11β-Hydroxysteroid Dehydrogenase Type 1 from Human Liver: Dimerization and Enzyme Cooperativity Support Its Postulated Role as Glucocorticoid Reductase[†]

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Received September 25, 2001; Revised Manuscript Received November 7, 2001

ABSTRACT: 11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD 1) is a microsomal enzyme that catalyzes the reversible interconversion of receptor-active 11-hydroxy glucocorticoids (cortisol) to their receptor-inactive 11-oxo metabolites (cortisone). However, the physiological role of 11β-HSD 1 as prereceptor control device in regulating access of glucocorticoid hormones to the glucocorticoid receptor remains obscure in light of its low substrate affinities, which is in contrast to low glucocorticoid plasma levels and low K_d values of the receptors to cortisol. To solve this enigma, we performed detailed kinetic analyses with a homogeneously purified 11β-HSD 1 from human liver. The membrane-bound enzyme was successfully obtained in an active state by a purification procedure that took advantage of a gentle solubilization method as well as providing a favorable detergent surrounding during the various chromatographic steps. The identity of purified 11β-HSD 1 was proven by determination of enzymatic activity, N-terminal amino acid sequencing, and immunoblot analysis. By gel-permeation chromatography we could demonstrate that 11β-HSD 1 is active as a dimeric enzyme. The cDNA for the enzyme was cloned from a human liver cDNA library and shown to be homologous to that previously characterized in human testis. Interestingly, 11β-HSD 1 exhibits Michaelis−Menten kinetics with cortisol and corticosterone (11β-dehydrogenation activity) but cooperative kinetics with cortisone and dehydrocorticosterone (11-oxoreducing activity). Accordingly, this enzyme dynamically adapts to low (nanomolar) as well as to high (micromolar) substrate concentrations, thereby providing the fine-tuning required as a consequence of great variations in circadian plasma glucocorticoid levels.

INTRODUCTION

It has long been assumed that the intracellular concentration of the corresponding receptor represents the primary mechanism for regulating the activity of a particular steroid hormone. However, in the past decade the role of tissue-specific hydroxysteroid dehydrogenases that control the amount of active steroid in target cells has been recognized as another important regulatory mechanism (*1*−*6*). In this context, 11β-hydroxysteroid dehydrogenase (11β-HSD)[1] (EC 1.1.1.146) catalyzes the 11β-oxidoreduction of glucocorticoids and is thus involved in the interconversion of receptor-active cortisol to receptor-inactive cortisone in mammals (corticosterone to dehydrocorticosterone in rodents) (*7*, *8*). By this action, 11β-HSD regulates glucocorticoid access to both the glucocorticoid receptor and mineralocorticoid receptor, thereby playing a key role in glucocorticoid homeostasis (reviewed in ref 9). Two different isoforms have been described so far, 11β-HSD 1 and 11β-HSD 2, which differ in their biological properties and tissue distribution.

The type 1 isozyme of 11β-HSD is a low-affinity NADP(H)-dependent dehydrogenase/oxoreductase, with an apparent K_m in the low micromolar range. Although the enzyme reaction catalyzed by 11β-HSD 1 in vitro is bidirectional, recent evidence suggests it to function primarily as reductase in vivo, reactivating inert 11-oxoglucocorticoids to the corresponding receptor-active 11β-hydroxy forms (*10*−*13*). This activity is likely to be of particular importance in tissues such as the liver, adipose tissue, and brain, where it may maintain high intracellular levels of glucocorticoids. For example, cortisol/cortisone affinity studies carried out on the expressed human 11β-HSD 1 isozyme reveal a lower K_m for cortisone than cortisol (*14*). Further, selective venous catheterization studies indicate low cortisone but high cortisol concentrations within the hepatic vein (*15*), in keeping with the observation that cortisone, taken by mouth, is rapidly converted to cortisol, with little or no change in circulating cortisone concentrations in the peripheral plasma (*16*). Finally, recent experiments on cultured rat and human hepatocytes have indicated high amounts of 11-oxoreductase activity, with little or no dehydrogenase activity in intact cells (*10*, *17*). Glucocorticoids play a key role both in the regulation of adipose tissue metabolism and in the differentiation of preadipocytes into adipocytes (*18*, *19*). In this context, Napolitano et al. (*13*) have shown that 11β-HSD 1 is expressed in adipocytes, where it has predominant reductase activity and may potentiate glucocorticoid action.

Type 2 11β-HSD is a high-affinity, unidirectional, NAD-dependent dehydrogenase with an apparent K_m for cortisol in the nanomolar range (*14*, *20*, *21*). This isozyme is found principally in mineralocorticoid target tissues such as the

[†] These studies were supported by the Deutsche Forschungsgemeinschaft (MA 1704/3-2) and the European Commission (to E.M.).

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[1] Abbreviations: 11β-HSD, 11β-hydroxysteroid dehydrogenase; SDR, short-chain dehydrogenases/reductases.

10.1021/bi015803t CCC: $22.00
Published on Web 01/22/2002

kidney and colon, where (by inactivating glucocorticoids) it protects the mineralocorticoid receptor from cortisol excess, thereby ensuring mineralocorticoid specificity of aldosterone (*22*, *23*).

In contrast to 11β-HSD 2, the functional role of 11β-HSD 1 is less clear at this time. These inconsistencies are due to discrepancies in low levels of circulating glucocorticoid hormones and high binding affinities of glucocorticoids to the glucocorticoid receptor (both in low nanomolar concentrations) on the one side, and the relatively low affinities of glucocorticoid substrates to the 11β-HSD 1 enzyme (in micromolar concentrations) on the other. It is clear that homogeneously purified enzymes are a prerequisite to elucidate their physiological functions, but 11β-HSD 1 is located within the membranes of the endoplasmic reticulum and many attempts failed to purify this enzyme in active form from human tissues. In previous studies, 11β-HSD 1 has been purified from rat (*24*) and mouse (*25*) liver. However, whereas the mouse enzyme showed both oxidase and reductase activities in purified form, rat liver 11β-HSD 1 failed to exhibit reductive activities toward its glucocorticoid substrates, which is remarkable since the physiological function of this enzyme has been proposed to be the 11-oxoreduction of glucocorticoids. This failure has later been discussed in terms of the sensitivity of this enzyme to changes in its three-dimensional architecture, as a consequence of alterations within the lipid or detergent environment during the isolation procedure (*1*).

To get further insight into the physiological activation and inactivation of glucocorticoids, we could finally isolate 11β-HSD 1 from human liver microsomes in an active state by a purification procedure that afforded a gentle solubilization method as well as providing a favorable detergent surrounding during the various chromatographic steps. We demonstrate that 11β-HSD 1 is active as a dimeric enzyme and that it retains all functional properties in its purified form, including the sensitivity to the specific inhibitor glycyrrhetinic acid. Most interestingly, enzyme cooperativity was shown for glucocorticoid 11-oxoreduction, thus emphasizing the important endocrinological role of 11β-HSD 1 in glucocorticoid activation, even at low substrate concentrations.

EXPERIMENTAL PROCEDURES

Chemicals. Nonlabeled glucocorticoids (cortisol, cortisone, corticosterone, and 11-dehydrocorticosterone) were purchased from Sigma Chemie GmbH (Munich, Germany). [1,2,6,7-$^3H_4$]Cortisol and [1,2,6,7-$^3H_4$]corticosterone (specific activities of 62 and 79 Ci/mmol, respectively) were obtained from Amersham Pharmacia Biotech. [1,2-$^3H_2$]Cortisone (specific activity 50 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Radiolabeled 11-dehydrocorticosterone was prepared by incubating purified 11β-HSD 1 from human liver with [^{3}H]corticosterone and NADP, followed by HPLC purification (see below) on a preparative reverse-phase column (Bio-Rad Bio-Sil C18 HL 90−10, 250 × 10 mm) and an eluent of 40% ethanol.

Purification of 11β-HSD 1. Human liver samples were obtained following routine surgical procedures and in accordance with German legislation. Samples were rinsed in an ice-cold isotonic solution of NaCl and homogenized in four volumes of 20 mM Tris-HCl buffer, pH 7.4, containing 250 mM sucrose, 1 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride (PMSF) with a glass−Teflon Potter-Elvehjem homogenizer. The homogenate was centrifuged at 600*g* for 10 min and at 10000*g* for 10 min to sediment nuclei, cell debris, and mitochondria. The supernatant at this stage was centrifuged at 170000*g* for 1 h to sediment the microsomes. The microsomal pellet was resuspended and washed with 0.15 M KCl to remove glycogen and then resuspended in the homogenization buffer without PMSF, finally yielding a protein concentration of about 20 mg/mL.

For solubilization of 11β-HSD 1 the microsomal suspension was diluted with an equal volume of a 10 mM sodium phosphate buffer, pH 7.2, containing 1 mM EDTA, 1 M NaCl, 40% glycerol (w/v), and 0.4% (w/v) of the nonionic detergent Emulgen 913. The solution was gently stirred for 45 min and subsequently centrifuged at 150000*g* for 60 min. The supernatant was adjusted to 0.4% (w/v) sodium cholate before being applied to the octyl-Sepharose CL-4B column.

To separate the enzyme from microsomal monooxygenase components, a hydrophobic interaction chromatographic step was performed. The following buffers were used: buffer A, 10 mM sodium phosphate, 1 mM EDTA, 500 mM NaCl, 20% (w/v) glycerol, and 0.5% (w/v) sodium deoxycholate, pH 7.4; buffer B, 10 mM sodium phosphate, 1 mM EDTA, 400 mM NaCl, 20% (w/v) glycerol, 0.4% (w/v) sodium cholate, and 0.1% (w/v) Emulgen 913, pH 7.4; buffer C, 10 mM sodium phosphate, 1 mM EDTA, 20% (w/v) glycerol, and 2% (w/v) Emulgen 913, pH 7.4. Solubilized microsomes (maximum 24 mL) were applied to the octyl-Sepharose CL-4B column (1.8 × 25 cm) previously equilibrated with 300 mL of buffer A. Elution was performed with buffer A until the end of peak 2, then with buffer B until the end of peak 3, followed by buffer C finally eluting peak 4. The elution profile was monitored by measuring the absorbance of the fractions at the wavelength of 417 nm. The column flow rate was 84 mL/h and the volume per fraction was 5 mL. Enzyme activity coincides only with peak 3, the fractions of which were collected, concentrated through an Amicon PM-10 membrane to about 20 mL, and dialyzed overnight against 5 mM sodium phosphate buffer, pH 7.4.

The dialyzed enzyme solution was applied to a column (1.6 × 20 cm) packed with Mono Q-Sepharose and previously equilibrated with 20 mM Tris-HCl buffer, pH 8. The column was washed with the equilibration buffer and the adsorbed enzyme was then eluted with stepwise increasing concentrations of NaCl in the equilibration buffer. Active fractions eluted with 75 mM NaCl at a flow rate of 36 mL/h and fraction volumes of 3 mL. Enzymatically active fractions were pooled, concentrated through an Amicon PM-10 membrane to about 2−3 mL, and supplemented with glycerol to a final concentration of 10%.

Fractions from Mono Q-Sepharose chromatography were directly applied on a red Sepharose A column (1.2 × 5 cm), previously equilibrated with a 10 mM potassium phosphate buffer, pH 7.4, containing 10% glycerol. The column was rinsed successively with the equilibration buffer, then with the equilibration buffer containing 0.8 M NaCl and 2 mM NADP, and then with the same buffer containing 1 M NaCl and 2 mM NADP. The enzyme was finally eluted with a 10 mM potassium buffer, pH 7.4, containing 1 M NaCl, 2 mM NADP, 0.1% Emulgen 913, and 10% glycerol. The fractions

with high enzyme activity were pooled, concentrated through an Amicon PM-10 membrane to about 2−3 mL, and stored in 0.2 mL aliquots at −70 °C. Throughout the purification procedure the temperature was kept at 4 °C. The purification was followed by increasing specific activities of 11β-HSD 1 toward metyrapone as model substrate and by monitoring the resulting protein fractions by SDS−PAGE and immunoblot analyses.

Gel-Permeation Chromatography. Gel-permeation chromatography was performed on a Pharmacia ÄKTA protein purifier system. Purified 11β-HSD 1 was applied to a Superdex-75 column (HiLoad 26/60, preparation grade) in 20 mM Tris-HCl buffer, pH 8.0, containing 0.2 M NaCl, 10% glycerol, and 0.1% Emulgen, and eluted with the same buffer at a flow rate of 0.5 mL/min. In a parallel experiment, 11β-HSD 1 active fractions after Mono Q-Sepharose chromatography were supplemented with 1% *n*-octyl β-D-glucopyranoside, applied to the Superdex-75 column, and eluted with 20 mM Tris-HCl buffer, pH 8.0, containing 0.2 M NaCl, 10% glycerol, and 0.1% *n*-octyl β-D-glucopyranoside. The molecular mass of 11β-HSD 1 was calculated by comparing its elution volume with that of standard proteins. In addition, fractions with 11β-HSD 1 activity (or those fractions where 11β-HSD 1 monomers could be expected) were pooled, desalted, and subjected to SDS−PAGE.

Enzyme Assays. Activity determination during the purification procedure was performed by measuring the carbonyl reduction of metyrapone, which is known to be a model substrate of 11β-HSD 1 (*26*). The assay was performed with 30 μL of protein extract, 10 μL of substrate (final concentration 1 mM), and 10 μL of a NADPH-regenerating system (final concentrations: $NADP^+$, 0.8 mM; glucose 6-phosphate, 6 mM; glucose-6-phosphate dehydrogenase, 0.35 unit; and $MgCl_2$, 3 mM). The mixture was incubated for 30 min at 37 °C. The reaction was stopped by adding 150 μL of acetonitrile. After vortexing and removal of proteins by centrifugation in a microfuge for 5 min, the supernatant was analyzed by HPLC (*26*).

Assay of 11β-HSD 1 dehydrogenation activity was performed by preincubating 20 μL of 10 mM sodium phosphate buffer, pH 9, 10 μL of $NADP^+$ (final concentration 2.8 mM), and 10 μL of substrate (2−150 μM of nonlabeled cortisol or 5−500 μM nonlabeled corticosterone) for 3 min at 37 °C. Steroids were dissolved in ethanol, which did not influence enzyme activity up to a concentration of 2%. The reaction was started by adding 10 μL of enzyme solution (corresponding to 0.12−0.24 mg of protein/assay). After 60 min incubation time the reaction was stopped, and glucocorticoid substrates and metabolites were extracted by adding 150 μL of ice-cold acetonitrile. The samples were centrifuged for 6 min at 8000*g* in the cold, and 20 μL of the supernatant served for the determination of glucocorticoids by HPLC analysis. Specific activities are expressed as nanomoles of oxidized glucocorticoids formed per minute per milligram of protein.

Assay of 11β-HSD 1 oxoreducing activity was performed by preincubating 20 μL of 10 mM sodium phosphate buffer, pH 7.4, 10 μL of an NADPH-regenerating system (final concentrations: $NADP^+$, 0.8 mM; glucose 6-phosphate, 6 mM; glucose-6-phosphate dehydrogenase, 0.35 unit; $MgCl_2$, 3 mM), and 10 μL of substrate (2−75 μM nonlabeled cortisone or 5−100 μM nonlabeled dehydrocorticosterone). Incubation conditions and metabolite detection were the same as described under 11β-HSD 1 dehydrogenation activity, except that incubation periods were 3 h. Specific activities are expressed as nanomoles of reduced glucocorticoids formed per minute per milligram of protein. Control experiments with heat-inactivated enzyme fractions were performed to determine nonenzymatic substrate conversions. Time and enzyme protein concentrations were chosen so that reaction velocities were time-linear.

Metabolite Determination by HPLC. After enzymatic conversion, oxidized or reduced metabolites were detected on a Merck reverse-phase HPLC system (Merck Hitachi L-6220 intelligent pump, Merck Hitachi AS-2000 A autosampler, Merck Hitachi L-4000 A UV detector), with a 4.6 mm × 25 cm LiChrosphere 100 RP18 column (Merck, Germany) and a Shimadzu C-R6A Chromatopac integrator. By use of a methanol/H_2O (58:42) eluent at a flow rate of 0.6 mL/min, the following retention times were achieved: cortisone, 13 min; cortisol, 17 min; dehydrocorticosterone, 14 min; and corticosterone, 23 min. Glucocorticoids were monitored at 262 nm and concentrations were calculated by referral to corresponding calibration curves.

Activity Determination with Radiolabeled Substrates. 11β-HSD 1 activity in low substrate concentrations was determined by measuring the conversion of radiolabeled substrates into their respective metabolites in the presence of the appropriate cofactor according to the procedure described above. Substrate concentrations were as follows: [^{3}H]-cortisol, 0.1 nM−25 μM (corresponding to 62 Ci/mmol−248 mCi/mmol); [^{3}H]cortisone, 0.1 nM−25 μM (corresponding to 50 Ci/mmol−200 mCi/mmol); [^{3}H]corticosterone, 0.1 nM−20 μM (corresponding to 79 Ci/mmol−395 mCi/mmol); and [^{3}H]-11-dehydrocorticosterone, 0.1 nM−20 μM (corresponding to 79 Ci/mmol−19.75 mCi/mmol). After termination of the enzyme reaction and addition of nonlabeled metabolite, substances were separated by HPLC (see above) and collected according to their respective retention times. The samples were transferred to vials containing liquid scintillant (Rotiszint eco plus, Roth, Karlsruhe, Germany) and counted in a β-counter (Beckman LS6000).

Kinetic Parameter Estimations. All kinetic measurements were performed at least four times, and the mean values were used for calculations or plots. The apparent kinetic parameters and statistical analyses were calculated by using the GraphPad Prism kinetic computer software.

N-Terminal Amino Acid Sequence Analysis. The active fractions from the red Sepharose A column were subjected to SDS−PAGE, blotted to a poly(vinylidene difluoride) membrane, and then subjected to Edman degradation in a gas-phase sequencer (Applied Biosystems, Inc.). The protein yielded a single amino acid sequence, indicating the purity of the preparation. Database searches and sequence comparisons were performed at the National Center for Biotechnology Information (NCBI) by using the BLAST program (*27*).

Cloning of Human Liver 11β-HSD 1 cDNA. The cDNA for human liver 11β-HSD 1 was obtained from a human liver cDNA library (Stratagene, Uni-ZAP XR cDNA library) by PCR. The primers used were designed to anneal at the 5′- and the 3′-ends of the coding region of 11β-HSD 1 described by Tannin et al. (*28*) in human testis. The 5′-primer sequence was 5′-ATGGCTTTTATGAAAAAATATCTC-3′; the 3′-

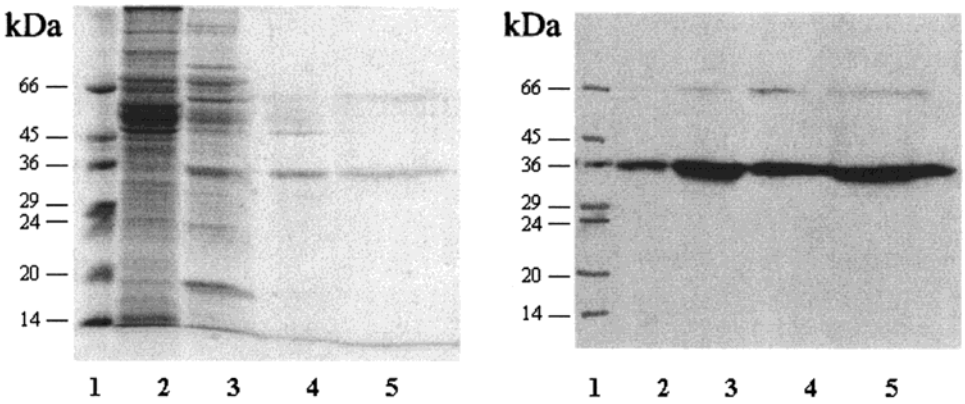

FIGURE 1: Purification of 11β-HSD 1 from human liver microsomes. 11β-HSD 1 was successfully obtained in an active state by a purification procedure that took advantage of a gentle solubilization method as well as providing a favorable detergent surrounding during the various chromatographic steps. The purification yielded an active enzyme with a single band of 34 kDa on an SDS−polyacrylamide gel (left). The identity of purified 11β-HSD 1 was proven by Western blot analysis (right). Note the comigrating band at approximately 68 kDa, indicating a dimeric form of 11β-HSD 1. Lane assignments are as follows: 1 = standard, 2 = solubilized microsomes, 3 = octyl-Sepharose, 4 = Mono Q-Sepharose, 5 = red Sepharose A.

Table 1: Purification Table for Human Liver 11β-HSD 1

step	total protein (mg)	total activity (nmol min^{-1})	specific activity (nmol mg^{-1} min^{-1})	recovery (%)	purification (*x*-fold)
solubilized microsomes	57.10	13.87	0.243	100.0	1.0
octyl-Sepharose CL-4B	3.04	7.56	2.490	54.5	10.3
Mono Q-Sepharose	0.28	3.85	13.690	27.8	56.3
red Sepharose A	0.035	3.22	91.360	23.2	376.0

primer sequence was 5′-CTTGTTTATGAATCTGTCCATATT-3′. The resulting cDNA sequence was determined by sequencing both strands with the Sequenase II kit (USB/Amersham) and the appropriate primers.

Immunoblotting of 11β-HSD 1. Immunization and preparation of antisera against 11β-HSD 1 of human liver was performed as described elsewhere (*29*). Electroblotting was performed in a semidry blotting system. Proteins were transferred to a nitrocellulose membrane, and antigen−antibody complexes were visualized by chemoluminescence (ECL Plus detection system, Amersham Pharmacia Biotech). Primary antisera were diluted 1/30 000; the secondary antibody (peroxidase-conjugated swine anti-rabbit immunoglobulin, DAKO) was used in a 1/2000 dilution.

RESULTS

Purification of 11β-HSD 1. Since 11β-HSD 1 is membrane-bound to the endoplasmic reticulum and remarkably sensitive to purification, it was first necessary to optimize the solubilization of 11β-HSD 1 by use of a favorable detergent surrounding and to prepare the protein for subsequent hydrophobic interaction chromatography. 11β-HSD 1 was solubilized by use of the nonionic detergent Emulgen 913. The addition of sodium cholate to the solubilized protein was critically important before it was subjected to the hydrophobic interaction chromatography on octyl-Sepharose. On one hand, sodium cholate turned out to decrease enzyme activity. On the other hand, without sodium cholate no separation of 11β-HSD 1 from the microsomal monooxygenase components could be achieved. In fact, it was not desirable for 11β-HSD 1 to bind very tightly to the octyl-Sepharose column, but it could be separated from the microsomal monooxygenase components by stepwise decreasing the sodium chloride concentration and simultaneously increasing the detergent content of the elution buffers. On subsequent Mono Q-Sepharose chromatography, 11β-HSD 1 eluted without additional detergents by simple application of stepwise increasing concentrations of sodium chloride in the elution buffer. In this case, the detergent concentration was sufficient from the previous chromatography step. From red Sepharose A, 11β-HSD 1 eluted with a combination of 1 M sodium chloride, 2 mM $NADP^+$, and 0.1% Emulgen 913 in a single step, yielding one prominent protein band in the 34 kDa region and a comigrating faint band at around 68 kDa (Figure 1). Compared to solubilized microsomes, the specific activity of homogeneously purified 11β-HSD 1 could finally be enhanced to about 376-fold (Table 1).

Rat liver				M	K	K	Y	L	L	P	V	L	G	L	C	L	G	Y	Y
Human liver*			?	F	M	K	Q	Y	L	L	P	I	L	?	L	F	M	A	Y
Human testis		M	A	F	M	K	K	Y	L	L	P	I	L	G	L	F	M	A	Y	Y

FIGURE 2: N-terminal sequences of 11β-HSD 1 from different species. Except for position 6, the amino acid sequence of human liver 11β-HSD 1 obtained in this study (*) shows the same composition as that from human testis (*28*) but differs in six residues from that of rat liver 11β-HSD 1 (*7*).

Analysis of the N-terminal sequence of the purified protein yielded the sequence <?FMKQYLLPIL?LFMAY> (Figure 2), which, except for one amino acid residue, was identical to 11β-HSD 1 cloned from human testis (*28*). A specific polyclonal antibody against recombinant human 11β-HSD 1 (*29*) was used to parallel the purification procedure and confirms that the purified protein indeed is 11β-HSD 1 (Figure 1). Interestingly, the comigrating band at 68 kDa was recognized by the antibody against 11β-HSD 1, supporting the view that 11β-HSD 1 exists as a dimer.

Dimeric Nature of 11β-HSD 1 As Determined by Gel-Permeation Chromatography. Upon gel-permeation chro-

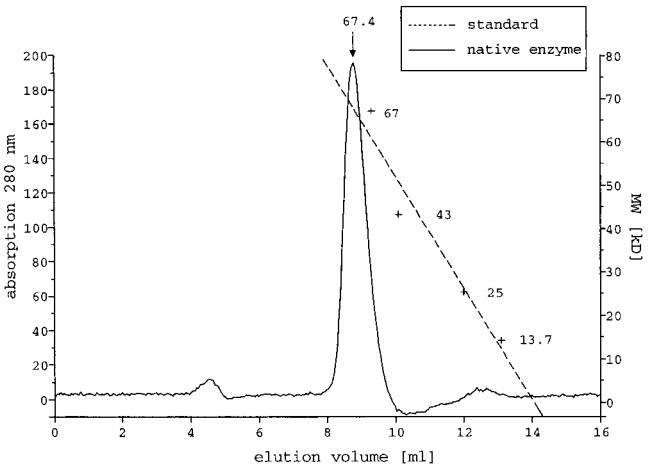

FIGURE 3: Gel-permeation chromatography: Evidence for the dimeric nature of 11β-HSD 1. Gel-permeation chromatography of purified 11β-HSD 1 was performed on a Pharmacia ÄKTA protein purifier system with a Superdex-75 column (HiLoad 26/60). The molecular mass of 11β-HSD 1 was calculated by comparing its elution volume with those of standard proteins. Purified 11β-HSD 1 eluted exclusively at 67.4 kDa as an active enzyme (shown by an arrow), thus revealing the dimeric nature of 11β-HSD 1 from human liver. Molecular mass markers (in kilodaltons): 13.7 = ribonuclease A; 25 = chymotrypsinogen; 43 = ovalbumin; 67 = bovine serum albumin.

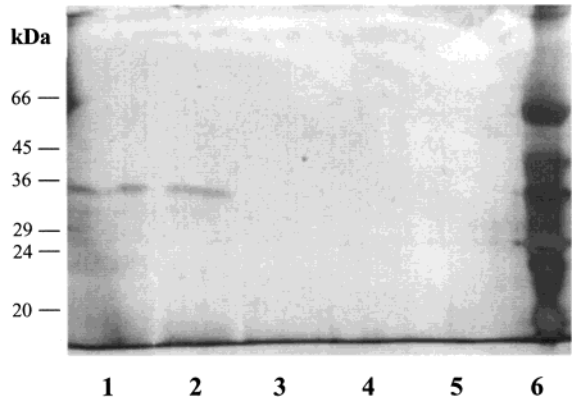

FIGURE 4: SDS−polyacrylamide gel electrophoresis after gel-permeation chromatography. Fractions with 11β-HSD 1 activity (or those fractions where 11β-HSD 1 monomers could be expected) after gel-permeation chromatography were subjected to reducing SDS−PAGE followed by silver staining. As can be seen in lanes 1 and 2 of this figure, 11β-HSD 1 eluted exclusively as dimeric protein upon gel filtration (at 67.4 kDa in Figure 3). No protein could be detected eluting as 11β-HSD 1 monomer (at 34 kDa in Figure 3; lanes 3−5 in this figure). Combined, these results proved the dimeric nature of 11β-HSD 1 in human liver.

matography, purified 11β-HSD 1 indeed eluted as an active enzyme with a molecular mass of 67.4 kDa, as calculated from the elution profile of standard proteins (Figure 3). Fractions with 11β-HSD 1 activity (or those fractions where 34 kDa 11β-HSD 1 monomers could be expected) were subjected to SDS−PAGE followed by silver staining. Figure 4 reveals that 11β-HSD 1 eluted exclusively as dimeric protein. No protein could be detected eluting as 11β-HSD 1 monomer. These results proved for the first time the dimeric nature of 11β-HSD 1 in human liver.

In a parallel experiment, 11β-HSD 1 active fractions were supplemented with 1% *n*-octyl β-D-glucopyranoside and eluted from a Superdex-75 column with a buffer containing 0.1% *n*-octyl β-D-glucopyranoside. The detergent, which is known to interfere with protein−protein interactions, resulted in the same elution profile as that shown in Figure 3 (not shown). Likewise, reducing SDS−PAGE followed by silver staining of these fractions gave the same separation pattern as that shown in Figure 4. This result indicates that dimerization may occur by disulfide bonding between two 11β-HSD 1 monomers, as has been proposed in ref *30*.

Table 2: Kinetic Constants of Purified Liver 11β-HSD 1

substrate[a]	V_{max} (nmol min^{-1} mg^{-1})	K_m (μM)	V_{max}/K_m	Hill coefficient
cortisone	5.3	13.9	0.38	1.6
cortisol	19.0	41.3	0.46	1.0
dehydrocorticosterone	4.0	19.7	0.20	2.7
corticosterone	10.6	42.8	0.25	0.7

[a] Final substrate concentrations ranged from 0.1 nM to 75−500 μM glucocorticoids (cf. Experimental Procedures). In contrast to calculations by the Michaelis−Menten equation, statistically significant values were obtained for cortisone ($p < 0.02$) and dehydrocorticosterone ($p < 0.0001$) when the sigmoidal dose−response kinetics were applied.

Kinetics of Purified 11β-HSD 1. At first, the kinetic data indicate that both 11-dehydrogenation and 11-oxoreduction of glucocorticoids can be catalyzed by purified 11β-HSD 1 from human liver (Table 2). Further, when Michaelis−Menten kinetics were considered, the K_m values for glucocorticoid oxidoreduction range in low micromolar concentrations, thus corresponding to literature results obtained from crude microsomal fractions (*31*−*34*). However, when the sigmoidal dose−response kinetics were applied, it was somewhat surprising to observe cooperative kinetics of 11β-HSD 1 with cortisone and dehydrocorticosterone (glucocorticoid 11-oxoreducing activity) with Hill coefficients of 1.6 and 2.7, respectively. Accordingly, the 11β-HSD 1 enzyme is obviously able to adapt to low (nanomolar) as well as to high (micromolar) substrate concentrations. The fact that this enzyme cooperativity was not observed for 11-dehydrogenation further emphasizes the physiological role of 11β-HSD 1 to act mainly as glucocorticoid reductase.

Inhibition of 11β-HSD 1 by Glycyrrhetinic Acid. Glycyrrhetinic acid, a constituent of licorice, is known as a classical inhibitor of 11β-HSD 1. With purified 11β-HSD 1, glycyrrhetinic acid exhibited inhibition constants (K_i for cortisol oxidation = 4.9 nM; K_i for cortisone reduction = 14.1 nM) corresponding to those obtained from 11β-HSD 1 inhibition experiments in crude cellular extracts. The fact that the purified enzyme retains the same K_i values as those forms in intact membranes supports the view that the present purification protocol yielded a preparation with a close to native enzyme.

cDNA Cloning. Since the N-terminal amino acid sequence obtained by Edman degradation exhibited some uncertainties compared to 11β-HSD 1 from testis (*28*), we cloned and sequenced the cDNA from a human liver cDNA library. The primary structure of human liver 11β-HSD 1 obtained revealed complete identity to that from human testis. The deduced protein has a molecular mass of 32 386 Da, which is in modest agreement with a molecular mass of 34 kDa of the purified protein estimated by SDS−PAGE and with the consideration that 11β-HSD 1 is a glycoprotein. The N-terminal sequence of human liver 11β-HSD 1 primary structure corresponded to that of the purified protein, thereby

rectifying the protein sequencing error at position 6 from Gln (Q) to Lys (K).

DISCUSSION

11β-HSD 1 is a glycosylated membrane-bound protein, which has proved difficult to purify in an active state. The purified enzyme typically loses the reductase properties seen in intact cells and shows principally dehydrogenase activity. This has led to some initial controversy over whether 11β-HSD 1 oxidase and reductase activity reside in one or two different proteins (*24*, *35*). However, studies on the purified enzyme from mouse liver proved the concept of a unique, reversible glucocorticoid oxidoreductase (*25*). Recently, it has been proposed that in vivo 11β-HSD 1 behaves predominantly as an oxoreductase by generating cortisol from cortisone (*10*−*13*).

The kinetic data of 11β-HSD 1 published so far demonstrate an enzyme with a micromolar affinity for glucocorticoids (*25*, *26*, *31*−*34*). This hardly seems compatible with a role of 11β-HSD 1 to regulate access of glucocorticoids to the glucocorticoid receptor since both endogenous free glucocorticoid levels as well as the dissociation constants of the glucocorticoid receptor for glucocorticoids range in low nanomolar concentrations. To solve this enigma, we have purified 11β-HSD 1 from human liver in active form, which is a prerequisite to perform functional studies and detailed kinetic analyses. Importantly, the purified enzyme exhibited both glucocorticoid 11-dehydrogenation and 11-oxoreducing activities. In addition, the classical 11β-HSD 1 inhibitor glycyrrhetinic acid inhibited the purified enzyme with the same K_i values as its membrane-bound forms in intact cells or cellular extracts.

Interestingly, our gel-permeation results clearly showed that active 11β-HSD 1 occurs as a dimeric enzyme in human liver. In previous studies, a second band corresponding to approximately 68 kDa has been observed in immunoblots with total protein from rat (*36*) and human (*37*) liver, suggesting for the first time that 11β-HSD 1 may exist as a dimer in this tissue. In addition, results from nonreducing gel electrophoresis and mutation of a nonconserved cysteine residue in recombinant 11β-HSD 1 led Walker et al. (*30*) to conclude that 11β-HSD 1 in human liver may be dimeric, where Cys272 is involved in dimerization via disulfide bonds between adjacent polypeptide chains of the enzyme. This assumption is corroborated by our results from gel-permeation chromatography and the use of the nonionic detergent *n*-octyl β-D-glucopyranoside, which is known to interfere with protein−protein interaction. Since *n*-octyl β-D-glucopyranoside did not affect the dimeric form of purified 11β-HSD 1 upon gel-permeation chromatography in the present study, it is suggested that dimerization indeed occurs via disulfide bonding.

11β-HSD 1 is a member of the short-chain dehydrogenase/reductase (SDR) protein superfamily which is comprised of a wide range of procaryotic and eucaryotic enzymes involved in the metabolism of sugars, steroids, aromatic hydrocarbons, and prostaglandins (*38*−*40*), and most SDR enzymes are active as either homodimers or homotetramers (*39*, *41*−*45*). In support of dimerization of SDR proteins, we have recently solved the crystal structure of 3α-HSD from *Comamonas testosteroni* (*45*). Interestingly, the quaternary structure of this SDR enzyme does not only appear as a homodimer but shows a novel type of dimerization pattern that has never been observed in other homodimeric SDRs crystallized so far. Dimerization has also been observed for 17β-HSD from the filamentous fungus *Cochliobolus lunatus*. This SDR enzyme has a monomer size of 28 kDa but occurs as a dimer of 60 kDa under native conditions (*46*). By using X-ray crystallographic structures of rat and human dihydropteridine reductase and *Streptomyces hydrogenans* 3α/20β-HSD as templates, Tsigelny and Baker (*42*, *43*) performed molecular modeling studies to understand structures important in mammalian SDRs, including 11β-HSD 1, 11β-HSD 2, and several 17β-HSDs. According to their results, these hydroxysteroid dehydrogenases are active as dimers, where the dimerization interface is formed by α-helices that bear the catalytic site.

Oligomeric enzymes often display kinetics other than that described by the Michaelis−Menten equation. To our surprise, when the sigmoidal−dose response calculation was applied, we could indeed find cooperative kinetics of 11β-HSD 1 action. The positive cooperativity observed deserves some comment. Endogenous glucocorticoid profiles are highly variable (ranging between 0.1 and 100 nM) in that they are triggered by the circadian rhythm and influenced by external challenges (e.g., stress). On the other hand, access of active glucocorticoids to the glucocorticoid receptor (K_d for glucocorticoids in the low nanomolar region) requires some fine-tuning, which could be best achieved by an enzyme that responds to these wide variations by dynamically adapting to low as well as to high endogenous glucocorticoid levels. The fact that 11β-HSD 1 shows this cooperative kinetics for 11-oxoreduction but not for 11-dehydrogenation of glucocorticoids is compatible with the concept of this enzyme having its real physiological role as glucocorticoid 11-oxoreductase.

A potential cooperativity among the subunits of another SDR enzyme has been discussed with respect to the crystal structure of the homotetrameric 3α,20β-HSD from *Streptomyces hydrogenans*, showing that residues from three subunits are lining up each substrate binding pocket (*47*, *48*). Further evidence for such cooperativity among SDR subunits was collected for two *cis*-retinol/3α-hydroxysterol SDR isoenzymes, which, interestingly enough, reveal both sigmoidal and Michaelis−Menten kinetics depending on the substrate (*49*, *50*). For example, cooperative kinetics were observed with 5α-androstan-3α,17β-diol (3α-hydroxysteroid dehydrogenase activity) and testosterone (17β-hydroxysteroid dehydrogenase activity), but Michaelis−Menten kinetics was obtained with androsterone (3α-hydroxysteroid dehydrogenase activity). This dual kinetic behavior resembles that found in the present study for 11-dehydrogenation (Michaelis−Menten) and 11-oxoreduction (cooperative) of glucocorticoid metabolism by purified 11β-HSD 1 from human liver.

Taken together, the present study reports the successful purification and functional characterization of 11β-HSD 1 from human liver, an enzyme that physiologically performs the regulation of access of glucocorticoids to the glucocorticoid receptor. The enzyme appears as homodimer and displays interesting kinetics in that it obeys Michaelis−Menten kinetics for glucocorticoid 11-dehydrogenation but exhibits cooperativity for glucocorticoid 11-oxoreduction.

This kinetic peculiarity may explain the enigma between the relatively low substrate affinities of 11β-HSD 1 observed in previous studies and the low levels of plasma glucocorticoids together with the low dissociation constants of active glucocorticoids from the glucocorticoid receptor.

REFERENCES

1. Monder, C. (1991) *FASEB J. 5*, 3047−3054.
2. Funder, J. W. (1997) *Annu. Rev. Med. 48*, 231−240.
3. Benediktsson, R., and Edwards, C. R. (1996) *Essays Biochem. 31*, 23−36.
4. Penning, T. M., Burczynski, M. E., Jez, J. M., Hung, C. F., Lin, H. K., Ma, H., Moore, M., Palackal, N., and Ratnam, K. (2000) *Biochem. J. 351*, 67−77.
5. Adamski, J., and Jakob, F. J. (2001) *Mol. Cell. Endocrinol. 171*, 1−4.
6. Baker, M. E. (2001) *Mol. Cell. Endocrinol. 171*, 211−215.
7. Agarwal, A. K., Monder, C., Eckstein, B., and White, P. C. (1989) *J. Biol. Chem. 264*, 18939−18943.
8. Monder, C., and White, P. C. (1993) *Vitam. Horm. 47*, 187−271.
9. Seckl, J. R. (1997) *Front. Neuroendocrinol. 18*, 49−99.
10. Jamieson, P. M., Chapman, K. E., Edwards, C. R., and Seckl, J. R. (1995) *Endocrinology 136*, 4754−4761.
11. Voice, M. W., Seckl, J. R., Edwards, C. R., and Chapman, K. E. (1996) *Biochem. J. 317*, 621−625.
12. Low, S. C., Chapman, K. E., Edwards, C. R., and Seckl, J. R. (1994) *J. Mol. Endocrinol. 13*, 167−174.
13. Napolitano, A., Voice, M. W., Edwards, C. R., Seckl, J. R., and Chapman, K. E. (1998) *J. Steroid Biochem. Mol. Biol. 64*, 251−260.
14. Stewart, P. M., Murry, B. A., and Mason, J. I. (1994) *J. Clin. Endocrinol. Metab. 79*, 480−484.
15. Walker, B. R., Campbell, J. C., Fraser, R., Stewart, P. M., and Edwards, C. R. (1992) *Clin. Endocrinol. Oxf. 37*, 483−492.
16. Stewart, P. M., Wallace, A. M., Atherden, S. M., Shearing, C. H., and Edwards, C. R. (1990) *Clin. Sci. 78*, 49−54.
17. Ricketts, M. L., Shoesmith, K. J., Hewison, M., Strain, A., Eggo, M. C., and Stewart, P. M. (1998) *J. Endocrinol. 156*, 159−168.
18. Gaillard, D., Wabitsch, M., Pipy, B., and Negrel, R. (1991) *J. Lipid Res. 32*, 569−579.
19. Bouchard, C., Despres, J. P., and Mauriege, P. (1993) *Endocr. Rev. 14*, 72−93.
20. Albiston, A. L., Obeyesekere, V. R., Smith, R. E., and Krozowski, Z. S. (1994) *Mol. Cell. Endocrinol. 105*, R11−R17.
21. Brown, R. W., Chapman, K. E., Edwards, C. R., and Seckl, J. R. (1993) *Endocrinology 132*, 2614−2621.
22. Edwards, C. R., Stewart, P. M., Burt, D., Brett, L., McIntyre, M. A., Sutanto, W. S., de Kloet, E. R., and Monder, C. (1988) *Lancet 2*, 986−989.
23. Funder, J. W., Pearce, P. T., Smith, R., and Smith, A. I. (1988) *Science 242*, 583−585.
24. Lakshmi, V., and Monder, C. (1988) *Endocrinology 123*, 2390−2398.
25. Maser, E., and Bannenberg, G. (1994) *J. Steroid Biochem. Mol. Biol. 48*, 257−263.
26. Maser, E., and Bannenberg, G. (1994) *Biochem. Pharmacol. 47*, 1805−1812.
27. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol. 215*, 403−410.
28. Tannin, G. M., Agarwal, A. K., Monder, C., New, M. I., and White, P. C. (1991) *J. Biol. Chem. 266*, 16653−16658.
29. Blum, A., Martin, H., and Maser, E. (2000) *Toxicology 144*, 113−120.
30. Walker, E. A., Clark, A. M., Hewison, M., Ride, J. P., and Stewart, P. M. (2001) *J. Biol. Chem. 276*, 21343−21350.
31. Diederich, S., Hanke, B., Burkhardt, P., Muller, M., Schoneshofer, M., Bahr, V., and Oelkers, W. (1998) *Steroids 63*, 271−277.
32. Pu, X., and Yang, K. (2000) *Steroids 65*, 148−156.
33. Agarwal, A. K., Tusie Luna, M. T., Monder, C., and White, P. C. (1990) *Mol. Endocrinol. 4*, 1827−1832.
34. Moore, C. C., Mellon, S. H., Murai, J., Siiteri, P. K., and Miller, W. L. (1993) *Endocrinology 133*, 368−375.
35. Monder, C., and Lakshmi, V. (1989) *J. Steroid Biochem. 32*, 77−83.
36. Monder, C., and Lakshmi, V. (1990) *Endocrinology 126*, 2435−2443.
37. Ricketts, M. L., Verhaeg, J. M., Bujalska, I., Howie, A. J., Rainey, W. E., and Stewart, P. M. (1998) *J. Clin. Endocrinol. Metab. 83*, 1325−1335.
38. Krozowski, Z. (1992) *Mol. Cell. Endocrinol. 84*, C25−C31.
39. Jörnvall, H., Persson, B., Krook, M., Atrian, S., Gonzalez-Duarte, R., Jeffery, J., and Ghosh, D. (1995) *Biochemistry 34*, 6003−6013.
40. Jörnvall, H., Hoog, J. O., and Persson, B. (1999) *FEBS Lett. 445*, 261−264.
41. Krook, M., Ghosh, D., Duax, W., and Jörnvall, H. (1993) *FEBS Lett. 322*, 139−142.
42. Tsigelny, I., and Baker, M. E. (1995) *J. Steroid Biochem. Mol. Biol. 55*, 589−600.
43. Tsigelny, I., and Baker, M. E. (1995) *Biochem. Biophys. Res. Commun. 217*, 859−868.
44. Penning, T. M. (1997) *Endocr. Rev. 18*, 281−305.
45. Grimm, C., Maser, E., Möbus, E., Klebe, G., Reuter, K., and Ficner, R. (2000) *J. Biol. Chem. 275*, 41333−41339.
46. Lanisnik, R. T., Moeller, G., Thole, H. H., Zakelj-Mavric, M., and Adamski, J. (1999) *Biochem. J. 337* (Part 3), 425−431.
47. Ghosh, D., Erman, M., Wawrzak, Z., Duax, W. L., and Pangborn, W. (1994) *Structure 2*, 973−980.
48. Ghosh, D., Wawrzak, Z., Weeks, C. M., Duax, W. L., and Erman, M. (1994) *Structure 2*, 629−640.
49. Chai, X., Zhai, Y., and Napoli, J. L. (1997) *J. Biol. Chem. 272*, 33125−33131.
50. Su, J., Chai, X., Kahn, B., and Napoli, J. L. (1998) *J. Biol. Chem. 273*, 17910−17916.

BI015803T