Comparative Enzymology of 11β-Hydroxysteroid Dehydrogenase Type 1 from Glucocorticoid Resistant (Guinea Pig) Versus Sensitive (Human) Species*

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Naeem Shafqat‡, Björn Elleby§, Stefan Svensson§, Jawed Shafqat‡, Hans Jörnvall‡, Lars Abrahamsson§, and Udo Oppermann‡¶

From the ‡Department of Medical Biochemistry and Biophysics, Karolinska Institutet, SE-171 77 Stockholm, Sweden and §Biovitrum AB, Department of Assay Development and Screening and Department of Structural Chemistry, SE-112 76 Stockholm, Sweden

Type 1 11β-hydroxysteroid dehydrogenase constitutes a prereceptor control mechanism through its ability to reduce dehydroglucocorticoids to the receptor ligands cortisol and corticosterone in vivo. We compared kinetic characteristics of the human and guinea pig 11β-hydroxysteroid dehydrogenase isozymes derived from species differing in glucocorticoid sensitivity. Both orthologs were successfully expressed as full-length enzymes in yeast and COS7 cells and as soluble transmembrane-deleted constructs in *Escherichia coli*. Both isozymes display Michaelis-Menten kinetics in intact cells and homogenates and show low apparent micromolar $K_m$ values in homogenates, which are lowered by approximately one order of magnitude in intact cells, allowing corticosteroid activation at physiological glucocorticoid levels. Recombinant soluble proteins were expressed and purified with high specific dehydrogenase and reductase activities, revealing several hundred-fold higher specificity constants than those reported earlier for the purified native enzyme. Importantly, these purified soluble enzymes also display a hyperbolic dependence of reaction velocity versus substrate concentration in 11-oxoreduction with $K_m$ values of 0.8 μM (human) and 0.6 μM (guinea pig), close to the values obtained from intact cells. Active site titration was carried out with the human enzyme using a novel inhibitor compound and reveals a fraction of 40–50% active sites/mol total enzyme. The kinetic data obtained argue against the involvement of 11β-hydroxysteroid dehydrogenase as a modulating factor for the glucocorticoid resistance observed in guinea pigs. Instead, the expression of 11β-hydroxysteroid dehydrogenase type 1 in the Zona glomerulosa of the guinea pig adrenal gland suggests a role of this enzyme in mineralocorticoid synthesis in this hypercortisolic species.

Glucocorticoid hormones regulate a multitude of physiological processes including carbohydrate and lipid metabolism, development, blood pressure, and immunity. Like other steroid hormones, glucocorticoids act through binding to specific nuclear receptors, thereby activating or repressing gene transcription (1).

In analogy to other steroid hormones, glucocorticoids are submitted to prereceptor control through metabolic conversion by enzymes acting as metabolic “switches,” producing “active” (i.e. binding to the receptor) or “inactive” (i.e. not binding to the receptor) hormone (2–6). The glucocorticoid “shuttle” mechanism consists of 11β-hydroxysteroid dehydrogenases (11β-HSDs),1 mediates the reversible oxoreduction/hydroxydehydrogenation at position C11 of cortisone and cortisol, respectively. At present, two 11β-HSD isozymes, the type 1 and 2 enzymes, have been characterized, and bothmediate activation or inactivation of the hormone in a tissue-specific manner (7, 8). Oxoreduction of 11-oxo-corticosteroids (cortisone in human, dehydrocorticosterone in rodents) is mediated by the NADPH-dependent type 1 11β-HSD (11β-HSD1), thus performing activation to the receptor ligand cortisol (corticosterone), which leads to the inactivation of glucocorticoid receptor ligands.

The two 11β-HSD isozymes of this enzymatic shuttle constitute important components within the glucocorticoid endocrine system. The type 2 enzyme has been shown to be involved in the “protection” of the mineralocorticoid receptor in peripheral tissues (such as distal renal tubules) against occupation by cortisol, exemplified by 11β-HSD2 gene defects leading to the “apparent mineralocorticoid excess syndrome” (9, 10). The physiological role of the type 1 form is less clear, but several studies established a critical role of 11β-HSD1 in the tissue-specific activation of corticosteroid ligands (11–14). This physiological role is established in hepatic and adipose tissue where the inhibition of enzyme activity might be beneficial in diseases such as obesity and non-insulin-dependent diabetes mellitus (12–15). However, the wide tissue distribution suggests that the enzyme has further specific functions.

In this study, we investigated enzymological properties of 11β-HSD1 from human and guinea pig. The latter species displays high circulating levels of glucocorticoids with lowered affinities of the glucocorticoid receptor for its ligand cortisol (16). The data obtained suggest that 11β-HSD1 is not a critical determinant of peripheral glucocorticoid resistance in guinea pig. Furthermore, we established an enzymological profile of recombinant human and guinea pig 11β-HSD1 and show for the first time high levels of reductive and oxidative activities of purified recombinant enzyme, demonstrating the suitability of this system for systematic investigations.

1 The abbreviations used are: 11β-HSD, 11β-hydroxysteroid dehydrogenase; ACTH, adrenocorticotropic hormone.

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¶ To whom correspondence should be addressed. Tel.: 46-8-7287680; Fax: 46-8-337462; E-mail: udo.oppermann@mbb.ki.se.

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**EXPERIMENTAL PROCEDURES**

Cloning of 11β-Hydroxysteroid Dehydrogenases—Guinea pig 11β-HSD1 was cloned by reverse transcriptase-PCR from a liver sample (Harletry strain). Total liver RNA was prepared by acid guanidinium thiocyanate/phenol extraction, and reverse transcriptase-PCR was carried out using sequence-specific primers (17). After digestion of the purified PCR product with nucleases SnaB I and NotI, the resulting cDNA was subcloned into pPIC3.5 (Invitrogen). The sequences of the constructs were verified by DNA sequencing. All clones obtained from guinea pig liver displayed identical sequences but show two differences from the published data (17). These exchanges comprise protein sequence position 22 (Pro → Ser) and position 103 (Ala → Val). All of the 11β-HSD1 sequences known to date show the same residues as the guinea pig sequence determined in this study. These structural details were reported to the Swiss Protein Data Bank. Human 11β-HSD1 was prepared as described previously (18).

Yeast Transformation, Selection, and Overexpression—After linearization of the plasmid DNA with endonucleases BgII or SacI, yeast strains KM71 or GS115 were transformed using the spheroplast method. Clones obtained after homologous recombination of the 11β-HSD-1 DNA into the Pichia alcohol oxidase locus were selected, and phenotyping for His" and "Mut" was carried out on appropriate media. Recombinant strains were confirmed by PCR of genomic DNA with gene-specific primers. Positive clones for each phenotype were selected, and expression trials were performed. Finally, a recombinant strain (His "Mut") for all further experiments was chosen. To exclude intrinsic activities, mock-transformed strains (vector alone, no insert) were used as background controls. Yeast clones were grown at 30 °C on 2% glucose buffered media (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4 × 10⁻⁵% biotin) containing 0.5% (v/v) methanol for induction of recombinant proteins.

Preparation of Yeast Fractions—Yeast subcellular fractions were prepared by mechanically disrupting the washed cells with glass beads (245–400 μm, Sigma) in 20 mM sodium phosphate buffer, 5% glyceral, 1 mM EDTA, pH 7.0, containing 1 mM PMSF. After the removal of cell debris by low speed centrifugation, an S9 fraction was prepared by a series of potassium phosphate buffers, pH optima were determined for sure liquid chromatography eluent and injected into the system. Using a series of potassium phosphate buffers, pH optima were determined for the purified enzymes, and kinetic measurements were subsequently carried out at pH 6.0 (reductase) and at pH 9.2 (dehydrogenase) in 50 mM potassium phosphate.

**Table I.** Comparative kinetic analysis for cortisone reduction of 11β-HSD1 expressed in different hosts

| Source     | V_{max} (µM) | K_{m} (µM) |
|------------|--------------|------------|
| Guinea pig |              |            |
| P. pastoris |              |            |
| COS intact | 420 ± 1.9    | 2.2 ± 0.7  |
| COS extract| 173 ± 10     | 0.28 ± 0.029 |
| Liver microsomes | 221 ± 15.9 | 1.2 ± 0.2 |
| Human      |              |            |
| P. pastoris | 30.3 ± 8.5   | 1.9 ± 0.2  |
| COS intact | 150 ± 66     | 0.33 ± 0.045 |
| Liver microsomes | 70 ± 3.0  | 2.5 ± 0.1  |

a Hepatic microsome data were taken from Ref. 22.

50 mM Tris-Cl, pH 7.4, at 37 °C for 10–60 min. Initial reaction velocities were measured, and conditions were chosen with no >20% of substrate conversion, and measurements were carried out in the linear range of product formation versus reaction time and enzyme concentration. At substrate concentrations over 5 µM, reactions were stopped by a 3-fold volume of ice-cold acetonitrile. After a brief centrifugation at 20,000 × g, supernatants were directly analyzed on reverse phase/high-pressure liquid chromatography consisting of a C18 stationary phase and an eluent of 30% ammonium acetate in 0.1% ammonium acetate, pH 7.0. UV detection of metabolites was achieved at 240 nm. Below 5 µM of substrate concentrations, reactions were stopped by the addition of 40 µl of phosphoric acid/ml, and samples were extracted by liquid phase extraction in ethyl acetate or by solid phase extraction using Oasis columns. After drying under nitrogen, samples were reconstituted in high pressure liquid chromatography eluent and injected into the system. Using a series of potassium phosphate buffers, pH optima were determined for the purified enzymes, and kinetic measurements were subsequently carried out at pH 6.0 (reductase) and at pH 9.2 (dehydrogenase) in 50 mM potassium phosphate.

Kinetic constants were calculated using Prism (GraphPad, San Diego, CA) software by linear or non-linear regression analysis and by fitting to different models describing either the Michaelis-Menten kinetics (V = V_{max} × S/K_{m} h + S/h; h = 1) or a cooperative kinetic behavior (h ≠ 1), respectively.

**Active Site Titration—**Fractional velocities (dehydrogenase reaction, 50 µM cortisol, 100 µM NADP⁺) were measured in the presence of increasing amounts of the aroylumidamidithioate inhibitor compound HVT.24,829 (Biovitrum) (15) at enzyme concentrations between 0.5 and 1 µM. Data obtained were fitted by non-linear regression to Equation (20).

\[
\nu/v_0 = 1 - \frac{(E + I + K_{I\text{app}})}{(E + I + K_{I\text{app}})^n} - \frac{((E + I + K_{I\text{app}} - (4 × E × I)^{1/2}) × (2 × E)^{1/2})}{(E + I + K_{I\text{app}})^n} - \frac{((E + I + K_{I\text{app}})^n - (4 × E × I)^{1/2}) × (2 × E)^{1/2})}{(E + I + K_{I\text{app}})^n}
\]

where \( v \) is the observed velocity in the presence of varied inhibitor concentrations, \( v_0 \) is the uninhibited velocity, \( E \) is the concentration of active enzyme, \( K_{I\text{app}} \) is the apparent inhibition constant, and \( I \) is the experimental inhibitor concentration.

**Protein Analysis—**Protein concentrations were determined using the Lowry method with bovine serum albumin as standard. Concentrations of purified samples were determined by compositional analysis on a Biochrom 20 Plus system (Amersham Biosciences) after hydroslysis of samples in 6 N HCl, 0.1% phenol.

**Immunohistochemistry—**Liver and adrenal glands from guinea pig (Harletry strain) were fixed in 1% buffered formaldehyde, dehydrated, and embedded into paraplast. Sections (about 5 µM) were processed for immunohistochemistry using established protocols. Primary antibodies used were anti mouse liver 11β-HSD1 affinity-purified through absorption on purified immobilized 11β-HSD1 (21) and corresponding immunization serum at 1:100 dilution in PBS. Western blot analysis of the antiserum revealed a single band at 32 kDa in guinea pig tissues (21) and non-reactivity with preimmunization serum. Detection was achieved using alkaline phosphatase-labeled secondary antibodies and...
Kinetic Analysis of Recombinant Full-length 11β-HSD1 from Guinea Pig and Human—Comparative kinetic analyses of recombinant human and guinea pig 11β-HSD1 were performed in homogenates prepared from Pichia pastoris strains and from COS7 cells (Table I and Fig. 1, A and B). Furthermore, kinetic constants were determined from intact monolayers of transiently transfected COS7 cells by analyzing supernatants of conditioned medium. These experiments yield an apparent $K_m$ in the low micromolar region measured in homogenates, similar for both guinea pig and human. The values range from 2.2 to 8.2 $\mu M$ for the guinea pig enzyme in $P$. pastoris and COS7 homogenates, respectively, whereas the human forms display $K_m$ values of 1.9 and 8.9 $\mu M$ for $P$. pastoris and COS7 homogenates, respectively. These values are in agreement with data obtained from previous studies (18, 22) using guinea pig and human liver microsomes (Table I). Specific activities obtained range from 30.3 ($P$. pastoris) to 150 (COS7) pmol/min $\times$ mg protein for the human enzyme, whereas guinea pig extracts have activities ranging from 42.0 ($P$. pastoris) to 221 (COS7) pmol/min $\times$ mg protein. In contrast, intact COS7 cells transiently transfected with plasmids expressing human or guinea pig 11β-HSD1 show a markedly higher affinity (approximately one order of magnitude) for cortisone reduction over homogenates with an apparent $K_m$ of 0.28 $\mu M$ for the guinea pig and 0.33 $\mu M$ for the human enzyme. Specific activities obtained in this system are similar and range from 173 pmol/10^6 cells $\times$ h to 191 pmol/10^6 cells $\times$ h for the guinea pig and human forms, respectively.

An analysis of COS7 extracts expressing human or guinea pig 11β-HSD1 using a wide range of cortisone substrate concentrations.

**RESULTS**

**Kinetic Analysis of Recombinant Full-length 11β-HSD1 from Guinea Pig and Human**—Comparative kinetic analyses of recombinant human and guinea pig 11β-HSD1 were performed in homogenates prepared from Pichia pastoris strains and from COS7 cells (Table I and Fig. 1, A and B). Furthermore, kinetic constants were determined from intact monolayers of transiently transfected COS7 cells by analyzing supernatants of conditioned medium. These experiments yield apparent $K_m$ values ranging from 2.2 to 8.2 $\mu M$ for the guinea pig enzyme in Pichia pastoris and COS7 homogenates, respectively, whereas the human forms display $K_m$ values of 1.9 and 8.9 $\mu M$ for Pichia pastoris and COS7 homogenates, respectively. These values are in agreement with data obtained from previous studies (18, 22) using guinea pig and human liver microsomes (Table I). Specific activities obtained range from 30.3 (Pichia pastoris) to 150 (COS7) pmol/min $\times$ mg protein for the human enzyme, whereas guinea pig extracts have activities ranging from 42.0 (Pichia pastoris) to 221 (COS7) pmol/min $\times$ mg protein. In contrast, intact COS7 cells transiently transfected with plasmids expressing human or guinea pig 11β-HSD1 show a markedly higher affinity (approximately one order of magnitude) for cortisone reduction over homogenates with an apparent $K_m$ of 0.28 $\mu M$ for the guinea pig and 0.33 $\mu M$ for the human enzyme. Specific activities obtained in this system are similar and range from 173 pmol/10^6 cells $\times$ h to 191 pmol/10^6 cells $\times$ h for the guinea pig and human forms, respectively.

An analysis of COS7 extracts expressing human or guinea pig 11β-HSD1 using a wide range of cortisone substrate concentrations.

**Table II**

Kinetic constants for purified soluble 11β-HSD1 from human and guinea pig

| Species | Enzyme | Cortisone | Cortisol |
|---------|--------|-----------|----------|
| **Km** | **Vmax** | **Kcat** | **Km/Vmax** |
| Human | s, Δtm | 0.8 ± 0.2 | 21.8 ± 5.1 | 1.6 ± 0.7 | 1.5 ± 0.2 | 2.9 ± 0.4 | 141.3 ± 18.9 | 11.1 ± 2.8 | 3.8 ± 0.3 |
| Guinea pig | s, Δtm | 0.6 ± 0.3 | 18.8 ± 2.5 | 0.54 ± 0.05 | 1.3 ± 0.5 | 3.1 ± 0.9 | 67.6 ± 20 | 2.1 ± 0.6 | 0.63 ± 0.02 |
| h (45) | rec, fl | 0.6 | 3.8 | 0.12 | 0.20 | 1.7 | 69.8 | 2.2 | 1.31 |
| h (42) | n, l | 13.9 | 5.3 | 0.17 | 0.01 | 41.3 | 19.0 | 0.6 | 0.01 |
| r (44) | n, l | na | na | na | na | 1.63 | 0.7 | 0.02 | 0.014 |

* $K_{cat}$ values, based on active site titration data.
* $K_m$ values derived by protein concentration obtained through hydrolysis.
* Literature values reported for purified 11β-HSD1.
* This paper postulates cooperative kinetics for the reductive reaction.
* Reaction direction not specified for activity data given in this paper.
centrations reveals in each case a hyperbolic character of the graph, indicating the Michaelis-Menten kinetics of the enzyme. In particular, no indication for a cooperative behavior was obtained (Fig. 1, A and B).

**Kinetic Analysis of Soluble and Purified 11β-HSD1 from Human and Guinea Pig**—Heterologous expression experiments with transmembrane-deleted soluble 11β-HSD1 enzymes carrying an NH2-terminal His6 tag (19) were carried out in E. coli. Both proteins could be produced to a level of 1–5 mg/l culture and were purified to homogeneity (Fig. 2) and analyzed by steady-state kinetic measurements (Table II, Fig. 3). Using the arylsulfonamidothiazole inhibitor BVT.24829, active site titration was carried out with the human form showing 40% of active sites/mol purified protein (Fig. 4). Corresponding experiments with the guinea pig enzyme were not successful as revealed by the lack of inhibition (80% residual activity at 25 μM) (data not shown). Analyzing the 11-oxo-reductase activity with cortisone as substrate, the $K_m$ values determined are 0.8 μM for human and 0.6 μM for guinea pig. The $k_{cat}/K_m$ values obtained are 1.5 (human) and 1.3 (guinea pig) $\text{M}^{-1} \text{min}^{-1} \times 10^6$ with both enzymes displaying hyperbolic kinetic behavior (Fig. 4). With cortisol as substrate, $K_m$ values are 2.9 μM (human) and 3.1 μM (guinea pig), whereas $k_{cat}/K_m$ values range from 3.8 (human) to 0.6 $\text{M}^{-1} \text{min}^{-1} \times 10^6$ (guinea pig).

**Immunohistochemistry of Guinea Pig Liver and Adrenal Gland**—Histochemical localization studies of 11β-HSD1 were carried out in guinea pig tissues. In liver, intense immunoreactivity around the central vein is noted (Fig. 5A), in agreement with histochemical data from the human (23). In the adrenal gland, strong staining is observed in subcapsular areas corresponding to the Zona glomerulosa, whereas decreasing intensities are noted in areas corresponding to the Zona fasciculata and Zona reticularis (Fig. 5B). No staining is observed in the adrenal medulla (data not shown).

**DISCUSSION**

The enzyme 11β-HSD1 has been suggested as a critical determinant protecting against hypercortisolism in the guinea pig.
pig model of glucocorticoid resistance (17). However, a comparison of the kinetic data obtained from the guinea pig and the human enzymes (Tables I and II) indicates no significant differences in substrate affinities. Combined with the observation of higher expression levels in guinea pig liver versus human liver, these data argue against a role of 11\beta-HSD1 as a crucial determinant protecting against hypercortisolism as suggested previously (17) in the guinea pig model of glucocorticoid resistance (16, 24–27). Thus, neither in the squirrel monkey (another natural model to study cortisol resistance) (28) nor in the guinea pig does 11\beta-HSD1 seem to contribute to the observed peripheral resistance to corticosteroids (28). Rather, altered glucocorticoid receptor affinities, ACTH-mediated cortisol secretion, lowered plasma protein binding, and other interactions (16, 24–27) but not 11\beta-HSD1 are concluded to be the factors mediating resistance to glucocorticoids. Consequently, the importance of 11\beta-HSD1 in mammalian physiology is underscored by the lack of excess states such as apparent mineralocorticoid excess or Cushing syndrome in species displaying both dehydrogenase and labile reductase activities (16, 24–27). However, a further evaluation of the kinetic behavior was not further corroborated. Nevertheless, a possible role of the 11\beta-HSD1 in the aldosterone-synthesizing compartment may contribute to the regulation of catecholamine synthesis (14, 23, 30, 31). Consequently, the expression of 11\beta-HSD1 in rodents, absent or low levels of 11\beta-HSD1 in humans (17, 28, 29) and apparent isozymes are apparent (17, 28), indicating a possible role in central feedback control. Interestingly, we detected the expression of 11\beta-HSD1 in the Zona glomerulosa of guinea pig adrenal gland and lower levels in Zona fasciculata and Zona reticularis. This is in contrast to the expression of 11\beta-HSD1 in human adrenal gland in which higher levels in segments close to the medulla are found, suggesting involvement in the regulation of catecholamine synthesis (23, 30, 31). Consequently, the expression of 11\beta-HSD1 in the aldosterone-synthesizing compartment suggests regulatory roles of glucocorticoids in mineralocorticoid secretion or synthesis in guinea pig, similar to data obtained in rat (32).

Interpretation of kinetic data obtained for 11\beta-HSD1 has been complicated by the occurrence of bidirectional activity (i.e., displaying both dehydrogenase and labile reductase activities) in vitro in homogenized or purified material, whereas intact cells (transfected or primary culture) display reductase activity with little or no dehydrogenase activity (14, 28, 33–39). This effect partially reflects intracellular cofactor availability, because 11\beta-HSD1 is clearly a NADPH-dependent reductase and localized to the lumen of the endoplasmic reticulum (40). In most studies, the kinetic analysis of homogenates reveals an apparent \( K_m \) value for cortisone/dehydrocorticosterone in the low micromolar range (7, 17, 18, 22), whereas some researchers report higher \( K_m \) values (41, 42) for recombinant homogenates or purified material. These latter in vitro data are clearly inconsistent with the circulating glucocorticoid levels, which are in the nanomolar concentration range. Therefore, cooperative kinetic behavior has been suggested recently as an explanation of how a micromolar \( K_m \) is compatible with the in vitro function at nanomolar substrate concentrations in humans (42). A Hill coefficient of 1.6 obtained through regression analysis was reported, but allosteric behavior was not further corroborated. However, a \( K_{Hill} \) of 10 μM obtained in the study reported by Maer et al. (42) is still ~100 times higher than the physiological glucocorticoid levels, thus making cooperative kinetics an unlikely explanation with these data obtained. Furthermore, as observed in our study and by others (14, 28, 33–39), intact cells, transfected or as primary culture, display an apparent \( K_m \) clearly around the physiological level, i.e., between 100 and 400 nM. The observed affinity difference to homogenate material therefore points to further explanations. These explanations could be that the intact intracellular environment, e.g., lipids, stabilizes the reductive enzyme activity or that protein modification occurs, that the glucocorticoid substrate is concentrated at the intracellular expression site, i.e., the lumen of the endoplasmic reticulum where the catalytic center is located (40), or that cellular factors inhibit activity upon disruption of cell material. Finally, cooperative kinetic behavior is not observed in intact cells or homogenates neither in our work nor other studies (14, 28, 33–39). To investigate the possibility of cooperative kinetics, we performed incubations with homogenates or purified enzyme at substrate concentrations spanning several orders of magnitude, indicating in no case cooperative kinetic characteristics. Importantly, the purified recombinant soluble 11\beta-HSD1 material employed in our study displays no sign of sigmoidal kinetics. Therefore, we believe that the most probable explanation for the postulated allosteric behavior (42) is the occurrence of partial inactivation of the purified native enzyme, thus suggesting cooperative behavior deduced from non-linear regression analysis. This is in line with the 150–380-fold lower \( k_{Hill} K_m \) values of material prepared from human liver versus our purified 11\beta-HSD1 forms (Table II, compare references and data). To our knowledge, active site titration of purified 11\beta-HSD1 has never been performed earlier. In this study, we show that ~40–50% active sites/mol enzyme can be obtained by the protocol used, suggesting that all earlier purification attempts reached lower ratios of active molecules. Apparent cooperativity is frequently observed at low substrate concentrations with labile enzymes (43), and instability of 11\beta-HSD1 reductase activity upon purification has been noted already previously (44) and has consistently been confirmed in all purification studies using native material. However, here we demonstrate that purified recombinant 11\beta-HSD1 from human and guinea pig displays \( K_m \) values close to those observed in intact mammalian cells. This indicates that during purification of native enzymes, either the conditions or intrinsic factors are employed that destabilize the enzyme or that unidentified cellular factors inhibiting activity copurify. Importantly, we demonstrate high level expression and maintenance of kinetic characteristics compared with that of the enzyme from its native cellular environment upon purification. Consequently, heterologous expression in E. coli of engineered constructs lacking the transmembrane domain is a promising route to derive at structural and functional details of 11\beta-HSD1.

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