Functional Expression, Characterization, and Purification of the Catalytic Domain of Human 11-β-Hydroxysteroid Dehydrogenase Type 1*

Elizabeth A. Walker‡, Anya M. Clark§, M. Hewison‡, Jon P. Ride§, and Paul M. Stewart‡¶

From the ‡Division of Medical Sciences and the §School of Biosciences, University of Birmingham, P. O. Box 363, Edgbaston, Birmingham B15 2TT

11-β-hydroxysteroid dehydrogenase type 1 catalyzes the conversion of cortisone to hormonally active cortisol and has been implicated in the pathogenesis of a number of disorders including insulin resistance and obesity. The enzyme is a glycosylated membrane-bound protein that has proved difficult to purify in an active state. Extracted enzyme typically loses the reductase properties seen in intact cells and shows principally dehydrogenase activity. The C-terminal catalytic domain is known to contain a disulfide bond and is located within the lumen of the endoplasmic reticulum, anchored to the membrane by a single N-terminal transmembrane domain. We report here the functional expression of the catalytic domain of the human enzyme, without the transmembrane domain and the extreme N terminus, in Escherichia coli. Moderate levels of soluble active protein were obtained using an N-terminal fusion with thioredoxin and a 6xHis tag. In contrast, the inclusion of a 6xHis tag at the C terminus adversely affected protein solubility and activity. However, the highest levels of active protein were obtained using a construct expressing the untagged catalytic domain. Nonreducing electrophoresis revealed the presence of both monomeric and dimeric disulfide bonded forms; however, mutation of a nonconserved cysteine residue resulted in a recombinant protein with no intermolecular disulfide bonds but full enzymatic activity. Using the optimal combination of plasmid construct and E. coli host strain, the recombinant protein was purified to apparent homogeneity by single step affinity chromatography. The purified protein possessed both dehydrogenase and reductase activities with a $K_m$ of 1.4 $\mu$M for cortisol and 9.5 $\mu$M for cortisone. This study indicates that glycosylation, the N-terminal region including the transmembrane helix, and intermolecular disulfide bonds are not essential for enzyme activity and that expression in bacteria can provide active recombinant protein for future structural and functional studies.

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In mammalian tissues, two isozymes of 11-β-hydroxysteroid dehydrogenase (11β-HSD) catalyze the interconversion of hormonally active C₁₁-hydroxylated corticosteroids (cortisol, corticosterone) and their inactive C₁₁-keto metabolites (cortisone, 11-dehydrocorticosterone). The 11β-HSD1 and 11β-HSD2 isozymes share only 14% identity and are separate gene products with different physiological roles, regulation, and tissue distribution (1). 11β-HSD2 is a high affinity NAD-dependent dehydrogenase that protects the mineralocorticoid receptor from glucocorticoid excess; mutations in the HSD11B2 gene explain an inherited form of hypertension, the syndrome of apparent mineralocorticoid excess in which cortisol acts as a potent mineralocorticoid (2). By contrast, 11β-HSD1 is a relatively low affinity NADP-dependent enzyme that acts predominantly as a reductase in vivo, although extracted enzyme typically shows predominant dehydrogenase activity (3). By converting cortisone to cortisol, 11β-HSD1 facilitates glucocorticoid hormone action in key target tissues such as liver and adipose tissue, and as such has been implicated in a number of disorders including insulin resistance and central obesity (4, 5).

11β-HSD1 is a member of the short chain alcohol dehydrogenase family, also known as the short chain dehydrogenase/ reductases (SDRs). SDRs typically exhibit residue identities only at the 15–30% level, indicative of early duplicatory origins and extensive divergence (6–8). However, in contrast to other SDR members, 11β-HSD1 is unusual in possessing a single transmembrane helix at the N terminus. This is intrinsic to the endoplasmic reticulum (ER) membrane, with a short 5-amino acid N-terminal region on the cytosolic side and the main catalytic domain of the protein facing the lumen of the ER (9, 10). The importance of the transmembrane domain on 11β-HSD1 activity has been studied but with inconclusive results. An N-terminally truncated variant of rat 11β-HSD1 was expressed in COS cells and reported to be inactive (11, 12). However, this construct encoded a protein that had lost more than just the transmembrane helix and therefore may have lost vital parts of the enzymatic domain. In addition, because the expression studies were performed in COS and Chinese hamster ovary cells, the truncated protein would have been targeted (because of the lack of signal sequence) to the cytosol and not the ER. The lumen of the ER promotes the formation of disulfide bonds, and studies have indicated that there are important intrachain disulfide bonds within the 11β-HSD1 protein (9).

The catalytic domain is glycosylated (13–15), which is in agreement with a luminal orientation. Experiments to resolve the importance of glycosylation have also yielded varying results. Enzymatic deglycosylation of rabbit (9) and human (13) 11β-HSD1 has indicated that glycosylation is not important for enzyme activity. However, partial inhibition of glycosylation of the rat enzyme by tunicamycin decreased dehydrogenase activity but not reductase activity (14), and mutation of the rat
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**Table I**

| Name               | Sequence                        | Restriction site |
|--------------------|---------------------------------|------------------|
| pET32CD 5'         | GACCCATGGGCAAGGAAATTCAAGTACAG   | NosI site        |
| pET32FL 5'         | GACCCATGCTTTTATGAAAAATATCTCTCT  | NosI site        |
| pET32 3'           | CAGCTGACGGCTCTGTTATGGAATGTCGCCAT| XhoI site        |
| pET21 5'           | TATGCTGACGGAGAATTCAAGTACAG      | NheI site        |
| pET21CD 3'         | CAGCTGACGGCTCTGTTATGGAATGTCGC   | XhoI site        |
| pET21CDH 3'        | CAGCTGACGGCTCTGTTATGGAATGTCGCCAT| XhoI site        |

Expression of human (and squirrel monkey) clones of 11β-HSD1 has been achieved in COS cells (11, 12), HEK cells (16), and the yeast *Pichia pastoris* (13, 17) using a variety of vectors. This has led to ambiguous kinetic results with over 10-fold variation in *Km* values and often significant differences in activity between whole cells and lysates. These systems have not yielded large amounts of pure recombinant protein, and no structural information has come from them. Overexpression of 11β-HSD1 in bacterial cells has been reported (17), but the resulting protein was inactive. Failure to obtain activity was attributed to either insolubility of the protein, and subsequent refolding problems, or a lack of glycosylation. In this study we sought to maximize the production of soluble recombinant human 11β-HSD1 within *Escherichia coli* by varying the expression construct, host strain, and the incubation conditions. In particular, because 11β-HSD1 is thought to contain disulfide bonds, we have assessed the value of *E. coli* strains that promote disulfide bond formation within the cytoplasm of the bacterium through mutations in the genes encoding thioredoxin reductase and/or glutathione reductase. We also tested the effect of thioredoxin fusions, histidine tags, glycosylation status, the presence of the transmembrane domain, and mutation of a nonconserved cysteine residue on the activity of human 11β-HSD1. Through these measures, we arrived at an optimal construct and *E. coli* host combination for producing sufficient protein for purification.

**Experimental Procedures**

**Materials**—Detergents, enzyme substrates, cofactors, chromatographic media, and chemicals were obtained from Sigma-Aldrich (Dorset, UK) unless otherwise stated. The [1,2,6,7-3H]cortisol (specific activity, 70.0 Ci/mmol) was supplied by PerkinElmer Life Sciences. Oligonucleotide primers were made by Alta Bioscience (Birmingham, UK). Restriction endonucleases were obtained from Promega (Southampton, UK).

Production of 11-β-Hydroxysteroid Dehydrogenase 1 Expression Vectors—The human 11β-HSD1 cDNA (3) was subcloned into pcDNA3.1 (15). This was further subcloned into pET21b (+) and pET32b (+) *E. coli* expression vectors (Novagen). Four distinct constructs bearing alterations to the N and C termini were synthesized (Fig. 1). Modifications to the inserts in the pET21b (+) vector were made to truncate the hydrophobic N terminus of 11β-HSD1, both with (pET21CDH) and without (pET21CD) a C-terminal 6-histidine tag (6xHis) sequence. Each of these modifications also introduced an Nhel restriction site coincident with the ATG start codon. The pET32b (+) vector was used to produce a N-terminal fusion protein between the vector-encoded thioredoxin gene (TrxA) and either the full-length 11β-HSD1 gene (pET32FL) or an N-terminally truncated version containing only the catalytic domain (pET32CD). This strategy also incorporated an N-terminal 6xHis tag to assist purification and introduced an Ncol site coincident with the ATG start codon. The original full-length cDNA in pcDNA3.1 was used as template for polymerase chain reaction amplification and the forward primers (pET32CD 5', pET32FL 5', and pET21 5'; see Table I) in conjunction with the appropriate reverse primers (which allowed the introduction of an XhoI restriction site downstream). The resulting polymerase chain reaction products were subcloned into pGEM-T Easy vector (Promega). After digestion with the appropriate restriction enzymes, fragments were gel-purified and ligated with the appropriate pET expression vector to give the final constructs (Fig. 1). The direction and nucleotide sequence of the inserted cDNA were confirmed by sequencing. For expression studies the plasmids were subcloned into the *E. coli* strains BL21(DE3), AD494(DE3), and Origami(DE3) (Novagen).

**Site-directed Mutagenesis**—A mutation was introduced into the expression construct pET21CD by polymerase chain reaction using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers were designed to mutate the cysteine at position 272 of the human type 1 sequence to the corresponding residue of 11β-HSD1 from the squirrel monkey, namely a serine (18). The oligonucleotides used were 5'-CAGAAATTCATCCAGGAATGATC-3' and 5'-GATCTCCTGGATTGATTCG-3' (the mutated nucleotides are in boldface). The resulting construct (designated pET21CD-C272S) was verified by sequencing.

**Expression of Recombinant 11β-HSD1**—Overtimes cultures of *E. coli* expressing the pET constructs in LB medium containing 50 μg/ml carbenicillin (for host strain BL21(DE3)), supplemented with 15 μg/ml kanamycin for AD494(DE3) and Origami(DE3), and 12.5 μg/ml tetracycline for Origami(DE3) were seeded (0.1%) into fresh LB medium containing appropriate antibiotics and then incubated at 37 °C for 6 h, at 15 °C unless otherwise stated, with shaking at 230 rpm.

**Preparation of Cleared Lysates**—Bacterial cultures from induced and uninduced *E. coli* cells were pelleted by centrifugation. The cells were disrupted by repeated freeze-thawing and sonication (Bio-Rad). The supernatant containing protease inhibitors (Mini-Complete EDTA-free, Roche Molecular Biochemicals) and benzonase DNase (Novagen) using 50 μl of lysis reagent/ml of original culture. For fuller lysis of the cells, lysosome was included to a final concentration of 200 μg/ml. After incubation at room temperature with shaking for 25–30 min, the cell debris was pelleted by centrifugation at 11,000 *g* for 10 min. The supernatant was removed for activity assay and protein determination. Protein was quantified using the Bio-Rad protein assay reagent (corresponding to an approximate absorbance at 600 nm of 1.0). Expression of recombinant 11β-HSD1 was then induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Control incubations without IPTG induction were also performed. Incubation was continued for 16 h, at 15 °C unless otherwise stated, with shaking at 230 rpm.

**Activity Assays**—Cleared lysates and other enzyme preparations (typically 1–10 μl) were incubated in 0.5 ml of phosphate buffer (0.1 M, pH 7.6) containing 50,000 cpm [3H]cortisol, 100 nM unlabeled cortisol, and 200 μM NADPH for 30 min at 37 °C to assess dehydrogenase activity or [3H]cortisone (generated as reported previously (19)), 100 nM unlabeled cortisol, 200 μM NADPH, and a regeneration system (10 mM MgCl2, 5 mM glucose-6-phosphate, and 10 units glucose-6-phosphate dehydrogenase) to assess reductase activity. Steroids were partitioned into 10 volumes of dichloromethane and separated by TLC using ethanol/chloroform (8:92) as the mobile phase. The TLC plates were analyzed on a BioScan radioimaging detector, and the fractional conversion of cortisol to cortisone or cortisone to cortisol was used to estimate enzyme activities.

Activity was also assessed in intact *E. coli* cells. Bacterial cultures (1 ml) were centrifuged, and the resulting pellet was resuspended in 0.5 ml of phosphate buffer (0.1 M, pH 7.6) containing either 100 nM cortisol plus [3H]cortisol tracer, to measure the levels of dehydrogenase activity, or 0.5 ml of phosphate buffer (0.1 M, pH 7.6) containing 100 nM cortisone and [3H]cortisone tracer, to measure reductase activity.

**Kinetic Analysis**—Enzyme activities were assayed in the standard reaction mixture containing appropriate cofactor (NADP or NADPH plus the regeneration system), and varying substrate concentrations (0.25–60 μM). In each case, linearity of enzyme activity versus time was ensured. *Km* value estimations were averaged from Lineweaver-Burke plots derived from three experiments as previously reported (21).

**Western Blot Analysis**—SDS-PAGE was performed using the Laemmli method (22) with 12.5% acrylamide minigels using a Bio-Rad
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Mini-Protean II apparatus. 10 μg of protein from bacterial cleared lysates, human liver homogenates, or mouse liver homogenates (produced as reported previously (23, 24)) were loaded either in sample buffer containing β-mercaptoethanol, to completely reduce any disulphide bonds, or in sample buffer without β-mercaptoethanol, to retain the disulphide bonds. Gels were stained with Coomassie Brilliant Blue (R-250) to investigate the purity and amount of protein in the extracts. Western blotting was performed as reported previously (23). Briefly, after electrophoresis, the proteins were transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). Nonspecific protein binding was blocked by incubating the membranes in 20% nonfat milk and 0.1% Tween 20 in phosphate-buffered saline at 25 °C for 1 h. The membranes were then incubated with a validated polyclonal antibody to human 11β-HSD1 (The Binding Site, Birmingham, UK) at a dilution of 1:1000 for 16 h at 4 °C. After three 10-min washes in phosphate-buffered saline/0.1% Tween 20, the membranes were incubated with a secondary antibody (goat anti-sheep IgG peroxidase conjugate (The Binding Site)) at a dilution of 1:75,000 for 1.5 h at room temperature. Bound peroxidase-conjugated IgG was visualized using an ECL detection kit (Amer sham Pharmacia Biotech) by exposing the membranes to x-ray film (Kodak). Relative band intensities were analyzed by laser scanning densitometry.

**Purification of Recombinant 11β-HSD1—** A further construct was designed for the purification of recombinant 11β-HSD. The plasmid pET21CD was digested with NcoI and XhoI to release the 11β-HSD insert and ligated to the similarly digested pT28h(+) vector (Novagen). This gave the construct pET28HCD, which contained the catalytic domain of 11β-HSD with an N-terminal 6xHis tag to aid purification. For expression, the construct was subcloned into E. coli strain BL21(DE3) and induced as described above. Cleared lysate (8 ml) was prepared from 200 ml of induced culture and mixed with 0.3 ml of nickel-nitrilotriacetic acid His-bind resin (Novagen) followed by gentle shaking at room temperature for 30 min. The mixture was loaded into an empty column, and unbound protein was removed by washing with 8 ml of 50 mM NaPO4 buffer, pH 8, containing 300 mM NaCl. Bound protein was eluted in a stepwise fashion with the same buffer containing increasing concentrations of imidazole (2 × 0.2 ml each of 40, 60, and 100 mM imidazole). The eluted fractions were tested for enzyme activity, and the purity was assessed by SDS-PAGE.

**RESULTS**

**Expression of Different Variants of 11β-Hydroxysteroid Dehydrogenase 1 in E. coli—** Four constructs were designed (Fig. 1) to test the effect of (a) removing the N-terminal transmembrane region of 11β-HSD1, (b) including a 6xHis tag at the C terminus, and (c) fusing the N terminus of the catalytic domain to thioredoxin, a procedure reported to increase the solubility of recombinant proteins (25), particularly those requiring disulphide bonds. Initial comparisons used a thioredoxin reductase-deficient strain of E. coli, AD494(DE3), that has been reported to enhance the formation of disulphide bonds within the bacterial cytoplasm (26), particularly for thioredoxin fusion proteins. In addition, because initial tests indicated that very little soluble protein was produced by incubation entirely at 37 °C, cultures were switched to 15 °C after the addition of IPTG to increase the possibility of producing soluble protein (26). The overall level of expression from each of the four constructs under these conditions was estimated using SDS-PAGE gels and subsequent densitometric analysis. All constructs produced protein of the expected size, with levels varying from 15% of the total cell protein for the thioredoxin full-length 11β-HSD1 fusion (pET32FL) to 35% for the nontagged catalytic domain (pET21CD).

The extraction of proteins by sonication indicated that none of the recombinant protein was in a soluble form (data not shown). Despite this, no enzyme activity could be detected in the sonicated lysates. However, incubation of intact bacterial cells (transformed with the pET32CD construct) with labeled cortisol and cortisone indicated that the bacteria had acquired both 11β-hydroxysteroid dehydrogenase and 11-oxo reductase activities (Fig. 2). This was specific to IPTG-induced cultures and hence indicative of the presence of active recombinant enzyme within the cells prior to sonication. Moreover, a gentler lysis of the cells by a mixture of a commercial detergent (Bug-Buster) and lysozyme produced an extract with clear 11β-HSD1 expression (data not shown). The diamond hatched region is the N-terminal region that includes not only the transmembrane domain but also the 5/6 amino acid extreme N terminus (amino acid sequence shown above). The diagonally hatched boxes represent the catalytic domain of 11β-HSD1 spanning amino acid residues 24–287. Additional amino acids (MAS), produced as a consequence of cloning, are shown by their single-letter codes at the N terminus of the pET21 construct. A mutation was made in the pET21CD construct at amino acid position 272, changing a cysteine residue to a serine, and is highlighted above.

**FIG. 1. Diagrammatic representation of 11β-HSD1 expression constructs.** (C) boxes represent the pET32 vector-encoded thioredoxin gene (TrxA), and the 6xHis-tag sequences are denoted by black boxes. The diamond hatched region is the N-terminal region that includes not only the transmembrane domain but also the 5/6 amino acid extreme N terminus (amino acid sequence shown above). The diagonally hatched boxes represent the catalytic domain of 11β-HSD1 spanning amino acid residues 24–287. Additional amino acids (MAS), produced as a consequence of cloning, are shown by their single-letter codes at the N terminus of the pET21 construct. A mutation was made in the pET21CD construct at amino acid position 272, changing a cysteine residue to a serine, and is highlighted above.
duced that encoded the full-length 11β-HSD1 sequence in pET21b (+) (i.e. not as a fusion), but these constructs repeatedly failed to express any recombinant protein in either a soluble or insoluble form (data not shown).

Expression of 11-β-Hydroxysteroid Dehydrogenase 1 in Different Strains of E. coli—The two constructs that demonstrated enzyme activity (pET21CD and pET32CD) were then used to compare the effect of E. coli host strain on the production of active protein, with the particular purpose of comparing strains reputed to enhance disulfide bond formation by mutations in either thioredoxin reductase or glutathione reductase genes (26). Thus the thioredoxin reductase-deficient strain, AD494(DE3), was compared with the thioredoxin reductase- and glutathione reductase-deficient strain Origami(DE3) and a strain deficient in neither enzyme, BL21(DE3). Surprisingly, for the pET21CD construct, both activity assays (Fig. 4C) and Western analyses (Fig. 4D) clearly indicated that BL21(DE3) cells gave better protein expression and activity than AD494(DE3) or Origami(DE3), with the latter giving negligible levels of both protein and activity. As before (Fig. 3), the thioredoxin fusion construct pET32CD yielded lower amounts of protein and enzyme activity compared with the nonfusion con-
which high enzyme activity was accompanied by a clear band in detergent alone, without lysozyme, resulting in a preparation in the presence of both dimer and monomer bands in the nonreducing conditions (Fig. 6 B). The results clearly showed that almost all the active 11β-HSD1 could be released by detergent alone (data not shown). This analysis showed that human 11β-HSD1 contains four cysteine residues, three of which are conserved across all mammalian 11β-HSD1 proteins, we tested for the presence of interchain disulfide bonds by probing Western blots of SDS-PAGE gels of lysates run under both reducing and nonreducing conditions (Fig. 6A). The results clearly showed the presence of both dimer and monomer bands in the nonreducing lanes, suggesting that some of the protein existed in an interchain disulfide-bonded dimeric form. Examination of human liver extracts indicated that the natural enzyme also consisted of a similar combination of monomeric and dimeric forms (Fig. 6B), although analysis of mouse liver extracts (Fig. 6C) showed the presence of only monomers. Because the human 11β-HSD1 contains an additional cysteine (Cys-272) when compared with the other mammalian sequences reported to date, we investigated the effect of mutating this residue to the corresponding residue of 11β-HSD1 from squirrel monkey, namely a serine (18). Interestingly, the expression of this mutant in E. coli produced very similar dehydrogenase and reductase activities to those observed with the wild type and no significant alteration in the respective \( K_m \) values. However, Western blots of nonreducing gels indicated that the ability of the protein to form disulfide-bonded dimers had been abolished (Fig. 6D).

**Mutational Analysis of the Enzyme**—Because human 11β-HSD1 contains four cysteine residues, three of which are conserved across all mammalian 11β-HSD1 proteins, we tested for the presence of interchain disulfide bonds by probing Western blots of SDS-PAGE gels of lysates run under both reducing and nonreducing conditions (Fig. 6A). The results clearly showed the presence of both dimer and monomer bands in the nonreducing lanes, suggesting that some of the protein existed in an interchain disulfide-bonded dimeric form. Examination of human liver extracts indicated that the natural enzyme also consisted of a similar combination of monomeric and dimeric forms (Fig. 6B), although analysis of mouse liver extracts (Fig. 6C) showed the presence of only monomers. Because the human 11β-HSD1 contains an additional cysteine (Cys-272) when compared with the other mammalian sequences reported to date, we investigated the effect of mutating this residue to the corresponding residue of 11β-HSD1 from squirrel monkey, namely a serine (18). Interestingly, the expression of this mutant in E. coli produced very similar dehydrogenase and reductase activities to those observed with the wild type and no significant alteration in the respective \( K_m \) values. However, Western blots of nonreducing gels indicated that the ability of the protein to form disulfide-bonded dimers had been abolished (Fig. 6D).

**Purification of Recombinant 11β-HSD1**—Several attempts at purification of recombinant pET21CD from BL21(DE3) cells were unsuccessful. Several combinations of gel filtration, ion exchange, and ADP-agarose methods failed to yield sufficiently pure protein. Because the addition of sequences at the N terminus of the catalytic domain of 11β-HSD did not seem to affect activity, a further expression construct based on our most active pET21CD plasmid was generated that incorporated an N-terminal 6xHis tag to allow purification by metal affinity chromatography. Use of this construct (pET28HCD) in BL21(DE3) cells resulted in lysates from which the enzyme could be purified to apparent homogeneity, as indicated by SDS-PAGE, in a single chromatographic step (Fig. 7). Activity measurements indicated that the recombinant human 11β-HSD1 had been purified 159-fold with an overall yield of 28%
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DISCUSSION

11-β-Hydroxysteroid dehydrogenase activity was first documented in the rat liver in the 1950s, but it was the studies of Tannin et al. (3) that resulted in the enzymatic characterization, purification, and cloning of the liver-type 11β-HSD isozyme. With the cloning of a second “kidney-type” 11β-HSD isozyme, the liver-type isozyme is now termed 11β-HSD1.

The importance of these isozymes in the metabolism and clearance of glucocorticoids is well established; in addition, these enzymes are intricately involved in the pathogenesis of human diseases. For example, 11β-HSD2 is implicated in hypertension and fetal growth retardation (1). Specifically, for 11β-HSD1, emerging data have highlighted the role of this enzyme in modulating insulin sensitivity and visceral adiposity. Thus mice lacking the HSD11B1 gene are resistant to hyperglycemia of stress/feeding because of a failure to activate glucocorticoid within the liver and stimulate gluconeogenesis (4). Improvements in insulin sensitivity in normal volunteers given the 11β-HSD1 inhibitor carbenoxolone support such a concept (27). Similarly in visceral adipose tissue, 11β-HSD1 expression may represent a novel mechanism to acts locally to generate active glucocorticoid concentrations, thereby stimulating adipogenesis (5, 28). Defect in the activity of 11β-HSD1 is also thought to underpin an inherited form of polycystic ovary syndrome, the syndrome of apparent cortisone to cortisol (cortisol to cortisone) directions, with the estimated $K_m$ for cortisol being 1.4 $\mu M$ (± 0.6 S.D.) and for cortisone being 9.5 $\mu M$ (± 0.9 S.D.), both values being similar to those reported from mammalian systems (reviewed in Ref. 1).

Fig. 6. Western analysis of SDS-PAGE gels under reducing (+ β-mercaptoethanol) and nonreducing (− β-mercaptoethanol) conditions. A, cleared lysates of pET21CD showing monomer (molecular mass, 29 kDa) and dimer (58 kDa) species of 11β-HSD1 under nonreducing conditions. B, human liver extracts showing dimeric (68 kDa) and monomeric (34 kDa) forms of 11β-HSD1. C, mouse liver extracts showing the presence of monomeric 11β-HSD1 only (32 kDa). D, cleared lysates from pET21CD-C272S showing monomeric 11β-HSD1 and the loss of the interchain disulfide-bonded dimeric forms.

Fig. 7. Gel electrophoresis of the purification steps for recombinant 11β-HSD1. Coomassie Blue-stained SDS-PAGE gel of various fractions from the purification of recombinant protein by His-bind affinity chromatography. Lane 1, initial cleared lysate from an induced culture of BL21(DE3) cells containing pET28HCD; lane 2, fraction eluted from the column with 40 mM imidazole; lane 3, fraction eluted with 60 mM imidazole; lane 4, final eluate using 100 mM imidazole.

11β-HSD1 belongs to the SDR superfamily, as defined on the basis of an N-terminal nucleotide binding motif, a central active site, and consensus sequence data. Sophisticated analytical approaches suggest that there are over 1000 members of this superfamily (6–8), with only one residue (Tyr) being strictly conserved. A lysine 4 residues downstream and a serine 14 residues upstream are also largely conserved; all these residues are present in 11β-HSD1. A model for catalysis of SDRs has been proposed on the basis of these residues (31).

Binding of the coenzyme, NAD(H) or NADP(H), is in the N-terminal part of the molecule involving a common protein folding arrangement of α- and β-strands (“Rossmann” fold) associated with a common GlyXaa₃GlyXaaGly motif (also found in 11β-HSD1). The critically important tyrosine seems to maintain a fixed position relative to the scaffolding of the Rossmann fold and the cofactor position, whereas the substrate-binding pocket alters in such a way that the dehydrogenation/reduction reaction site is brought into bonding distance of the tyrosine hydroxyl group. The tyrosine therefore acts as a basic catalyst, the lysine binds to NAD(P)H and lowers the $pK_a$ value of the tyrosine, and the serine plays a subsidiary role of stabilizing substrate binding (31, 32).

(4) 42.47 pmol/h/g of protein. The purified enzyme had activity in both dehydrogenase (cortisol to cortisone) and reductase (cortisone to cortisol) directions, with the estimated $K_m$ for cortisol being 1.4 $\mu M$ (± 0.6 S.D.) and for cortisone being 9.5 $\mu M$ (± 0.9 S.D.), both values being similar to those reported from mammalian systems (reviewed in Ref. 1).
Several groups have evaluated the importance of the N-terminal domain of 11β-HSD1. Recently, it has been shown that the orientation of the enzyme within the ER is determined by sequences close to the N terminus (10). Chimeric proteins where the N-terminal regions, including the membrane anchors, of the 11β-HSD1 and 11β-HSD2 enzymes were exchanged adopted inverted orientations in the ER membrane (10). Neither protein was catalytically active. However, mutation of a single lysine residue close to the N terminus of type 1 resulted in an inverted orientation without loss of activity. These results suggest that the N-terminal anchor is required for both activity and correct orientation, although it should be noted that the sequences exchanged in the chimeras included much more than just the transmembrane helix. Mercer et al. (12) reported that expression of an N-terminally truncated 11β-HSD1 did not produce a soluble protein. However, these studies and others have employed mammalian expression systems where such constructs would not be appropriately targeted to the ER, and hence correct folding and disulfide bond formation may not have been facilitated.

In this study, using a series of bacterial expression constructs, we have shown that the activity of human 11β-HSD1 does not depend on the N-terminal domain. Constructs where the N-terminal region had been removed (pET32CD and pET21CD) exhibited higher levels of expression and activity than constructs containing the entire 11β-HSD1 sequence. Moreover, inclusion of the transmembrane domain, either with or without the thioredoxin fusion partner, failed to produce soluble active protein. This is in agreement with a study carried out by Blum et al. (13), in which the complete human 11β-HSD1 sequence was expressed in E. coli and resulted in a protein that was virtually insoluble, difficult to purify, and completely inactive.

We also investigated expression systems in which thioredoxin, the product of the E. coli TrxA gene (25), was a fusion partner. In many cases heterologous proteins produced as thioredoxin fusion proteins are correctly folded and display full biological activity (25, 33–35). This has been thought to be caused by the small, highly soluble nature of thioredoxin, which also has robust folding characteristics (36). However, in our study proteins produced as a fusion with thioredoxin at the N terminus (pET32CD and pET32FL) showed no overall increase in the levels of soluble protein when compared with nonfusion constructs (pET21CD and pET21CDH), indicating that such fusions are not always profitable. Similarly, fusion of a 6xHis tag at the C terminus, as a means to simplify purification, was also detrimental to the solubility, and particularly the activity, of the enzyme. Residues close to the C terminus of SDRs may frequently be important in substrate binding (37), and modifications in this region may thus affect protein structure to the detriment of enzyme activity.

This study also clearly resolves the issue of whether glycosylation is required for the activity of the human enzyme. Studies on the rat 11β-HSD1 enzyme indicated that partial inhibition of glycosylation with tunicamycin inhibited dehydrogenase activity by 50% but had no effect on reductase activity (14). Mutagenesis of the first of two potential N-glycosylation sites reduced dehydrogenase and reductase activities by 75 and 50%, respectively, whereas mutagenesis of the second site completely abolished activity (15). Conversely, studies carried out on the rabbit enzyme, which like the human homologue contains three potential glycosylation sites, suggest that glycosylation is not important for enzyme activity. No alteration in activity could be observed after complete deglycosylation of rabbit 11β-HSD1 (9). Conflicting studies on the human enzyme have also been reported. Recently, human 11β-HSD1 has been expressed in E. coli, where the biosynthesis of N-linked glycoproteins does not occur. This resulted in a recombinant protein that was completely devoid of enzyme activity (17). The same group also investigated the effects of deglycosylation on human 11β-HSD1 purified from liver and recombinant protein produced by the yeast P. pastoris (13). Site-directed mutagenesis of the three potential glycosylation sites yielded an inactive protein from yeast cells as assessed using metyrapone and metyrapol as the substrates. However, the enzyme purified from human liver, upon complete deglycosylation, remained fully active. The results here agree with the latter experiment and clearly show that nonglycosylated enzymatically active 11β-HSD1 can be generated within E. coli, with the recombinant enzyme possessing both reductase and dehydrogenase activities with similar kinetic properties to those reported previously from mammalian expression systems. Glycosylation is therefore not required for activity or protein folding, although it could still be important for protein stability within the endoplasmic reticulum.

All the constructs used in this study gave only moderate levels of soluble protein but a high proportion of protein in an insoluble form. The lack of protein solubility in E. coli is a complex event with many contributing factors. Although fusion with heterologous proteins may sometimes help to redress many solubility problems, another factor that may be important is the inability of the recombinant protein to form key disulfide bonds in the reducing environment of the bacterial cytoplasm (38). Rabbit 11β-HSD1 is known to contain an intrachain disulfide bond (9), and therefore we investigated the expression levels and activity of our recombinant proteins in a variety of host E. coli strains, some of which have been developed to promote disulfide bond formation. Within E. coli at least two systems are responsible for reducing disulfide bonds that form in the cytoplasm; the thioredoxin system that consists of thioredoxin reductase and thioredoxin and the glutaredoxin system that includes glutathione reductase, glutathione, and glutaredoxins. We evaluated this using three separate E. coli strains. It was anticipated that disulfide bonds, and therefore solubility and activity of the soluble protein, would be enhanced by the use of AD494(DE3) and particularly the Origami(DE3) strain. In effect, the reverse was observed with the highest levels of soluble protein and enzyme activity being observed using BL21(DE3) as the host strain. This result could imply that the intramolecular disulfide bond observed in the rabbit 11β-HSD1 protein (9) is not present in the human enzyme, although this awaits experimental confirmation.

We also tested for the presence of interchain disulfide bonds by probing Western blots of SDS-PAGE gels of bacterial lysates.

### Table II

| Sample            | Volume | Protein concentration | Specific activity | Fold purification yield |
|-------------------|--------|-----------------------|-------------------|-------------------------|
| Cleared lysate    | 8      | 23.4                  | 0.27              | 100                     |
| Imidazole eluate 1 (40 mM) | 0.4 | 0.358 | 8.10 | 30 | 2 |
| Imidazole eluate 2 (60 mM) | 0.4 | 0.469 | 10.02 | 37 | 4 |
| Imidazole eluate 3 (100 mM) | 0.4 | 0.817 | 42.47 | 157 | 28 |
run under both reducing and nonreducing conditions. Both dimer and monomer bands were identified in the nonreducing lanes, suggesting that some of the recombinant protein exists in an interchain disulfide-bonded form. Examination of human liver extracts indicated that the native 11β-HSD1 enzyme existed in a similar combination of monomeric and dimeric forms, proving that the heterogeneity was not a consequence of expression in the bacterial system. However, this heterogeneity not only complicates the purification of 11β-HSD1, as has been noted previously for native enzyme (9), but could also hinder crystallographic analysis because crystal growth requires pure protein in a homogeneous form. Tests on extracts of mouse liver, however, detected no intermolecular disulfide bridges.

Because previous reports also suggested that rabbit 11β-HSD1 contains no intermolecular disulfide bonds (9), we investigated the effect of mutating the additional cysteine (Cys-272), found only in the human sequence, to the corresponding residue from the most closely related 11β-HSD1 sequence (squirrel monkey). Expression of the resulting mutant (pET21CD-C272S) in the optimized bacterial expression system resulted in a protein with kinetic properties indistinguishable from the wild-type recombinant protein. However, nonreducing gels showed that the ability to form the interchain disulfide bond had been abolished. Previous structural studies on other SDRs suggest that they exist naturally as nondisulfide-bonded dimers or tetramers (reviewed in Ref. 37). The results here suggest that Cys-272 of human 11β-HSD1 may be involved in disulfide bonds between adjacent polypeptide chains of the enzyme, possibly stabilizing the dimeric form. However, this property does not seem to be vital to the activity of the enzyme.

Using a modified expression construct that included an N-terminal 6×His tag, we developed a simple purification protocol that allowed 157-fold purification of recombinant human 11β-HSD1 in one step from crude cell lysates. The purified protein demonstrated activity in both dehydrogenase and reductase directions with \( K_m \) values of 1.4 \( \mu M \) for cortisol and 9.5 \( \mu M \) for cortisone.

In conclusion this study has shown that, despite reports to the contrary, bacterial expression systems have the potential to produce active soluble 11β-HSD1 protein. The results also demonstrate conclusively that the N-terminal region, containing the transmembrane domain, glycosylation, and interchain disulfide bonds are not essential for the activity of this enzyme. For the first time, active soluble 11β-HSD1 has been produced in vitro and purified to apparent homogeneity. This will now allow detailed functional analysis of the enzyme using \( E. \ coli \)-produced protein and facilitate future structure/crystallographic studies.

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