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TYR-179 AND LYS-183 ARE ESSENTIAL FOR ENZYMATIC ACTIVITY OF 11ß- HYDROXYSTEROID DEHYDROGENASE

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Summary: Tyr-179 and Lys-183 are likely to be functionally important residues in 11ß-hydroxysteroid dehydrogenase, as these amino acids are absolutely conserved in all members of the "short chain dehydrogenase" family. We modified these residues by site-directed mutagenesis of rat cDNA and transfected these constructs into CHO cells. A highly but not absolutely conserved residue, Asp-110, was also studied. Mutation of Tyr-179 to Phe or Ser completely abolished enzymatic activity (interconversion of corticosterone and 11-dehydrocorticosterone), as did Lys-183→Arg. Asp-110→Asn affected activity only mildly. Tyr-179 and Lys-183 may be directly involved in the catalytic function of this class of enzymes.

The interconversion of cortisol and cortisone (or corticosterone and 11-dehydrocorticosterone in rodents) is catalyzed by 11β-hydroxysteroid dehydrogenase (11-HSD, EC 1.1.1.146) using NADP+ or NADPH as cofactors in the dehydrogenase and oxoreductase directions respectively. The dehydrogenase activity may be of considerable physiological importance in the kidney; since the renal Type 1 (mineralocorticoid) receptor has identical *in vitro* affinities for cortisol and aldosterone, it has been suggested that 11-HSD provides ligand specificity to this receptor by deactivating cortisol to cortisone, allowing aldosterone to regulate sodium homeostasis (1,2).

An enzyme with 11-HSD activity was purified from rat liver microsomes and shown to be a glycoprotein with a molecular weight of 34,000 (3). The purified enzyme catalyzed dehydrogenation of corticosterone to 11-dehydrocorticosterone but did not display the reverse oxoreductase activity. A cDNA clone encoding this enzyme was then isolated by screening a rat liver cDNA library in λgt11 with an antiserum against 11-HSD. The complete cDNA contained an insert of 1265 base pairs (bp) with an 861 bp open reading frame. This clone was successfully expressed in cultured mammalian cells (4). The encoded enzyme was found to be

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active both as a dehydrogenase and an oxoreductase with similar kinetic constants for both activities (5).

A search of sequence databases revealed that 11-HSD is structurally related to other "short-chain dehydrogenases" from mammals and lower organisms. Such enzymes all have molecular weights of about 30kD. Highly conserved amino acids in these enzymes are clustered near the amino terminus in a region proposed to constitute part of the binding site for the cofactor, which in the case of 11-HSD is NADP+ or NADPH. An alignment of ten short chain dehydrogenases (6) suggested the presence of three absolutely conserved residues distal to this area. In rat 11-HSD, these are Asp-110, Tyr-179 and Lys-183. To determine if these residues were important to the catalytic function of the enzyme, we introduced conservative substitutions at these positions and expressed the mutant enzymes in cultured mammalian cells.

MATERIALS AND METHODS

Mutagenesis. Mutations were introduced into rat 11-HSD cDNA (4) in two consecutive rounds of PCR (7). Primers used corresponded to the 5' and 3' ends of the cDNA and included sites for restriction enzymes *Bgl* II and *Hind* III respectively (TGG AGA TCT GTT ATG AAA AAA TAC CTC C and AAG AAG CTT CAC CAG GCC TCA GGA G). Primers necessary to introduce the mutations Asp-110→Asn, Tyr-179→Ser, Tyr-179→Phe and Lys-183→Arg included G GGT GGA CTG *A*AC ATG CTC AT, ATT GCT TCC T*C*C TCT GCA AGC, ATT GCT TCC T*T*C TCT GCA AGC, and TCT GCA AGC A*G*G TTT GCT CTG respectively, and their complements. All were synthesized using an Applied Biosystems 391EP DNA synthesizer.

Amplified normal and mutant cDNA segments were digested with *Bgl* II and *Hind* III, gel purified and subcloned into pCMV4 (8). Plasmid DNA was purified either by banding in ethidium bromide-CsCl gradients or by PEG precipitation. The cDNA insert of each construct was fully sequenced (9).

Expression in Mammalian Cells. Chinese hamster ovary (CHO) cells were cultured in 60 mm plates in Ham's F12 medium supplemented with 10% fetal calf serum and grown to about 85% cell confluence. Transfection was accomplished by incubating the cells in 3 ml of Opti-MEM (Gibco BRL Life Technologies, Gaithersburg, MD) containing 30μl of Lipofectin cationic liposomes (Gibco BRL) (10) and 10μg of plasmid DNA followed by culturing the cells for 48 hours in Ham's F12 medium with 20% fetal calf serum.

Enzymatic activities of transfected cells were determined by adding 1 μCi of [^{3}H]-corticosterone or dehydrocorticosterone to the medium. Unlabelled steroids were also added to a final concentration of 10 nM. Cells were incubated for another 8 hours. The medium was extracted using methylene chloride, dried under N_2 and applied to silica gel thin layer chromatography plates. Plates were developed in 20:1 chloroform:methanol and radioactivity detected with a Bioscan System 200 Imaging Scanner (Bioscan, Washington, D.C.).

Immunoprecipitation of Radiolabelled Proteins. Cells were transfected as described above. After 48 hours of incubation in Ham's F12 medium with 20% fetal calf serum, the medium was changed to a methionine/cysteine free medium with Tran^{35}S-Label (ICN Biochemicals, Irvine, CA), and cells were incubated for two additional hours. Cells were then washed once in phosphate buffered saline and lysed in 250 μl of 0.01M Tris HCl, pH 8.0/ 1%Triton X-100/ 0.1% sodium dodecyl sulfate/ 1mM EDTA/ 100 μg/ml phenylmethylsulfonyl fluoride. Lysates were transferred to micro-centrifuge tubes and 300 μl of dilution buffer (0.1M Tris HCl, pH 8.0/ 0.14M NaCl/ 0.1% Triton X-100) was added. Lysates were precleared by successive 1/2 hour incubations on ice with 5 μl of normal rabbit serum and 1:10 volume of protein A-Sepharose (Pharmacia, Piscataway, NJ). After centrifugation 2 μl of rabbit antirat 11-HSD

serum (a gift from C. Monder (3)) was added to the supernatant and incubated for 2 hours at 4°C. Proteins were then precipitated with protein A-Sepharose and washed three times in dilution buffer, once in 0.1 M Tris HCl, pH 8.0/ 0.14 M NaCl, and twice in 0.05 M Tris HCl, pH 6.8 (11). Samples were boiled in Laemmli sample buffer and subjected to SDS-polyacrylamide gel electrophoresis in a 12% gel followed by autoradiography.

RESULTS

Asp-110→Asn, Tyr-179→Ser, Tyr-179→Phe and Lys-183→Arg all permitted expression of an adequate amount of protein when transfected into CHO cells, as detected by immunoprecipitation (Fig.1).

When expressed in CHO cells, wild type 11-HSD displayed more prominent oxoreductase than dehydrogenase activity resulting in the conversion of most dehydrocorticosterone to corticosterone in an 8 hour incubation. Tyr-179→Ser, Tyr-179→Phe and Lys-183→Arg abolished both dehydrogenase and oxoreductase activities (Fig 2).

Asp-110→Asn retained most of its activity in both directions. No significant difference in dehydrogenase activity could be detected after 8 hours of incubation with the substrates (29% conversion from corticosterone to 11-dehydrocorticosterone by the wild type enzyme vs. 27% for Asp-110→Asn). There was a more significant difference in the oxoreductase direction (62% conversion by the wild type vs. 35% for Asp-110→Asn).

DISCUSSION

Our original multiple sequence alignment of short chain dehydrogenases (6) led us to speculate that Asp-110, Tyr-179 and Lys-183 might all be critical for catalytic activity of this class of enzymes in a manner reminiscent of the His, Asp and Ser triad of serine proteases. Further evidence supported the possible functional importance of Tyr-179 and Lys-183 but not

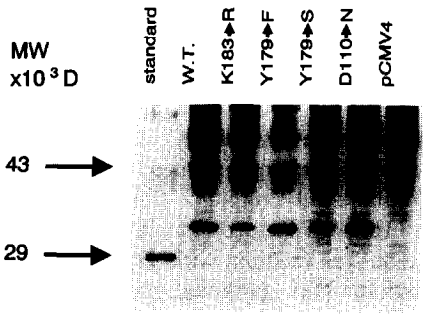

Figure 1. Immunoprecipitates of [^{35}S]-labeled cell lysates with anti-11-HSD serum, resolved by SDS-polyacrylamide gel electrophoresis. Molecular weight markers are indicated by arrows. **W.T.**, cells transfected with wild type 11-HSD cDNA; **K183R**, cells transfected with Lys-183→Arg mutant cDNA; **Y179F**, Tyr-179→Phe; **Y179S**, Tyr-179→Ser; **D110N**, Asp-110→Asn. **pCMV4** without an insert was used as a negative control. The 11-HSD polypeptide migrates as being 34 kDa in size.

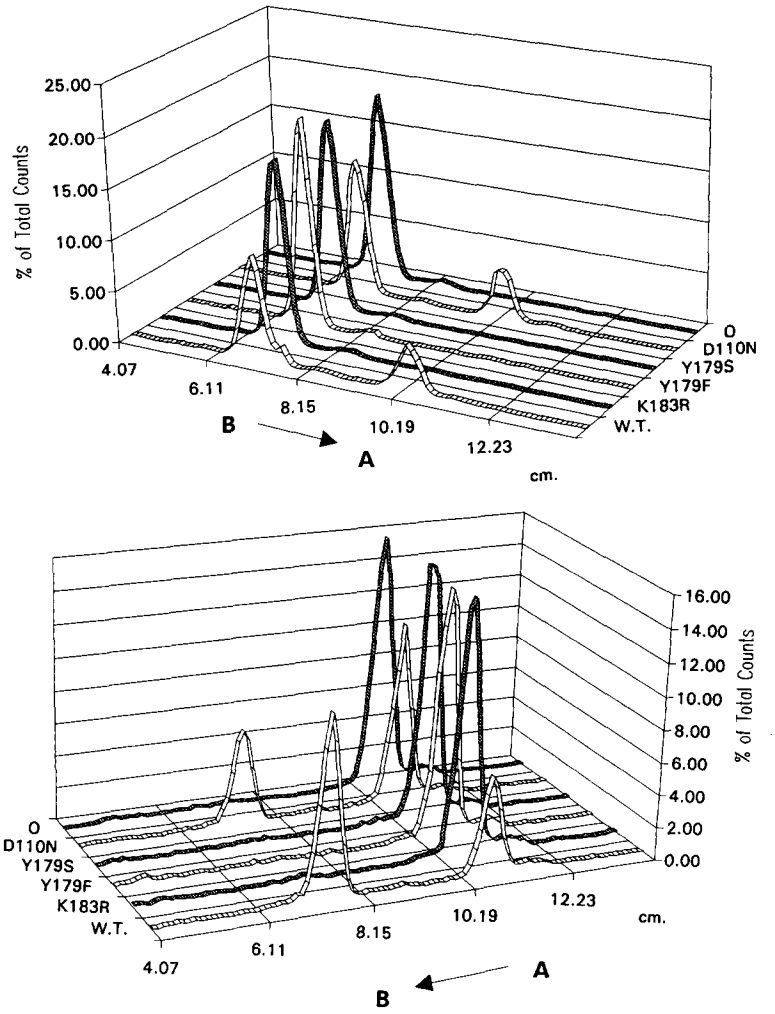

Figure 2. Enzymatic activities of transfected cells. Steroids were resolved by thin layer chromatography; numbers at bottom of each panel represent cm of from the origin of the plate. *Top*, dehydrogenation of corticosterone to 11-dehydrocorticosterone (**B→A**; the letters are at the positions to which the corresponding standards migrate). *Bottom*, reduction of dehydrocorticosterone to corticosterone (**A→B**). Abbreviations are the same as in Figure 1; **0** represents cells transfected with pCMV4 without an insert.

that of Asp-110. In particular, other alignment studies (12,13) confirmed the invariant nature of Tyr-179 and Lys-183, but residues aligning with Asp-110 were not absolutely conserved.

Furthermore, X-ray crystallographic studies of a related enzyme, 3α,20β-hydroxysteroid dehydrogenase, demonstrated that the residues corresponding to Tyr-179 and Lys-183 were located near the pyridine ring of the cofactor in a cleft presumed to be the substrate binding site (14) and could therefore be involved in catalytic activity. In contrast, the residue corresponding to Asp-110 occupied a peripheral position.

If Tyr-179 and Lys-183 were critical for catalytic function, even conservative substitutions at these positions should not be tolerated, whereas Asp-110 should tolerate at least

some degree of modification if its role is not central. This is what was observed in the present study; in Tyr-179→Ser the hydroxyl group is preserved, as is the aromatic ring in Tyr-179→Phe and the positive charge in Lys-183→Arg, yet all of these mutations abolish activity.

Supportive evidence of the functional importance of Tyr-179 and Lys-183 comes from other mutagenesis studies on corresponding residues in this family of enzymes. Substitution of Tyr-151 of 15-hydroxyprostaglandin dehydrogenase with alanine yielded a catalytically inactive enzyme (15). Substitution of Tyr-152 in *Drosophila* alcohol dehydrogenase with Phe, Glu and His completely abolished its activity when recombinant proteins were expressed in *E. coli*, as did Lys-156→Ile. Tyr-152→Cys and Lys-156→Arg dramtically decreased activity (M. Baker, personal communication).

Thus, available data support the hypothesis that Tyr-179 and Lys-183 play a major role in the catalytic site of this enzyme and probably other enzymes in its class. These two residues are believed to participate in the hydride ion transfer from corticosterone to $NADP^+$. This reaction may be facilitated by Tyr-179 if its deprotonated phenolic group acts to remove a proton from the 11ß-hydroxyl group of the steroid. Although deprotonation of a phenolic group normally has a pKa of about 10, Lys-183, which is located along with Tyr-179 in the cleft forming the substrate binding site, may provide a local alkaline milieu lowering the apparent pKa of tyrosine's phenolic group into the physiologic range.

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