Site-directed mutagenesis of the putative active site of human 17β-hydroxysteroid dehydrogenase type 1

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Several amino acid residues (Cys^{54}, Tyr^{155}, His^{210}, His^{213} and His^{221}) at a putative catalytic site of human 17β-hydroxysteroid dehydrogenase type 1 were mutated to Ala. Replacement of His^{221} by Ala remarkably reduced the catalytic activity, which resulted from a change of both the K_m and the $V_{max.}$ values of the enzyme. Compared with the wild-type enzyme, the catalytic efficiency of the His^{221}→Ala mutant was reduced 20-fold for the oxidative reaction and 11-fold for the reductive reaction. With similar mutations at His^{210} or His^{213}, no notable effects on the catalytic properties of the enzyme were detected. However, a simultaneous mutation of these amino acid residues decreased the $V_{max.}$ values of both oxidation and reduction by about 50% from those measured for the wild-type enzyme. Although Cys^{54} has been localized in the cofactor-binding region of the enzyme, a Cys^{54}→Ala mutation did not lead to changes in the enzymic activity. The most dramatic effects on the catalytic properties of the enzyme were achieved by mutating Tyr^{155}, which resulted in an almost completely inactivation of the enzyme. The decreased enzymic activities of the Tyr^{155}→Ala, His^{210}→Ala + His^{213}→Ala and His^{221}→Ala mutations were also reflected in a reduced immunoreactivity of the enzymes. The results thus suggest that the lowered catalytic efficiency of the mutant enzymes is due to an exchange of catalytically important amino acid residues and/or remarkable alterations in the three-dimensional structure of the enzyme. The recently detected polymorphisms (Ala^{237}↔Val and Ser^{312}↔Gly) were not found to affect either the catalytic or the immunological properties of the type 1 enzyme.

INTRODUCTION

17β-Hydroxysteroid dehydrogenases (17-HSDs) catalyse the reversible reaction between neutral and phenolic 17-oxosteroids and 17-hydroxysteroids. Thus 17-HSD activity is essential for both oestradiol and testosterone biosynthesis. In addition to being present in steroidogenic tissues, 17-HSD activity is also present in several peripheral tissues (Martel et al., 1992). 17-HSD type 1 (EC 1.1.1.62), which was first purified from human placenta, catalyses primarily the interconversion between oestradiol and a less active oestrogen, oestrone (Langer and Engel, 1958). In contrast with the type 1 enzyme, the recently characterized 17-HSD type 2 catalyses the reactions between testosterone and androstenedione and between oestradiol and oestrone. As has been predicted from the kinetic constants measured *in vitro*, the oxidative reaction is predominant for the type 2 enzyme (Wu et al., 1993). 17-HSD type 1 has instead been demonstrated to favour the reductive reaction from oestrone to oestradiol in cultured cells (Poutanen et al., 1993). Thus the intracellular concentration of oestrogens could be regulated by the expression of different forms of 17-HSD isoenzymes.

17-HSD type 1 is expressed in breast-cancer tissue (Luu-The et al., 1990; Poutanen et al., 1992b), and the enzyme might therefore be responsible for the relatively high intracellular oestradiol concentrations detected in breast-cancer tissues of post-menopausal women (Beranek et al., 1985; Bonney et al., 1986). As oestradiol is a mitogenic factor towards breast epithelium (Vihko and Apter, 1989; Cullen and Lippman, 1989), the interconversion of oestrone and oestradiol might play a crucial role in promoting the growth of hormone-dependent breast carcinomas. Therefore this reaction seems to be a logical site for inhibiting oestradiol synthesis in the peripheral tissues of post-menopausal women, and specific inhibitors for its activity may have significant clinical use in reducing or eliminating the oestrogen stimulation of oestrogen-sensitive breast-tumour cells.

17-HSD type 1 protein exists in a dimer of identical subunits (Burns et al., 1972; Nicolas and Harris, 1973; Lin et al., 1992) each with a predicted molecular mass of 34853 Da (Peltoketo et al., 1988). The enzyme belongs to the superfamily of short-chain-alcohol dehydrogenases (SCADs). The family consists of prokaryotic and eukaryotic proteins involved in nitrogen fixation, in the metabolism of sugars, steroids, aromatic hydrocarbons and prostaglandins, and in the synthesis of antibiotics (Krozowski, 1992). Apart from six conserved regions (A–F), the members of the SCAD family share low overall sequence similarity. Of these regions, those suggested to be involved in the binding of the cofactor [NAD(P)/NAD(P)H] and those suggested to have a role in the conformation of the secondary and tertiary structure of the proteins are also conserved in human 17-HSD type 1. In addition, a Tyr residue in the region D (Tyr^{155} in human 17-HSD type 1) is totally conserved in members of the SCAD family (Krozowski, 1992). This Tyr residue has been suggested to form a hydrogen bond with the substrate in holo-3α,20β-HSD (Ghosh et al., 1991). Results from previous affinity-labelling studies have also revealed that three histidine residues (His^{210}, His^{213} and His^{221}) are located in the steroid-binding region, and might thus be involved in the catalytic event of human 17-HSD type 1 (Murdock et al., 1986, 1991). In addition, inhibition studies have demonstrated that Cys^{54} is not reactive with *N*-ethylmaleimide and *p*-chloromercuribenzoate in the presence of the cofactor (Nicolas and Harris, 1973), suggesting that the cysteine might be located in the cofactor-binding site in 17-HSD type 1.

In the present work, Cys^{54}→Ala-, His^{210}→Ala-, His^{213}→Ala-, His^{221}→Ala-, His^{210}→Ala + His^{213}→Ala- and Tyr^{155}→Ala-

Abbreviations used: AcNPV, *Autographa californica* nuclear-polyhedrosis virus; 17-HSD, 17β-hydroxysteroid dehydrogenase; SCAD, short-chain-alcohol dehydrogenase; Sf9, *Spodoptera frugiperda*.

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mutated proteins were produced to obtain more detailed information about their role in the catalytic event of 17-HSD type 1. In addition, the possible effects of the previously detected (Normand et al., 1993; Mannermaa et al., 1994) protein polymorphism ($Ala^{237} \leftrightarrow Val$ and $Ser^{312} \leftrightarrow Gly$) on the catalytic properties of human 17-HSD type 1 were investigated.

EXPERIMENTAL

Site-directed mutagenesis

Eight 17-HSD type 1 mutants, $Cys^{54} \rightarrow Ala$, $Tyr^{155} \rightarrow Ala$, $His^{210} \rightarrow Ala$, $His^{213} \rightarrow Ala$, $His^{221} \rightarrow Ala$, $His^{210} \rightarrow Ala + His^{213} \rightarrow Ala$, $Ala^{237} \rightarrow Val$ and $Ser^{312} \rightarrow Gly$, were generated using the overlap-extension technique described by Ho et al. (1989). The method utilizes partially complementary primer pairs containing a codon of an altered amino acid (Table 1). The entire coding region of the 17-HSD type 1 cDNA (Peltoketo et al., 1988; Luu-The et al., 1989) was amplified using the flanking primers 1 (5′-TTATATTAGCGGCCGCACCATGGCCCG-CACCGTG-3′) and 2 (5′-TATATGAATTCAGGAAGCCTTT-ACTGCGGGGC-3′), which also contained recognition sites for *Not*I and *Eco*RI respectively. The mutated fragments were subcloned into the pBluescript I SK$^+$ vector, and the complete coding regions of the mutated cDNAs were sequenced (Sanger et al., 1977) to confirm the presence of the desired mutation and to ensure that no other mutations had occurred.

Expression in *Spodoptera frugiperda* (Sf9) cells

The *Eco*RI fragment of the cDNA (Peltoketo et al., 1988) containing the entire coding region for 17-HSD type 1 and the mutated *Not*I–*Eco*RI fragments were subcloned into the pVL1392 non-fusion transfer vector, in which the proteins were expressed under control of the polyhedrin promoter. The baculovirus expression system has been recently used successfully in our laboratory (Vihko et al., 1993). Therefore, wild-type and mutated 17-HSD-AcNPVs were also produced in this study in Sf9 insect cells (Invitrogen, San Diego, CA, U.S.A.) by using the BaculoGold (Pharmingen, San Diego, CA, U.S.A.) transfection system. The Sf9 cells were grown in complete TNM-FH (Sigma, St. Louis, MO, U.S.A.) medium containing 10% foetal-calf serum. To produce recombinant proteins, Sf9 cells were grown in 600 ml tissue-culture flasks at a density of 25×10^6 cells/flask. The cells were infected with different 17-HSD-AcNPVs at a multiplicity of infection of 1. After 72 h incubation at 27 °C, the infected cells were harvested by centrifugation (1000 *g* for 10 min) and suspended in buffer A [10 mM potassium phosphate buffer (pH 7.5)/1 mM EDTA/0.5 mM phenylmethanesulphonyl fluoride/0.02% NaN_3/20% glycerol]. The cells were disrupted by sonication (2×10 s at 1 min intervals) in an ice-bath, followed by centrifugation of the cell lysates (1000 *g* for 10 min). The supernatants were then stored at −70 °C until used.

Immunochemical measurement of the wild-type and the mutated proteins

The concentrations of the 17-HSD type 1 proteins in Sf9 cell lysates were measured using a time-resolved immunofluorometric assay (Mäentausta et al., 1991). With the immunoassay, both the wild-type and the mutated proteins were detected to a similar extent, as evaluated by an immunoblot after SDS/PAGE and by the expected expression level after infection. For immunoblotting, cytosolic proteins from Sf9 cell lysates were separated by SDS/PAGE or native PAGE (Mini-PROTEANII apparatus; Bio-Rad, Richmond, CA, U.S.A.). After electrophoresis, the proteins were electrophoretically transferred on to a nitrocellulose membrane (Towbin et al., 1979). The membranes were immunostained as previously described by Poutanen et al. (1992a), using rabbit polyclonal antibodies raised against human 17-HSD type 1 (Mäentausta et al., 1990). The immunoreactive proteins were revealed with the use of 4-chloro-1-naphthol as a substrate.

Table 1 Internal primers used for site-directed mutagenesis

Mutation into human 17-HSD type 1 cDNA were constructed by PCR, using the overlap-extension method described by Ho et al. (1989). The mutated codons of the primer pairs are underlined

Mutation	Primer	Sequence	Annealing temperature (°C)
$Cys^{54} \rightarrow Ala$	Reverse	5′-TCCCGGAGGTGCTGCCAGG-3′	66
	Forward	5′-CCTGGCAGCACCTCCGGGA-3′	66
$Tyr^{155} \rightarrow Ala$	Reverse	5′-GGCGCAGGCAACGTCATTG-3′	62
	Forward	5′-GACGTTGCCTGCGCCAGCAAG-3′	70
$His^{210} \rightarrow Ala$	Reverse	5′-GAAGGTGGCGATGTCCGTGC-3′	66
	Forward	5′-GCACGGACATCGCCACCTTCC-3′	70
$His^{213} \rightarrow Ala$	Reverse	5′-GAAGCGTGCGAAGGTGTGG-3′	62
	Forward	5′-CCACACCTTCGCACGCTTCTAC-3′	70
$His^{210} \rightarrow Ala + His^{213} \rightarrow Ala$	Reverse	5′-AGCGTGCGAAGGTGGCGATGTC-3′	72
	Forward	5′-CATCGCCACCTTCGCACGCTTC-3′	72
$His^{221} \rightarrow Ala$	Reverse	5′-CTTGCTTGCTGGCGGCGAGGT-3′	70
	Forward	5′-CCTCGCCGCCAGCAAGCAAG-3′	68
$Ala^{237} \rightarrow Val$	Reverse	5′-GAAGACCTCCACCACCTCC-3′	62
	Forward	5′-GGAGGTGGTGGAGGTCTTCCTC-3′	72
$Ser^{312} \rightarrow Gly$	Reverse	5′-CCCCACCGCACCGCGCCCG-3′	72
	Forward	5′-GGGCGCGGTGCGGTGGGGG-3′	72

Measurement of 17-HSD type 1 activity

The activities of the wild-type and the mutated 17-HSD type 1 enzymes were determined using the method described by Tseng and Gurpide (1974). Briefly, samples of 400 μl, which were diluted in buffer B [10 mM potassium phosphate buffer (pH 7.5)/1 mM EDTA/0.02% NaN_3/20% glycerol/0.1% BSA], were first mixed with [^{3}H]oestradiol or [^{3}H]oestrone (2×10^6 c.p.m./ml; Amersham, Arlington Heights, IL, U.S.A.) and with a non-labelled steroid (Steraloids Inc., Wilton, NH, U.S.A.) to a final substrate concentration of 37 μM. The reactions were started by adding 50 μl of 14.3 mM NAD^+ (NADH), and the samples were incubated for 1–10 min at 37 °C. Thereafter the reactions were stopped by adding 450 μl of buffer B containing 0.93 μM of the end product (oestrone or oestradiol) and freezing the reaction mixture in solid CO_2. The reaction products were separated from the substrates by using an acetonitrile/water (9:11, v/v) solution as a mobile phase in a Sephasil C18 reverse-phase-chromatography column (0.21 cm × 10 cm) connected to a SMART system (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Lineweaver–Burk plots were used to determine the K_m and $V_{max.}$ values. In these kinetic measurements, substrate concentrations of 0.73, 1.45, 3.6 and 7.3 μM were used, and the reaction times were 20 and 40 s. The kinetic values represent the average ± S.D. for at least three independent experiments. A unit of enzyme activity is defined as 1 μmol of product formed/min.

RESULTS AND DISCUSSION

Expression of the wild-type and the mutated proteins in Sf9 cells

The baculovirus expression system was used to produce wild-type and mutated 17-HSD type 1 proteins in Sf9 cells, in which no endogenous 17-HSD activity could be detected. The expressed 17-HSD type 1 enzymes remained primarily inside the cells and were expressed at a concentration of 5 μg/1.0 $\times$ 10^6 Sf9 cells. The kinetic values for the wild-type and the mutated 17-HSD type 1 enzymes were measured from 1000 *g* supernatants of the cell homogenates using oestradiol and oestrone as substrates. The specific activities of the wild-type protein were 11.5 and 26.6 units/mg for the oxidative and reductive reaction respectively. The catalytic efficiency, $V_{max.}/K_m$, for the reduction (72.7) was 2-fold compared with that measured for the oxidation (37; Table 2). Thus these kinetic measurements show that the reductive reaction, from oestrone to oestradiol, is predominant for the recombinant 17-HSD type 1 *in vitro*. This is also in line with the predominantly reductive activity of the enzyme in cultured COS-m6 cells (Poutanen et al., 1993). Immunoblot analyses of the wild-type and the mutated proteins were performed after separation of the cytosolic proteins by SDS/PAGE or native PAGE (Figure 1). The actual molecular mass of 38 kDa for the monomer was determined for both the intact 17-HSD type 1 and the mutated proteins (Figure 1a). In the denatured form, after SDS/PAGE, all the proteins were similarly recognized by rabbit polyclonal anti-(17-HSD type 1) antibodies (Figure 1a). However, as discussed in detail below, in the native form the mutated enzymes were differently recognized by the antibodies (Figure 1b).

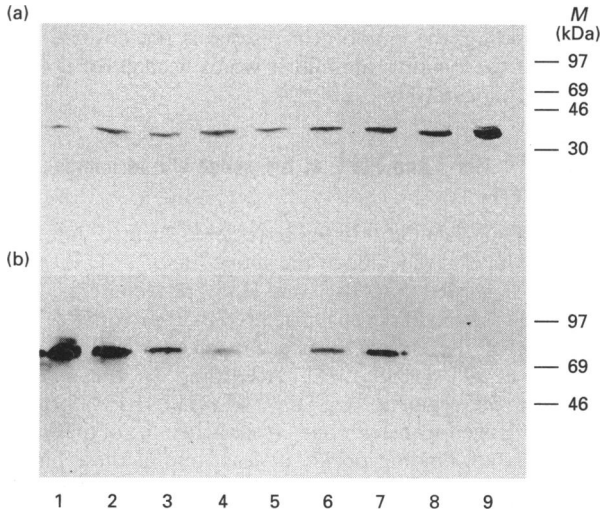

Figure 1 Immunoblot analysis of the wild-type and mutated 17-HSD type 1 proteins

Immunoblot analysis of intact 17-HSD type 1 (lane 1) and the $Cys^{54}\rightarrow$Ala- (lane 2), $His^{210}\rightarrow$Ala- (lane 3), $His^{213}\rightarrow$Ala- (lane 4), $His^{221}\rightarrow$Ala- (lane 5), $Ala^{237}\rightarrow$Val- (lane 6), $Ser^{312}\rightarrow$Gly- (lane 7), $His^{210}\rightarrow$Ala + $His^{213}\rightarrow$Ala- (lane 8) and $Tyr^{155}\rightarrow$Ala- (lane 9) mutated proteins after SDS/PAGE (**a**) and native PAGE (**b**). The amount of 17-HSD type 1 applied to the gel was 100 ng (**a**) or 200 ng (**b**) as measured by a time-resolved immunofluorometric assay of 17-HSD type 1. After electrophoreses on the MiniPROTEAN II apparatus, the proteins were transferred on to a nitrocellulose membrane and immunostained as described by Poutanen et al. (1992a).

Role of Cys^{54} in NAD^+ binding

Cys^{54} or its immediate neighbours in the primary structure of human 17-HSD type 1 have been suggested to be part of the cofactor-binding site (Nicolas and Harris, 1973). On the other hand, the first conserved region (A-region) among the SCAD members, including the amino acids 2–31, has been suggested to constitute the cofactor-binding region of the enzymes (Yamada and Saier, 1987). However, the amino acid sequence of this region do not follow exactly the fingerprint designed for the $\beta\alpha\beta$-structures involved in NAD^+ binding (Wierenga et al., 1986). Nevertheless, the importance of the A-region for catalysis is further confirmed by the three-dimensional structure of holo-3α,20β-HSD. In this enzyme, the binding environment of NAD(H) has been shown to be formed partly by this conserved N-terminal region (Ghosh et al., 1991).

In the present work, Cys^{54} was mutated to an Ala residue, and no effects on the specific activity were observed (Table 2). In addition, the $Cys^{54}\rightarrow$Ala-mutated and the wild-type proteins were equally well recognized by rabbit polyclonal anti-(17-HSD type 1) antibodies (Figure 1b). It can hence be concluded that,

Table 2 Specific activities and kinetic parameters of human recombinant 17-HSD type 1 (wild-type) and mutated enzymes

The units of specific activity, K_m and $V_{max.}$, are units/mg, μM and units/mg respectively. The specific activities were determined using a method described by Tseng and Gurpide (1974). The kinetic constants for the wild-type and mutated 17-HSD type 1 enzymes were determined using substrate concentrations of 0.73–7.3 μM at 37 °C. Results are means ± S.D. for at least three independent experiments. The ratio $V_{max.}/K_m$ represents the catalytic efficiency.

Substrate...	17β-Oestradiol				Oestrone			
Enzyme	Specific activity	K_m	$V_{max.}$	$V_{max.}/K_m$	Specific activity	K_m	$V_{max.}$	$V_{max.}/K_m$
Wild-type	11.5	1.84 ± 0.33	68.0 ± 13.1	37.0	26.6	1.08 ± 0.36	78.5 ± 6.4	72.7
$His^{221}\rightarrow$Ala	1.4	4.10 ± 0.63	7.6 ± 1.2	1.9	4.8	3.86 ± 0.86	25.9 ± 5.8	6.7
$His^{210}\rightarrow$Ala + $His^{213}\rightarrow$Ala	8.3	1.99 ± 0.34	34.1 ± 10.1	17.1	13.3	0.97 ± 0.11	44.0 ± 9.3	45.4
$Tyr^{155}\rightarrow$Ala	0.028	9.78 ± 2.04	0.40 ± 0.19	0.041	0.012	2.91 ± 0.22	0.02 ± 0.002	0.007
$Cys^{54}\rightarrow$Ala	10.6				24.7			
$His^{210}\rightarrow$Ala	13.5				25.9			
$His^{213}\rightarrow$Ala	10.6				27.2			
$Ala^{237}\rightarrow$Val	10.2				25.3			
$Ser^{312}\rightarrow$Val	12.9				28.7			

even if Cys^{54} is located at a region thought to be involved in cofactor binding, the amino acid residue is not critical for the structure of the binding site and it is not needed for cofactor-binding during catalysis.

Role of His^{210}, His^{213} and His^{221} at the active site of human 17-HSD type 1

Previous affinity-labelling studies have demonstrated that His^{210}, His^{213} and His^{221} are located at the active site of 17-HSD type 1. It has been suggested that His^{210} and His^{213} proximate the D-ring of the bound steroid in the enzyme, and His^{221} proximates the A-ring region of the steroid during the reversible binding step (Murdock et al., 1983, 1986). According to the suggested mechanism, the segment Arg-His^{210}-Phe-Thr-His^{213} forms the catalytic hydrogen-transfer 'zone' at the active site of the enzyme. In this substrate-binding pocket at least one of these histidine residues directly participates in hydrogen transfer (Murdock et al., 1988, 1991). Thus the reaction mechanism would be similar to that shown for the 2-hydroxyacid dehydrogenases (Rao and Rossman, 1973; Garavito et al., 1977). According to Pons et al. (1977), however, only one histidine residue has a role in binding the substrate. This amino acid has been localized very close to the A-ring of the substrate, and it is therefore most probably the His^{221} also labelled by Murdock et al. (1983). However, this region shows low sequence similarity among the SCAD members, and these histidine residues cannot be found in any other members of the superfamily, including 17-HSD type 2.

To find out the importance of these three histidines in the catalysis of human 17-HSD type 1, the residues His^{210}, His^{213} and His^{221} have each been separately mutated to an Ala residue. The $His^{210}\rightarrow$Ala- and $His^{213}\rightarrow$Ala-mutated proteins have no effects on the enzymic properties of 17-HSD type 1 (Table 2). However, in contrast with the $His^{210}\rightarrow$Ala mutation, the reactivity of our polyclonal antibodies against the $His^{213}\rightarrow$Ala-mutated protein was reduced as evaluated by native PAGE (Figure 1b). It is likely, therefore, that a mutation of His^{213} to Ala changes the structure of the protein without affecting its catalytic activity. Even though it seemed that His^{210} and His^{213} had no effects on the catalytic event of the enzyme, these studies could not rule out the possibility that, while one of the His residues was mutated, the other could still have participated in the transfer of hydrogen. For this reason, His^{210} and His^{213} were simultaneously mutated to an Ala residue. The results in Table 2 indicate that only small differences could be detected in the specific activity and kinetic parameters as compared with the wild-type enzyme. This double mutation, $His^{210}\rightarrow Ala + His^{213}\rightarrow$Ala, has no effect on the K_m values, indicating that the binding of the substrate was not disturbed. Instead, the $V_{max.}$ values decreased about 2-fold compared with those measured for the intact enzyme. In native PAGE, the $His^{210}\rightarrow Ala + His^{213}\rightarrow$Ala- mutated protein was nearly non-detectable with polyclonal anti-(17-HSD type 1) antibodies (Figure 1b), which suggests that the lowered activity of the mutated enzyme may be a consequence of a change in the three-dimensional structure of the protein. Thus, even if the amino acids are located at the active site of the enzyme, it is unlikely that the residues directly participate in the catalytic reaction of human 17-HSD type 1. However, the His^{210} and His^{213} residues are critical for the proper folding of the protein.

Replacement of the third His residue, His^{221}, by an Ala residue significantly reduced the specific activity of the enzyme (1.4 units/mg for oestradiol as a substrate and 4.8 units/mg for oestrone). With this mutation, an 8-fold decrease was observed in the oxidative activity and a roughly 6-fold decrease in the reductive activity. A lowered enzymic activity was also observed with changes in the K_m and $V_{max.}$ values. The K_m value increased 2-fold for the oxidation and about 4-fold for the reductive reaction, and the $V_{max.}$ value decreased 9- and 3-fold respectively (Table 2). According to these values, the catalytic efficiency, $V_{max.}/K_m$, of the mutant enzyme decreased 20-fold (1.9) for the oxidative reaction and 11-fold (6.7) for the reductive reaction compared with the wild-type enzyme. In addition, the $His^{221}\rightarrow$Ala mutant was almost non-detectable after a native PAGE with the antibodies used (Figure 1b). This further indicates the critical role of the His residues in the three-dimensional structure of the enzyme. The structural change might also contribute to the lowered activity of the $His^{221}\rightarrow$Ala mutant. However, the $His^{221}\rightarrow$Ala-mutated protein still retains about 15% of its activity. This, together with previous findings (Pons et al., 1977; Murdock et al., 1983, 1986), shows that, although His^{221} does not directly participate in hydrogen transfer, it might play a role in substrate binding or orientation in human 17-HSD type 1.

Role of Tyr^{155} in human 17-HSD type 1

In human 17-HSD type 1, the amino acid residues 155–172 make up a region called the D-domain, which is one of the six conserved regions among the SCAD members (Krozowski, 1992). Tyr, being the first amino acid of this region, is entirely conserved in all the members of the SCAD family. This Tyr residue has been localized in the subunit binding region in glucose dehydrogenase (Jany et al., 1984). It is also one of the amino acid residues which line the steroid-binding pocket of holo-3α,20β-HSD (Ghosh et al., 1991).

In the present study Tyr^{155} in human 17-HSD type 1 was mutated to an Ala residue. The replacement of the Tyr residue inactivated the enzyme almost completely. A lowered specific activity resulted from a change in both the K_m and the $V_{max.}$ values. The K_m increased 5.5-fold (9.78 μM) for the oxidative reaction and about 3-fold (2.91 μM) for the reductive reaction (Table 2). However, more remarkable changes were detected in the $V_{max.}$ values. With this mutation, the $V_{max.}$ values for the oxidative and reductive reactions were 0.6% (0.40 unit/mg) and 0.03% (0.02 unit/mg) of the values measured for the wild-type enzyme (68.0 and 78.5 units/mg respectively). This resulted in a significantly decreased catalytic efficiency of the enzyme. $V_{max.}/K_m$ for the mutant enzyme decreased 900-fold for the oxidative reaction and over 10000-fold for the reductive reaction. Interestingly, the $Tyr^{155}\rightarrow$Ala mutated protein catalysed the oxidative reaction 10-fold better than the reductive one *in vitro*, although the catalytic efficiencies decreased significantly in both cases. The fact that the $Tyr^{155}\rightarrow$Ala mutation strongly affected the catalytic efficiencies and also changed the preferred reaction direction of the enzyme suggests that this Tyr residue plays an essential role in determining the structure and the function of the active site. However, structural studies are needed to clarify the reaction mechanism of the enzyme, and studies of this kind could also explain the low residual oxidative activity of the $Tyr^{155}\rightarrow$Ala-mutated enzyme. Similarly to the other mutated proteins with a lowered enzymic activity, the immunological properties of the $Tyr^{155}\rightarrow$Ala-mutated protein were affected, and the protein was non-detectable with polyclonal anti-(17-HSD type 1) antibodies after native PAGE. Therefore, proper dimerization of the mutated protein could not be confirmed. However, the fact that the enzymes with a lowered specific activity could not be detected after native PAGE with the antibodies either as a dimer or as a monomer suggests that significant changes have occurred in the three-dimensional structure of the proteins (Figure 1b).

The results clearly indicate the importance of this Tyr for the

activity of human 17-HSD type 1. This, together with the previous findings, supports the hypothesis that the conserved Tyr residue may have a key role in the catalytic event of the enzymes in the SCAD superfamily. On the basis of the three-dimensional structure of the 3α,20β-HSD, it has been suggested that the amino acid might participate in the transfer of a hydride radical from the steroid to the cofactor (Tannin et al., 1991). It is also possible, however, that the lowered catalytic efficiency observed in the present study is due to the critical role of the amino acid in the three-dimensional structure of the enzymes. This essential Tyr may thus play a role in determining the structure of the protein and hence its function.

Protein polymorphism detected in human 17-HSD type 1

In addition, the effects of Ala^{237}→Val and Ser^{312}→Gly substitutions on the activity of the enzyme were investigated. These amino acid variants are protein polymorphisms recently detected in human 17-HSD type 1 (Normand et al., 1993; Mannermaa et al., 1994). Neither Ala^{237}↔Val nor Ser^{312}↔Gly polymorphisms was found to affect the specific activity of the enzyme (Table 2). In addition, the Ala^{237}→Val, Ser^{312}→Gly and wild-type proteins were all equally well recognized by 17-HSD type 1 antibodies in native PAGE (Figure 1b). The results thus suggest that these protein variants do not possess any functional significance in human 17-HSD type 1.

In conclusion, the notable effect of the Tyr^{155}→Ala mutation on the catalytic activity of human 17-HSD type 1 clearly shows the importance of this amino acid residue for the activity of the enzyme. However, without the three-dimensional structure of this oxidoreductase, the structure of the active site and all the amino acid residues involved in catalysis cannot be completely clarified. The exact role of the conserved Tyr (catalytic and/or structural) therefore remains unknown. The replacement of His^{210} and His^{213} by Ala, separately or simultaneously, had no critical effect on the specific activity of 17-HSD type 1. Therefore, in contrast with previous findings, our results suggest that these amino acids are not necessary for the binding of the substrate and do not participate directly in the catalysis of 17-HSD type 1. Instead, the results show that His^{221} is critical for both the activity and the folding of the enzyme. This is in line with previous findings, which have indicated that His^{221} might play a role in the orientation of the substrate into the active site by binding to the A-ring of the substrate (Murdock et al., 1983, 1986).

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