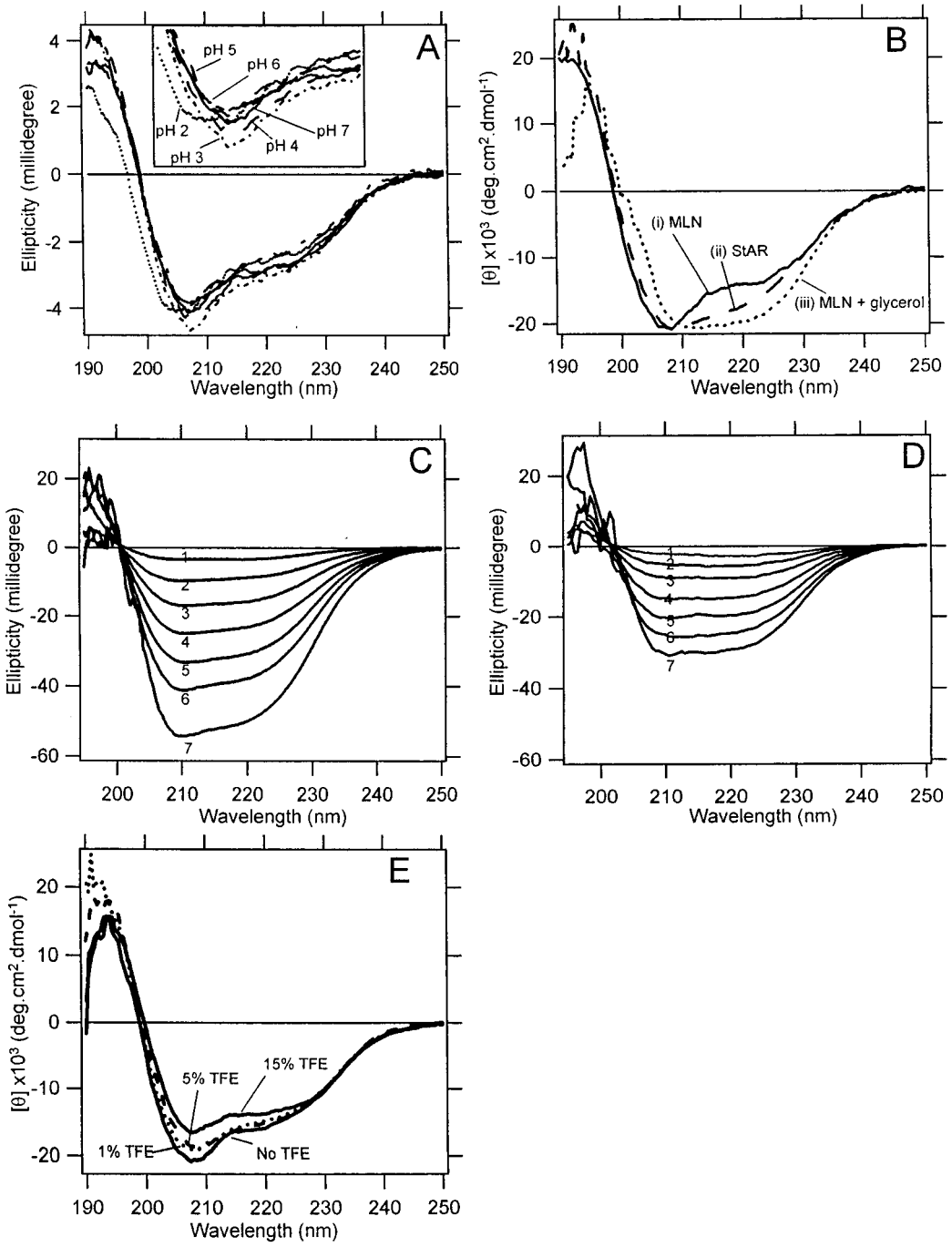

FIGURE 5: Unsmoothed far-UV CD spectra of N-218 MLN64 in 25 mM NaH_2PO_4 buffer. Panel A: CD spectra of N-218 MLN64 at a concentration of 50 μg/mL (~20 μM) at pH 2 (.....), pH 3 (-·-·-), pH 4 (– – –), pH5 (–·–·–), pH6 (— - —) and pH 7 (—). The region from 200 to 220 nm is expanded in the insert to clarify the identities of each curve. Note that the data are presented in millidegrees, as measured. Panel B: CD spectra of (i) N-218 MLN64 at pH 7 recorded as in panel A; (ii) N-62 StAR; (iii) N-218 MLN64 in the presence of 40% glycerol; note that data are presented as molar ellipticity. Panel C: CD spectra at pH 4.5 in the presence of 40% glycerol. Lines 1–6 show the spectra of protein at 61.3, 92, 138, 206, 310, and 476 μg/mL recorded in a 1-mm path-length cuvette; line 7 shows the spectrum of protein at 4.9 mg/mL recorded in a 0.1-mm path-length cuvette. Panel D: CD spectra at pH 7.0 in the presence of 40% glycerol. Lines 1 to 6 show the spectra of protein at 39, 78, 156, 197, and 235 μg/mL (path-length, 1 mm) and line 7 shows the spectrum of protein at 2.61 mg/mL (path-length, 0.1 mm). Panel E: Far-UV CD spectra of N-218 MLN64 at 50 μg/mL in 20 mM NaH_2PO_4 without glycerol at pH 7.0 without and with trifluoroethanol (TFE) at 1, 5, and 15%.

media could only be collected at low concentrations (20 μM or less) (Figure 5A). To emphasize the weak signals obtained, the ordinate in Figure 5A shows the measured ellipticity in millidegrees, rather than the derived quantity, molar ellipticity. Under these conditions, we did not observe a spectral shift at pH 4 that would suggest a transition to a molten globule, as we observed with N-62 StAR (*12*). However, although some of the variation among the various curves is probably due to minor technical differences, the curve for pH 2 is clearly shifted to lower wavelengths, indicating a loss of secondary structure. Furthermore, the curves for pH 3 and pH 4 show deeper minima at 208 nm than do the curves for pH 5 to 7 (Figure 5A, insert). These changes at 208 nm are consistent with a loss of tertiary structure and an increase in the content of α-helix that we noted previously with N-62 StAR in association with molten globule formation

Table 1: Secondary Structural Analysis

% struct	N-62 StAR (aq)[a]	N-218 MLN64 (aq)	N-218 MLN64 (40% glycerol)	N-216 MLN64 (X-ray struct)[b]
α-helix	26	31	(41)	26
β-sheet	23	9	(19)	38

[a] From CD data of Bose et al. (*12*). [b] Tsujishita and Hurley (*35*).

(*12*). At or below pH 4, more concentrated solutions of N-218 MLN64 begin to aggregate, also suggesting a pH-dependent conformational change with exposure of hydrophobic surfaces that are normally buried in the protein.

The CD spectrum of N-218 MLN64 at pH 7 suggests a lower proportion of β-sheet than is observed for N-62 StAR under equivalent conditions (Figure 5B, curves i and ii). To increase the solubility of N-218 MLN64 and hence increase the signal-to-noise ratio of the CD data, we added 40% glycerol to the buffer, as the bioactivity, and hence presumably the folding, of many enzymes is maintained in 40% glycerol (*30*). Although glycerol increased solubility up to 5 mg/mL and prevented aggregation at low pH, the CD spectrum in 40% glycerol at pH 7 showed an increased signal at 218−222 nm, indicating a conformational shift to higher β-sheet composition (Figure 5B, curve iii). Secondary structural analysis by the variable selection method (*31*) suggested that 40% glycerol had increased the β-sheet content from 9 to 19% (Table 1), but the increased absorption and erratic signals at wavelengths below 200 nm make this quantitation fairly unreliable. In the presence of 40% glycerol, N-218 MLN64 retained a constant conformation over a 50−60-fold range of protein concentrations of up to 4.9 mg/mL at pH 4.5 (Figure 5C). The far-UV CD spectrum of N-218 MLN64 in 40% glycerol at pH 7.0 was virtually indistinguishable from that at pH 4.5, but at pH 7.0, the protein was less soluble so that spectra could not be obtained at concentrations greater than 2.6 μg/mL (Figure 5D). Thus in 40% glycerol N-218 MLN64 is more soluble under acidic than under neutral conditions.

Changing the ionic environment of N-218 MLN64 from NaH_2PO_4 buffer to NaOAc buffer at pH 7.0 to 4.0 had only a slight effect, suggesting that the ionic environment has little direct effect on the conformation of the protein; a far-UV CD spectrum could not be measured below pH 3.75 due to the strong absorption of acetate ion at the lower wavelengths (data not shown). High ionic strength may open the core of a protein, causing partial denaturation, but adding 10 mM to 1.0 M NaF to 4 μM N-218 MLN64 in phosphate buffer at pH 7.0 had no visible effect on the far-UV CD spectrum (data not shown).

Protein conformation may also be influenced by solvent conditions, e.g., strongly polar solvents such as dichloroacetic acid favor coiled states. Weakly bonding solvents such as the fluorinated alcohols, including trifluoroethanol (TFE) and hexafluoroisopropanol, do not compete for hydrogen bonds as effectively as water, thus they lower the dielectric constant appreciably and strengthen the intramolecular hydrogen bonds that stabilize helical structure (*33*). Although this can promote helical structure, these effects may be primarily applicable to isolated peptides and such solvents may behave differently with individual proteins, e.g., they may destabilize globular proteins (*34*). The far-UV CD spectrum of N-218 MLN64 was moderately affected by the addition of up to 15% TFE with a minor decrease in the α-helical signal (Figure 5E). This observation for a protein that displays a mostly α-helical spectrum suggests that the degree of secondary structure observed by CD is highly stable and truly representative of the normal state of the protein and that there are no other regions of incipient helicity.

We attempted to measure the thermal stability of N-218 MLN64 by recording the far-UV CD spectra at temperatures from 20 to 70 °C and over a range of pH values. At pH 7.4, the spectra remained constant up to 50 °C then showed a progressive loss of signal up to 65 °C, with a midpoint at ∼55 °C. However, although N-218 MLN64 was clearly unfolding due to the heating effect, once the temperature had exceeded 55 °C, the CD spectrum did not recover upon cooling, indicating that the protein failed to refold it to its original conformation. Thus, it was impossible to separate a loss of signal due to unfolding from a loss of signal due to thermally induced aggregation. This effect was more pronounced at lower pH, and below pH 4 the signal was lost at ∼40 °C.

Proteolytic Digestion of N-218 MLN64. To gain a better understanding of the folding of N-218 MLN64 we sought to determine whether this protein contains domains that are differentially protected from proteolysis. Trypsin is a highly specific enzyme that cleaves after lysine and arginine under physiological or mildly alkaline conditions. We partially digested N-218 MLN64 with trypsin at pH 8.0 at room temperature and at 4° C for different lengths of time and at varying concentrations of trypsin (Figure 6). Following electrophoresis through SDS−20% polyacrylamide gel, the digestion patterns were analyzed by silver staining and

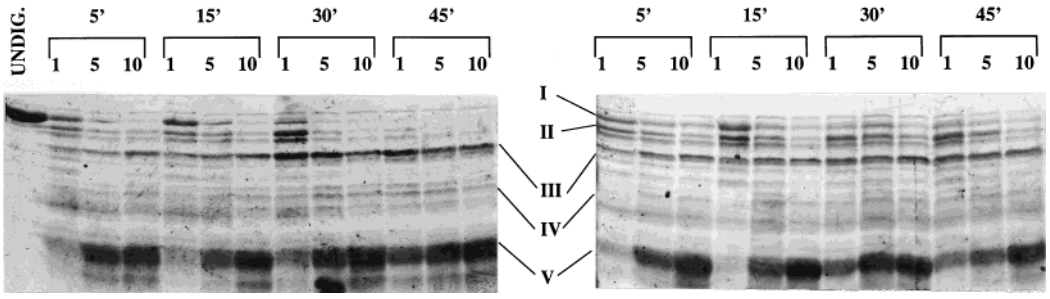

FIGURE 6: Proteolytic digestion of N-218 MLN64. A total of 250 ng of N-218 MLN64 was incubated with 1, 5, or 10 units of trypsin for 5−45 min as indicated at 20 °C (left panel) and at 4 °C (right panel). The proteolytic digestions were terminated with 1× sample buffer containing 2 mM PMSF and electrophoresed in SDS−20% polyacrylamide gel and stained with silver nitrate. A 250 ng sample of undigested N-218 MLN64 is shown at the left of the gel of the digestions at 20 °C and corresponds to band I. Bands II, III, IV, and V were excised from the gel and subjected to mass spectrometric analysis. Note that band III remains relatively resistant to proteolysis, even with 10 units of trypsin at 20 °C for 45 min.

Table 2: Mass Spectrometric Analysis of N-218 MLN64 Peptides

	digest peptide mass (M+H)+				
sequence residues	calculated	MLN64 protein (N-218)	conserved band II pH 8.0	conserved band III pH 8.0	conserved band IV pH 8.0
353−355	417.246	417.254	417.249	417.253	
261−263	423.224	423.232	423.228	423.227	423.227
378−380	437.251	437.254	437.250	437.253	
352−355	573.347		573.346	573.346	
237−240	580.310	580.313	580.313	580.312	580.317
282−286	621.398	621.395	621.404	621.407	621.404
393−398	631.316	631.317	631.320	631.328	
438−444	745.421	745.420	745.422		
346−351	749.395	749.392	749.395	749.398	749.396
307−312	790.429	790.430	790.431	790.428	790.432
307−312 met-ox[b]	806.424	806.423	806.424	806.432	806.424
230−236	824.390	824.394	824.386	824.392	824.386
229−236	952.485	952.494	952.484	952.486	952.480
381−392	1187.642	1187.632	1187.640	1187.640	
313−322	1189.636	1189.627	1189.637	1189.632	1189.630
18′ + 219−228[a]	1208.511	1208.502			1208.506
18′ + 219−228[a] met-ox	1224.505	1224.500			
18′ + 219−229[a]	1336.606	1336.594			
399−411	1608.846	1608.832	1608.836	1608.834	
360−377	1881.982	1881.973	1881.981	1881.972	1881.970
244−260	1943.971	1943.945	1943.970	1943.962	1943.973
264−281	2082.977	2082.961	2082.976	2082.978	2082.976
323−345	2277.136	2277.111	2277.144	2277.130	2277.138
217−235	2295.175	2295.160	2295.185		
287−306	2402.243	2402.208	2402.250	2402.245	2402.282
3′-18′ + 219−228[a]	2907.174	2907.142			
3′-18′ + 219−228[a] met−ox	2923.169	2923.140			
3′-18′ + 219−229[1]	3035.269	3035.234			
3′-18′ + 219−229[a] met−ox	3051.264	3051.234			

[a] Leader sequence (1′-18′) MRGSHHHHHHGSDDDDKM. [b] Met-ox: Methionine sulfoxide residues.

Western blotting with antiserum to human N-62 StAR. At pH 8.0, a major fragment of 21.3 kDa was protected for up to 45 min of incubation with up to 10 units of trypsin at either 4 or 20° C. This fragment as well as three other fragments were excised from the gel and digested further with trypsin. The resulting tryptic peptides were analyzed by HPLC-electrospray ionization MS (Table 2). Identification of the peptides generated from fragment II showed that this 26.4-kDa fragment had lost only 11 amino acids from the amino terminus of N-218 MLN64 to yield N-229 MLN64. Fragment III (21.3 kDa), which is the most abundant and hence the most stable large fragment, had also lost 11 amino acids from the amino terminus and had also lost 29 amino acids from the carboxy terminus, and thus residues 229 to 414 of MLN64 were protected. Fragment IV (17 kDa) had lost 68 amino acids from the carboxy-terminus but none from the amino terminus, i.e., the protected domain is residues 218−377. Fragment V (11 kDa) was a mixture of two smaller fragments covering most of N-218 MLN64; their analysis was not considered further. These data establish the identities of the protected fragments and show that the protease-resistant domain of N-218 MLN64, encompassing residues 229−414, is somewhat larger than the protease-resistant 63−193 domain of StAR (*12*) (Figure 7).

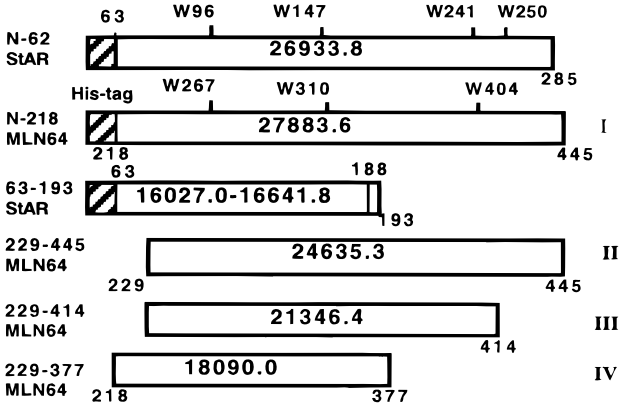

FIGURE 7: Interpretation of the mass spectrometric data in Table 2 showing the regions of N-218 MLN64 that are protected from proteolysis at pH 8.0. The N-218 MLN64 peptides from bands I, II, III, and IV in Figure 6 are designated with the corresponding Roman numbers, shown at the right of the figure. The protease-resistant fragments of N-218 are compared to those of N-62 StAR, as determined previously (*12*).

DISCUSSION

Although the function of MLN64 has yet to be defined, this molecule is of considerable interest as it might promote steroidogenesis in tissues in which StAR is not expressed. Our data confirm the report of Watari et al. (*14*) that truncated forms of MLN64 have some StAR activity, and we provide evidence that cellular processing indeed produces a major truncated form of MLN64 in the placenta. Thus we propose that MLN64 is processed in vivo to a product similar to N-218 MLN64, which then acts to promote steroidogenesis by the same mechanism as StAR. Consistent with this proposal, our proteolysis experiment demonstrates that the 229−414 domain is not accessible to solvent at physiological pH. However, as is the case with StAR, the extreme C-terminus is flexible, solvent-accessible, and able to promote steroidogenesis.

Because N-62 StAR and N-218 MLN64 have similar activities, sequences, and antigenic epitopes, we expected to find that these two proteins were folded in the same fashion and would exhibit very similar physical behavior. However, the similarities were less than predicted. The far-UV CD spectra of dilute solutions of recombinant N-218 MLN64 at physiological pH were very similar to those for N-62 StAR (*12*, *18*). However, increasing the concentration above 20 μM resulted in loss of signal due to aggregation, a phenomenon not observed with N-62 StAR. Aggregation did not occur with N-218 MLN64 preparations that were refolded and concentrated in the presence of glycerol, but these displayed a different CD spectrum characteristic of less α-helix and more β-sheet. As these CD spectra were unlike those of either N-218 MLN64 or N-62 StAR in purely aqueous buffers, we concluded that they had folded in a fashion that does not occur in vivo, and hence these preparations were not used for further biophysical studies. The contributions of α-helix and β-sheet to the structure of N-218 MLN64 cannot be calculated accurately from the CD data because of the highly erratic data below 200 nm. Nevertheless, both visual inspection and our rough calculations show substantially less β-sheet contribution for N-218 MLN64 than for N-62 StAR (Figure 5B and Table 1). Even in 40% glycerol, the apparent β-sheet content was only 19%, which is in stark contrast to the recently determined crystallographic structure of N-216 MLN64, which contains 38% β-sheet (*35*). The reasons for these major differences are not clear but may be related to the crystallization at pH 9.5 from a very hypertonic buffer.

Reducing the pH of dilute aqueous solutions of N-218 MLN64 did not induce a major increase in secondary structure at pH 3−4 as we had found for N-62 StAR (*12*). However, a modest increase in the amount of α-helix was seen at pH 3−4 associated with a loss of tertiary structure, which became more dramatic when the pH was reduced to 2.0. Aggregation was seen at higher protein concentrations or when the protein was unfolded by raising the temperature, particularly at lower pH. Thus monomeric N-218 MLN64 is less stable than N-62 StAR. Although the solubility of the full-length protein was not studied, it appears that the removal of 218 amino-terminal amino acids to produce the truncated protein exposes a hydrophobic domain that is responsible for aggregation. It is not clear whether the exposure of such a hydrophobic domain is relevant to the steroidogenic action of N-218 MLN64; such a domain might increase the protein's association with the outer mitochondrial membrane; however, N-62 StAR, which has greater activity than N-218 MLN64, is more soluble.

The proteolysis and mass spectrometric data indicate that the protease-sensitive and protease-resistant domains of N-62 StAR and N-218 MLN64 are similar. Full-length 37 kDa StAR has a typical mitochondrial leader sequence that is cleaved upon entering the mitochondria to yield the 30-kDa form (*2*, *36*). In the case of StAR, we have suggested that the protease-resistant region comprising residues 63−193 acts as a "pause-transfer" sequence, increasing the residency time of the biologically active carboxy-terminus on the outer mitochondrial membrane, thus increasing activity (*12*). However full-length MLN64 does not have a mitochondrial leader but instead has four amino-terminal transmembrane domains that anchor it to cytoplasmic structures and prohibit its entry into mitochondria (*13*). Thus, its protease-resistant domain comprising residues 229−414 cannot serve a similar mitochondrial pause-transfer function, yet N-218 MLN64 also acts in association with the outer mitochondrial membrane (*14*). If the protease-resistant domain of N-218 MLN64 is required for the protein's association with the outer membrane, then the protease-resistant 63−193 domain of StAR may serve a similar function. Thus, we suggest that the protease-resistant domain of StAR may function both as a pause-transfer sequence as well as in some other fashion required StAR's biological activity, possibly by associating with the outer mitochondrial membrane itself.

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