Functional Characterization of the Human 11β-Hydroxysteroid Dehydrogenase 1B (11β-HSD 1B) Variant[†]

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ABSTRACT: 11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD 1) catalyzes the interconversion of inactive into active glucocorticoids and has been shown to play a key role in metabolic disorders such as obesity and diabetes. 11β-HSD 1 belongs to the short chain dehydrogenases/reductases (SDR) and shares all common structural motifs typically for this protein superfamily. Unlike common SDRs, 11β-HSD 1 is N-terminally extended by a hydrophobic domain that anchors this enzyme in the endoplasmic reticulum (ER) membrane. Interestingly, the occurrence of 11β-HSD 1 transcripts lacking the N-terminal hydrophobic domain has repeatedly been reported in a variety of tissues, and the corresponding protein has been named 11β-HSD 1B. So far, no activity of 11β-HSD 1B has been observed, such that a physiological role could not be ascribed. In the present investigation, we showed for the first time that the truncated human 11β-HSD 1B form, expressed in the yeast *Pichia pastoris*, may indeed be active. However, this activity was prevented by the fact that 11β-HSD 1B is still kept attached to the ER membrane. Via computer assisted simulation and modeling, we identified a putative domain within the 11β-HSD 1 structure that could be responsible for this additional membrane attachment. By performing site-directed mutagenesis, heterologous expression, immunoblot analysis, and activity assays, we verified that this hydrophobic domain could indeed interact with the ER membrane and that some of the introduced mutations (V149R, V149E) led to a release of 11β-HSD 1B from membrane attachment without affecting its enzymatic activity. However, the activity of 11β-HSD 1B proved to be very unstable and was lost within hours after solubilization and release from the ER membrane. Importantly, 11β-HSD 1 constructs lacking the first 15 N-terminal amino acids and bearing additional amino acid substitutions (t15-V149R, t15-V149E) were then found to be soluble and to be stable in terms of enzyme activity. Combined, despite its occurrence in mammalian tissues, 11β-HSD 1B has obviously no physiological role since it is either inactive while being attached to the ER or it is rapidly losing activity once being released from intracellular membranes. Our findings with the t15-V149R and t15-V149E constructs are promising to further understand the complex mechanistical and structural properties of 11β-HSD 1.

Glucocorticoid hormone action in human cells is regulated via a pre-receptor control mechanism that is accompanied by interconversion of active cortisol to cortisone, the inactive 11-keto metabolite of glucocorticoids (*1*). This oxidoreduction of the hydroxy/oxo group in the 11 position of the glucocorticoid scaffold is catalyzed by two enzymes named 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD 1)[1] and type 2 (11β-HSD 2) (*2*). Both enzymes share common structural motifs e.g., a catalytically active triad of amino acids consisting of Ser, Tyr, and Lys residues and a glycine-rich N-terminal cosubstrate binding site with the consensus sequence Gly-X-X-X-Gly-X-Gly. Both motifs identify 11β-HSD type 1 and 2 as members of the short-chain dehydrogenase/reductase protein superfamily (SDR) (*3*). Despite their affiliation to the same protein superfamily and the same glucocorticoid substrate (cortisol), they share only 14% identity on the amino acid level (*4*).

The distribution of these enzymes depends on the function of the tissue. 11β-HSD 2 is found mainly in mineralocorticoid target tissues such as kidney and colon but has also been found in placenta. In these tissues, 11β-HSD 2 acts only as a dehydrogenase producing inactive cortisone, thereby protecting the mineralocorticoid receptor and the fetus from high levels of receptor-active cortisol (*5*). 11β-HSD 1 is found in a wide range of tissues, and although being bidirectional in tissue homogenates, acts predominantly as a reductase in intact cells and tissues, regenerating active cortisol from cortisone (*6*). Furthermore, in recent investigations it could be shown that 11β-HSD 1 plays an important role in xenobiotic carbonyl reduction (*7*).

11β-HSD 1 is anchored within the endoplasmic reticulum (ER) membrane with one single N-terminal hydrophobic domain. This domain is also responsible for the retention of 11β-HSD 1 in the ER (*8*, *9*). Interestingly, the active site protrudes into the lumen of the ER, and because of this lumenal orientation, 11β-human HSD 1 is glycosylated at three different glycosylation sites. However, glycosylation

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[1] Abbreviations: 11β-HSD, 11β-hydroxysteroid dehydrogenase; ER, endoplasmic reticulum; SDR, short-chain dehydrogenase/reductase.

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Table 1: Sequences of Sense Primer Oligonucleotides for Mutagenesis[a]

primer	
t-V148R	5′-TTC CTC AGT TAC **CG**G GTC CTG ACT GTA GCT GCC-3′
t-V149R	5′-CTC AGT TAC GTG **CG**C CTG ACT GTA GCT-3′
t-V149E	5′-CTC AGT TAC GTG G**AA** CTG ACT GTA GCT-3′
t-L150R	5′-AGT TAC GTG GTC C**G**G ACT GTA GCT GCC TTG CCC-3′

[a] Antisense primers were complementary to the above sequences. Nucleotides mutated from the wild-type sequence are in bold face and underlined.

is not necessary for proper enzyme function, as could recently be shown in several investigations (*10*, *11*).

Alignment of 11β-HSD 1 with other related SDR enzymes revealed that the SDR-core region is extended N-terminally in 11β-HSD 1 by the hydrophobic membrane anchor. This has led to the assumption that a truncated 11β-HSD 1, lacking the N-terminal hydrophobic domain, could be soluble and active. This assumption was further supported by the observation that the hydrophobic domain is part of a sequence of 30 amino acid residues that exactly match the first exon of the human 11β-HSD 1 gene. Moreover, the second exon starts with a codon for methionine, raising the possibility that this codon may serve as an alternative start site of 11β-HSD 1 gene transcription. Indeed, immunological studies suggested that truncated 11β-HSD 1 forms, commonly referred to as 11β-HSD 1B, might arise from alternative transcripts of the 11β-HSD 1 gene (*12*). In addition, Krozowski et al. (*13*) and Obeid et al. (*14*) reported the occurrence of truncated transcripts, mainly in kidney, where they appear to be about as abundant as the mRNA corresponding to the nontruncated 11β-HSD 1.

So far, many working groups have investigated the importance of the transmembrane domain in relation to activity (*14*, *15*). Various human 11β-HSD 1B forms, shortened by the amino acid residues encoded by exon 1, expressed in bacterial and yeast systems have been reported recently (*15*). Neither the constructs expressed in *Escherichia coli* with a N- or C-terminal His-tag nor the recombinant enzymes expressed in the yeast *Pichia pastoris* exhibited any activity. Furthermore, the 11β-HSD 1B constructs expressed in *E. coli* turned out to be insoluble like the full-length protein expressed in the same system, indicating that deletion of the hydrophobic domain did not improve the solubility. This observation is in accordance with the results published by Mercer et al. (*16*), who reported that expression of a N-terminally truncated 11β-HSD 1 in COS cells did not produce a soluble protein. Hydrophobicity plots confirmed the observation that 11β-HSD 1 is a very hydrophobic protein that is hardly soluble (*15*). In this respect, we could clearly show that 11β-HSD 1B expressed in *P. pastoris* remained still attached to the microsomes although lacking the hydrophobic membrane spanning domain.

Recently, Walker et al. (*11*) reported the bacterial expression and purification of 11β-HSD 1 constructs that were lacking the first 23 amino acid residues and that were found to be soluble and active. This finding raised the question of whether the seven amino acid residues (residues 24−30) of the N-terminus preserve the solubility and activity of the truncated 11β-HSD 1 constructs.

In the present investigation, we found that solubilization of *P. pastoris* microsomes by detergents led to an activation of human 11β-HSD 1B, which we suspected was due to a release from the ER membrane. However, soluble 11β-HSD 1B rapidly lost its activity casting doubt on a putative physiological role of the corresponding transcript. By computer assisted calculation of a three-dimensional 11β-HSD 1 model, amino acid residues critical for membrane affinity of the 11β-HSD 1 protein to the ER could be identified and were subsequently confirmed by site-directed mutagenesis, heterologous expression in *P. pastoris*, and activity determination. Furthermore, by generating 11β-HSD 1 constructs that were shortened by the first 15 amino acid residues and that were found to exhibit stable activities, we highlighted the importance of the amino acid sequence encoded by the second part of exon 1 for enzyme stability and activity. Combined, we succeeded in producing a soluble and active 11β-HSD 1B mutant, which is a further step in understanding the mechanistic and structural properties of this important enzyme in more detail.

EXPERIMENTAL PROCEDURES

Hydrophobicity Plots and Molecular Modeling. Prediction of hydrophobic membrane attaching domains within the 11β-HSD 1 primary structure was performed with the TMAP program (*17*) using multiple sequence alignments. This program was also used for our theoretical mutagenesis studies. The three-dimensional structure of 11β-HSD 1 was calculated with the SWISS-MODEL program (GlaxoSmith-Kline) and refined with the Weblab Viewer program (Molecular Simulations Inc.). The model structure was obtained based on an alignment of the 11β-HSD 1 primary structure with the known structures of 3α,20β-HSD from *Streptomyces hydrogenans* and of tropinone reductase 1 from *Datura stramonium*.

Site-Directed Mutagenesis. Mutations were introduced into the human 11β-HSD 1 cDNA by polymerase chain reaction (PCR). Primers corresponding to the 5′ and 3′ ends of the N-terminally truncated 11β-HSD 1B coding sequence contained restriction sites for *Sna*BI and *Avr*II, respectively, as has been described previously (*15*). The primer for generating the 11β-HSD 1 constructs shortened by the first 15 amino acid residues was 5′-ATGCCGCCGGTACGTAATGGC-CTACTACTACTA-3′. Primers used to introduce additional point mutations within the 11β-HSD 1 primary structure are shown in Table 1. In all cases (positions 148, 149, 150), the amino acids chosen were substituted by arginine, with the exception of valine in position 149, which was additionally substituted by glutamate. *Pfu*-DNA-polymerase, an enzyme with high proofreading activity, was used for PCR. After double restriction with *Sna*BI and *Avr*II, the respective cDNAs were introduced into the *P. pastoris* expression vector pPIC3.5K. The fragments inserted were completely sequenced to ensure that only the desired substitutions and no other mutations had taken place during the entire procedure.

Overexpression of Different Human 11β-HSD 1 Constructs in the Yeast P. pastoris. The overexpression of different

human 11β-HSD 1 constructs in the yeast *P. pastoris* was performed as described recently (*18*). The expression vector containing the mutant cDNA of 11β-HSD 1 was linearized with endonuclease *Sal*I for transformation of the *P. pastoris* strain GS115 by the spheroplasting method (*19*). After initial selection for His^+ transformants, a second selection was performed with the antibiotic G418 for recombinant strains with multiple plasmid integrations. Because of the linearization with endonuclease *Sal*I, *P. pastoris* strains with the phenotype His^+Mut^+ were generated. Six clones of each mutant with the highest resistance to G418 were tested for their expression of 11β-HSD 1. Each clone was grown in 150 mL minimal methanol medium (1.34% yeast nitrogen base, 4×10^{-5} % biotin, 1% methanol) at 30 °C for 5 days. After harvesting and mechanical disruption by using glass beads, crude yeast cell extracts were tested for 11β-HSD 1 activity and analyzed by Western blot as described below.

Preparation of Subcellular Fractions. The yeast was harvested by low speed centrifugation (Sorvall GSA, 4000 rpm, 2600*g*, 10 min, 4 °C) and washed once with 20 mL of water. After removal of the water by centrifugation (Sorvall SS-34, 5000 rpm, 3000*g*, 10 min, 4 °C), the yeast pellet obtained was resuspended in homogenization buffer (20 mM Tris/HCl, 250 mM sucrose, 1 mM EDTA, 0.1 mM PMSF, pH 7.4). Per gram of yeast cells (wet weight), 1.5 mL of homogenization buffer was used. After adding 0.5 g of glass beads per gram of yeast, the cells were mechanically disrupted for 1 min under CO_2 cooling in a Braun homogenizer (type 853032). The yeast cell extract was subsequently centrifuged at 4 °C (Sorvall SS-34, 3500 rpm, 1500*g*, 15 min) and filtered through a glass fiber filter to remove the class beads and cell debris. The preparation was then ultra-centrifuged at 4 °C (Beckmann SW28, 100 000*g*, 1 h). The supernatant represented the cytosolic fraction, and the microsomal pellet was resuspended in homogenization buffer (1 mL per 0.5 g of microsomes).

Enzyme Solubilization. For solubilization of membrane associated proteins, the microsomal suspension was diluted with an equal volume of 10 mM sodium phosphate buffer, pH 7.4, containing 1 mM EDTA, 40% glycerol (w/v), and 0.4% of the nonionic detergent Emulgen (Kao). The solution was gently stirred for 1 h at 4 °C and subsequently centrifuged at 140 000*g*. The supernatant was immediately used to determine enzyme activities because of a rapid decrease in activity of 11β-HSD 1B upon storage.

Determination of Protein Concentration. Determination of protein concentration was performed using the Bradford method (*20*) with bovine serum albumin as standard.

Analysis of Enzyme Activity. For quantitative analysis of 11β-HSD 1 activity, the oxidation of cortisol and the reduction of cortisone were monitored. In addition, the carbonyl reduction of the 11β-HSD 1 model substrate metyrapone was measured. Because of its sterical demand, metyrapone is not recognized by the majority of dehydrogenases/reductases and serves as a suitable substrate for 11β-HSD 1. The assay used was 30 μL of protein extract, 10 μL of 5 mM metyrapone (final concentration: 1 mM), 10 μL of a NADPH-regenerating system (2 mg of NADP, 6 mg of glucose-6-phosphate, 5 μL of glucose-6-phosphate dehydrogenase, 100 μL of 20 mM phosphate buffer, pH 7.4, 100 μL of 0.1 M magnesium chloride). The mixture was incubated for 30 min at 37 °C. The reaction was stopped by addition of 150 μL of acetonitrile. After vortexing and removal of proteins by centrifugation in a microfuge for 5 min, the supernatant was analyzed by HPLC. The oxidation of cortisol was performed with 10 μL of 10 mM NADP instead of the NADPH-regenerating system. The reaction times for cortisone were 180 min and for cortisol 60 min.

Metabolite Determination by HPLC. After enzymatic conversion, oxidized or reduced metabolites were detected on a Merck (Darmstadt, Germany) reversed phase HPLC system, with a LiChrosphere 100 RP-18 (5 μm) column, 4.5 $\times$ 25 mm. The following conditions were used for (a) metyrapone: the eluent was a solution of 0.1% ammonium acetate and 30% acetonitrile in water, pH 7.4, with a flow rate of 1 mL/min, absorbance was monitored at 254 nm and (b) cortisone/cortisol: the eluent was a methanol/H_2O (58:42, v/v) mixture at a flow rate of 0.5 mL/min, absorbance was monitored at 262 nm. The following retention times were achieved, metyrapone: 13 min; reduced metyrapone (metyrapol): 8 min; cortisone: 16 min; and cortisol: 20 min. Concentrations were calculated referring to corresponding calibration curves.

Kinetic Parameter Estimations. All kinetic measurements were performed at least two to four times, and deviations from the mean were less than 10%. The apparent kinetic parameters and statistical analyses were calculated by using the Graph-Pad Prism kinetic computer software.

Western Blot Analysis. SDS−PAGE was performed by the method of Laemmli (*21*) with 12% acrylamide gels. Ten micrograms of protein, either cytosolic fractions or resuspended microsomal fractions, were loaded to the gels in sample buffer. Electroblotting was performed in a semi-dry blotting system. Proteins were transferred to a nitrocellulose membrane, and antigen−antibody complexes were visualized by chemoluminescence (ECL PLUS-detection system, Amersham Pharmacia Biotech). Blots were probed with antisera raised against purified recombinant 11β-HSD 1 in a dilution of 1:50 000. The secondary antibody (peroxidase conjugated swine anti-rabbit immunoglobulin, DAKO) was used in a 1:4000 dilution.

RESULTS

Hydrophobicity Plots of the Complete Human 11β-HSD 1 Protein. The hypothesis that other domains, in addition to the N-terminal domain, anchor 11β-HSD 1 within the membranes of the ER is supported by hydrophobicity plots, characterizing 11β-HSD 1 as a very hydrophobic protein. By using the TMAP program, we identified two other putative domains located at amino acid residues 136−158 (domain b) and 197−220 (domain c) that could be responsible for membrane attachment of human 11β-HSD 1B (Figure 1). Furthermore, with this program theoretical mutagenesis studies were performed by exchanging hydrophobic amino acid residues in domain b against arginine, the most hydrophilic amino acid. For example, the substitution of valine in position 149 by arginine considerably decreased the hydrophobicity of the putative membrane attachment site b (Figure 1), which may affect membrane affinity of the entire protein.

Calculation of the Three-Dimensional Structure of Human 11β-HSD 1. To infer where the exchanged arginine is located within the 11β-HSD 1 structure, a theoretical three-

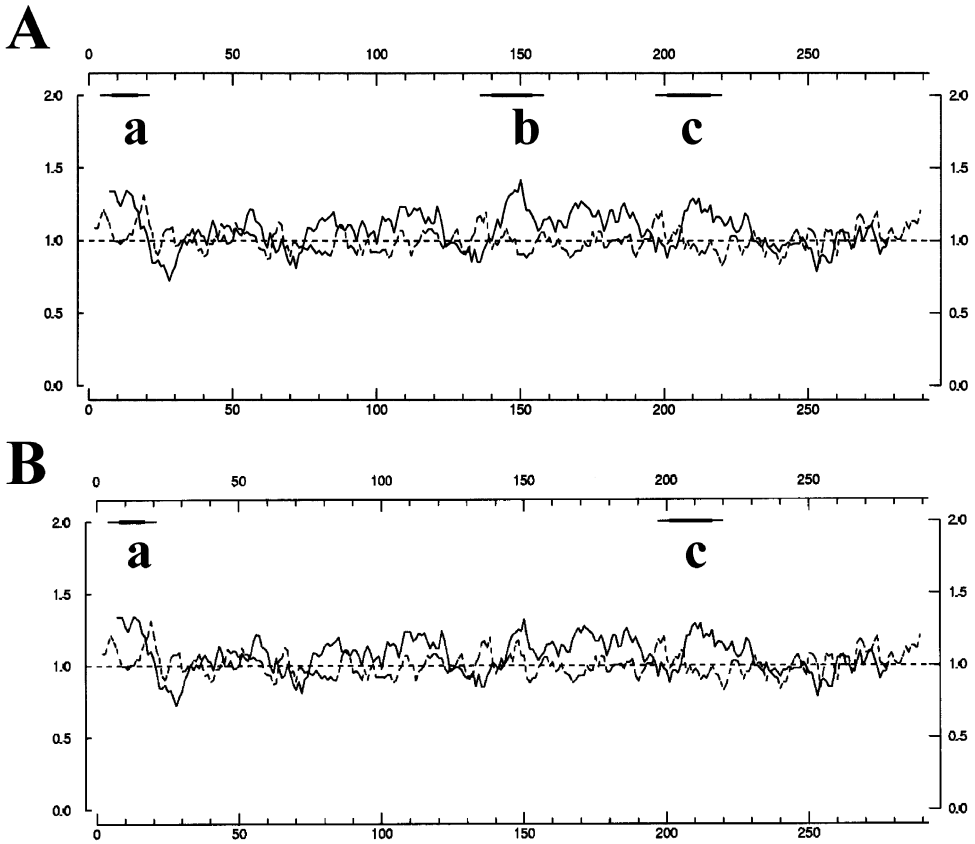

FIGURE 1: Hydrophobicity plot of human 11β-HSD 1. Hydrophobicity plot of native (A) and mutant (B) 11β-HSD 1 using the TMAP program. Letters a, b, and c indicate putative membrane attachment sites. (A) In addition to the N-terminal anchor (domain a), two further hydrophobic domains at amino acid positions 136−158 (domain b) and 197−220 (domain c) have been identified, which may be responsible for membrane attachment of 11β-HSD 1. (B) In the mutant 11β-HSD 1, valine in position 149 has been exchanged against arginine (V149R). This substitution decreases hydrophobicity in domain b and is suspected to affect membrane affinity of the entire protein.

dimensional structure of human 11β-HSD 1 was modeled by using the SWISS MODEL program (Figure 2). To affect membrane affinity and to improve solubility, the substituted arginine 149 should be located on the surface of the enzyme. On the other hand, if arginine 149 is directed to the interior of the protein, it may have an impact on the overall conformation of the enzyme that, in turn, could influence its activity. The structure of 11β-HSD 1 was modeled based on the alignment of the primary structure of 11β-HSD 1 with the known three-dimensional structures of 3α,20β-hydroxysteroid dehydrogenase from *S. hydrogenans* and tropinone reductase 1 from *D. stramonium*. As can be seen in Figure 2, valine 149 is indeed located on a surface helix of the 11β-HSD 1 protein that may serve as an additional membrane attaching domain. It should be mentioned that the model lacks N-terminally the first 28 and C-terminally the last 77 amino acid residues, but the calculation provides evidence that domain b (Figure 1) should be located on the surface of 11β-HSD 1. The putative membrane attaching domain c is represented by a β-strand in our model and contains the third of three glycosylation sites in human 11β-HSD 1.

Site-Directed Mutagenesis of Putative Membrane Attachment Sites within the 11β-HSD 1 Structure. On the basis of the above simulation and modeling calculations, site-directed mutagenesis experiments were performed to proof the in silico data by wet chemistry. A sequence motif of three amino acids within domain b was chosen for these experiments (valine 148, valine 149, and leucine 150) that represented the most hydrophobic part of domain b (Figure 3). The mutations introduced were V148R, V149R, V149E, and L150R. As mentioned above, arginine as the most hydrophilic amino acid was chosen to replace the original amino acid residues. In addition, glutamate, which is almost as hydrophilic as arginine but which is negatively charged, was introduced at position 149. This negative charge should disturb the interaction of domain b with the phospholipids of the ER membrane to a higher extent than the positively charged arginine. Likewise, amino acid residues adjacent to position 149 were substituted (V148R, L150R) because the underlying three-dimensional structure of 11β-HSD 1 only represents a theoretically calculated model. Normally, three to four amino acid residues form one turn in an α-helix. Therefore, the side-chain of at least one of the chosen amino acids should protrude onto the surface of 11β-HSD 1 and should be targeted by our approach.

Expression and Activities of 11β-HSD 1 Mutants in the Yeast P. pastoris. Truncated 11β-HSD 1, lacking the first 30 amino acid residues and commonly referred to as 11β-HSD 1B, could be successfully expressed in *P. pastoris*. However, no 11β-HSD 1 activity could be found in crude cell extracts, although the recombinant protein was synthesized as revealed by immunoblot analysis (not shown). Corresponding to the results with *E. coli* (*15*), 11β-HSD 1B appeared as a prominent band of approximately 29 kDa (Figure 4). Accordingly, this form does not undergo glycosylation in the yeast *P. pastoris*, as was the case with the complete protein. Interestingly, Western blot analysis gave clear evidence that 11β-HSD 1B in *P. pastoris* remained

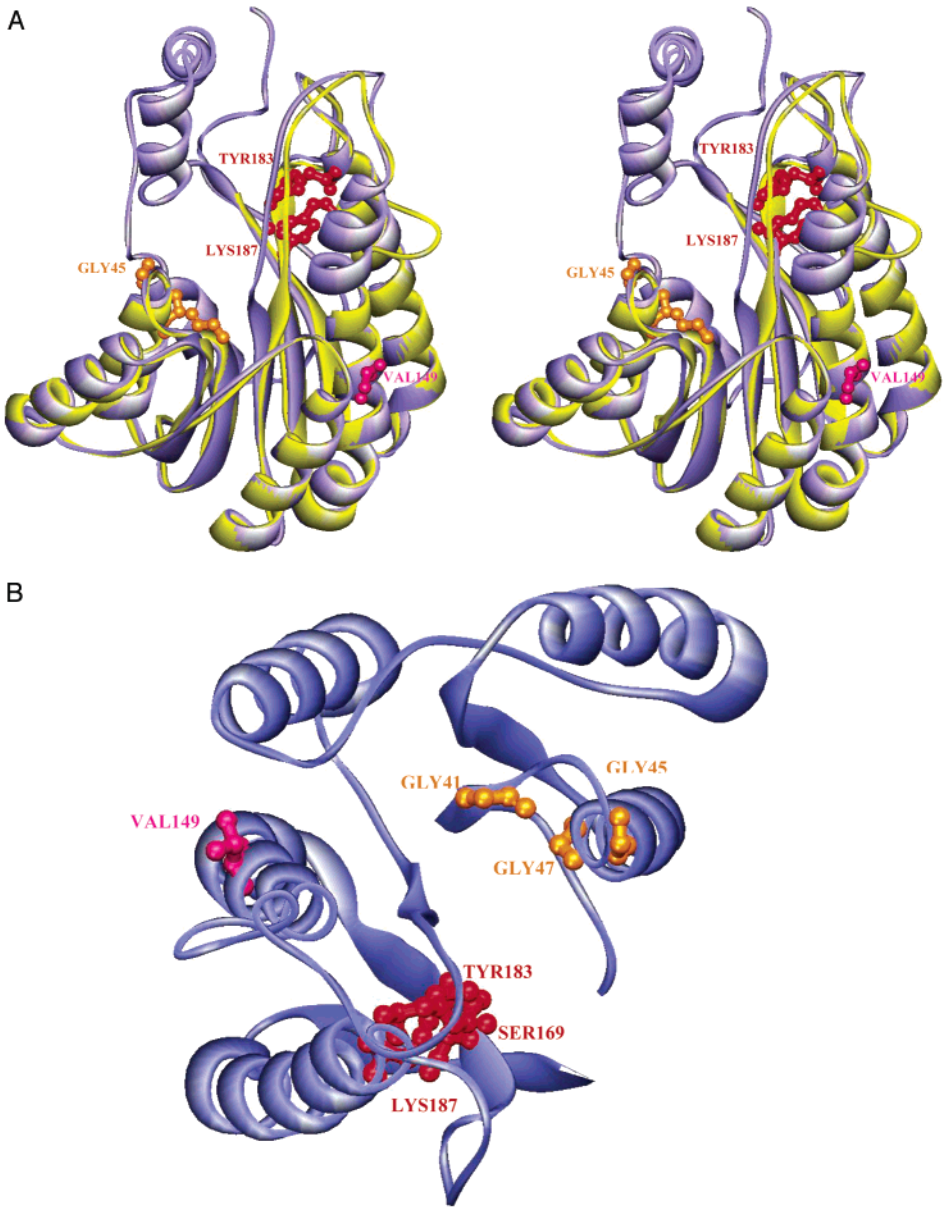

FIGURE 2: Calculated structure of human 11β-HSD 1. The structure of human 11β-HSD 1 was calculated by using the SWISS MODEL program and arranged with the Weblab Viewer (Molecular Simulations Inc.) program based on the alignment of its primary structure with the known three-dimensional structures of 3α,20β-HSD from *Streptomyces hydrogenans* and Tropinone reductase 1 from *Datura stramonium*. (A) Superposition stereo comparison of 11β-HSD 1 (yellow) and Tropinone reductase 1 (blue). (B) Enlargement of the 11β-HSD 1 critical domains. These are the catalytically active triad (Ser169, Tyr183, Lys187), the cofactor binding motif (Gly41, Gly45, Gly47), and Val149, the latter located on a surface helix of 11β-HSD 1 that may serve as an additional membrane attaching domain. Note that the model lacks N-terminally the first 28 and C-terminally the last 77 amino acid residues.

attached to the ER membrane after ultracentrifugation but that the respective microsomal fractions exhibited no activity (Figure 4). Surprisingly, solubilization of these fractions with Emulgen led to an activation of the truncated enzyme, apparently because of its release from the ER membranes by the detergent (Figure 4). In cytosolic fractions, neither 11β-HSD 1B protein nor activity could be detected. It should be noted that, for some unknown reason, 11β-HSD 1B activity after solubilization turned out to be unstable. Whereas the microsomal fractions could be stored for several months without effect, after solubilization the activity declined within a few hours at 4 °C; it was even lost within days, when the fractions were stored at −80 °C.

All mutant 11β-HSD 1B forms were expressed in the yeast *P. pastoris*. Western blot analysis of microsomal and cytosolic extracts from *P. pastoris* strains, expressing these 11β-HSD 1 constructs (Figure 4), revealed that only the truncated mutants t-V149R and t-V149E appeared to be soluble because of their appearance in the cytosolic fraction. From these soluble forms, only t-V149R was found to be enzymatically active. Interestingly, solubilization of the membrane-bound mutants t-V149R and t-L150R with Emulgen led to their activation, like nonmutant 11β-HSD 1B (Figure 4). In contrast, the mutant t-V148R was found only in the microsomes and turned out to be inactive, even when Emulgen was used to get the protein soluble. The results of these experiments clearly showed that the considered domain b could indeed interfere with the ER membrane and that the calculated structure is a valuable approach to the tertiary structure of 11β-HSD 1.

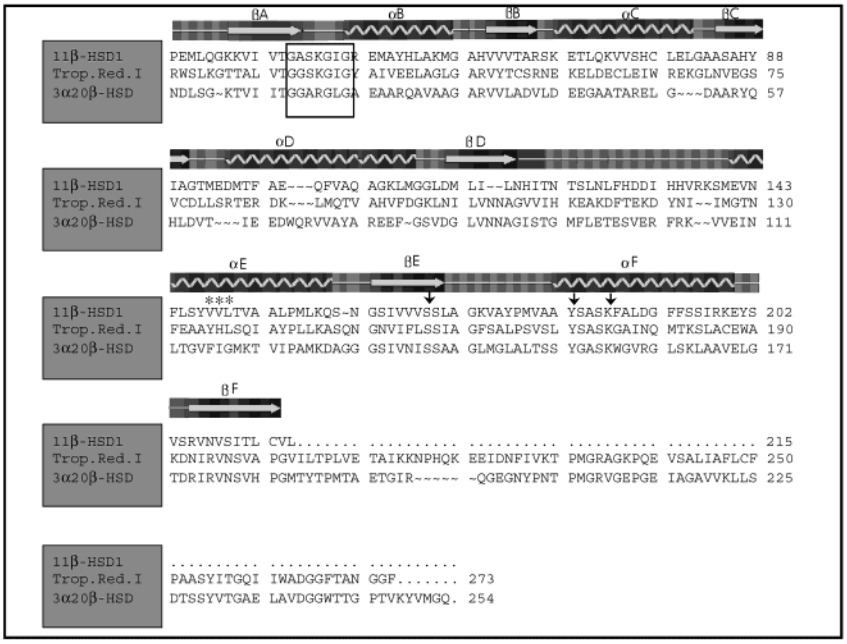

FIGURE 3: Alignment of human 11β-HSD 1 and two further representatives of the SDR protein superfamily. The alignment is based on the results with the SWISS MODELL program (cf. Figure 2). The 11β-HSD 1 secondary structure elements are shown on the top of the alignment. The cofactor binding domain is boxed, and the catalytically active triad is marked by arrows. The hydrophobic amino acid residues that were exchanged in the course of this study are indicated by asterisks. 3α,20β-HSD, 3α,20β-HSD from *S. hydrogenans*; Trop.Red. I, Tropinone reductase 1 from *D. stramonium.* Note that the sequence of 11β-HSD 1 lacks N-terminally the first 28 and C-terminally the last 77 amino acid residues.

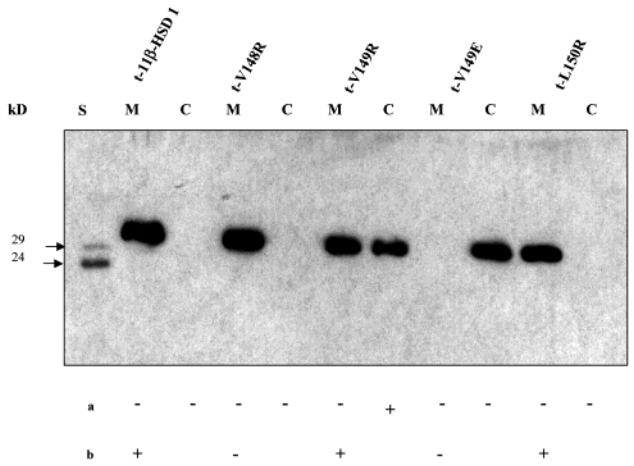

FIGURE 4: Immunoblot analysis and activity determination of 11β-HSD 1B mutants. Upper panel: Microsomal (M) and cytosolic (C) fractions from *P. pastoris* strains, expressing various 11β-HSD 1B mutants, were analyzed by immunoblot. Only mutants t-V149R and t-V149E appear as soluble forms in the cytosolic fraction. S = molecular mass standard. Lower panel: Enzymatic activity of 11β-HSD 1B mutants. Line a shows the activity of microsomal or cytosolic preparations. From these, only the soluble mutant t-V149R exhibits activity (+). Line b shows the activity of the microsomal extracts after solubilization with Emulgen as detergent. Here, t-11β-HSD 1 (=11β-HSD 1B) as well as its mutant forms t-V149R and t-L150R gain activity.

Solubilized 11β-HSD 1B mutants that exhibited activities were tested with different glucocorticoid and metyrapone concentrations as substrates (Figure 5). A clear concentration dependency becomes evident, although enzyme activities could only be detected at relatively high substrate concentrations (cortisol 140−400 μM; metyrapone 0.25−1 mM). In addition, whereas the respective 11β-HSD 1B mutants show cortisol dehydrogenation and metyrapone carbonyl reducing activities, no activity could be detected with cortisone as substrate. This was apparently because of the long incubation periods (180 min at 37 °C) required for detecting cortisol formation, during which the labile 11β-HSD 1B mutants had lost their activities. Nevertheless, these findings raised the question if the extension of amino acid residues 24−30 at the N-terminus determine stability and activity, as has been reported by Walker et al. (*11*).

In focusing on the critical role of the N-terminus of 11β-HSD 1 for enzyme stability, two constructs, shortened by the first 15 amino acid residues, were designed: the first carrying the V149R substitution (t15-V149R) and the second carrying the V149E substitution (t15-V149E). The t15-deletion was chosen since amino acid residue 16 is a methionine in the 11β-HSD 1 primary structure. The subcellular localization and activity of the resulting constructs were compared with respective mutants of full-length 11β-HSD 1 and those of 11β-HSD 1B (Figure 6; Table 2). The full-length proteins were found only in the microsomal fraction and turned out to be active with kinetic parameters very similar to the recombinant wild-type 11β-HSD 1 (Table 2, ref *18*). These results emphasize the fact that introducing a substitution at position 149 does not affect activity. Both 11β-HSD 1 mutants, lacking the first 15 amino acid residues (t15-V149R and t15-V149E), behave like the truncated protein shortened by the domain encoded by the entire first exon (t-V149R and t-V149E) with regard to the subcellular localization. Both mutant V149R forms were found in the microsomal as well as in the cytosolic fraction. However, only the cytosolic fraction appeared to be active, whereas the microsomal fraction turned out to be inactive. Releasing V149R constructs from the ER membrane, because of solubilization with Emulgen as detergent, led to an activation of the microsomal bound enzymes. In contrast to the 11β-

Table 2: Kinetic Parameters of Nontruncated and Truncated Human 11β-HSD 1 Forms, Expressed in the Yeast *P. pastoris*, toward Its Physiological Glucocorticoid Substrates[a]

	11β-HSD1-V149R		11β-HSD1-V149E		t15−11β-HSD1-V149R		t15−11β-HSD1-V149E	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
cortisone	109	0.4	113	0.4	99	0.7	95	0.5
cortisol	103	1.9	99	2.1	90	1.9	93	2.3

[a] Glucocorticoid oxidoreduction was determined as described under Experimental Procedures. Data obtained are the means from $n = 4$ independent experiments. $K_m = \mu M$; V_{max} = nmol/(mg min).

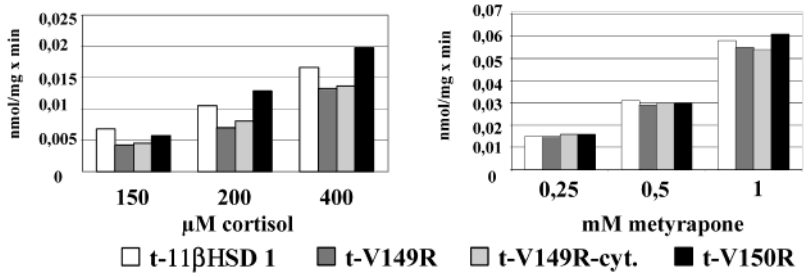

FIGURE 5: Activity testing with soluble or solubilized 11β-HSD 1B mutants. Soluble (t-V149R-cyt.) or solubilized (t-11β-HSD 1, t-V149R, t-L150R) 11β-HSD 1B mutants that exhibited 11β-HSD 1 activity were tested for enzyme activity. A clear concentration dependency for cortisol dehydrogenation (left) and metyrapone carbonyl reduction (right) becomes evident.

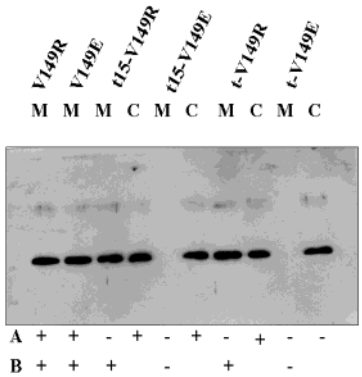

FIGURE 6: Expression of various truncated 11β-HSD 1 forms in the yeast *P. pastoris*. Three 11β-HSD proteins, differing in length of their N-terminus, were expressed in the yeast *P. pastoris*. All constructs bear a substitution of valine 149 either against arginine or against glutamate. Protein identification was performed by Western blot analysis of the microsomal (M) and cytosolic (C) fractions. Line A shows the activity of microsomal or cytosolic preparations and line B the activity of the microsomal extracts after solubilization with Emulgen as detergent.

HSD 1B mutant V149R, the activity of t15-V149R was stable and could be stored. This is also the case with the mutant form t15-V149E that exhibited a stable activity, whereas the t-V149E mutant was completely inactive. Both V149E mutants were found only in the cytosolic fraction. No V149E constructs remained bound to the microsomes as indicated by Western blot analysis. The kinetic values of the t15-11β-HSD 1 forms are in good agreement with the kinetic parameters found with the full-length proteins (Table 2).

DISCUSSION

Since Monder and Lakshmi per immunoblot analysis have identified a 26 kDa species of 11β-HSD 1 in rat brain (*12*) and Krozowski et al. described multiple forms of 11β-HSD 1 mRNA in rat kidney (*13*) (which was confirmed by an investigation of Moisan et al. (*22*)), several working groups focused on the question of whether a truncated form of 11β-HSD 1, lacking the entire peptide sequence encoded by the first exon, does exist and whether this truncated enzyme could play a physiological role. In a further study, Krozowski et al. (*23*) confirmed the tissue-specific expression of the 11β-HSD 1 gene in rat kidney by S1 nuclease protection assays and sequencing the obtained mRNA species. By this approach, they could indeed identify a transcript of the 11β-HSD 1 gene lacking the sequence corresponding to the first exon. Furthermore, they, for the first time, named this predicted protein 11β-HSD 1B, in contrast to the full-length enzyme that was designated 11β-HSD 1A. The latter study was supported by investigations performed by Moisan et al. (*24*), who were concerned with the cis-acting regulatory elements of the rat 11β-HSD 1 gene. Using primer extension and RNA protection analysis, they found a predominant transcription start site in the rat liver 11β-HSD 1 gene, resulting in a full-length 11β-HSD 1 protein, whereas in rat kidney a further transcription start site within intron A could be identified. The predicted protein turned out to be truncated by the first 26 amino acid residues and was found to be identical to 11β-HSD 1B.

In vitro transcription/translation experiments as well as expression of rat 11β-HSD 1B in Chinese hamster ovary (CHO) (*14*) and COS (*16*) cells yielded a recombinant protein with a molecular mass of approximately 26 kDa, suggesting lack of glycosylation. Importantly, the recombinant 11β-HSD 1B form failed to exhibit any activity. The lack of activity could have several reasons (e.g., the absence of the carbohydrate residues caused by incorrect posttranslational processing or a decrease in stability of the truncated protein, as has been discussed by the authors). Unfortunately, neither the role of the N-terminal transmembrane domain as a targeting signal was investigated, which is responsible for the retention of 11β-HSD 1 in the ER membrane, nor was tested whether the truncated enzyme is soluble. Only it was hypothesized that a truncated 11β-HSD 1 may be directed to an intracellular compartment other than the ER.

The state of knowledge regarding the question of whether a 11β-HSD 1B enzyme does exist and of whether such a protein could play a physiological role was summarized by Stewart and Krozowski in 1994 (*25*). At that time, the interest

on this topic tired because of the facts that expression experiments led to inactive enzymes only and that truncated 11β-HSD 1B forms had never been detected in human tissues.

Nevertheless, the question has still been considered in some recent publications (*26*−*28*). Walker et al. (*11*) reported the bacterial expression and purification of a 11β-HSD 1 form that was truncated by the first 23 amino acid residues and that was enzymatically active. Simultaneously, we reported the expression of human 11β-HSD 1B in *E. coli* and *P. pastoris* (*15*). We found that deleting the amino acid residues encoded by the first exon did not improve solubility. In contrast, the protein expressed in *E. coli* was found to be packed in inclusion bodies, and furthermore, like the full-length protein could only be purified under denaturating conditions (i.e., by using guanidinium hydrochloride as detergent (*18*)). Likewise, human 11β-HSD 1B expressed in *P. pastoris* turned out to be inactive, and as expected, was not found in the cytosolic fraction. To our surprise, we, by immunoblot analysis, could clearly show that the truncated 11β-HSD 1B still remained attached to the ER membrane. This finding has never been reported before and was the first indication for the existence of at least one more domain within the 11β-HSD 1 protein that could interact with the ER membrane. These results, together with those published by Walker et al. (*11*), prompted us to focus on possible interferences of the truncated human 11β-HSD 1B with the microsomal membrane in *P. pastoris*, and moreover, arose the question if the seven amino acid residues 24−30 on the N-terminus determine stability and activity of the catalytic domain of human 11β-HSD 1.

Indeed, release from the ER membrane by solubilization with Emulgen led to an activation of the truncated enzyme. This indicates that attachment to the ER membrane prevents 11β-HSD 1B from being enzymatically active. Furthermore, we showed for the first time that 11β-HSD 1B exhibits activity, but it should be noted that the activity was very unstable. The microsomal fraction could be stored for several months, but after solubilization the activity declined in a few hours. Combined, the conclusion could be drawn that 11β-HSD 1B does not play a physiological role. If such a protein indeed is expressed in normal cells, it should not exhibit enzymatic activity because of either membrane attachment or instability of the free enzyme.

The mechanism of inactivation of the truncated enzyme caused by membrane attachment remains speculative. Two explanations seem possible: First, the substrate and/or cofactor do not have access to the active site of 11β-HSD 1B because of a sterical hindrance of this domain through the ER membrane. Second, the truncated enzyme is tightly fixed to the ER membrane, such that the protein cannot undergo the conformational changes required for the catalytic process. Although hydrophobic substrates such as the glucocorticoids should be able to freely cross the ER membrane, the membrane bound form of 11β-HSD 1B was not found to exhibit any glucocorticoid oxidoreduction activity. This observation leads to the assumption that, if the first hypothesis is correct, the failure of cofactor binding to the active site is critical for activity. On the other hand, the second hypothesis would be in agreement with results published recently, where 11β-HSD 1 was shown to be active as a homodimer that exhibits enzyme cooperativity (*29*).

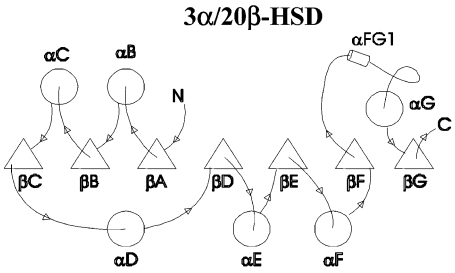

FIGURE 7: Folding topology of SDR enzymes. The common folding topology of SDR enzymes is shown for 3α,20β-HSD from *S. hydrogenans* as an example (derived from Ghosh et al. (*31*)). α-Helices are represented as circles and β-strands as triangles. Human 11β-HSD 1 is supposed to have the same folding pattern as 3α,20β-HSD. If this is true, the hydrophobic membrane attaching domain b is located within α-helix E, and therefore, is positioned on the surface of 11β-HSD 1. In contrast, domain c is part of β-strand E, and therefore, protrudes to the interior of the whole protein. It is clear from these calculations that amino acid substitutions in domain b alter the membrane affinity of 11β-HSD 1B, whereas mutations in domain c would affect the entire three-dimensional architecture.

To answer the question of how 11β-HSD 1B is attached to the ER membrane, we took advantage of computer based simulation and modeling programs. By using the TMAP program, we identified two hydrophobic domains located at amino acid residues 136−158 (domain b) and 197−220 (domain c) that could be responsible for membrane attachment of human 11β-HSD 1B. To infer where the considered domains are positioned within the three-dimensional architecture, the structure of human 11β-HSD 1 was modeled based on the alignment of its primary structure with the known three-dimensional structures of 3α,20β-HSD from *S. hydrogenans* and Tropinone reductase 1 from *D. stramonium*.

Like 3α,20β-HSD and Tropinone reductase 1, 11β-HSD 1 belongs to the SDR protein superfamily. All members of this protein superfamily have some common structural features, although the amino acid sequence identities are very low, ranging around 15−30% (*30*). One of these structural features is known as the Rossmann fold, a sequence of alternating α-helices and β-strands that has been found in all tertiary structures of SDR enzymes solved so far (Figure 7). The β-strands form a four- or five-stranded parallel β-sheet with two or three α-helices residing on either side, which is characteristic for many NADH- and NADPH-binding domains. A comparison of five SDR structures revealed that, although there are only 11 fully conserved residues common to the five structures, the three-dimensional conformation of these SDR members is highly conserved (*31*). Furthermore, SDR enzymes have a catalytically active triad consisting of Ser, Tyr, and Lys residues and a glycine-rich N-terminal cofactor binding site with the consensus sequence Gly-X-X-X-Gly-X-Gly.

3α,20β-HSD from *S. hydrogenans* was the first SDR enzyme whose structure was solved (*32*). Interestingly, glycyrrhizic acid and carbenoxolone, two potent inhibitors of both 11β-HSD isozymes, do also act as competitive inhibitors of 3α,20β-HSD (*33*). This finding suggests similarities between the catalytic centers of 3α,20β-HSD and the two 11β-HSD isozymes, a fact that may be extrapolated to the entire three-dimensional structure of 11β-HSD 1 (although the 11β-HSD 1 model structure lacks N-terminally the first 28 and C-terminally the last 77 amino acid residues).

Interestingly, the model structure gives some evidence that the putative membrane attaching domain b should be an α-helix and should locate on the surface of 11β-HSD 1. Simulation of mutagenesis studies, performed with the TMAP-program, revealed that substituting valine in position 149 by a hydrophilic amino acid should best disturb membrane attachment and improve solubility of 11β-HSD 1. Furthermore, together with the neighboring valine in position 148 and leucine in position 150, these three amino acid residues represent the most hydrophobic motif within the putative membrane attaching domain b. Indeed, immunoblot analysis of microsomal and cytosolic fractions from *P. pastoris*, expressing various mutant 11β-HSD 1B proteins, revealed that only mutants t-V149R and t-V149E appeared to be soluble because of their occurrence in the cytosolic fraction. From these soluble forms, only t-V149R was found to be enzymatically active.

The lack of activity of the soluble 11β-HSD 1B mutant t-V149E might be explained by the observation that active 11β-HSD 1B mutants and nonmutant 11β-HSD 1B lose their activity within a few hours. Apparently, initial association to the ER membrane is necessary for correct protein folding to yield an active enzyme. Upon release of the various forms (nonmutant 11β-HSD 1B, t-V149R, and t-L150R) from the ER membrane by detergent solubilization, the activity disappears over a period of a few hours. In contrast, mutant t-V149E is only found in the cytosolic fraction. Obviously, the negatively charged glutamate completely prevents membrane attachment, a condition that may not allow correct protein folding and resulting in an inactive enzyme. In contrast, t-V149R is found both in the cytosol as well as in the microsomes. Here, it is possible that the positively charged arginine allows initial protein folding at the ER membrane and active t-V149R is then released to the cytosol upon preparation of the cell extracts.

The mutant form t-V148R was found exclusively in the microsomes and turned out to be inactive, even when Emulgen was used to get the protein soluble. Hence, site-directed mutagenesis experiments clearly showed that valine in position 148 is not interchangeable without disrupting the architecture of the Rossmann fold, which is not the case with valine 149 and leucine 150. These findings corroborate the prediction from Duax et al. (*34*) that, despite little sequence similarity, the overall architecture of the Rossmann fold within the SDR protein superfamily may tolerate some mutations at various sites without loss of activity.

Furthermore, this prediction was the reason for why we focused our studies on domain b and not on domain c. In the model structure of human 11β-HSD 1, domain c corresponds to the β-strand E within the Rossmann fold (Figure 7). Introducing mutations into this β-strand should have considerable impact on the entire three-dimensional structure of 11β-HSD 1.

On the other hand, our results provide evidence that the first 30 N-terminal amino acid residues within the enzyme may be important for stabilizing the overall 11β-HSD 1 architecture. To prove this hypothesis, two 11β-HSD 1 mutants shortened by the first 15 amino acid residues were compared concerning their stability and kinetic properties with full-length 11β-HSD 1 and truncated 11β-HSD 1B forms. Both truncated mutant forms, lacking only the first 15 amino acid residues (t15-V149R and t15-V149E), exhibited activity upon expression in *P. pastoris*. Importantly, the activity turned out to be stable, a fact that emphasizes the role of the hydrophilic amino acids 24−30 of the N-terminal peptide to ensure enzyme function.

In conclusion, this study has shown for the first time that the truncated 11β-HSD 1B enzyme, lacking the complete peptide sequence encoded by the first exon, exhibits activity. This activity, however, is prevented by attachment to the ER and is seen only upon release of 11β-HSD 1B from the membrane by detergent solubilization. Unfortunately, solubilized 11β-HSD 1B turned out to be unstable, such that the activity declines within a few hours. Interestingly, computer based simulations and modeling of the 11β-HSD 1 structure, combined with wet chemistry (site-directed mutagenesis, heterologous expression, and activity determination), led to the identification of a hydrophobic domain that could interact with the ER membrane and that may be responsible for additional membrane attachment of truncated 11β-HSD 1 forms. Substitution of a critical hydrophobic residue by a hydrophilic amino acid within this domain, combined with a deletion of the first 15 hydrophobic amino acids at the N-terminus, led to the soluble and active 11β-HSD 1 mutants t15-V149R and t15-V149E. The results of the present study clearly shows that, although occurring in mammalian tissues, 11β-HSD 1B could not play a physiological role. A critical research on the N-terminus and additional membrane attachment sites might constitute an useful approach to further understand the complex mechanistical and structural properties of 11β-HSD 1.

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