Purification, Reconstitution, and Steady-state Kinetics of the Trans-membrane 17β-Hydroxysteroid Dehydrogenase 2*

Received for publication, December 9, 2001, and in revised form, March 21, 2002
Published, JBC Papers in Press, April 8, 2002, DOI 10.1074/jbc.M111726200

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Human membrane 17β-hydroxysteroid dehydrogenase 2 is an enzyme essential in the conversion of the highly active 17β-hydroxysteroids into their inactive keto forms in a variety of tissues. 17β-hydroxysteroid dehydrogenase 2 with 6 consecutive histidines at its N terminus was expressed in Sf9 insect cells. This recombinant protein retained its biological activity and facilitated the enzyme purification and provided the most suitable form in our studies. Dodecyl-β-D-maltoside was found to be the best detergent for the solubilization, purification, and reconstitution of this enzyme. The overexpressed integral membrane protein was purified with a high catalytic activity and a purity of more than 90% by nickel-chelated chromatography. For reconstitution, the purified protein was incorporated into dodecyl-β-D-maltoside-destabilized liposomes prepared from L-α-phosphatidylcholine. The detergent was removed by adsorption onto polystyrene beads. The reconstituted enzyme had much higher stability and catalytic activity adsorption onto polystyrene beads. The reconstituted droprogesterone as substrates were also determined.

The members of the 17β-hydroxysteroid dehydrogenase (17β-HSD) family are crucial in the biosynthesis and metabolism of active steroid hormones in a variety of tissues. Estrogens and androgens in turn control a variety of important physiological functions such as growth, reproduction, and differentiation. Using NAD as cofactor, 17β-HSD2, with its predominantly oxidative activity, primarily converts the highly active 17β-hydroxysteroids such as estradiol, testosterone, and dihydrotestosterone into their inactive keto forms. Furthermore, studies carried out in vitro indicate that 17β-HSD2 is able to use C20-steroids as substrates, namely to catalyze the oxidation of 20α-dihydroprogesterone to progesterone. The expression of the mRNA of human 17β-HSD2 has been detected in a large variety of tissues. Its 1.5-kb mRNA is highly expressed in the endometrium, placenta, liver, and small intestine and also in smaller amounts in the pancreas, colon, kidney, and prostate (1–5). Human 17β-HSD2 mRNA has also been found to be present in human breast, endometrial, and prostate cancer cell lines (3). In addition, both rodent and human 17β-HSD2 enzymes are widely distributed in the gastrointestinal and urinary tracts, in the liver, as well as in the adrenals of adults and developing fetuses (2–5). Recently, the correlation between 17β-HSD2 and colonic cancer was reported (6). The broad tissue distribution, together with the predominant oxidative activity of 17β-HSD2, suggests that the enzyme plays an essential role in the inactivation of highly active 17β-hydroxysteroids. It may have a protective role by lowering the active steroid concentrations and reducing excessive sex hormone action in target tissues.

17β-HSD2 is a trans-membrane protein, which is demonstrated by its subcellular distribution in the endoplasmic reticulum (7). 17β-HSD2 cDNA encodes a predicted protein of 387 amino acids with a molecular mass of 42,782 daltons. The primary structure shows that it belongs to the type II signal anchor membrane protein, which is characterized by possessing a cluster of positively charged amino acids and followed by a hydrophobic core of about 33 nonpolar amino acids close to the N terminus of the protein (1, 8, 9). The carboxyl terminal has a luminal carboxyl-terminal endoplasmic reticulum retention motif (KKK) (1). Based on the trans-membrane helices prediction using a hidden Markov model (10), there are two proposed trans-membrane helices close to the N terminus of 17β-HSD2, the first one situated in amino acids 5–27 and the second one in 34–56. The latter is much more hydrophobic than the former. The enzyme is thus suggested to be an integral membrane protein (7).

Up to now, most of the information obtained for 17β-HSD2 is about genes and mRNA studies. Although an N-29 amino acid truncated form, in which the first proposed transmembrane helix was deleted, retained about 60% of its catalytic activity as compared with wild type in the intact cells and was purified using a detergent β-octyl glucoside, knowledge about the purification of the enzyme is still limited (7). In order to elucidate the structure and function of the protein, we carried out the overproduction, purification, reconstitution, and characterization of N-terminal His6-tagged full-length 17β-HSD2, which are reported here.
EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and modifying enzymes used in molecular biology experiments were purchased from Amersham Biosciences and Roche Molecular Biochemicals. Taq DNA polymerase and Pfu DNA polymerase were from PerkinElmer Life Sciences and Stratagene (La Jolla, CA), respectively. Spodoptera frugiperda (Sf9) cells, the Bac-N-Blue transfection kit, and the pBlue Bac4.5 transfer vector were from Invitrogen. Schistosoma mansoni, Drosophila melanogaster, tobacco insect cell culture medium, yeastolate, and lactalbumin hydrolysate were from Invitrogen. β-Octyl glucoside, decyl-β-d-maltoside, dodecyl-β-d-maltoside (β-DMM), polyoxyethylene 8-lauryl ether (C12E8), and Triton X-100 were from Anatrace (Maumee, OH). Radiosioptically labeled steroids were from PerkinElmer Life Sciences. His bind resin was from Novagen. SM2 Bio-Beads was from Bio-Rad. Steroids, bis-sulfosuccinimidyl suberate (BS), L-α-phosphatidylcholine (PC), L-phosphatidylethanolamine, L-α-phosphatidylinositol, and all other chemicals were from Sigma.

Construction and Production of Recombinant Baculovirus—The full-length human 17β-HSD2 cDNA coding sequence was obtained by PCR amplification from PCMV17βHSD2 (11). A nucleotide sequence coding for 6 histidine residues followed by a Factor Xa cleavage site was added to the 5' terminus of the 17β-HSD2 cDNA. The forward primer contained a BamHI site (underlined), 5'-CGGATTCTCTGGAAGCCCATCA-TACTACATCATCATTGAGGATGCAGGCGCTGTCGCTGAGCATGAAAGCTATGAG-CACTTCTTCTCCCGGACTGAGATCC-3', and the reverse primer contained an EcoRI site (underlined), 5'-GGGATCTCTGGAAGCCCATCA-TACTACATCATCATTGAGGATGCAGGCGCTGTCGCTGAGCATGAAAGCTATGAG-CACTTCTTCTCCCGGACTGAGATCC-3'. The 1.2-kb amplified products were digested with the appropriate enzymes and subcloned into the corresponding sites of the pBlue Bac4.5 vector. Using this method, we also constructed N-terminal His6-tagged 17β-HSD2 and the enzyme lacking the first 38, 52, and 61 amino acids of the N terminus (N-38, N-52, and N-61-deleted 17β-HSD2). The recombinant vectors were identified using dideoxynucleotide sequencing (Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit, PerkinElmer Applied Biosystems, 373 sequencer with XL Upgrade). Linearized AcMNPV DNA (Bac-N-Blue DNA) (0.5 μg) was used to co-transfect monolayers of Sf9 cells in the presence of Lipofectamine, sterile insect cell culture medium, yeastolate, and 20% lactalbumin hydrolysate were from Invitrogen. β-Octyl glucoside, decyl-β-d-maltoside, dodecyl-β-d-maltoside (β-DMM), polyoxyethylene 8-lauryl ether (C12E8), and Triton X-100 were from Anatrace (Maumee, OH). radiosioptically labeled steroids were from PerkinElmer Life Sciences. His bind resin was from Novagen. SM2 Bio-Beads was from Bio-Rad. Steroids, bis-sulfosuccinimidyl suberate (BS), L-α-phosphatidylcholine (PC), L-phosphatidylethanolamine, L-α-phosphatidylinositol, and all other chemicals were from Sigma.

Cell Culture and Virus Infection—Sf9 cells were grown as monolayers in flasks containing Grace's insect cell culture medium with 5% fetal bovine serum and maintained at 27 °C. The wild type baculovirus and the recombinant virus carrying 17β-HSD2 were used to infect the 90% confluent cells at a multiplicity of infection of 0.1–0.5 for virus amplification and an multiplicity of infection of 5–10 for protein overproduction. The infected cells were harvested 72 h postinfection, washed with cold phosphate-buffered saline, pelleted, and stored at −80 °C for later use.

Solubility and Stability Test of N-terminal His6-tagged 17β-HSD2—Cell pellets containing overexpressed N-terminal His6-tagged 17β-HSD2 were fractionated in 1.5 ml of buffer A (40 mM Tris, pH 8.0, 20% glycerol, 20 μM NAD, 0.4 mM phenylmethylsulfonyl fluoride, 0.15 mM NaCl, and 1 μg/ml each of the following protease inhibitors: leupeptin, chymostatin, antipain, aprotinin, and pepstatin A) containing detergents. β-Octyl glucoside, decyl-β-d-maltoside, β-DMM, Triton X-100, sodium cholate, and C12E8 were used with concentrations 0.1–1.2%. The samples were sonicated on ice by a sonic dismembranator (Fisher Scientific) and incubated for 1 h at 4 °C. 0.1 ml of each homogenate was transferred to separate tubes as control, and the remaining homogenate was centrifuged for 45 min at 180,000 × g at 4 °C. The aliquots from the homogenates, supernatants, and pellets were analyzed by electrophorese, immunoblotting, and activity assay.

Recombinant His6-tagged 17β-HSD2—The cell pellets from 6–8 × 10^7 cm^2 flasks (which represents about 1.6–2 × 10^8 cells) were suspended in 50 ml of buffer A. The remaining procedures were carried out at 4 °C or on ice unless otherwise specified. The cells were lysed by sonication. The suspension was incubated for 15 min and centrifuged for 30 min at 180,000 × g. The pellets were then solubilized in 100 ml of buffer B (40 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, 8% polyethylene glycol 8000, 0.4 mM phenylmethylsulfonyl fluoride, 0.5% β-DMM, 1 μg/ml each of the protease inhibitors) and incubated by rotating for 1 h. The supernatants were collected after centrifugation for 45 min at 180,000 × g, adjusted to 300 mM NaCl, mixed with 3 ml of nickel-chelated resin pre-equilibrated with buffer B (containing 300 mM NaCl), and incubated by rotating for 1 h. The mixture was loaded onto the column. The column was washed with 10 column volumes of buffer C (buffer B containing 300 mM NaCl, 0.3% β-DMM, 15 mM imidazole, and 15% glycerol) and 10 column volumes of buffer D (buffer B containing 45 mM imidazole, 200 mM NaCl, 0.3% β-DMM, and 20% glycerol). Bound proteins were eluted with buffer E (40 mM Tris, pH 7.5, 150 mM NaCl, 20% glycerol, 0.2% β-DMM, 250 mM imidazole, 40 μM NAD, 0.4 μM phenylmethylsulfonyl fluoride, and 1 μg/ml protease inhibitors). The fractions were collected in 1 ml each and concentrated in liquid nitrogen, and stored at −80 °C. The purified enzyme without adding cofactor NAD in the purification procedures was used to detect cofactor kinetic constants.

Preparation of Lipozymes and Protein Reconstitution—The reconstitution method basically depended on the strategies described by Rigaud et al. (10). The appropriate enzymes and subcloned into the corresponding sites of the pBlue Bac4.5 vector. Using this method, we also constructed N-terminal His6-tagged 17β-HSD2 and the enzyme lacking the first 38, 52, and 61 amino acids of the N terminus (N-38, N-52, and N-61-deleted 17β-HSD2). The recombinant vectors were identified using dideoxynucleotide sequencing (Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit, PerkinElmer Applied Biosystems, 373 sequencer with XL Upgrade). Linearized AcMNPV DNA (Bac-N-Blue DNA) (0.5 μg) was used to co-transfect monolayers of Sf9 cells in the presence of Lipofectamine, sterile insect cell culture medium, yeastolate, and 20% lactalbumin hydrolysate were from Invitrogen. β-Octyl glucoside, decyl-β-d-maltoside, dodecyl-β-d-maltoside (β-DMM), polyoxyethylene 8-lauryl ether (C12E8), and Triton X-100 were from Anatrace (Maumee, OH). Radiosioptically labeled steroids were from PerkinElmer Life Sciences. His bind resin was from Novagen. SM2 Bio-Beads was from Bio-Rad. Steroids, bis-sulfosuccinimidyl suberate (BS), L-α-phosphatidylcholine (PC), L-phosphatidylethanolamine, L-α-phosphatidylinositol, and all other chemicals were from Sigma.

Steady-state Kinetics—The kinetic constants of 17β-HSD2 were determined using purified and reconstituted N-terminal His6-tagged 17β-HSD2. The reaction mixture contained 50 mM sodium phosphate buffer, pH 7.4, 1 mM constant concentration of cofactor NAD, with different steroid substrates ([14C]estradiol, [14C]testosterone, [14C]dihydrotestosterone, and [3H]20α-dihydrotestosterone), which were incorporated from 0.14 to 4 μM of the kinetic constants of steroids. The same buffer system contained a 10 mM constant concentration of steroids (estradiol and testosterone) with various concentrations of cofactor NAD (0.05–1 mM) for the kinetic constants of NAD. The same buffer with 10 μM of constant concentration of 4-dione and estrone and with various concentrations of cofactor NAD (0.001–0.2 mM) was used for the kinetic...
constants of NADH. The kinetic constants of NADP were determined with a 10 μM constant concentration of estradiol and various concentrations of cofactor NADP (0.6–10 μM). The initial velocity was measured with less than 5% substrate conversion. The reactions were carried out at 37 °C and stopped by removing 0.5 ml of reaction mixture to the cold diethyl ether at four different time intervals (0, 20, 40, and 80 s). The steroids were extracted with ethanol in dry ice and dried by evaporation. They were then dissolved in dichloromethane, applied onto thin layer chromatograms (TLC), separated by toluene/acetone (4:1, v/v), and quantified by Storm 860 Laser Scanner (Molecular Dynamics, Inc., Sunnyvale, CA; ImageQuant software). At least three independent experiments were carried out for each kinetic constant. The kinetic results were fitted for the Michaelis-Menten equation and calculated using a Lineweaver-Burk plot. The values of the catalytic constant, kcat, were calculated from the Vmax values with the homodimer molecular mass of 90 kDa (kcat is the turnover number, i.e. the number of moles of substrate transformed per second per mole of enzyme).

Glycerol Gradient—The apparent functional molecular mass of N-terminal His14-tagged 17β-HSD2 was estimated by cosedimentation with protein standards on 8–30% glycerol gradients. Glycerol gradients (13 ml; Beckman SW 40Ti rotor) were prepared by using a gradient maker with equal volumes of 8 and 30% glycerol gradients. Glycerol gradients for about 8 h before loading the samples. The samples contained 20 μg of purified and reconstituted 17β-HSD2 and were incubated at 4°C for 30 min before adding glycine to a final concentration of 30 mM. The samples were then loaded on top of the gradient and centrifuged at 40,000 rpm for 1 h at 4°C. The gradients were fractionated from the bottom into 0.3-ml fractions. 17β-HSD2 fractions were verified by the enzyme activity assay and Western blot. The positions of the standard markers were determined by SDS-PAGE.

Chemical Cross-linking of 17β-HSD2—This was performed according to the method described by Knoller (14) with modifications. 60 μl of reaction buffer contains 50 mM potassium phosphate, pH 7.4, 0.15% NaCl, 40 μM NAD, 1 mM EDTA, 0.5 mM DTT, and 0.1% Triton X-100. The glycerol gradients were equilibrated at 4°C for about 8 h before loading the samples. The samples contained 20 μg of purified and reconstituted 17β-HSD2, 100 μg of each protein standard (rabbit skeletal muscle aldolase, bovine serum albumin, and chicken ovalbumin), and the same buffer as in the gradient, but the glycerol concentration was less than 8%. The samples were then loaded on top of the glycerol gradients and centrifuged at 40,000 rpm for 1 h at 4°C. The gradients were fractionated from the bottom into 0.3-ml fractions. 17β-HSD2 fractions were verified by the enzyme activity assay and Western blot. The positions of the standard markers were determined by SDS-PAGE.

SDS-PAGE and Western Blot Analysis—SDS-PAGE was performed according to the method of Laemmli (15) using 12% polyacrylamide gel or 5–15% gradient SDS-PAGE. The samples in reducing loading buffer were incubated at 4°C for 30 min instead of boiling before loading to prevent the aggregation of the membrane protein (16). The gel after electrophoresis was stained with Coomassie Blue. For Western blot analysis, the proteins were transferred to nitrocellulose membranes according to the method of Laemmli (15) using 12% polyacrylamide gel. The blots were probed with rabbit polyclonal antibody raised against human 17β-HSD2, and cross-linking reagent BS with concentrations of 0, 0.25, 1, and 3 mM, respectively. The reaction proceeded for 30 min at 25°C and stopped by adding glycine to a final concentration of 30 mM. The samples were analyzed by 5–15% gradient SDS-PAGE followed by Western blot.

Protein Concentration Determination—Protein concentrations without detergent were determined using the Bradford reagent (Bio-Rad). The concentrations of proteins with detergents or with phospholipid were determined by the method of microgram quantities of protein determination (17) to prevent the alteration of detergents and phospholipids in the protein concentration by conventional methods.

RESULTS

Overproduction of Various Recombinant 17β-HSD2—Human 17β-HSD2 cDNA with a 6-histidine coding sequence and a Factor Xa cleavage site at its 5’ terminus was subcloned into the baculovirus transfer vector pBlueBac 4.5. The incorporation of the Factor Xa cleavage site allowed the removal of the His tag after purification of the recombinant protein. Leave only two adjacent glycines at the N terminus of 17β-HSD2. Sf9 cells were co-transfected with Bac-N-Blue DNA and the above transfer vector of 17β-HSD2 to produce the recombinant baculovirus. Protein expression was optimized by evaluating the expression levels of the infection at different time intervals. The activity was first detected 24 h postinfection and reached a maximum between 60 and 72 h postinfection (Fig. 1, A and B), whereas no activity could be detected in wild-type AcMNPV virus-infected cells. Thus, the protein expression conditions were set as follows: infection of the cells at a multiplicity of infection from 5 to 10 and harvest in 72 h postinfection. Under these conditions, the overexpressed 17β-HSD2 constitutes about 3% of the total protein in the insect cell lysate, with a specific activity of 0.012 units/mg in the cell homogenate.

Using the same method, we also overproduced N-38-, N-52-, and N-61-deleted 17β-HSD2, as well as N-terminal His14-tagged and C-terminal His8-tagged 17β-HSD2. The truncated N-38 form was expressed at about 2% of the total protein in the insect cell lysate, with a specific activity of 0.005 units/mg in the cell homogenate. Although this form could be solubilized to a higher level with detergent from membrane vesicles (solubilized 45% of the enzyme in the presence of 0.4% β-DDM) than that of N-terminal His8-tagged 17β-HSD2 (solubilized 30.5% of the enzyme in the presence of 0.4% β-DDM), it was unstable in the solubilized state with detergents even in intact cells and showed a very strong tendency to degrade and aggregate in the cell homogenate. The truncated N-52 form in fresh cultured cells still retained a little activity, but it completely lost activity in several hours at 4°C. Moreover, the N-61 form was totally inactive in fresh cultured cells. The N-terminal His8-tagged 17β-HSD2 was expressed to a high level (about 5% of the total protein); however, it retained a quite lower specific activity (about 0.001 units/mg) than that of the N-terminal His8-tagged form (about 0.012 units/mg). The C-terminal His8-tagged 17β-HSD2 was expressed at about 2% of the total protein in the insect cell lysate, with a specific activity of 0.005 units/mg in the cell homogenate.
Finally, N-terminal His6-tagged 17β-HSD2 was purified with a purity of more than 90% based on Coomassie Blue staining and densitometric analysis. There were two bands with molecular masses of about 44 and 90 kDa on the SDS gel, which were confirmed to be a monomer form and a dimer form, respectively, by Western blot analysis. The yield of the purification was typically about 1 mg of homogeneous protein with a specific activity of about 0.9 unit/mg using estradiol as substrate from 2 × 10⁶ cells. We found that the enzyme activity in both the homogenate and the supernatant using β-DM as detergent could be kept for several days at 4 °C without significant loss (data not shown). However, the β-DM-solubilized and purified 17β-HSD2 had so strong a tendency to denature that the protein stored at 4 °C for 3 and 24 h would lose half and total activity, respectively (data not shown). The fractions of the elution had to be frozen by liquid nitrogen as quickly as possible and stored at −80 °C or immediately used for reconstitution.

Liposomes from the mixtures of PC, phosphatidylethanolamine, and l-a-phosphatidylinositol and from single PC were tested to compare their ability to reconstitute 17β-HSD2 activity. The proteoliposomes formed from single PC demonstrated the highest activity and were thus chosen for the protein reconstitution. To follow the physical state of the liposomes, the absorbance at 540 nm was measured at various concentrations of β-DM. Purified 17β-HSD2 was tested for the incorporation into four different stages of liposomes with β-DM (i.e. the liposomes before saturation, saturated (onset solubilization), halfway through the breakdown of the liposomes, and fully solubilized (micellar state)). The concentrations of β-DM at these four points corresponded to 0.2, 0.4, 0.5, and 0.9%, respectively. The physical states of the liposomes with β-DM and the activities of the reconstituted 17β-HSD2 are shown in Fig. 3. The highest activity was obtained when the liposomes saturated with β-DM. Typically the liposomes with a slight oversaturation (0.42% β-DM) were used in the reconstitution system.

The activities in the proteoliposomes depended not only on the physical state of the liposomes at the beginning of the reconstitution but also on the lipid/protein ratios, the concentration of glycerol, and the ionic strength. Different liposome/protein ratios were tested, and the optimal ratio was around 14:1 (w/w). Using a lower lipid/protein ratio in the reconstitution system, the enzyme was not able to incorporate into the liposomes totally, and using a higher ratio, the harvested proteoliposomes were less active or totally inactive. The presence of glycerol at less than 15% concentration in protein oversaturation (0.42% β-DM) were used in the reconstitution system.

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The reconstituted protein was also purer than before due to the activity of 0.009 units/mg in the cell homogenate. This recombinant was solubilized to a lower level from membrane vesicles (solubilized 21% of the enzyme in the presence of 0.4% β-DM) than that of N-terminal His₆-tagged 17β-HSD2 and exhibited a very high tendency to aggregate, as seen in SDS gel and Western blot analysis. Indeed, the majority of the protein presented as a polymer staying in the sample-loading place or as a dimer (data not shown). These findings suggest that there is a stronger membrane interaction in C-terminal His₆-tagged 17β-HSD2 than in N-terminal His₆-tagged 17β-HSD2. Furthermore, several purification tests using various detergents demonstrated that the C-terminal His₆-tag of this form was not able to be effectively bound to nickel-chelated affinity matrix. Finally, N-terminal His₆-tagged 17β-HSD2, although still highly labile, was found to be able to retain full biological activity, to be expressed in a fairly good amount in the baculovirus expression system, and to facilitate its purification. Therefore, this form was chosen in our study.

### Table I

| Detergents | Detergent concentration | Solubility | Activity in homogenate | % | % | units/ml |
|------------|------------------------|------------|------------------------|---|---|---------|
| No detergent | 0.4 0.6 | 0.014 | 
| Sodium cholate | 0.8 1.3 | 0.014 | 
| β-octyl glucoside | 1.2 2.3 | 0.015 | 
| C₁₂E₈ | 0.8 13.4 | 0.011 | 
| Decyl-β-D-maltoside | 0.8 14.5 | 0.012 | 
| β-DDM | 0.8 21.8 | 0.010 | 
| Triton X-100 | 0.4 32.9 | 0.009 | 
| Sodium cholate | 0.8 36.2 | 0.006 | 
| Decyl-β-D-maltoside | 1.2 38.9 | 0.004 | 
| Triton X-100 | 0.4 25.1 | 0.016 | 
| Sodium cholate | 0.8 27.6 | 0.015 | 
| Decyl-β-D-maltoside | 1.2 30.2 | 0.009 | 
| β-DDM | 0.4 30.5 | 0.016 | 
| Decyl-β-D-maltoside | 0.8 32.6 | 0.015 | 
| Decyl-β-D-maltoside | 1.2 35.7 | 0.013 | 

The purification was carried out in a single affinity chromatography step using β-DDM as detergent. The results are summarized in Table I and are presented in Fig. 2, A and B. Most contaminants were removed by washing the column with 45 mM imidazole, and N-terminal His₆-tagged 17β-HSD2 was purified with a purity of more than 90% based on Coomassie Blue staining and densitometric analysis. There were two bands with molecular masses of about 44 and 90 kDa on the SDS gel, which were confirmed to be a monomer form and a dimer form, respectively, by Western blot analysis. The yield of the purification was typically about 1 mg of homogeneous protein with a specific activity of about 0.9 unit/mg using estradiol as substrate from 2 × 10⁶ cells. We found that the enzyme activity in both the homogenate and the supernatant using β-DM as detergent could be kept for several days at 4 °C without significant loss (data not shown). However, the β-DM-solubilized and purified 17β-HSD2 had so strong a tendency to denature that the protein stored at 4 °C for 3 and 24 h would lose half and total activity, respectively (data not shown). The fractions of the elution had to be frozen by liquid nitrogen as quickly as possible and stored at −80 °C or immediately used for reconstitution.
Characterization of the Biochemical and Catalytic Properties of the Purified and Reconstituted 17β-HSD2—The apparent subunit mass of this protein was calculated based on the relative mobility of protein standards and the 17β-HSD2 on the reducing SDS-PAGE gel (A) and Western blot (B). M, standard protein molecular weight marker; lane 1, supernatant of β-DMM-extracted membranes after ultracentrifugation; lane 2, suspended pellet after removing supernatant; lane 3, flow-through; lane 4, washed with 45 mM imidazole; lane 5, eluent from 250 mM imidazole (10 μg of protein).

The Western blot results confirmed that the protein was present exclusively in the fractions corresponding to the peak of 17β-HSD2 activity. No 17β-HSD2 bands were detected in the rest of the fractions. To further clarify the association of the subunits, we performed a cross-linking experiment using BS as reagent. There was one major band at monomer and one minor band at dimer positions in the control sample (Fig. 5). In the samples with 0.25, 1, and 3 mM of BS, the rather defused dimer band intensity increased with increasing BS concentration while the monomer intensity decreased. There were no clear bands of trimer and tetramer. The polymer form of the protein presented in every sample loading position. With the BS concentration increasing, the polymer bands became stronger. These results demonstrate that 17β-HSD2 presents as a homodimer in the natural state.

The kinetic constants for different substrates (testosterone, estradiol, dihydrotestosterone, and 20α-dihydroxyprogesterone) and those for cofactors NAD(H) and NADP are summarized in Tables III and IV. The $K_{m}^{app}$ values for steroid substrates were close to those published results measured in cell homogenates (1) and in purified N-29-deleted 17β-HSD2 (7). We also measured the kinetic constants for diphosphate cofactors NAD(H) with saturated concentrations of steroids (estradiol, testosterone, estrone, and 4-dione) and triphosphate cofactor NADP with saturated concentration of estradiol. Similar to the $K_{m}^{app}$ values for substrates, the $K_{m}^{app}$ values for NAD between two oxidative substrates (estradiol and testosterone) and the $K_{m}^{app}$ values for NADH between two reductive substrates (estrone and 4-dione) were also very close. Although the $K_{m}^{app}$ values for NAD were 20–30-fold higher and the apparent $V_{max}$ values were also 10–15-fold higher than those for NADH, the enzyme had almost the same apparent catalytic specificity for both oxidation and reduction. However, it was almost unidirectional in favor of oxidative reaction in intact Sf9 cells (Fig. 6), and the same results were reported in cultured HEK293 cells (7, 11). The $K_{m}^{app}$ for NADP with the estradiol as substrate at a saturating level, however, reached 9600 μM, more than 80-fold higher than that for NAD with the same substrate. This finding suggests that the cofactor concentration in the cells is a key factor to decide the reaction direction. It is well known that the reductase uses NADPH as cofactor and the dehydrogenase uses NAD as cofactor in vivo. As a result of the enzyme’s kinetic property and since the intracellular concentration of NAD is remarkably higher than that of NADH, with a ratio of about 1000 (18), the reaction will be in the oxidation direction by using NAD as cofactor for this enzyme in vivo.

TABLE II  

| Cell homogenate | Total protein | Total activity | Specific activity | Yield | Purification |
|----------------|--------------|---------------|------------------|-------|-------------|
|                | mg           | units         | units/mg         | %     | fold        |
| Supernatant    | 107.5        | 2.35          | 0.022            | 92.3  | 1           |
| Ni²⁺ column eluted | 1.1  | 0.91          | 0.83             | 35.7  | 39.5        |
| Reconstituted  | 0.48         | 1.20          | 2.50             | 47.1  | 119.6       |

DISCUSSION

As described in the Introduction, there are two proposed trans-membrane helices located at the N terminus of 17β-HSD2, but the more hydrophobic domain centers on the second proposed trans-membrane helix. We have devoted considerable effort to find a truncated form, which was able to retain enzyme activity as well as having better solubility. We found that the truncated N-38 form retained about 40% of its catalytic activity as compared with the full-length enzyme, but this form was extremely unstable either in the cell homogenate or in the detergent-solubilized state. For the other recombinants, the N-52 form only retained some activity in fresh cultured cells, and the N-61 form was totally inactive. It was reported that the truncated N-29 17β-HSD2 (in which the first proposed trans-membrane helix was deleted) retained about 60% of its cata-

FIG. 2. Purification results of His₆-tagged 17β-HSD2 overexpressed in Sf9 cells. Comassie Brilliant Blue-stained 12% SDS-PAGE gel (A) and Western blot (B). M, standard protein molecular weight marker; lane 1, supernatant of β-DMM-extracted membranes after ultracentrifugation; lane 2, suspended pellet after removing supernatant; lane 3, flow-through; lane 4, washed with 45 mM imidazole; lane 5, eluent from 250 mM imidazole (10 μg of protein).

FIG. 3. Solubilization of PC liposomes by β-DMM and 17β-HSD2 activity in proteoliposomes. The state of the liposomes was monitored by measuring the absorbance at 540 nm upon stepwise addition of β-DMM. The activities of the proteoliposomes are measured with buffer (0.05 M sodium carbonate, pH 9.2, 25 μM testosterone, and 1 mM NAD). The histograms represent the activities of the proteoliposomes using four different physical states of the liposomes with β-DMM, which correspond to 0.2% β-DMM (before saturating state), 0.4% β-DMM (saturated state, onset), 0.5% β-DMM (halfway solubilized state), and 0.9% β-DMM (fully solubilized state or micelle state), respectively.
maintaining the enzyme functions.

We found that N-terminal His6-tagged 17β-HSD2 was the most suitable form to study among our three overexpressed His-tagged 17β-HSD2 recombinants. Our experiments demonstrated that C-terminal His6-tagged 17β-HSD2 was much more hydrophobic than the N-terminal His6-tagged form. This suggests that the soluble His tag on the N terminus not only facilitates its purification but also weakens the hydrophobicity centered on the N-terminal region of the enzyme. Therefore, the N-terminal His6-tagged protein should be less hydrophobic than the full-length one without His tag protein. However, the N-terminal His10-tagged protein retained only about 10% of the wild type catalytic activity, and this indicates that the longer His tag may perturb the enzyme structure. We also found that the overproduction level of the N-terminal His6-tagged form was higher than the C-terminal His6-tagged form and lower than the N-terminal His10-tagged form. This suggests that less hydrophobicity close to the N terminus of the protein will overproduce a higher level of the protein.

According to the primary structure of human 17β-HSD2, there is a strong hydrophobic core possessing 33 nonpolar amino acids close to its N terminus and a quite hydrophilic motif in the other region (1), which indicates that 17β-HSD2 has a high tendency to aggregate especially in the detergent-solubilized state. Based on this consideration, several precautions had been taken in the purification and reconstitution procedures. First, an appropriate concentration of glycerol was required when treating 17β-HSD2. We found that glycerol played an important role in stabilizing the protein, but high concentration of glycerol reduced the binding capacity of the His6-tagged 17β-HSD2 with Ni²⁺ matrix. Thus, 10% glycerol was used in the sample buffer when the protein bound with Ni²⁺ matrix, and 20% glycerol was used in the other steps. Second, we found that the proper ionic strength (0.15–0.2 M NaCl) could strengthen the detergent acting on the hydrophobic region of the protein, so that it could stabilize the protein. High ionic strength could increase the specific binding of the protein with Ni²⁺ resin, but excessive high ionic strength (>0.5 M NaCl) caused serious enzyme aggregation and degradation. A concentration of 0.3 M NaCl was thus used when the protein bound with Ni²⁺ matrix, and a lower concentration of NaCl was used in the other steps. Third, the proper amount of cultured cells and Ni²⁺ resin was required. We tested different amounts of the cultured cells and Ni²⁺ resin for purification. Using a large scale of cultured cells and large amount of Ni²⁺ resin or using a pressure on the column always led to the protein aggregation and to its denaturation. Finally, we found that cofactor NAD had an effect on stabilizing the enzyme. Using NAD in the purification procedure helped to obtain an enzyme preparation with higher activity.

The purified full-length 17β-HSD2 was unable to keep its activity, and the protein could not be solubilized by 1 M potas-
Kinetic constants of substrates in the oxidation reaction with purified reconstituted His₆-tagged 17β-HSD2

The $K_{\text{m}}^{\text{app}}$ and $V_{\text{max}}$ values represent mean ± S.D. of three independent experiments. The $k_{\text{cat}}$ values were calculated from the $V_{\text{max}}$ values with the homodimer molecular mass of 90 kDa.

| Substrate (varied) | Cofactor (constant) | $K_{\text{m}}^{\text{app}}$ (μM) | $k_{\text{cat}}$ (s⁻¹) | $k_{\text{cat}}/K_{\text{m}}^{\text{app}}$ (s⁻¹·μM⁻¹) |
|--------------------|--------------------|-------------------------------|-----------------|-------------------|
| Estradiol          | NAD                | 0.35 ± 0.09                  | 3.9 ± 0.5       | 11                |
| Testosterone       | NAD                | 0.61 ± 0.06                  | 3.4 ± 0.4       | 5.5               |
| Dihydrotestosterone| NAD                | 0.25 ± 0.11                  | 2.4 ± 0.4       | 10                |
| 20α-Dihydroprogesterone P | NAD | 0.53 ± 0.22                  | 0.78 ± 0.20     | 1.5               |

Kinetic constants of cofactors with purified reconstituted His₆-tagged 17β-HSD2

The $K_{\text{m}}^{\text{app}}$ and $V_{\text{max}}$ values represent mean ± S.D. of three independent experiments. The $k_{\text{cat}}$ values were calculated from the $V_{\text{max}}$ values with the homodimer molecular mass of 90 kDa.

| Cofactor (varied) | Substrate (constant) | $K_{\text{m}}^{\text{app}}$ (μM) | $k_{\text{cat}}$ (s⁻¹) | $k_{\text{cat}}/K_{\text{m}}^{\text{app}}$ (s⁻¹·μM⁻¹) |
|-------------------|----------------------|-------------------------------|-----------------|-------------------|
| NAD               | Estradiol            | 110 ± 10                      | 4.7 ± 0.4       | 0.04              |
| NAD               | Testosterone         | 167 ± 32                      | 2.9 ± 0.5       | 0.02              |
| NADH              | Estrone             | 5.6 ± 1.2                     | 0.33 ± 0.05     | 0.06              |
| NADH              | 4-Dione             | 5.3 ± 1.0                     | 0.27 ± 0.01     | 0.05              |
| NADP              | Estradiol           | 9600 ± 100                    | 1.3 ± 0.3       | 0.0001            |

![Fig. 6. 17β-HSD2 activities in the intact SF9 cells. Five different substrates, three (estradiol (E2), testosterone (T), and dihydrotestosterone (DHT)) for the oxidation reaction and two (E1 and 4-dione) for the reduction reaction were used to detect the enzyme's substrate specificity. The results demonstrate that the enzyme is almost unidirectional in favor of the oxidation reaction with the same specific activity among those three substrates.](image-url)
is the best detergent in both solubilizing the protein and maintaining the enzyme in an active state. 17β-HSD2 was proved to be a homodimer with a molecular mass of 90.4 ± 1.2 kDa in the presence of a 2-kDa His tag. Our purification and reconstitution procedures provide a new and advanced way to obtain homogeneously and functionally reconstituted 17β-HSD2. This will permit us to further scale up the cell culture volume and recombinant protein production, thereby yielding sufficient homogeneous protein to approach crystallization and further structure studies. The methods we have introduced here may be applicable for other membrane steroid enzymes.

Acknowledgments—We thank Dr. F. Labrie for interest in this work. We also thank Dr. V. Luu-The for providing the pCMV/17β-HSD2 and the polyclonal antibody for 17β-HSD2 and M. Losier for the editing of the manuscript.

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Purification, Reconstitution, and Steady-state Kinetics of the Trans-membrane 17β-Hydroxysteroid Dehydrogenase 2
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J. Biol. Chem. 2002, 277:22123-22130.
doi: 10.1074/jbc.M111726200 originally published online April 8, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M111726200

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