Kinetic alteration of a human dihydrodiol/3α-hydroxysteroid dehydrogenase isoenzyme, AKR1C4, by replacement of histidine-216 with tyrosine or phenylalanine

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Human dihydrodiol dehydrogenase with 3α-hydroxysteroid dehydrogenase activity exists in four forms (AKR1C1–1C4) that belong to the aldo–keto reductase (AKR) family. Recent crystallographic studies on the other proteins in this family have indicated a role for a tyrosine residue (corresponding to position 216 in these isoenzymes) in stacking the nicotinamide ring of the coenzyme. This tyrosine residue is conserved in most AKR family members including AKR1C1–1C3, but is replaced with histidine in AKR1C4 and phenylalanine in some AKR members. In the present study we prepared mutant enzymes of AKR1C4 in which His-216 was replaced with tyrosine or phenylalanine. The two mutations decreased 3-fold the K_m for $NADP^+$ and differently influenced the K_m and k_{cat} for substrates depending on their structures. The kinetic constants for bile acids with a 12α-hydroxy group were decreased 1.5–7-fold and those for the other substrates were increased 1.3–9-fold. The mutation also yielded different changes in sensitivity to competitive inhibitors such as hexoestrol analogues, 17β-oestradiol, phenolphthalein and flufenamic acid and 3,5,3′,5′-tetraiodothyropropionic acid analogues. Furthermore, the mutation decreased the stimulatory effects of the enzyme activity by sulphobromophthalein, clofibric acid and thyroxine, which increased the K_m for the coenzyme and substrate of the mutant enzymes more highly than those of the wild-type enzyme. These results indicate the importance of this histidine residue in creating the cavity of the substrate-binding site of AKR1C4 through the orientation of the nicotinamide ring of the coenzyme, as well as its involvement in the conformational change by binding non-essential activators.

Key words: aldo–keto reductase, coenzyme binding, non-essential activator, substrate-binding cleft.

INTRODUCTION

Dihydrodiol dehydrogenase (DD, EC 1.3.1.20) catalyses the $NADP^+$-linked oxidation of *trans*-dihydrodiols of aromatic hydrocarbons to the corresponding catechols. At least four isoenzymes, composed of 323 amino acid residues with 83–98 % sequence identities, have been isolated as enzyme proteins or cDNA species from human tissues and cultured cells [1–6]. The four isoenzymes belong to the aldo–keto reductase (AKR) superfamily and have recently been named AKR1C1–AKR1C4 [7]. AKR1C1, AKR1C2 and AKR1C4 correspond to the three isoenzymes DD1, DD2 and DD4 respectively, purified from human liver [1], and AKR1C3 has been characterized with its recombinant enzyme [8,9] but their properties are different. AKR1C1 and AKR1C3 show dual specificity for hydroxysteroids and are thought to act as 3(20)α-hydroxysteroid dehydrogenase (HSD) and 3α(17β)-HSD respectively; AKR1C3 is also classified as one type, 17HSD/KSR5, of the 17β-HSD/17-ketosteroid reductase family [10]. AKR1C2 and AKR1C4 exhibit 3α-HSD activity but the former isoenzyme oxidizes some 3α-hydroxysteroids and the latter shows broad specificity and high 3α-HSD activity towards various steroids including bile acids. Except for AKR1C4, the isoenzymes show additional oxidoreductase activity towards prostaglandins D_2 and $F_{2\beta}$ [9,11]. They also differ in their inhibitor sensitivities; AKR1C4 is uniquely activated by sulphobromophthalein [12], clofibric acid derivatives [13], anti-inflammatory 2-arylpropionic acids [14] and thyroxine [15], which kinetically show non-essential activation.

Previously we identified amino acid residues at position 54 (before the catalytic residue, Tyr-55) as determinants for the differences in substrate specificity and inhibitor sensitivity between AKR1C1 and AKR1C2, which show only seven amino acid differences [16]. In addition, a study with chimaeric enzymes, in which the C-terminal 39 residues were exchanged between AKR1C1 and AKR1C4, has suggested that residues in the C-terminal domains of the isoenzymes are involved in the binding of substrates, inhibitors and some activators [17]. Site-directed mutagenesis and crystallographic studies of aldose reductase and aldehyde reductase [18–20] have also indicated the crucial role of the C-terminal domain in substrate specificity and inhibitor specificity. However, the replacement of the C-terminal residues did not produce complete conversion of AKR1C4 into AKR1C1 [17], which suggests that further amino acid residues in the other regions are responsible for determining the substrate specificity and modulating the inhibitor binding.

Recent crystallographic studies of the AKR family proteins have shown or predicted many residues responsible for the binding of the coenzyme and substrate [20–25]. One of the proposed functional residues, tyrosine (corresponding to position 216 in AKR1C1–1C4) has been shown to have a role in stacking the nicotinamide ring of the coenzyme in the AKR structures. This residue is conserved in most AKR family proteins including

Abbreviations used: AKR, aldo–keto reductase; DD, dihydrodiol dehydrogenase; HSD, hydroxysteroid dehydrogenase; WT, wild-type AKR1C4.

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AKR1C1, AKR1C2 and AKR1C3; however, the corresponding residue is phenylalanine or tryptophan in some members of this family and is histidine in only two members, AKR1C4 and *Malus domestica* sorbitol phosphate dehydrogenase [26]. Either of the residues with an aromatic ring at this position in the AKR family members is expected to have the same role as the tyrosine residue in the known AKR structures because of the high degree of amino acid sequence similarity between the members of this family [25]. However, the molecular size and hydrophobicity of the above four amino acid residues are different. Replacement of a residue at this position with one of the other residues would influence the active-site cavity of AKR proteins and might affect the substrate specificity. In the present study we prepared the AKR1C4 mutant enzymes His-216 → Tyr (H216Y), His-216 → Phe (H216F), His-216 → Trp (H216W) and His-216 → Leu (H216L), of which H216Y and H216F were purified, and we characterized their alterations in terms of their affinities for the coenzyme, their specificities for substrates and inhibitors and their non-essential activation, which is a unique property of this isoenzyme.

EXPERIMENTAL

Materials

Pfu DNA polymerase and a Genepure kit for isolation of DNA were purchased from Stratagene and Nippongene (Tokyo, Japan) respectively. Restriction endonucleases were obtained from Takara (Kusatsu, Japan); plasmids, *Escherichia coli* host strain JM109, Sephadex G-100 and Q-Sepharose were from Pharmacia; and steroids, anti-inflammatory drugs and thyroxine analogues were from Sigma-Aldrich Co. (*R*)-Ibuprofen was obtained from Research Biochemicals (Natick, MA, U.S.A.). Expression plasmid pKK 223-3 containing the cDNA for AKR1C4 (pKKDD4), previously constructed [27], was used.

Construction of expression plasmids

Four AKR1C4 mutants, H216Y, H216F, H216W and H216L, were generated by the overlap-extension method [28], with *Pfu* DNA polymerase and the following primer pairs, each of which was composed of forward and reverse oligonucleotides. The sequences of the mutagenic 21-mer primers synthesized corresponded to positions 637–657 of AKR1C4 cDNA, except that the original codon, CAC, was replaced with TAT, TTC, TGG or CTC for H216Y, H216F, H216W or H216L respectively. The mutated cDNA species were prepared by the method described for the preparation of other mutant AKR1C4 cDNA species [29]. The entire coding regions of the cDNA species in the expression plasmids were sequenced as described previously [30] to confirm the presence of the desired mutation and to ensure that no other mutation had occurred.

Expression of the recombinant mutant enzymes in the *E. coli* cells and their purification were performed by the methods described for wild-type AKR1C4 (referred to as WT hereafter) [27].

Enzyme assay

Dehydrogenase activity was assayed fluorimetrically by recording the production of NADPH as described [1]. The standard reaction mixture consisted of 0.1 M potassium phosphate, pH 7.4, 0.25 mM $NADP^+$, 5 μM (for WT) or 50 μM (for the mutant enzymes) androsterone and enzyme, in a total volume of 2.0 ml. One unit of enzyme activity was defined as the amount catalysing the formation of 1 μmol of NADPH/min at 25 °C.

Kinetic constants for the substrates were determined by Lineweaver–Burk analyses with at least five different substrate concentrations at a saturating $NADP^+$ concentration of 0.25 mM. The kinetic study in the presence of inhibitor was performed in a similar manner; the inhibition constant, K_i, for the competitive inhibitor was determined as described [31]. The mutual exclusivity of the two inhibitors was examined by Dixon plots of $1/v$ against the concentration of one inhibitor at a fixed concentration of another inhibitor, as described by Segel [32]. Unless otherwise noted, these kinetic values are the means of triplicate determinations.

Determination of K_d values for NAD(P)$^+$

The K_d values for the binding of $NADP^+$ to WT and the mutant enzymes were determined by measuring protein fluorescence as described for the determination of the values for thyroxine derivatives [15], except that an excitation wavelength of 295 nm was chosen to minimize photodecomposition of the enzymes. Although the K_d for $NADP^+$ was obtained by incremental addition of the coenzyme (0.05–2.0 μM) to the enzyme solution (1.0 μM in 0.1 M potassium phosphate, pH 7.0), the enzyme was not saturated with 0.5 mM NAD^+ because of its low affinity. The K_d for NAD^+ was determined from competitive inhibition studies in NADPH-linked reductase activities of the enzymes. The reductase activity was assayed at 25 °C by recording the NADPH oxidation rate (at 340 nm) in a reaction mixture (total volume of 2.0 ml) consisting of 0.1 M potassium phosphate, pH 7.0, 4.0 mM pyridine-3-aldehyde, NADPH (1–10 μM) and enzyme.

Other methods

Protein concentration was determined by the method of Bradford [33]. SDS/PAGE on a 12.5% (w/v) slab gel [34] and Western blot analysis with antibodies against AKR1C4 [27] were performed as described. The effects of activators on the fluorescence of the enzyme (1.0 μM) were determined at 25 °C in 0.1 M potassium phosphate buffer, pH 7.4 [13,15].

RESULTS AND DISCUSSION

Expression and purification of the mutant enzymes

The expression plasmids for the mutant AKR1C4 enzymes H216Y, H216F and H216W were prepared because other AKR-family proteins have these aromatic amino acids at position 216. In addition, an expression plasmid for the mutant H216L was constructed to determine the effect of the removal of the aromatic ring from the residue at this position. All of the mutant enzymes were expressed in *E. coli* cells that had been transfected with the constructs for the mutant enzymes: 36 kDa recombinant enzymes were detected by Western blot analysis of the cell extracts (Figure 1A). The extracts of H216F and H216Y showed androsterone dehydrogenase activities of 0.015 and 0.010 unit/mg respectively; however, the activity was low (0.001 unit/mg) in the extract of H216W and undetectable in the extract of H216L. H216F and H216Y were therefore purified to homogeneity on the basis of an SDS/PAGE analysis (Figure 1B). The overall yields of H216Y and H216F were 34% and 29% respectively; the respective specific activities were 0.45 and 0.25 unit/mg, which are high in comparison with 0.07 unit/mg for the purified WT.

Khurana et al. [35] have reported the crystal structure of a complex of 2,5-dioxo-D-gluconic acid reductase, a member of the AKR family, with NADPH, in which tryptophan residue at the position corresponding to His-216 is involved in a stacking interaction with the nicotinamide ring of the coenzyme. The

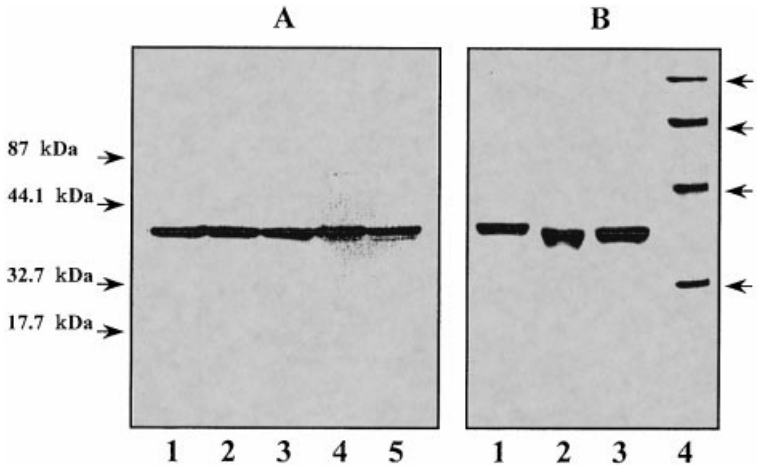

Figure 1 Western blot analysis of WT and mutated enzymes in the extracts of *E. coli* cells, and SDS/PAGE of the purified enzymes

(**A**) The *E. coli* extracts (each containing 20 μg of proteins) were analysed by Western blotting with antibodies against AKR1C4. Lane 1, WT; lane 2, H216Y; lane 3, H216F; lane 4, H216W; lane 5, H216L. The positions of molecular-mass markers are indicated at the left. (**B**) The purified enzymes (each 1 μg) of WT (lane 1), H216Y (lane 2) and H216F (lane 3) were analysed by SDS/PAGE and stained for protein with Coomassie Brilliant Blue R-250. Molecular-mass marker proteins in lane 4 (arrowed) were 94, 67, 43 and 30 kDa, from the top.

Table 1 Effects of the mutation on the K_d values and kinetic constants for the coenzymes

Results shown are means; S.E.M. values were less than 15% except where indicated with an asterisk (less than 20%). Numbers in parentheses are ratios of the value for the mutant enzyme to that for WT. The K_d for NAD^+ was determined from inhibition studies.

Coenzyme	Constant	WT	H216Y	H216F
$NADP^+$	K_d (μM)	0.32	0.21 (0.6)	0.27 (0.8)
	K_m (μM)	1.7	0.5 (0.3)	0.6 (0.4)
	k_{cat} (min^{-1})	2.4	15 (6.3)	9.6 (4.0)
	k_{cat}/K_m ($min^{-1} \cdot \mu M^{-1}$)	1.4	30 (21)	16 (11)
NAD^+	K_d (mM)	0.89	0.88 (0.9)	1.0 (1.1)
	K_m (mM)	1.2*	1.0 (0.8)	1.2 (1.0)
	k_{cat} (min^{-1})	12	35 (2.9)	20 (1.7)
	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)	10*	35 (3.5)	17 (1.7)

enzyme exhibits a lower binding affinity for the coenzyme than human aldose reductase, which has a tyrosine residue at this position. The low affinity for the coenzyme has been explained as resulting from the smaller number of interactions between the residues of the enzyme and the coenzyme molecule compared with that in human aldose reductase. Although the properties of H216W could not be characterized because of its low activity, the enzyme preparation partly purified by Sephadex G-100 filtration showed K_m values for $NADP^+$ (6.7 μM) and androsterone (7.2 μM) that were higher than the respective values of WT, H216Y and H216F (Tables 1 and 2). The introduction of the larger tryptophan residue in place of His-216 might impair the interactions of other residues with the coenzyme molecule or the spatial arrangement of C-4 of the nicotinamide ring of $NADP^+$ and the catalytic residue Tyr-55 [16] via a change in the orientation of the nicotinamide ring of $NADP^+$. The production of inactive enzyme by the loss of the aromatic ring in H216L might produce a more drastic structural change in the coenzyme-binding site and/or the active site, which suggests the importance of residues with an aromatic ring at this position in stacking the nicotinamide ring of the coenzyme.

Alteration of coenzyme binding by mutagenesis

The K_d values for coenzymes were first compared between WT and H216Y or H216F because His-216 was expected to be involved in the binding of the coenzyme, as described above. The mutations slightly decreased the K_d value for $NADP^+$, but no apparent change was observed in the value for NAD^+ (Table 1). The small increases in the affinity for $NADP^+$ by mutations of H216Y and H216F correspond to increases of 1.0 and 0.4 kJ/mol respectively in ground-state binding energy, which was calculated from:

$$\Delta\Delta G_b = -RT \ln [K_{d(WT)}/K_{d(mutant)}]$$

A phenylalanine or a tyrosine residue, being more hydrophobic than histidine, might exert a larger π–π stacking force on the nicotinamide ring of $NADP^+$ than does His-216. When the steady-state kinetic properties of WT and the mutant enzymes were compared, the K_m values for $NADP^+$ were decreased approx. 3-fold and k_{cat} values were increased more than 4-fold, resulting in changes in catalytic efficiency of more than 11-fold. The thermodynamic effects of H216Y and H216F on the transition-state binding energy in the $NADP^+$-linked reaction were calculated to be 7.6 and 6.0 kJ/mol respectively, from

$$\Delta\Delta G_t = -RT \ln [(k_{cat}/K_m)_{WT}/(k_{cat}/K_m)_{mutant}]$$

The larger values in comparison with the effects on the ground-state binding energy suggest that the residue at position 216 is also important in maintaining the transition-state binding energy and that the presence of tyrosine or phenylalanine instead of His-216 is suitable for allowing the $NADP^+$-linked reaction to proceed. In contrast, the effects of mutations on the NAD^+-linked activities were also not pronounced and only the k_{cat} values increased 2–3-fold. The mutations probably elicit small conformational changes around the coenzyme-binding site to decrease the transition-state energy of the $NADP^+$-linked reaction; however, the microenviroment change might not significantly influence the binding of the low-affinity coenzyme, NAD^+. Alternatively, $NADP^+$ and NAD^+ might bind differently as

Table 2 Comparison of kinetic constants for substrates between WT and mutated enzymes

Kinetic constants are shown as means; S.E.M. values are < 20%. Abbreviation: tetralol, 1,2,3,4-tetrahydronaphth-1-ol.

		WT			H216Y			Ratio of H216Y/WT			H216F			Ratio of H216F/WT		
Group	Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min$^{-1}\cdot$mM^{-1})	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min$^{-1}\cdot$mM^{-1})	K_m	k_{cat}	k_{cat}/K_m	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min$^{-1}\cdot$mM^{-1})	K_m	k_{cat}	k_{cat}/K_m
Alicyclic alcohols	(*S*)-Indan-1-ol	146	6.1	0.04	420	24	0.06	2.8	3.9	1.4	280	16	0.06	1.9	2.6	1.5
	(*S*)-Tetralol	110	12	0.11	290	26	0.09	2.6	2.2	0.8	240	16	0.07	2.2	1.3	0.6
	(*R*)-Tetralol	160	11	0.07	680	46	0.07	4.2	4.2	1.0	380	23	0.06	2.4	2.1	0.9
Androstanes	Androsterone	0.5	2.6	5.2	2.7	17	6.3	5.3	6.4	1.3	2.2	9.2	4.2	3.9	3.5	0.9
	5β-Androstan-3α-ol-17-one	0.9	2.9	3.2	1.8	11	6.1	1.2	3.6	3.2	1.4	8.1	5.8	1.2	2.5	2.2
	5α-Androstane-3α,17β-diol	0.8	5.2	6.2	2.6	21	8.1	4.5	4.9	1.2	3.1	18	5.8	5.3	4.5	0.9
	5β-Androstane-α,17β-diol	1.2	2.8	2.3	2.2	11	5.0	2.1	3.4	1.6	2.1	9.9	4.7	2.3	3.1	1.4
Bile acids	Lithocholic acid	1.0	1.9	1.9	1.9	2.6	1.4	1.9	1.4	0.7	1.8	2.4	1.3	1.8	1.3	0.7
	Chenodeoxycholic acid	1.1	1.9	1.7	2.9	8.0	2.8	2.6	4.2	1.6	2.3	6.3	2.7	2.1	3.3	1.6
	Deoxycholic acid	9.7	5.1	0.53	1.3	1.1	0.85	0.1	0.2	1.6	1.4	0.7	0.54	0.1	0.1	0.9
	Cholic acid	59	12	0.20	41	3.0	0.07	0.7	0.3	0.4	32	1.8	0.06	0.5	0.2	0.3

Table 3 Comparison of inhibitor sensitivity between WT and mutated enzymes

Inhibition constants are shown as means; S.E.M. values were less than 15% except where indicated with an asterisk (less than 25%). Numbers in parentheses are ratios of the values for mutant enzyme to that for WT. Abbreviations: T_4P, 3,5,3′,5′-tetraiodothyropropionic acid; T_3P, 3,5,3′-tri-iodothyropropionic acid; T_3A, 3,5,3′-tri-iodothyroacetic acid.

	K_i (nM)		
Inhibitor	WT	H216Y	H216F
Hexoestrol	120	2* (0.02)	2* (0.02)
Dienoestrol	430	17 (0.04)	13* (0.03)
Diethylstilboestrol	900	48 (0.05)	50 (0.05)
Phenolphthalein	18	6* (0.3)	8* (0.4)
17β-Oestradiol	360	440 (1.3)	480 (1.3)
T_4P	40	9* (0.2)	11 (0.3)
T_3P	60	12 (0.2)	20 (0.3)
T_3A	1500	78 (0.05)	80 (0.05)
Flufenamic acid	22000	14000 (0.7)	16000 (0.8)

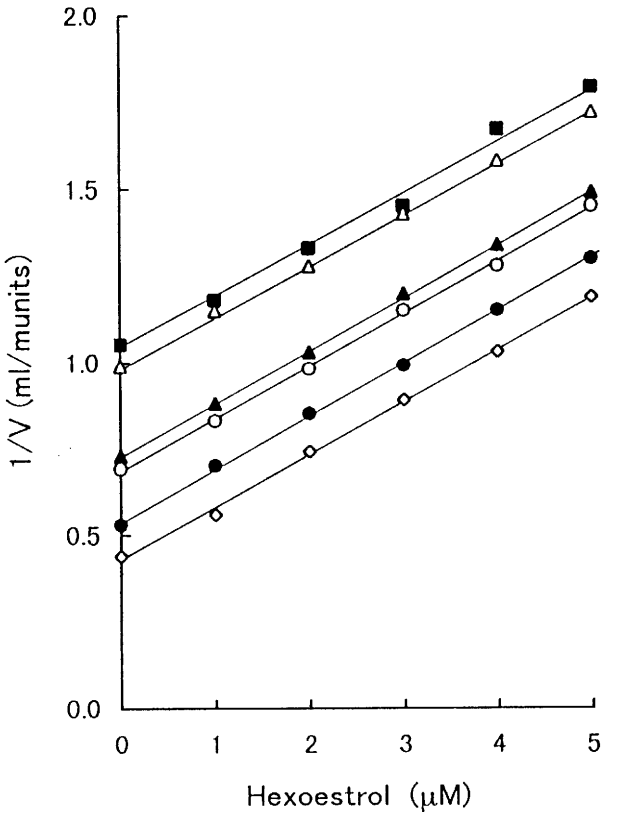

Figure 2 Dixon plots of the combined inhibition of WT by hexoestrol and other inhibitors

The activity was assayed with 5 μM androsterone as the substrate in the absence (◇) or presence of the following mixed inhibitors: 100 nM 3,5,3′,5′-tetraiodothyropropionic acid (●), 80 μM flufenamic acid (○), 1 μM 3,5,3′-tri-iodothyroacetic acid (▲), 8 μM 17β-oestradiol (△) and 50 nM phenolphthalein (■).

reported recently by Ma et al. [36], who suggested differential binding modes for NADP(H) and NAD(H) with rat 3α-HSD by site-directed mutagenesis of four nicotinamide pocket residues, including Tyr-216.

Table 4 Effects of activators on WT and mutated enzymes

Values are means for two determinations; numbers in parentheses are ratios of the values of the activities with activator to those without activator.

Activator	Concentration (μM)	Relative activity (%)			K_m for $NADP^+$ (μM)			K_m for androsterone (μM)			k_{cat} (min^{-1})		
		WT	H216Y	H216F	WT	H216Y	H216F	WT	H216Y	H216F	WT	H216Y	H216F
None		100	100	100	1.6	0.5	0.5	0.5	2.2	2.6	2.6	18	9.2
Sulphobromophthalein	5	328	169	158	1.9 (1.2)	2.5 (5.0)	1.4 (2.8)	1.1 (2.2)	23 (10)	23 (8.8)	27 (10)	50 (2.8)	29 (3.2)
(*R*)-Ibuprofen	50	167	122	115	1.7 (1.1)	1.0 (2.0)	0.6 (1.2)	0.7 (1.4)	5.0 (2.3)	8.3 (3.2)	10 (3.8)	23 (1.3)	13 (1.4)
Clofibric acid	100	240	107	112	1.8 (1.1)	0.9 (1.8)	1.1 (2.2)	0.8 (1.6)	11 (5.0)	10 (3.8)	6.4 (2.5)	26 (1.4)	15 (1.6)
L-Thyroxine	1	237	137	127	1.9 (1.2)	1.8 (3.6)	1.3 (2.6)	1.0 (2.0)	20 (9.1)	14 (5.4)	17 (6.5)	40 (2.2)	19 (2.1)
D-Thyroxine	1	260	142	127	1.7 (1.1)	1.9 (3.8)	1.5 (3.0)	1.2 (2.4)	13 (5.9)	13 (5.0)	20 (7.7)	37 (2.1)	19 (2.1)

Effect of mutagenesis on binding of substrate

Because the reaction catalysed by AKR1C4 follows an Ordered Bi Bi mechanism with $NADP^+$ binding first [27], the alteration in the K_m for the coenzyme by the mutations of H216Y and H216F also affect the kinetic constants for the substrate. The mutations influenced the apparent kinetic constants differently for various substrates, depending on their structures (Table 2). The two mutants increased both K_m and k_{cat} values for alicyclic alcohols, androstanes and bile acids without a 12α-hydroxy group 1.3–7-fold, whereas the values for deoxycholic acid and cholic acid were decreased 1.4–10-fold. Although the changes in k_{cat}/K_m values for the substrates by the mutations were low, the values for 5β-androstan-3α-ol-17-one more than doubled and those for cholic acid decreased to less than half. In addition, the specificity for 5α- and 5β-androstanes was slightly affected by the mutations. WT showed higher k_{cat}/K_m values for 5α-androstanes than for 5β-androstanes, whereas the values for H216Y and H216F towards 5α- and 5β-androstanes were almost the same, mainly because of the larger increase in the K_m values for 5α-androstanes compared with their 5β isomers. The different effects of the mutations on the kinetic constants for the respective substrates suggest that the replacement of His-216 with tyrosine or phenylalanine alters the substrate-binding cavity through the change in the orientation of the coenzyme.

Effect of mutagenesis on binding of competitive inhibitors

To make a further assessment of the change in the substrate-binding cleft by mutagenesis, the K_i values for various competitive inhibitors were compared between WT and H216Y or H216F. In addition to known competitive inhibitors (hexoestrol, phenolphthalein, flufenamic acid, 3,5,3′,5′-tetraiodothyropropionic acid and 3,5,3′-tri-iodothyropropionic acid) [2,15,17,27], dienoestrol, diethylstilboestrol, 17β-oestradiol and 3,5,3′-tri-iodothyroacetic acid inhibited both WT and the mutant enzymes competitively with respect to the substrate (Table 3). The mutations produced different effects on the K_i values for the competitive inhibitors depending on the inhibitors, although the sensitivities of H216Y and H216F to inhibitors were almost the same. The inhibitors are structurally divided into two groups, phenolic compounds and carboxylic acids. AKR family enzymes contain an anionic binding site that is formed by C-4 of the nicotinamide ring of the coenzyme and active-site residues corresponding to Tyr-55, Lys-84 and His-117 of AKR1C4 [25,37]; Jez et al. [25,38] have indicated that the carboxylic group of inhibitory compounds and an appropriate electronegative phenolic group of hexoestrol interact with the anionic binding site of rat 3α-HSD. The Dixon plots of $1/v$ against hexoestrol concentration at a fixed concentration of another inhibitor with AKR1C4 were parallel, as the representative results with WT show in Figure 2. Thus the carboxylic and phenolic groups of the present inhibitors might also bind to the identical anionic binding site in the active site of the human enzyme; the differential effects of mutations on inhibitor binding suggest not only that the binding methods of the other parts of the inhibitors in the substrate-binding cleft are important for determining inhibitory potency, but also that the substrate-binding cleft is altered by the mutations.

The mutations had a large effect on the K_i values for the synthetic oestrogens of the phenolic inhibitors. The synthetic oestrogens structurally mimic 17β-oestradiol but lack a methyl group corresponding to C-19 of 17β-oestradiol and are more flexible than the 17β-oestradiol molecule. Either or both of the structural differences might be related to the high affinity of the mutant enzymes for the synthetic oestrogens. In addition, the

extents of the decrease in the K_i values were hexoesterol > dienoestrol > diethylstilboestrol, which differ structurally only in the central aliphatic chain of two carbons between the two phenyl rings. The change in the substrate-binding cleft induced by the mutations might extend beyond the phenolic ring of the synthetic oestrogens that bind to the active site. For the carboxylic acid inhibitors examined, only the K_i for 3,5,3′-tri-iodothyroacetic acid was significantly decreased by the mutations, similarly to the different effects on the binding of the phenolic inhibitors. The previous inhibition study of 3,5,3′,5′-tetraiodothyropropionic acid and 3,5,3′-tri-iodothyropropionic acid [15] suggested that, in addition to the hydrophobic interaction between the iodinated phenyl rings and the residues of AKR1C4, the carboxylic group of the inhibitors binds to the anion-binding site in the active site of the enzyme. The inhibition pattern and the significant decrease in the K_i value for 3,5,3′-tri-iodothyroacetic acid suggest that the interaction of the carboxy group of this inhibitor with the anion-binding site of the enzyme is made tighter by the mutation. Because the molecular size of this inhibitor is shorter by a carbon than that of 3,5,3′-tri-iodothyropropionic acid, the large change in the affinity for this inhibitor is clear evidence for alteration of the cavity around the active site of the enzyme by the mutation.

The above kinetic data collectively show not only the role of His-216 in stacking interaction with the nicotinamide ring of the coenzyme but also its importance for controlling the substrate specificity of a human 3α-HSD isoenzyme, AKR1C4. Previous functional studies on the substrate-binding site of the AKR family proteins have focused on residues that are directly involved in the binding of the substrates and/or inhibitors (cited in [25,39]). The present study is the first to show the important role of a residue responsible for the orientation of the coenzyme in serving indirectly to create the substrate-binding cavity.

Effects of mutagenesis on non-essential activation

AKR1C4 is uniquely activated by several drugs and thyroxines with a sulphonyl or carboxy group [12–15]. Because the activation is abolished by the mutation of Lys-270 and Arg-276, which are responsible for the binding of the 2′-phosphate of NADP(H) [29], the negatively charged group of the activators has been suggested to interact with Lys-270 and/or Arg-276 of the enzyme, and the activation has been thought to result from the elevation of catalytic-centre activity caused by the increase in the K_m values for the coenzyme and substrate. Lys-270 and Arg-276 are conserved in the other human AKR1C enzymes, which are not activated by the activators. Therefore the other residues of AKR1C4, which differ from those of the other isoenzymes, might be involved in the binding of the activators. Although hydrophobic interaction of the aromatic ring of the activator molecule with the enzyme [12–15] and the involvement of the C-terminal loop of the enzyme in the binding of some activators [17] have been suggested, the residues responsible for the interactions have not been identified definitely. Because His-216 of AKR1C4 is replaced with a tyrosine residue in the other human AKR1C enzymes, and its replacement with tyrosine or phenylalanine affected the kinetic constants for the coenzyme and substrates, the effect of the mutation on non-essential activation was examined. Both the H216Y and H216F mutations significantly decreased the stimulatory effects of all the activators (Table 4). However, the intrinsic fluorescence of the mutant enzymes, similarly to that of WT [12,15], was quenched by the addition of sulphobromophthalein and L-thyroxine (results not shown), which indicates that the activators bind to the free forms of the mutant enzymes. The activation of the mutant enzymes resulted in increases in the K_m values for $NADP^+$ (1.2–5-fold) and androsterone (2.3–10-fold) that were larger than those for the coenzyme (1.1–1.3-fold) and substrate (1.4–2.2-fold) determined with WT. Conversely, the increase in k_{cat} caused by the activation of the mutants was smaller than that for WT. The small increase in k_{cat} brought about by the addition of the activators to the mutants, despite the large extent of the increase in the K_m values, suggests that the activation mechanism is not simple, as has previously been reported [12,29]. Considering the alteration of the orientation of $NADP^+$ and the substrate-binding cleft by the mutations, the conformation of the mutant enzymes induced by the binding of the activator might differ from that of the activated form of WT. His-216 might be required for creating the conformational change after the binding of the activator. It should be noted that monkey liver 3α-HSD, which has a high sequence similarity (including His-216) to AKR1C4, is activated by the above activators (T. Kamiya and A. Hara, unpublished work). Therefore the human and monkey 3α-HSD isoenzymes, in contrast with the other AKR1C enzymes, have an activator-binding site that is composed of Lys-270 and Arg-276, residues in the C-terminal loop, and probably other unidentified residues. Although His-216 is not involved directly in the binding of the activators, the presence of this residue is one of the structural determinants for non-essential activation.

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