Mutation of tyrosine-194 and lysine-198 in the catalytic site of pig $3\alpha/\beta,20\beta$ -hydroxysteroid dehydrogenase

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Pig $3\alpha/\beta,20\beta$ -hydroxysteroid dehydrogenase is an NADPH-dependent enzyme that catalyses the reduction of ketones on steroids and aldehydes and ketones on various xenobiotics, like its homologue carbonyl reductase. $3\alpha/\beta,20\beta$ -Hydroxysteroid dehydrogenase and carbonyl reductase are members of the short-chain dehydrogenases/reductase family, in which a tyrosine residue and a lysine residue have been identified as catalytically important. In pig 20β -hydroxysteroid dehydrogenase these residues are tyrosine-194 and lysine-198. Here we report the effect on the reduction of two ketone and two aldehyde substrates by pig $3\alpha/\beta,20\beta$ -hydroxysteroid dehydrogenase in which tyrosine-194

has been mutated to phenylalanine and cysteine, and lysine-198 has been mutated to isoleucine and arginine. Mutants with phenylalanine-194 or isoleucine-198 are inactive. Depending on the substrate, the mutant with cysteine-194 has a catalytic efficiency of 0.4–1 % and the mutant with arginine-198 has a catalytic efficiency of 4–23 % of the wild-type enzyme. We also mutated tyrosine-81 and tyrosine-253 to phenylalanine. Although both tyrosines are conserved in $3\alpha/\beta,20\beta$ -hydroxysteroid dehydrogenase and carbonyl reductase, depending on the substrate, the mutant enzymes are as active as, or more active than, wild-type enzyme.

INTRODUCTION

Pig $3\alpha/\beta,20\beta$ -hydroxysteroid dehydrogenase ($3\alpha/\beta,20\beta$ -HSD) was first isolated from the testes and shown to catalyse the reduction of various progestins and androgens, suggesting that it might regulate the concentration of these steroid hormones during development [1–3]. However, later studies with antibodies against $3\alpha/\beta,20\beta$ -HSD revealed that it is expressed in many other tissues including kidney, liver, brain, lung, thymus, heart, spleen and adrenal gland, suggesting a broader role in steroid hormone action [4]. The cloning and sequencing of $3\alpha/\beta,20\beta$ -HSD [5] showed that it belongs to the short-chain dehydrogenases/reductase (SDR) superfamily [6–12], which includes 11β -hydroxysteroid dehydrogenase, 17β -hydroxysteroid dehydrogenase and 15 -hydroxyprostaglandin dehydrogenase.

Unexpectedly, analysis of the sequence of $3\alpha/\beta,20\beta$ -HSD revealed that it is 85 % identical with carbonyl reductase, an enzyme first isolated from human brain by Wermuth [13], who showed that carbonyl reductase metabolizes steroids, prostaglandins and a variety of carbonyl-containing compounds that are xenobiotics. Indeed, carbonyl reductase has an important role in quinone detoxification in man [14]. Pig $3\alpha/\beta,20\beta$ -HSD also metabolizes prostaglandins and xenobiotics [15], leaving open the possibility of multiple functions of $3\alpha/\beta,20\beta$ -HSD and carbonyl reductase depending on the tissue in which they are expressed [4,13–21].

Substantial information about the structure and function of homologues of $3\alpha/\beta,20\beta$ -HSD has been obtained from the determination of the three-dimensional structures of several SDRs [9,21–25] and site-specific mutagenesis studies of these enzymes [26–32]. A key insight from these studies is that a tyrosine residue and lysine residue separated by three amino acids residues (i.e. Tyr-Xaa-Xaa-Lys) are important in catalysis. Also important is a serine residue that is approx. 12

residues upstream from the catalytic tyrosine [8–11]. In $3\alpha/\beta,20\beta$ -HSD these residues are serine-140, tyrosine-194 and lysine-198. The much larger distance between serine and tyrosine in $3\alpha/\beta,20\beta$ -HSD compared with almost all other SDRs is also characteristic of carbonyl reductase and plant protochlorophyllide reductase [6,7,9,11].

Another distinguishing feature of $3\alpha/\beta,20\beta$ -HSD and carbonyl reductase is that they are monomers [1,13], in contrast with other SDRs, which are either dimers or tetramers [8,22,33,34] stabilized by a four- α -helix bundle. In this structure each monomer contributes two α -helices to form an interface that stabilizes the dimers, which in some cases can associate further into tetramers. One of these stabilizing α -helices contains the catalytically important tyrosine and lysine residues. Disruption of the dimer interface on *Drosophila* alcohol dehydrogenase, an SDR, leads to a loss of catalytic activity [35], which is probably due to distortion in the α -helix containing the catalytic tyrosine and lysine residues owing to the interaction of solvent with the α -helix. This suggests that most SDRs are not active as monomers and that the activity of $3\alpha/\beta,20\beta$ -HSD and carbonyl reductase as monomers is exceptional. The extra segment between serine-140 and tyrosine-194 on $3\alpha/\beta,20\beta$ -HSD and carbonyl reductase has been proposed to form a stabilizing interface with the α -helix that contains tyrosine-194 and lysine-198 [33].

Our interest in the mechanism of action of pig $3\alpha/\beta,20\beta$ -HSD led us to construct mutants in which the catalytically important tyrosine residue was changed to either phenylalanine or cysteine, and the lysine residue changed to either isoleucine or arginine. These mutants were tested for reductive activity with two ketone and two aldehyde substrates. Under these conditions we could examine the importance of the unique structural properties of $3\alpha/\beta,20\beta$ -HSD on the roles of tyrosine and lysine residues in catalysis with substrates with different structures and chemical properties. Our experiments confirm the importance of tyrosine-

Abbreviations used: $3\alpha/\beta,20\beta$ -HSD, $3\alpha/\beta,20\beta$ -hydroxysteroid dehydrogenase; SDR, short-chain dehydrogenases/reductase.

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194 in the catalytic site of SDRs but indicate that lysine-198 can be replaced by arginine and yield an enzyme that has reasonable catalytic activity.

EXPERIMENTAL

Chemicals

4-Nitrobenzaldehyde and 4-benzoylpyridine were obtained from Nacalai Tesque (Kyoto, Japan). Octanal and 4-nitroacetophenone were obtained from Wako Chemicals (Tokyo, Japan). NADPH and DE-52 were obtained from Oriental Yeast Co. (Tokyo, Japan) and Whatman International Ltd. (Maidstone, Kent, U.K.) respectively.

Enzyme assay

Aldo-keto reductase activity was determined spectrophotometrically by measuring the oxidation rate of NADPH with an enzyme rate calculator apparatus (Hitachi 200-0045) attached to a spectrophotometer (Hitachi 200-20) at 37 °C. For the determination of kinetic constants for the ketone and aldehyde substrates, the assay mixture consisted of 60 mM potassium phosphate buffer, pH 6.5, 80 μ M NADPH, substrate at various concentrations and enzyme (50 μ g) in a total volume of 1.2 ml. For the determination of kinetics for the cofactor, enzyme assays were performed with 4-nitrobenzaldehyde at fixed concentrations (156, 313 and 1250 μ M) and the NADPH concentration was changed from 2.5 to 240 μ M. The reaction was initiated by the addition of NADPH to the assay mixture. Blanks without substrate or enzyme were included routinely. Substrates were dissolved in ethanol. The final concentration of the solvent in the assay mixture did not exceed 1% (v/v). The kinetic parameters were calculated from Lineweaver–Burk plots.

Expression and isolation of $3\alpha/\beta,20\beta$ -HSD mutants

Site-directed mutagenesis was performed by using a Mutant-Express Km kit and the protocol (Takara Biomedicals, Tokyo, Japan) in accordance with the oligonucleotide-directed dual amber method [36]. The following oligonucleotides were used: Y81F, 5'-CGGAAGGAGTTCGGTGGGCTG-3'; Y194F, 5'-GATTTCGACCTTCGGAGTGACA-3'; Y194C, 5'-GATTTCGACCTTCGGAGTGACA-3'; Y253F, 5'-ACCCCGTGTCTTGGCCCTT-3'; K198R, 5'-GGAGTGACAAGAATTGGGGTCTC-3'; K198I, 5'-GGAGTGACAATAATTGGGGTCTC-3'.

All mutant forms of $3\alpha/\beta,20\beta$ -HSD were sequenced to confirm the presence of the correct codon substitutions for Y81F, Y194F, Y194C, Y253F, K198R and K198I. The vector pKK223-3 [37] was used for the construction of different mutants and their expression in *Escherichia coli* JM105 cells. *E. coli* cells transformed with the different vectors were seeded into Luria–Bertani medium containing 50 μ g/ml of ampicillin. Isopropyl β -D-thiogalactoside was added to the medium for 18 h at 37 °C to induce the expression of $3\alpha/\beta,20\beta$ -HSD. The *E. coli* cells were collected by centrifugation and suspended in KPB buffer (3 mM K⁺, pH 7.4) with 0.1 mM PMSF, 1 mM pepstatin, 0.1 mM EDTA and 0.1 mM dithiothreitol. The *E. coli* cells were disrupted by sonication and the homogenate was centrifuged for 60 min at 105000 g. The supernatant was dialysed with KPB buffer containing 0.1 mM EDTA and dithiothreitol. The enzyme was purified with DE-52 column chromatography by the method of Nakajin et al. [38]. Wild-type $3\alpha/\beta,20\beta$ -HSD and its mutants were identified in column fractions by an enzyme assay or a Western blot specific for $3\alpha/\beta,20\beta$ -HSD [4]. The purified enzyme was concentrated with an ultrafiltration apparatus using a PM-10 Diaflo membrane (Amicon, Beverly, MA, U.S.A.) and stored

Figure 1 PAGE of purified pig $3\alpha/\beta,20\beta$ -HSD and various mutants

Electrophoresis was performed in 10% (w/v) polyacrylamide gels with the separation gel at pH 8.8. Protein was stained with Coomassie Brilliant Blue R-250. Approx. 1 mg of protein was applied to each lane. The applied samples were: lane M, marker proteins (molecular masses indicated at the left in kDa); lane a, wild-type; lane b, Y81F; lane c, Y194F; lane d, Y194C; lane e, Y253 F; lane f, K198R; lane g, K198I.

at –30 °C until use. Figure 1 shows SDS/PAGE by the method of Laemmli [39]. The protein markers used for SDS/PAGE were phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa).

Sequence alignment

The Feng–Doolittle algorithm [40] was used to construct a multiple alignment of pig $3\alpha/\beta,20\beta$ -HSD and other mammalian carbonyl reductases and *Schistosoma mansoni* carbonyl reductase.

RESULTS

Strategy for mutagenesis of $3\alpha/\beta,20\beta$ -HSD

Our initial goal was to examine the effect of mutations of tyrosine-194 and lysine-198 on the activity of $3\alpha/\beta,20\beta$ -HSD under the assumption that, by analogy with its homologues, these two residues are at the catalytic site of $3\alpha/\beta,20\beta$ -HSD. However, a search of GenBank revealed that Davis et al. [41] had determined the sequence of a carbonyl reductase from the invertebrate *Schistosoma mansoni*. *S. mansoni* carbonyl reductase has approx. 40% sequence identity with $3\alpha/\beta,20\beta$ -HSD. Because vertebrates and invertebrates diverged from a common ancestor at least 550 million years ago, amino acids conserved in the fluke and mammalian carbonyl reductases are likely to be important. As shown in the multiple alignment of these carbonyl reductases in Figure 2, tyrosine-81 and tyrosine-253 are absolutely conserved in the vertebrate and invertebrate enzymes. Thus, in addition to mutating tyrosine-194 to phenylalanine (Y194F), we decided to mutate tyrosine-81 (Y81F) and tyrosine-253 (Y253F) to phenylalanine. This would test the importance of the phenolic group in these three conserved tyrosines. We also mutated tyrosine-194 to cysteine (Y194C) to determine whether the thiol group could function like the phenolic group on tyrosine in reduction of substrates. Similarly, lysine-198 was mutated to isoleucine (K198I), which lacks a positively charged side chain, and to arginine (K198R), which has a positively charged side chain that might be able to replace the positively charged side chain on lysine in catalysis.

Pig 3 α / β -20 β -HSD		1 M S S N T R V A L V T G A N K G I G F A I V R D L C R Q F A G - - - D V V L T A R
Mouse CR		1 . . . S R P T K . K . S . - - - A . .
Rat CR		1 . . . D R P K . K . L . - - -
Human CR		1 . . . G H G L L . S . - - -
Rabbit CR		1 . P . D R V T . A . . . L . S . - - - Q
<i>S. mansoni</i> CR		 1 M K L F S Y S . E K * A E F Y G A S G E W * I Y
Pig 3 α / β -20 β -HSD		39 D V A R G Q A A V K Q L Q A E G L S P R F H Q L D I D L Q S I R A L C D F L R K E
Mouse CR		39 . B E . . . T . Q K L Q A E G L S P R F H Q L D I D N P Q S I R A L C D F L R K E
Rat CR		39 . E S . . E T D N P L Q .
Human CR		39 . T Q . R
Rabbit CR		39 . E . Q Q . R . . . R A
<i>S. mansoni</i> CR		39 N * E L . R E . . . K . S N K . . D V K T . Q N * R K V F L T * V E R N
Pig 3 α / β -20 β -HSD		81 X - G G L D V L V N N A A I A F L O D N P T P F H I Q A E L T M K T N F M G T R N V
Mouse CR		81 . V F
Rat CR		81 N G K V V D V F . . . Q D
Human CR		81 N G K V A D V F . . . D .
Rabbit CR		81 N V K M E D T V V . . .
<i>S. mansoni</i> CR		81 . P N * I N I A G . Y K A N S A A . . G E . . R V * V N T S . I D F
Pig 3 α / β -20 β -HSD		122 C T E L L P L I K P Q G R V V N V S S T E G V R A L N E C S P E L Q Q K F K S E T I
Mouse CR		122 . K M V S L K N . R L R
Rat CR		122 . K P I K S L R
Human CR		122 . I M S R
Rabbit CR		122 M R . G M T C L K S R
<i>S. mansoni</i> CR		122 M E * S I . . L A K H A T S I S L T S * K K L . D D . Y G . . V * - P *
Pig 3 α / β -20 β -HSD		164 T E E L V L G L M N K F V E D T K N G V H R K E G W S D S T X G V T X I G V S V L S
Mouse CR		164 E L V L G L M N K F K A E P N . A T . . .
Rat CR		164 I . A . K A P N . A T . . .
Human CR		164 . K Q P S . A T . . .
Rabbit CR		164 . K Q T P T A M V T . .
<i>S. mansoni</i> CR		164 S L L . . R K H V S E - . K S A E D . T Y S E K . . P S N A S . I A L Q S . I
Pig 3 α / β -20 β -HSD		206 R I Y A R K L R E Q R A G D K I L N A C C P G W V R T D M G G P K A P K S P E V G
Mouse CR		206 L N R E A T
Rat CR		206 L N E R E A T
Human CR		206 H S K V A T
Rabbit CR		206 Q H . S . H G . N T
<i>S. mansoni</i> CR		206 - Y F G E M * K D D P R E - . . V I N S Y C D S S H K G T K T A D E *
Pig 3 α / β -20 β -HSD		248 A E T P V X L A L L P S D A E G P H G Q F V T D K K V V E W G V P P E S Y P W V N A
Mouse CR		248 . Q E P . .
Rat CR		248 . P G Q E P
Human CR		248 . P E Q .
Rabbit CR		248 . P E R . E Q
<i>S. mansoni</i> CR		244 D F I G S K E . I N Y E R R . . K . S K

Amino acids that are identical with those in pig $3\alpha,20\beta$ -HSD are denoted by ●. The tyrosine residues and the lysine residue that were mutated are in bold type and underlined. The sequences of the pig [5], mouse [42], rat [19], human [16], rabbit [43] and fluke [41] carbonyl reductase (CR) were obtained from the GenBank server at the National Center for Biotechnology Information.

Figure 3 shows the structures of the four substrates that were used in assays with the mutant enzymes. We chose these compounds for three reasons: first, aldehydes and ketones have different chemistries; secondly, the substrates have different

The K198R mutant retained some activity for all four substrates, with catalytic efficiencies from 4 % to 23 % of wild-type $3\alpha/\beta,20\beta$ -HSD. Three substrates, 2-benzoylpyridine, 4-nitrobenzaldehyde and octanal yielded catalytic efficiencies of 23 %, 15 % and 13 % respectively with wild-type $3\alpha/\beta,20\beta$ -HSD.

Table 1 Catalytic efficiency of $3\alpha/\beta,20\beta$ -HSD and its mutants

K_m and V_{max} values are means $\pm$ S.D. Abbreviation: n.a., no activity.

Substrate	Enzyme	K_m (μ M)	V_{max} (nmol/min per mg)	V_{max}/K_m	Catalytic efficiency (%)
4-Nitrobenzaldehyde	Wild-type	22.8 $\pm$ 0.7	222 $\pm$ 1	9.74	100
	Y81F	21.4 $\pm$ 0.7	237 $\pm$ 1	11.07	114
	Y194F	n.a.	n.a.	n.a.	0
	Y194C	2060.0 $\pm$ 923	230 $\pm$ 58	0.11	1.1
	Y253F	33.9 $\pm$ 2.0	296 $\pm$ 4	8.73	90
	K198R	239.0 $\pm$ 29	363 $\pm$ 13	1.52	15.5
	K198I	n.a.	n.a.	n.a.	0
2-Benzoylpyridine	Wild-type	177 $\pm$ 40	179 $\pm$ 12	1.01	100
	Y81F	324 $\pm$ 107	207 $\pm$ 27	0.64	63.4
	Y194F	n.a.	n.a.	n.a.	0
	Y194C	1467 $\pm$ 510	5.9 $\pm$ 0.8	0.004	0.4
	Y253F	384 $\pm$ 165	298 $\pm$ 52	0.77	76
	K198R	882 $\pm$ 101	206 $\pm$ 13	0.23	22.8
	K198I	n.a.	n.a.	n.a.	0
Octanal	Wild-type	28.4 $\pm$ 1.7	179 $\pm$ 2	6.30	100
	Y81F	23.2 $\pm$ 2.7	163 $\pm$ 4	7.03	112
	Y194F	n.a.	n.a.	n.a.	0
	Y194C	n.a.	n.a.	n.a.	0
	Y253F	29.1 $\pm$ 3.0	249 $\pm$ 4	8.56	136
	K198R	289.0 $\pm$ 44	243 $\pm$ 10	0.84	13.3
	K198I	n.a.	n.a.	n.a.	0
4-Nitroacetophenone	Wild-type	83.6 $\pm$ 6.8	163 $\pm$ 3	1.95	100
	Y81F	89.6 $\pm$ 3.5	157 $\pm$ 2	1.75	90
	Y194F	n.a.	n.a.	n.a.	0
	Y194C	n.a.	n.a.	n.a.	0
	Y253F	97.1 $\pm$ 5.8	233 $\pm$ 4	2.40	123
	K198R	1514.0 $\pm$ 346.0	126 $\pm$ 15	0.08	4
	K198I	n.a.	n.a.	n.a.	0

Table 2 Catalytic efficiency of NADPH for pig $3\alpha/\beta,20\beta$ -hydroxysteroid dehydrogenase and its mutants

All incubations were conducted in the presence of the cofactor NADPH. K_m and V_{max} values are means $\pm$ S.D. Abbreviation: n.a., no activity.

Enzyme	K_m (μ M)	V_{max} (nmol/min per mg)	V_{max}/K_m	Catalytic efficiency (%)
Wild-type	22.5 $\pm$ 4.4	236 $\pm$ 12	10.5	100
Y81F	26.6 $\pm$ 4.6	250 $\pm$ 13	9.40	89.5
Y194F	n.a.	n.a.	n.a.	0
Y194C	13.6 $\pm$ 1.4	136 $\pm$ 5.1	10.0	95
Y253F	37.1 $\pm$ 5.6	358 $\pm$ 17	9.65	92
K198R	51.5 $\pm$ 7.9	357 $\pm$ 18	6.93	66
K198I	n.a.	n.a.	n.a.	0

4-Nitroacetophenone yielded only 4 % of the wild-type catalytic efficiency. As seen in Table 1, the K_m increased by 5–18-fold depending on the substrate. However, in some cases this was compensated for by an increase in V_{max} , leading to good catalytic efficiency.

The Y81F and Y253F mutants retained good activity towards three of the four substrates. Mutant Y81F had 114 % and 112 % of the wild-type catalytic efficiency towards 4-nitrobenzaldehyde and octanal respectively, and 90 % and 63 % of the wild-type $3\alpha/\beta,20\beta$ -HSD catalytic efficiency towards 4-nitroacetophenone and 2-benzoylpyridine respectively. Compared with wild-type $3\alpha/\beta,20\beta$ -HSD, the catalytic efficiency of Y253F was 90 % for 4-

nitrobenzaldehyde, 76 % for 2-benzoylpyridine, 123 % for 4-nitroacetophenone and 136 % for octanal.

Cofactor binding

The results of binding of NADPH to wild-type $3\alpha/\beta,20\beta$ -HSD and the different mutants are shown in Table 2. The Y194F and K198I mutants were inactive and could not be used to determine cofactor binding. Interestingly, the overall catalytic efficiency was relatively unchanged for mutants Y81F, Y194C and Y253F. The catalytic efficiency with NADPH was lowered by 34 % for mutant K198R.

DISCUSSION

Our studies of $3\alpha/\beta,20\beta$ -HSD mutants confirms work with other SDRs and also yields some new information that might be related to properties unique to $3\alpha/\beta,20\beta$ -HSD.

Most of the previous mutations of the active-site tyrosine residue have been to amino acids such as alanine, phenylalanine or isoleucine that lack the chemistry of the phenolic group of tyrosine. These mutants are inactive, which is consistent with an analysis of the configuration of tyrosine and substrates and cofactor in the catalytic site on SDRs [9,21–25]. Thus our finding that the replacement of tyrosine-194 with phenylalanine leads to an inactive $3\alpha/\beta,20\beta$ -HSD is not surprising.

However, we also constructed a Y194C mutant, which had been studied only in *Drosophila* alcohol dehydrogenase by Chen

et al. [27] and protochlorophyllide reductase by Wilks and Timko [29]. The Y152C mutant of *Drosophila* alcohol dehydrogenase has approx. 0.25 % activity of the wild-type enzyme; the Y275C mutant in protochlorophyllide reductase is inactive. We find that the Y194C mutant has approx. 1 % and 0.4 % of the wild-type $3\alpha/\beta,20\beta$ -HSD activity towards 4-nitrobenzaldehyde and 2-benzoylpyridine respectively. However, mutant Y194C is inactive towards 4-nitroacetophenone and octanal.

Interestingly, there is no distinct pattern regarding better catalytic efficiency with the two aldehydes, 4-nitrobenzaldehyde and octanal, compared with the two ketones, 2-benzoylpyridine and 4-nitroacetophenone. This indicates that it is the substrate structure and not a difference in aldehyde or ketone chemistry that is responsible for the changes in activity. A similar pattern is found for the K198R mutant, as discussed below.

Consistent with other studies of mutation of the catalytically important lysine residue, we find that replacement of lysine-198 with isoleucine leads to an inactive mutant. However, the K198R mutant of $3\alpha/\beta,20\beta$ -HSD is active with all four substrates. This occurs despite the lower affinity of NADPH for K198R. The K198R mutant has an activity as high as 23 % of wild-type $3\alpha/\beta,20\beta$ -HSD, which is very different from the four SDRs tested with this mutation. In these studies, the mutation of lysine-183 to arginine in 11β -hydroxysteroid dehydrogenase type I [26], lysine-199 to arginine in *Leishmania* pteridine reductase I [32] and lysine-279 to arginine leads to inactive enzymes. The mutation of lysine-156 to arginine in *Drosophila* alcohol dehydrogenase [27] leads to an enzyme with an activity 2.2 % of that of the wild-type enzyme. Thus $3\alpha/\beta,20\beta$ -HSD seems to be unusual in retaining good activity for the K199R mutant.

In other SDRs this lysine residue is part of an α -helix on the dimer interface, which might limit the conformational flexibility of the lysine in these SDRs. In contrast, the monomeric $3\alpha/\beta,20\beta$ -HSD could have more flexibility in the catalytic site, which might account for the broad substrate specificity of this enzyme. This flexibility could allow the longer positively charged side chain of arginine to assume the role of lysine-198 in the wild-type enzyme.

The absence of substantial changes in catalytic activity in either Y81F or Y253F indicates that the phenolic group at these two positions is not involved in either catalysis or binding of cofactor. The Y81F mutant has essentially the same or a slightly higher catalytic efficiency than three of the four substrates. Only 2-benzoylpyridine shows a lower activity due to the mutation. Similarly, the Y253F mutant has an activity increased by approx. 23 % and 35 % towards 4-nitroacetophenone and octanal respectively, which is due to an increase in $V_{\max}$. The lack of a substantial loss of activity in these mutants is perplexing because these two tyrosine residues are conserved in all carbonyl reductases. It might be that these amino acids are important in the reduction of some other substrate or in conferring stability on $3\alpha/\beta,20\beta$ -HSD.

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