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Chaperones Rejuvenate Folding and Activity of 3-β Hydroxysteroid Dehydrogenase 2

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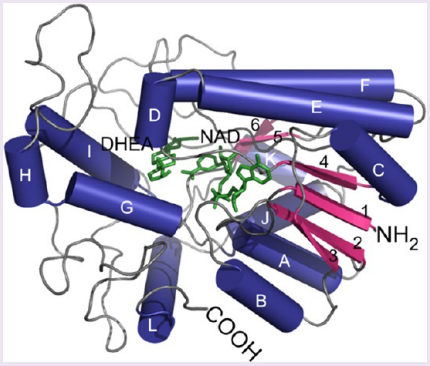
Supporting Information

ABSTRACT: The steroidogenic enzyme 3-β hydroxysteroid dehydrogenase 2 (3βHSD2) mediates the conversion of pregnenolone to progesterone and dehydroepiandrosterone to androstenedione through both its dehydrogenase and isomerase activities, making it necessary for the protein to undergo a reversible conformational change. We hypothesized that chaperones assist 3βHSD2 in switching between the conformations to initiate, enhance, and maintain activity. In the presence of the chaperone lauryl maltoside (LM), 3βHSD2 immediately converted pregnenolone to progesterone, with a 6.4-fold increase in synthesis. Using far-UV circular dichroism (CD), we found that addition of LM increased 3βHSD2's α-helical content, which over time reverted to control levels, suggesting the formation of a stable but reversible conformation possibly due to hydrophobic interactions of the protein with LM micelles. We also found that LM increased fluorescence resonance energy transfer (FRET) about 11-fold between 3βHSD2 and fluorescing ANS molecules. This observation supports the idea that detergent(s) act as chaperones to assist 3βHSD2 in forming stable complexes, which in turn promotes proper folding. Mass spectrometric fingerprinting illustrated that LM incubation resulted in an ordered fragmentation of molecular mass from 39 to 13 kDa, as compared to limited or no proteolysis in the absence of LM. In addition, space-filling modeling demonstrated that 3βHSD2 association with detergents likely exposed the hydrophobic region, leading to its proteolysis. We conclude that detergents help 3βHSD2 to refold in order to rejuvenate, contributing to the ability of cells to rapidly produce steroids when needed.



All mammals need steroids for survival, and steroid secretion requires steroid synthesis. Cells that produce polypeptides for export synthesize them and then store large amounts of protein in secretory vesicles, allowing for rapid release when necessary. By contrast, steroidogenic cells, mostly found in adrenal and gonadal tissues and to a limited extent in the brain, do not store steroids and thus a rapid steroidogenic response requires rapid synthesis of steroid.[1] The rate-limiting step in steroidogenesis is the movement of cholesterol from the outer to inner mitochondrial membrane. Cytochrome P450 side chain cleavage (SCC) enzyme catalyzes cholesterol to pregnenolone using a pair of electrons from NADPH, ferrodoxin, and ferrodoxin-reductase.[1] Next, 3-β hydroxysteroid dehydrogenase 2 (3βHSD2) acts as a dehydrogenase to convert pregnenolone to 5-pregnene-3,20-dione.[2] The same enzyme then works as an isomerase to catalyze 5-pregnene-3,20-dione to 4-pregnene-3,20-dione or progesterone.[2] Dehydroepiandrosterone (DHEA) is converted to androstenedione by the same 3-β hydroxysteroid dehydrogenase/isomerase enzyme. Human 3βHSD1 and 3βHSD2 are encoded by two distinct genes that are expressed in a tissue-specific pattern.[2] These isoforms are expressed in the mitochondrion and smooth endoplasmic reticulum.[2] We have recently shown that in steroidogenic cells, 3βHSD2 resides at the inner mitochondrial membrane (IMM)[3,4] where it interacts with two different mitochondrial translocases, and that this interaction is necessary for its activity.[3] As a resident of the IMM, 3βHSD2 faces the mitochondrial proton pump, and we have demonstrated that changes in pH correlate with reversible conformational changes and enhanced enzyme activity.[4] IMM proteins have an internal, positively charged "presequence"-like signal, often preceded by a hydrophobic sequence,[5] which leads to the arrest of translocation in the IMM and the subsequent lateral release into the lipid phase of the membrane.[6] Therefore, to reach the appropriate conformation, these IMM proteins generally undergo multiple intermediate states with decreasing free energies.[7] 3βHSD2 N-terminal sequence is not cleaved following mitochondrial import;[3,8] therefore, we hypothesize

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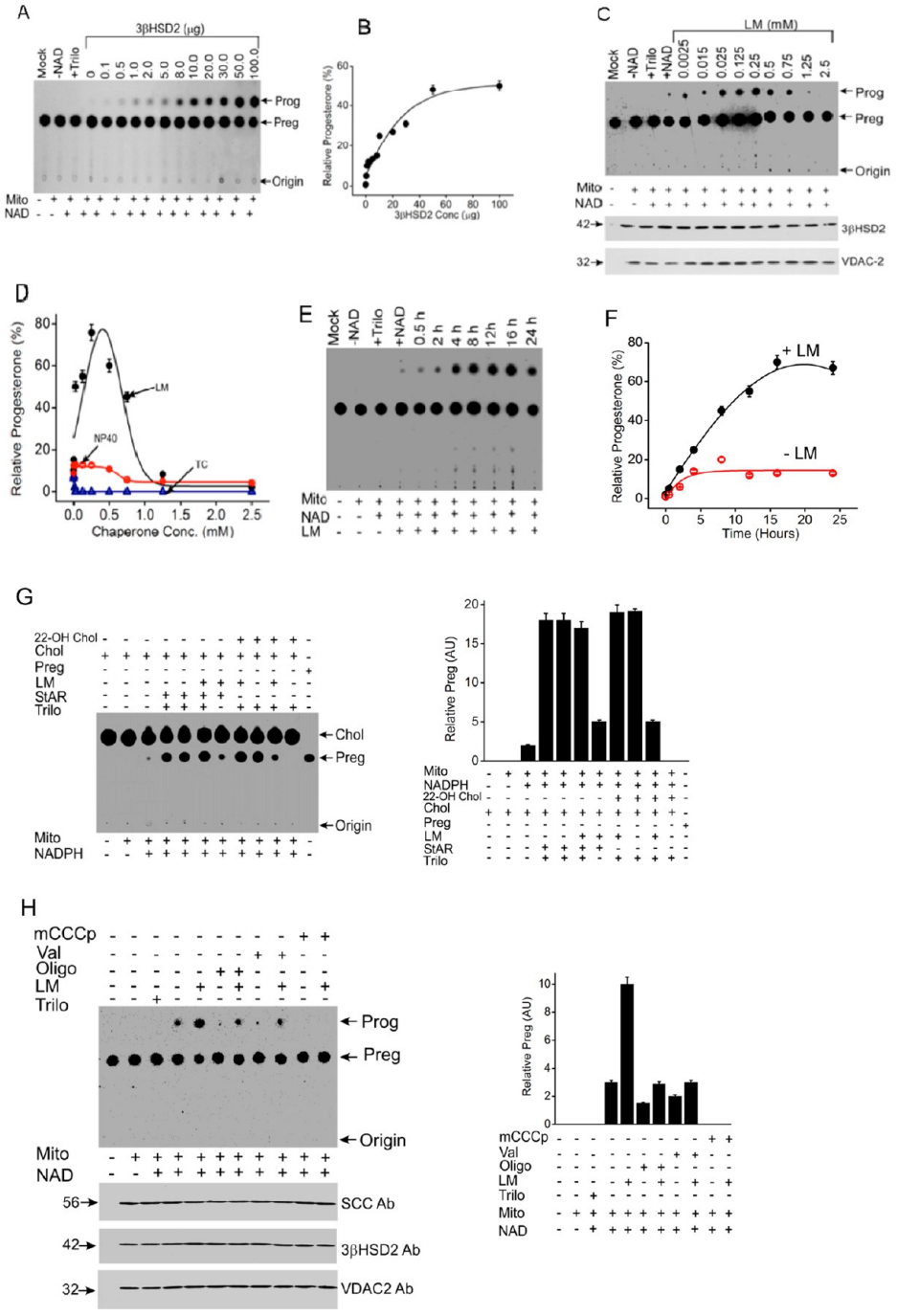

Figure 1. Measurement of metabolic activity. (A) ^{3}H-Pregnenolone to ^{3}H-progesterone conversion with increasing concentrations of 3βHSD2 protein. (B) Quantitative analysis of the progesterone in panel A. (C) Endogenous pregnenolone conversion using 3βHSD2 incubated with various concentrations of LM. The bottom panels show the Western blot with 3βHSD2 and VDAC2 antibodies. (D) Quantitative estimation of relative progesterone after incubation with LM (Panel C), NP40, and TC (see also Supplementary Figure 3A,B). (E) Pregnenolone to progesterone by endogenous 3βHSD2 from 30 min to 24 h with and without LM. (F) Quantitative estimation of relative progesterone synthesized derived from panel E and Supplementary Figure 3C. (G) Left, metabolic conversion of ^{14}C-cholesterol to pregnenolone in the presence and absence of LM. Right, quantitative analysis of the spot intensity from the left-hand panel. (H) Left, measurement of pregnenolone to progesterone by endogenous 3βHSD2 with the indicated inhibitors with and without addition of LM. The bottom panels show the Western blot with the indicated antibodies. Right, quantitative estimation of the spot intensity from the left-hand panel. Data presented in panels B, D, F, G-right and H-right are the mean ± SEM from three independent experiments performed in triplicate.

that steroidogenic activity of 3βHSD2 requires the protein to exist in different conformational states.

To survive an injury, illness, or sudden accident, the body must immediately synthesize cortisol, and so 3βHSD2 must be in a position to act rapidly to produce sufficient quantities of the enzyme. However, the IMM cannot support a high concentration of 3βHSD2 molecules, and thus the protein must be able to quickly cycle between the conformations needed to support the immediate and continuous metabolic activity of 3βHSD2. Most likely, the IMM environment

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supports the reactivation of 3βHSD2, thereby allowing one molecule to catalyze multiple reactions.

In the complex cellular environment, newly synthesized proteins are at risk for misfolding and aggregation,[9] so to prevent these risks, cells utilize the action of molecular chaperones.[10] Chemical chaperones, such as nonionic detergents or poly(ethylene glycol), are known to increase activity in vitro.[11] Members of the chaperone family include stress proteins or heat-shock family of proteins (HSPs). HSP70 and HSP90, which help in de novo protein folding, act through an ATP-dependent mechanism that promotes folding through ATP- and cofactor-regulated binding and release cycles. Cellular stress often up-regulates chaperones due to the tendency of proteins to form aggregates in this environment. While the chaperone(s) for 3βHSD2 is currently unknown, we have mimicked this environment by applying nonionic detergents, which are also called chemical chaperones. For clear presentation we have described these nonionic detergents as detergents throughout the manuscript.[12] In this study, we describe a mechanism by which detergents assist IMM resident 3βHSD2 to promote conformational changes and activation of the enzyme, resulting in its endogenous activity.

RESULTS AND DISCUSSION

Influence of Detergents on 3βHSD2 Activity. To address the role of detergents in the activity of 3βHSD2, we measured the metabolic conversion of pregnenolone to progesterone using mitochondria isolated from mouse Leydig MA-10 cells in the presence of LM, NP40, and TC. We chose MA-10 cells as they express abundant levels of 3βHSD2. The conversion was initiated in the presence of NAD (Figure 1A and Supplementary Figure 1A,B). The 3βHSD2 inhibitor trilostane was added in a separate reaction as a control (Figure 1A). Increasing the concentration of 3βHSD2 from 0.1 to 50 μg continuously increased progesterone synthesis with a plateau reached at 50 μg (Figure 1A,B). The accuracy of the measurements of steroid metabolic conversion was confirmed through GC−MS analysis (Supplementary Figures 2 and 3). To evaluate if detergents have an effect on the expression of 3βHSD2, we measured cell viability (Supplementary Figure 1C) and endogenous progesterone synthesis of MA-10 mitochondria after the addition of increasing concentrations of LM from 2.5 μM to 2.5 mM for 4 h (Figure 1C). Progesterone synthesis started after addition of LM (Figure 1C) and reached maximum level with a concentration of 0.25 mM LM (Figure 1C), but there was minimal or no change in activity with NP40 or TC (Supplementary Figure 3A,B). Quantitative analysis of the progesterone conversion with respect to detergent concentration showed maximum activity at about 0.38 mM LM (Figure 1D). Repurification of the mitochondria by centrifugation followed by Western staining showed expression of 3βHSD2 and VDAC2 was unchanged (Figure 1C, bottom panels). In the presence of 0.25 mM LM, this metabolic activity started after 30 min and reached its maximum level after 16 h (Figure 1E,F), after which activity leveled out. However, in the absence of LM the endogenous activity was minimally achieved at 2 h and marginally increased at 4 h (Supplementary Figure 4C). Therefore LM activated progesterone synthesis almost 6.4-fold (Figure 1D,F). Metabolic activity was increased when MA-10 cells were incubated with LM for 24 h (Supplementary Figure 1A). However, metabolic activity was only reduced in the presence of LM, when the mitochondrial proton pump was disturbed by the addition of uncouplers (Supplementary Figure 1B). In summary this experiment validated the active state of the organelles in the presence of detergents.

To confirm that indeed LM influenced the activity of 3βHSD2 and not any other proteins, we measured cholesterol to pregnenolone conversion after incubation of MA-10 mitochondria with LM. Steroidogenic Acute Regulatory Protein (StAR) fosters cholesterol from the outer to inner mitochondrial membrane, and thus we also incubated with a fixed amount of biosynthetic StAR (8 μg) (Figure 1G, left panel).[15,16] A quantitative estimation of pregnenolone synthesis showed no increase with LM (Figure 1G, right panel) as compared to a 6.4-fold increase in progesterone with endogenous 3βHSD2 with the MA-10 mitochondria (Figure 1D). Numerical values are listed in Supplementary Table-1. We next examined if the presence of LM altered the expression of 3βHSD2, StAR, VDAC2, and SCC. StAR is expressed as a 37 kDa precursor and imported into the mitochondria as a 30 kDa protein. Western blotting showed that expression of 3βHSD2, StAR, VDAC2, and SCC was unchanged after addition of LM or NP40 (Supplementary Figure 4D−G).

To determine if the addition of LM changes the mitochondrial electrochemical gradient, we measured metabolic conversion of the MA-10 mitochondria incubated with various inhibitors: oligomycin (OLIGO) inhibits the H^+-translocating ATP, and valinomycin (VAL) collapses the electrochemical potential across the inner membrane.[17,18] OLIGO and VAL inhibited the conversion of pregnenolone to progesterone (Figure 1H, left panel), suggesting that the inhibition of proton pump blocked metabolic conversion. A quantitative estimation of the intensity of the bands (Figure 1H, right panel) showed that mitochondria incubated with VAL and OLIGO produced about 30% progesterone. As a control for OMM-associated expression, we probed with VDAC2 antibody and found that VDAC2 expression remained unchanged (Supplementary Figure 4F), suggesting that the addition of these inhibitors did not reduce mitochondrial architecture. In summary, LM did not change the mitochondrial architecture but rather enhanced 3βHSD2 metabolic activity.

3βHSD2 was embedded in the micelle due to hydrophobic interactions between the protein and detergent. NP40 (149 residues per micelle) forms larger micelles than LM (98 residues per micelle); thus, the interaction of 3βHSD2 with NAD^+ was likely blocked by the bigger micelles of NP40 but facilitated by the smaller micelles of LM. LM forms a monodisperse population (PDI $\ll$ 0.1) of spherical micelles and thus preserves activity and conformation for an extended time.[19−21] In summary, our results indicate that the endogenous conversion of pregnenolone to progesterone is facilitated by LM. In fact, external addition of 50 μg of 3βHSD2 resulted in progesterone levels similar to that observed when mitochondria were incubated with LM.

Thermal Unfolding of 3βHSD2. To understand the chaperone-assisted structural stabilization of 3βHSD2, we measured the thermal unfolding (T_m) in the presence of the detergents LM, NP40, and TC. The change in ellipticity at 222 nm was measured by increasing the temperature from 4 to 90 °C (Supplementary Figure 5). The midpoint of the curve is defined as the melting temperature (T_m). Derivatives ($d\theta/dt$) were also plotted as a function of temperature to yield the exact T_m shown in the inset (Supplementary Figure 5). The protein in the absence of detergent exhibited a change in ellipticity at approximately 40 °C and complete denaturation at 60 °C

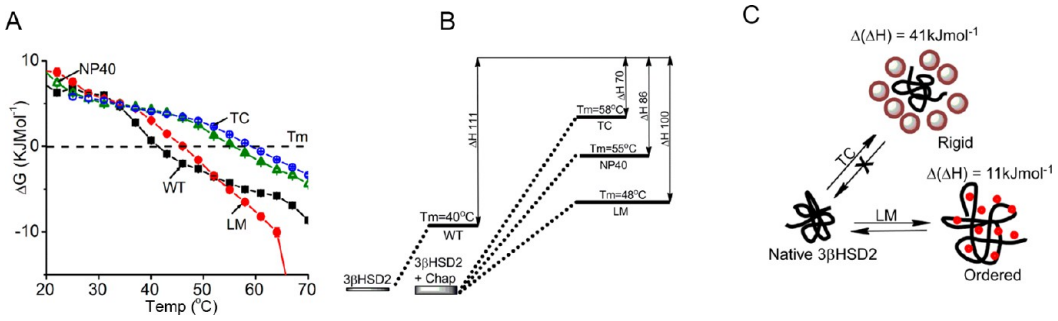

Figure 2. Thermal unfolding of 3βHSD2 in the presence of different detergents. (A) Thermal stability analysis of 3βHSD2 as identified by plotting ΔG at varying temperature. (B) Analysis of the stability by determining ΔH. (C) Schematic presentation of reversibility of 3βHSD2 in the presence of smaller micelles (LM) but not with the large micelles (TC). The $\Delta\Delta H$ values are subtracted from the 3βHSD2 protein in the absence of chaperones.

(Supplementary Figure 4A). We calculated the T_m to equal 40 °C in the absence of detergent. In the presence of LM, the T_m increased to 48 °C and the protein underwent complete denaturation at 70 °C (Supplementary Figure 5B). The T_m also significantly increased from 40 to 55 °C in the presence of NP40 (Supplementary Figure 5C) and to 58 °C in the presence of TC (Supplementary Figure 5D). These results suggest that detergents promote increased stability within the protein core, possibly due to the interaction of 3βHSD2 with these chaperones. The flexible conformation of 3βHSD2 makes the apparent binding possible.[14] A comparison of the thermal unfolding (Figure 2A) at 37 °C, where ΔG at the T_m is zero, shows a ΔG of 5 kJ mol^{-1} in the presence of chaperones and a ΔG of 2.6 kJ mol^{-1} in the absence of detergents. The addition of detergents initially increased ΔH, and thus the T_m of 3βHSD2 reflected the overall change in ΔH. A summary describing the change in ΔH (Figure 2B) with the addition of chaperones at the respective temperature shows a ΔH of 100.0 kJ mol^{-1} with LM, 86.5 kJ mol^{-1} with NP40 and 70.0 kJ mol^{-1} in the presence of TC and in the absence of chaperone ΔH was 111.0 kJ mol^{-1}. The smaller change in ΔH with the addition of the detergent LM helps 3βHSD2 attain its reversible conformation as compared to the higher stability with the larger micelles of NP40 or TC, resulting in a more rigid, nonreversible conformation (Figure 2C). Tm and ΔG values are listed in Supplementary Table-2.

Effect of Detergents on the 3βHSD2 Conformation. The previous experiments provided evidence that detergents assist endogenous 3βHSD2 activity through the hydrophobic interactions facilitated by the smaller micelles. To understand the mechanism of enhanced stabilization, we determined the conformational changes associated with detergents. Circular dichroism (CD) is a powerful technique that can be used to distinguish secondary structural components of proteins. The presence of minima near 208 and 222 nm indicate the existence of an α-helix, while minima near 218 nm indicate a β-sheet and minima near 198 nm indicate a random coil.[22] For the CD experiments, we chose two different detergent concentrations: a concentration just above the critical micelle concentration (CMC) and a concentration 5-fold higher than the CMC. At the CMC, weak hydrophobic interactions with the protein are most apparent; thus, we used a detergent concentration 5-fold higher than the CMC to check the effect of molecular crowding.[10] Addition of LM (200 μM) at the CMC resulted in a significant increase of ellipticity at both the $\pi-\pi^*$ (208 nm) and n−p* (222 nm) positions (Figure 3A). Indeed, we determined the helical content of each spectrum using CD-pro

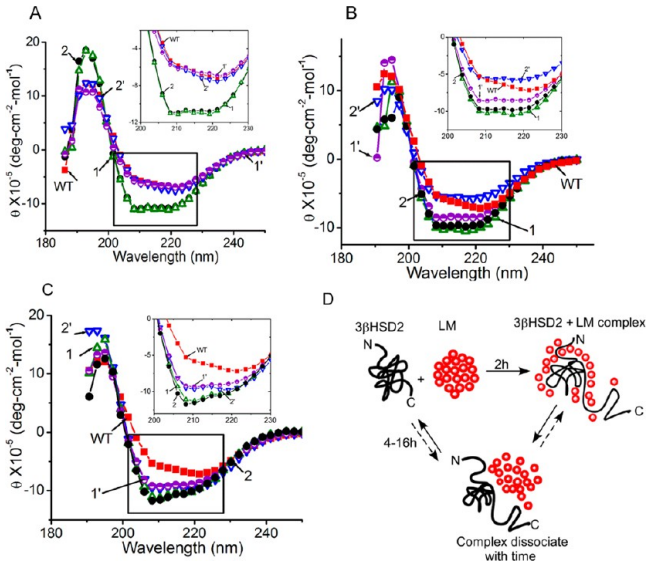

Figure 3. Effect of detergents on protein conformation. Far-UV CD of 3βHSD2 recorded between 190 and 250 nm after addition of different concentrations of detergents. (A) The far-UV CD spectra of 3βHSD2 (red solid square) was shifted after addition of LM at its CMC (green triangle 1). The initial conformation was restored after overnight incubation (violet half circle 1′). Here, 2 indicates addition of 5-fold higher CMC of each detergent, and 2′ indicates the CD spectrum obtained after incubating for 16 h with the indicated detergent. The inset shows the enlargement of the indicated section. (B and C) Similar far-UV CD spectra as in panel A in the presence of NP40 (B) and TC (C) under identical conditions. The inset shows the difference in conformational change among the three detergents. (D) Cartoon depicting the reversible conformation facilitated by incubation with LM over 16 h.

analysis and found that α helical content increased from 18% to 32% and β sheet increased from 19% to 22% in the presence of LM at the CMC. An increase in helical content increases stability.[23] The protein conformation, so-called ellipticity, is independent of concentration, suggesting that the increase in ellipticity in the presence of LM helped the protein to adopt a more stable conformation, possibly due to the hydrophobic interaction of the protein with detergent micelles.

Increasing the LM concentration 5-fold higher than the CMC did not significantly alter the ellipticity as compared with LM at the CMC (Figure 3A). However, we observed that incubation of the protein with LM for longer time resulted in the spectra overlapping with the sample that lacked detergent

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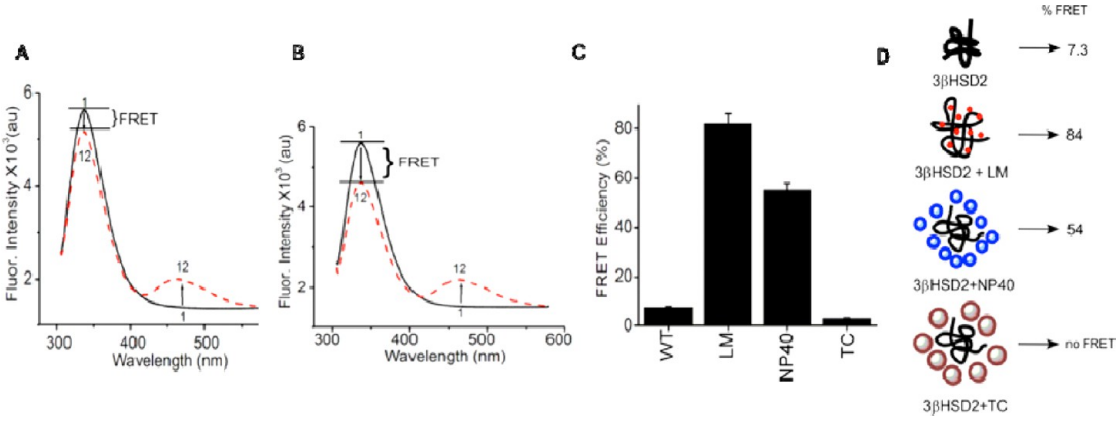

Figure 4. Adsorption of 3βHSD2 to chaperones as assessed by FRET. (A) The fluorescence emission spectra, after excitation at 295 nm, was acquired independent of detergents. The black solid line shows the fluorescence spectrum of 3βHSD2 in the absence of ANS when excited at 295 nm, and the red dashed line represents the fluorescence spectrum of 3βHSD2 in the presence of ANS when excited at 295 nm. (B) FRET after addition of LM. Black and red lines indicate the fluorescence spectra in the absence and presence of ANS, respectively, when excited at 295 nm similar to panel A. (C) FRET with detergents LM, NP40, and TC. Data presented is the mean ± SEM from three independent experiments performed in triplicate. (D) Diagram describes the influence of micelle size on the FRET efficiency of 3βHSD2. When the micelle size is increased, ANS molecules cannot approach Trp residues of the protein, decreasing FRET efficiency. 3βHSD2 had no FRET with the larger micelles of TC.

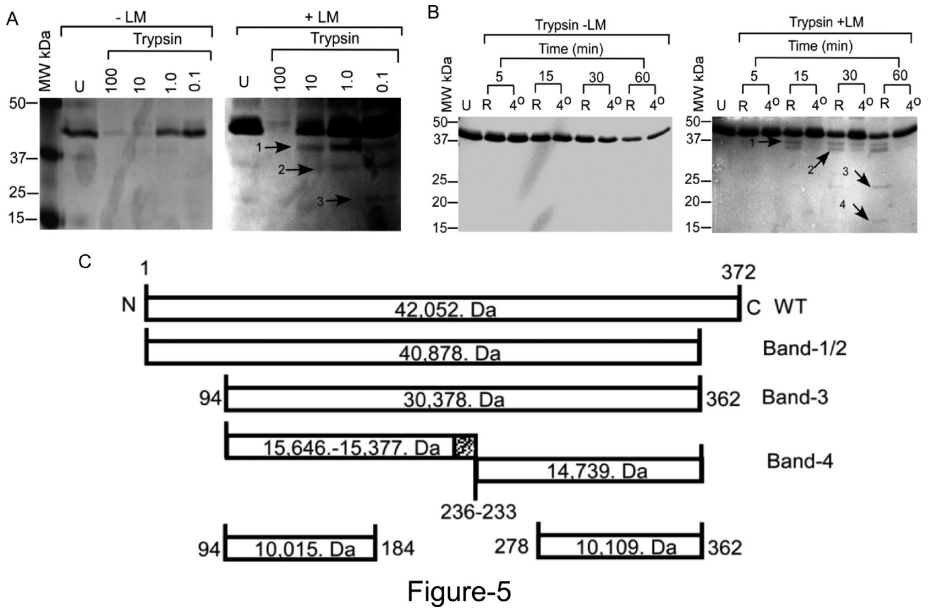

Figure-5

Figure 5. Kinetic analysis of 3βHSD2 in the presence and absence of LM. (A) 3βHSD2 was incubated with indicated concentrations of trypsin for 30 min in the presence (right) or absence (left) of LM. (B) Left, 3βHSD2 (5 μg) was incubated with 1 units of trypsin at RT (R) or 4 °C (4°) for 5, 15, 30, and 60 min in the presence (right) and absence (left) of LM. The first lane shows the undigested (U) protein applied for each digestion. The protected bands, indicated with arrowheads, were excised for mass spectrometry analysis. (C) Diagram describing the summary of the mass spectrometry analysis of the trypsin-protected bands.

(Figure 3A), suggesting a transient chaperone-assisted stabilization of 3βHSD2. The transient nature may be important because the protein must be able to simultaneously interact with OMM-associated Tom22, which faces the IMS, and with IMS resident Tim50 as a requirement for conformational changes for metabolic activity.[3]

A similar trend in structural stabilization was observed in the presence of the nonionic detergent NP40 (Figure 3B) and TC (Figure 3C). After addition of 200 μM NP40, which is a concentration just above the CMC, the $\pi-\pi^*$ position at 208 nm and the n$-\pi^*$ position at 222 nm both showed a significant increase in ellipticity (Figure 3B). However, only LM restored the original conformation after 16 h. However, in the presence of TC at a concentration 5-fold higher than the CMC, which actually increased the crowding significantly, the maxima at 192 nm showed a significant loss in ellipticity, indicating partial unfolding of the α helix in 3βHSD2. A similar trend in the ellipticity change was observed at the other chaperone concentrations. These results clearly indicate that 3βHSD2 interacted strongly with detergents at their CMC in a time-dependent fashion to form a more stable intermediate and that the 3βHSD2 core eventually released from the detergent due to the absence of the free energy necessary for it to remain associated for a longer time (Figure 3D). The 4-h refolding time may be crucial for 3βHSD2 refolding to its initial conformation and insertion to the IMM, a conformational change necessary for the activity of 3βHSD2.

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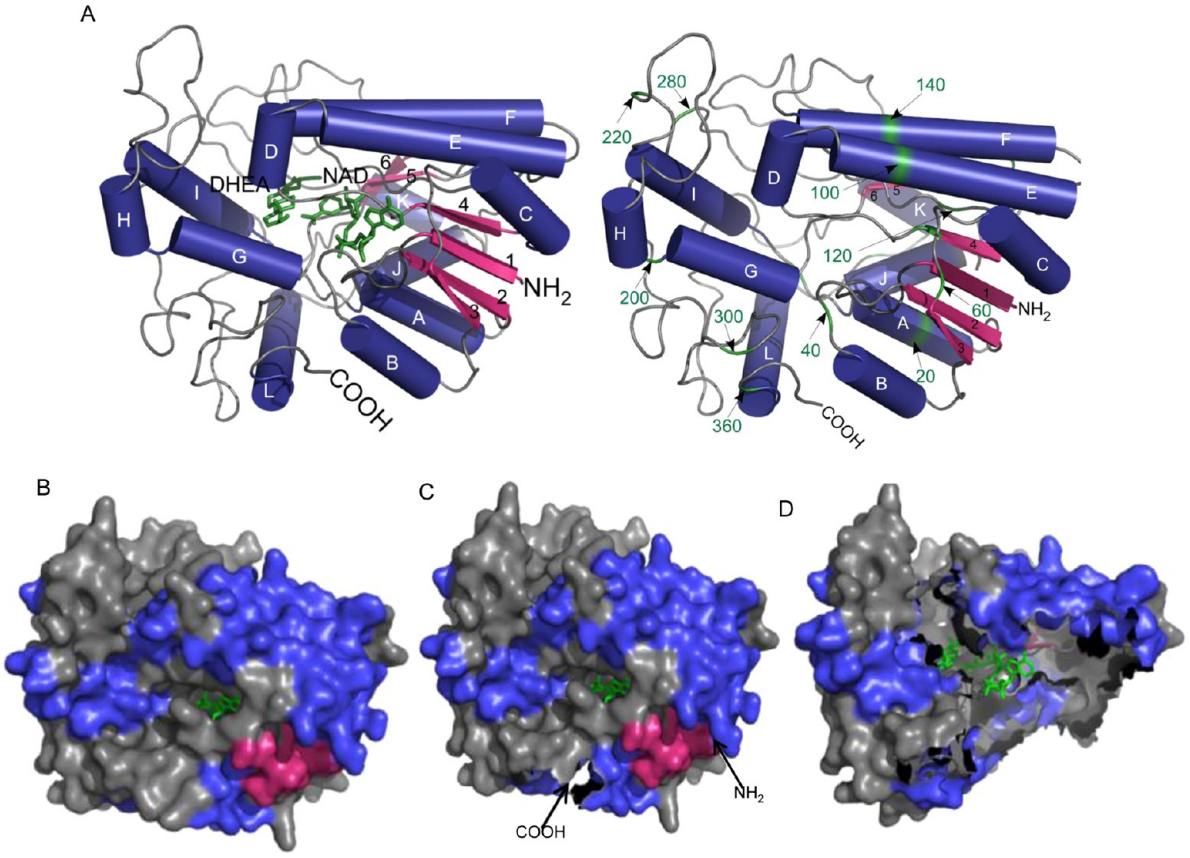

Figure 6. Model of the 3βHSD2 isomerase regenerated by the Pymol molecular graphics program. (A) Ribbon diagram of 3βHSD2 isomerase using mass spectrometric data: (left) without numbers and (right) with numbers. The protein consists of 6 β-sheets: 1 (residues 1−7), 2 (31−36), 3 (57−60), 4 (78−82), 5 (182−184), and 6 (265−267), which are colored in red. There are also 12 α-helices: A (residue 15−26), B (43−56), C (66−75), D (88−93), E(96−115), F (154−174), G(191−200), H(202−207), I (225−238), J (245−253), K (331−335) and L (346−360), which are shown in blue. Peptide segments with nonregular random coils are in gray. The active site bound with NAD^+ and DHEA are shown in green. (B) The β-sheet regions are colored red, the α-helix regions are colored blue, and the random coil is colored gray. The cofactors DHEA and NAD in the active site are shown in green. (C) Illustration of trypsin digest of 3βHSD2 in the presence of LM. The space-filling model of protected segment identified in band 2 (panel B, right) shows the 1−362 amino acid segment that resulted from the cleavage of the 10 amino acids from the C-terminus, while the cavity shows the cleavage site. (D) Fragments identified by the mass spectrometric analysis after trypsin incubation for 30 min. In band 3 (panel B, right , LM protected α-helix regions 94−102, 164−174, and 352−362, while amino acids 1−93 from the N-terminus and 10 amino acids from the C-terminus were cleaved (space-filling model). The cavity shows the opening segment of the active site, where most of the N-terminus is cleaved by the protease and thus shows the protected regions in the presence of LM.

Measurement of FRET in the Presence of Detergents. We further analyzed the influence of detergents on 3βHSD2 by monitoring the transfer of energy from Trp (tryptophan) residues of 3βHSD2 to ANS using fluorescence resonance energy transfer (FRET). In this technique, a donor chromophore, initially in its electronically excited state, transfers energy to a nearby acceptor chromophore through nonradiative dipole−dipole coupling. The dependency of FRET efficiency on the inverse of the distance, to the sixth power, between acceptor and donor pairs makes this technique useful for monitoring adsorption of molecules to a surface. In these experiments, 3βHSD2 tryptophan (Trp) residues, which are excited at 295 nm, served as the donor, while ANS served as the acceptor. ANS was in a different environment because of the presence of detergent. As a baseline, we first collected the fluorescence spectra of 3βHSD2 in the presence of detergents but in the absence of ANS, by exciting the Trp residues at 295 nm. We observed peak maxima at 340 nm (Figure 4 A,B). We then added increasing concentrations of ANS, and after an equilibration period of 3 min, the mixture was excited at 295 nm. The detergent concentration was 250 μM throughout the experiment. The binding of ANS resulted in a decrease in intensity of peak maxima at 340 nm followed by an increase at 470 nm, indicating sufficiently effective FRET between the protein and ANS molecules (Figure 4A,B). To compare the effects of different detergents, we determined the percentage of energy transfer efficiency for each scenario and found that it equaled 7.3 in the absence of detergents, 84 in the presence of LM, 55 in the presence of NP40, and 3 in the presence of TC (Figure 4C, Supplementary Table-3). These results indicate that ANS molecules came into closer proximity to Trp residues in the presence of LM than in the presence of NP40 or in the absence of detergent (Figure 4C,D).

Fingerprinting of 3βHSD2. To understand 3βHSD2 folding in greater detail, we sought to determine whether detergents protect 3βHSD2 domains from proteolysis. Trypsin is a highly specific enzyme that cleaves proteins after lysine and arginine residues under mildly alkaline conditions (pH $\geq$7.0). We used trypsin at pH 7.4 to partially digest 3βHSD2 in the presence and absence of chaperones at RT and at 4 °C. The digestion patterns were analyzed by staining with Coomassie blue in a 15% SDS-PAGE and further confirmed by Western

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blotting with 3βHSD2 antiserum along with mass spectrometry (Supplementary Table 4). In the absence of any detergent, the 3βHSD2 protein, especially at lower trypsin concentrations, remained fairly insensitive to digestion (Figure 5A, left panel) but underwent complete proteolysis with concentrations of 10 units or higher of trypsin (Figure 5A, left panel). The proteolysis in the absence of LM suggests that the protein maintained a closed conformation, making the trypsin sites not readily accessible. Theoretical prediction of trypsin digestion of 3βHSD2 was made by the Protein Prospector program (http://prospector.ucsf.edu), showing multiple fragments. In contrast, the addition of LM resulted in protease-resistant bands in the presence of 10 U of trypsin (Figure 5A, right panel). We next performed a kinetic analysis to identify the shielding of the chaperones after longer incubation times with trypsin, up to 60 min. We incubated 3βHSD2 with 1 U of trypsin from 5 to 60 min at either RT or 4 °C. As expected, in the absence of LM, we did not observe any protected bands, regardless of incubation time (Figure 5B, left panel). However, in the presence LM, incubation with 1.0 U of trypsin for 60 min resulted in four protected bands at approximately 40, 39, 24, and 13 kDa in size (Figure 5B, right panel), suggesting that LM association with 3βHSD2 exposed the N-terminal β-sheet-rich hydrophobic region, making it easily accessible to trypsin. Figure 5B shows separation by 17.5% SDS-PAGE to evaluate smaller fragments. The two closely sized bands at 40 and 39 kDa appeared within 5 min of incubation at RT and remained at the same intensity throughout the time span of the analysis. Both the 24 and 13 kDa bands appeared after 60 min of trypsin incubation. The appearance of the 24 and 13 kDa bands after a longer incubation time suggests a stronger association with LM, limiting the access of trypsin to the core of 3βHSD2. Mass spectrometric analysis of the protease-resistant bands showed that the 40 kDa band 1 consisted of amino acids 2−362 of 3βHSD2 and band 2 consisted of the same amino acid range, suggesting the deletion of several amino acids from the C-terminal region (Figure 5C). To our surprise, band 3, which ran at 24 kDa, contained amino acids spanning a mass range of 30 kDa. Similarly, band 4 shows an approximately 10 kDa protein containing two overlapping bands covering the region of amino acids 94−362. theoretical analysis of 3βHSD2 shows that both of these peptides contained numerous potential cleavage sites for trypsin; thus, the failure of trypsin digestion to cleave these sites after addition of chaperones attests to a substantial change in protein folding and protection and the possibility that these cleavage sites were buried within the detergent-3βHSD2 interaction sites. The diagram shows that 3βHSD2 proteolyzed into discrete amino acids, resulting in no protected regions of amino acids. Addition of detergents generated a gradual but ordered fragmentation resistant to proteolysis for 1 h.

Modeling of Proteolysis Experiments. We next generated a ribbon diagram and space-filling model of 3βHSD2. Full-length 3βHSD2 was digested with trypsin in the presence of LM, NP40, or TC at the CMC for each detergent. After the protected bands were separated by SDS-PAGE, excised, and sequenced by mass spectrometry, we used Pymol to create the models and correlated our findings with the developed model (Figure 6A), with 6 β-sheets colored red, 12 α-helices colored blue shown as cylinders, and the random coil colored gray. The substrate DHEA and cofactor NAD in the active site are shown in green (Figure 6A). The space-filling model of the trypsin-digested protein in the presence of LM is shown in Figure 6B−D. Figure 6A (right panel) shows the protected band 1 (Figure 5B, right panel), which appeared within 5 min of digestion and included β-sheet region 3−18 as well as α-helix regions 22−27, 93−103, 135−159, 337−350, and 353−363 (Figure 6A, right panel) but lacked the 10 amino acids from the C-terminus random coil. When the protein was incubated for 30 min at RT in trypsin, another two bands (bands 2 and 3, Figure 5B, right panel) appeared. Mass spectrometry analysis of band 3 identified α-helix fragments 94−102, 164−174, and 352−362 (Figure 6A, right panel). Thus, the region from 94 to 362 was protected, while the first 93 amino acids of the catalytically important N-terminus were cleaved, leaving an open cavity as shown in Figure 6D. The hydrophobic region that is rich in β-sheets was entirely cleaved. So we conclude that in the presence of detergent micelles, the hydrophobic regions are more exposed, while the other regions of the proteins are well protected (Figure 6C and D).

Discussion. We found that the steroidogenic enzyme 3βHSD2 is not cleaved upon import into the mitochondrion.[3] Rather, the mitochondrial N-terminal leader sequence directs the protein to enter the mitochondria. 3βHSD2 does so in a state in which it remains to interact with translocases that face the IMS. The cell must be able to recycle 3βHSD2 to catalyze the multiple reactions needed to produce steroids on demand. This led us to consider the possibility that 3βHSD2 continuously moves between a folded and unfolded state to function as facilitated by the IMM environment. Our studies suggest that the pH gradient[4] and lipids[14] both play significant roles in the unfolding of 3βHSD2. The most intriguing part of the addition of LM was that it showed the activation, maintenance, and increase in activity of 3βHSD2 for 16 h. As it appears that 3βHSD2 lacks a receptor, thus it requires environmental factors to fold it properly to have activity. In this study, we posit that chaperones, as yet unidentified in vivo, are present at the IMM, and once the protein is unfolded through the mitochondrial proton pump or vesicles, the chaperones help in the refolding process in order for 3βHSD2 to catalyze more reactions.

The observed detergent-mediated changes in CD spectra of the 3βHSD2 may be described reasonably well by a simple, three-state binding, stability of conformation, and recirculation model (Figure 3C). The detergents, especially LM, induced a higher structural organization, indicating the presence of a stable folding intermediate to rejuvenate enzyme activity for further catalysis. This intermediate retained a significant amount of increased secondary structure, suggesting that the native state of the protein was an unfolded conformation.

Several scenarios could account for the presence of a 3βHSD2 stable intermediate following the unfolded state. The simplest explanation is that interaction of 3βHSD2 with the LM micelles increased the thermal unfolding from 40 to 48 °C, while the other detergents increased the T_m even further, to 55 or 58 °C. The LM-mediated faster recirculation of 3βHSD2 may have resulted from the binding with these detergents, with the first transition producing the observed state that remained for up to 2 h, followed by a gradual release to the unfolded state (Figure 3D). In the case of detergents, the hydrophobic interaction may have resulted in a very high activity of 3βHSD2 as compared to no or low activity in the absence of detergents (Figure 1C,H and Supplementary Figure 3A,B).

A second scenario is that detergents stabilize an intermediate to an increasingly active state for recirculation after a pseudoequilibrium. At 37 °C, the ΔG of the 3βHSD2 was 2.6 kJ mol^{-1}, while the addition of detergents increased the ΔG

to 6 kJ mol^{-1}. This demonstrates that in the physiological environment the protein was energetically equally stable, but the variation in helical conformation may possibly serve as factor for the overall change in free energy. However, in the presence of larger micelles the steric hindrance resulted in a conformation with a higher ΔH, which is thus irreversible.

Protein fingerprinting followed by mass spectrometric analysis identified 4 protected bands in the presence of chaperones. The formation of a tightly packed hydrophobic core represents a critical step in the folding pathway of globular proteins. Hydrophilicity analysis shows that the N-terminal 3βHSD2 sequence is more hydrophobic up to amino acid 120 and the amino acid sequence of the rest of the protein is weakly hydrophobic and does not form an amphiphilic helix, resulting in a favored association with the membrane. The C-terminal sequence is most likely not cleaved after import through the mitochondrial membrane but instead remains associated with the chaperones. The association may be the result of an electrostatic interaction between the positively charged residues of the C-terminus of the protein and the nonpolar micelles of the chaperones. In the presence of the detergents, the N-terminal region of 3βHSD2 interacted with the detergents to open its conformation. As identified by the modeling of the fingerprinting, once the protein interacted with the chaperones, the initial N-terminal 93 amino acids became exposed to the surface and thus were proteolyzed. Our data suggest that the active form of the 3βHSD2 structure could be preserved in the membrane-associated form. The random coil region of the C-terminus is membrane bound leaving the N-terminus associated with the detergents. This might be the critical reason that allows 3βHSD2 to have two different activities at two different steroidogenic steps.

The 3βHSD2 N-terminal mitochondrial leader sequence is in the most hydrophobic portion of 3βHSD2, and secondary structure predictions suggest that all regions of the protein are highly structured. Being at the IMM, where the mitochondrion is continuously moving, the 3βHSD2 protein must be very flexible. Thus, a transient electrostatic interaction could occur between the positively charged residues of 3βHSD2 and nonionic chaperones, possibly forming a molten-disc conformation.[24] Very high activity of the steroidogenic mitochondria was seen immediately after introduction to the chaperone environment (Figure 1C). The detergents at the IMM are likely to help the protein to become stable transiently and then go back to the active conformation. Alternatively, small structured domains of 3βHSD2 might interact with an as-yet unidentified receptor on the IMM or at the IMS. In either event, the physical data suggest that the helices in the soluble active 3βHSD2 structure could be preserved in the IMM,[23] suggesting that the membrane-bound protein retains its flexible conformation. This must be a rapid process as steroid production must be rapid to meet physiological demands, suggesting that a chaperone-assisted conformation of 3βHSD2 is a physiological requirement. This will allow 3βHSD2 to interact continuously and directly with neighboring proteins as described by our detergents model.

METHODS

Biological Activity. Cells were maintained at 37 °C in a humidified incubator under 5% CO_2. The cell viability was determined before and after incubation of LM following identical procedure as described recently.[13] Metabolic conversion assays and CD were conducted following our standard procedure.[3,4]

Determination of ΔG of the Unfolding in Presence of Detergents. Gibb's free energy of the thermal unfolding process was calculated from the following equation:

$$\Delta G = \Delta G^{\circ} - RT \text{ in } \Sigma u/\Sigma f$$

where Σf and Σu are the binding polynominals of the native (folded) and denatured (unfolded) forms of the protein. Detailed procedure is in the Supporting Information.

Gas Chromatography, Mass Spectrometry, Fingerprinting, and Modeling. We have performed these experiments following procedures described before.[14−16]

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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