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Identification of a determinant for strict NADP(H)-specificity and high sensitivity to mixed-type steroid inhibitor of rabbit aldo–keto reductase 1C33 by site-directed mutagenesis

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ABSTRACT

In rabbit tissues, hydroxysteroid dehydrogenase belonging to the aldo–keto reductase (AKR) superfamily exists in six isoforms (AKRs: 1C5 and 1C29–1C33), sharing >73% amino acid sequence identity. AKR1C33 is strictly NADPH-specific, in contrast to dual NADPH/NADH specificity of the other isoforms. All coenzyme-binding residues of the structurally elucidated AKR1C5 are conserved in other isoforms, except that S217 (interacting with the pyrophosphate moiety) and T273 (interacting with the 2′-phosphate moiety) are replaced with F217 and N272, respectively, in AKR1C33. To explore the determinants for the NADPH specificity of AKR1C33, we prepared its F217S and N272T mutant enzymes. The mutation of F217S, but not N272T, converted AKR1C33 into a dually coenzyme-specific form that showed similar k_{cat} values for NAD(P)H to those of AKR1C32. The reverse mutation (S217F) in dually coenzyme-specific AKR1C32 produced a strictly NADPH-specific form. The F217S mutation also abolished the activity towards 3-keto-5β-cholestanes that are substrates specific to AKR1C33, and markedly decreased the sensitivity to 4-pregnenes (such as deoxycorticosterone and medroxyprogesterone acetate) that were found to be potent mixed-type inhibitors of the wild-type enzyme. The results indicate the important role of F217 in the strict NADPH-dependency, as well as its involvement in the unique catalytic properties of AKR1C33.

Introduction

The aldo–keto reductases (AKRs) are a growing superfamily [1–3], which includes more than 150 primary structures annotated in various sequence databases. The majority of the functionally characterized members in this superfamily are NAD(P)(H)-dependent enzymes that metabolize carbohydrates, steroids, and other endogenous aldehydes and ketones, as well as xenobiotic compounds. The members of this superfamily are classified into 15 families, in which proteins with >60% sequence identity are further divided into several subfamilies. The crystallographic studies show that AKRs adopt a $(\alpha/\beta)_8$-barrel structure characterized by a deep hydrophobic active-site located near the C-terminal end of the β-barrel [1,2,4–10]. The coenzyme is situated at the base of the active-site cavity, in which a conserved catalytic tetrad (D50, Y55, K83 and H117; shown as the residue numbers of AKR1C5) forms an oxyanion binding site with the nicotinamide ring of the coenzyme via a hydrogen-bonding network. The optimal substrate positioning for a specific activity is dictated by the configuration of the residues forming the substrate-binding cavity, in which some substrate-binding residues are highly conserved in the enzymes, but other residues and their positions are different depending on the diverse functions of the enzymes.

Most AKRs utilize NADP(H) or NAD(H) as the coenzyme, NADP(H) being preferred. Previous studies have shown that salt bridges between K270 and/or R276 and the NADPH 2′-phosphate are critical for the preference for NADP(H) [1,4]. The opposite preference for NAD(H) to NADP(H) is observed in several AKRs, in which the K270 and/or R276 in the NADP(H)-preferring AKRs are replaced with Q and E, respectively [11–15]. The key roles of Q270 and E276 in the NAD(H) preference are demonstrated by site-directed mutagenesis of the residues in rat NAD^+-dependent

Abbreviations: 5αAD, 5α-androstane-3α,17β-diol; AKR, aldo–keto reductase; DOC, deoxycorticosterone; HSD, hydroxysteroid dehydrogenase; P3A, pyridine-3-aldehyde.

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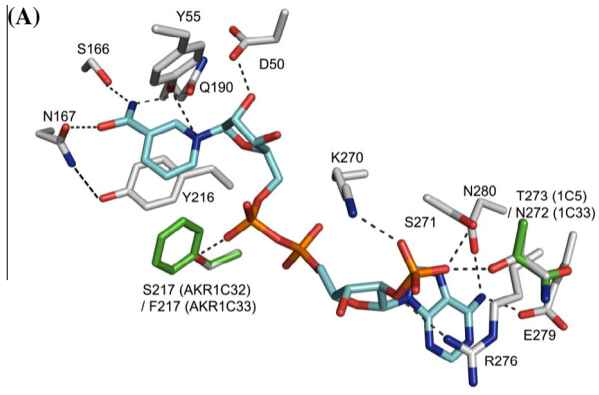
3α-hydroxysteroid dehydrogenase (HSD) [13]. This superfamily also includes several AKRs, which exhibit absolute coenzyme specificity for either NADP(H) or NAD(H). Such NADPH-specific enzymes are AKRs (1A1, 1B8, 1B13, 1C8, 2E3, 3F2 and 3F3) [16–20], whereas only one NAD(H)-specific AKR11B2 was reported [20]. The NADH specificity of AKR11B2 is shown to be due to D232 that forms interaction with hydroxyl groups of the adenine ribose and electrostatic repulsion against the NADPH 2′-phosphate [20]. However, the structural basis of the absolute NADPH specificity of the above AKRs remains unknown.

In our previous studies, rabbit HSD belonging to the AKR1C subfamily was found to exist in nine forms, sharing more than 73% amino acid sequence identity. They are AKR1C5 (17β/20α-HSD) [7,21], AKRs (1C26, 1C27 and 1C28) acting as 3α/17β-HSDs [22], AKR1C29 (3α/3β/17β/20α-HSD) [23], AKR1C30 (17β-HSD), AKR1C31 (3α/17β/20α-HSD), AKR1C32 (3α/20α-HSD) and AKR1C33 (3α/17β/20α-HSD) [24]. Among the isoforms, AKR1C33 shows the broadest substrate specificity for various steroids including bile acids and 3-keto-5β-cholestanes, and is selectively inhibited by hexestrol, zearalenone and isolithocholic acid [24]. These rabbit isoforms are divided into three groups differing in coenzyme specificity. (1) NAD(H)-preferring AKRs (1C26, 1C27 and 1C28), which possess E276, one of the determinants of the NAD(H) preference [13]. (2) NADP(H)-preferring AKRs (1C5, 1C29, 1C30, 1C31 and 1C32), in which K270 and R276, the determinants for NADP(H) preference, are conserved. (3) Strictly NADP(H)-specific AKR1C33. Although K270 and R276 are conserved in AKR1C33, other residues are presumed to be involved in its NADP(H) specificity.

The coenzyme-binding residues observed in the $NADP^+$-bound AKR1C5 crystal structure [7] are all conserved in other rabbit NADP(H)-preferring AKRs (1C29–1C32), whereas S217 and T273, that interact with the pyrophosphate and 2′-phosphate moieties of $NADP^+$ [7], are replaced with F217 and N272, respectively, in strictly NADPH-specific AKR1C33 (Fig. 1). In order to clarify the structural features that confer the absolute NADPH specificity, we have prepared mutant enzymes by exchanging the two residues between AKR1C33 and AKR1C32, and analyzed the alterations in the kinetic constants for the coenzymes, substrates and inhibitors including 4-pregnenes that were found to be potent and mixed type inhibitors in this study.



(B)

	50	55	166	167	190	216	217	270	273	280
1C5	D	Y	S	N	Q	Y	S	KSFTEKRIKEN		
1C29-1C32	D	Y	S	N	Q	Y	S	KSFTEKRIKEN		
1C33	D	Y	S	N	Q	Y	F	KSFNEKRIKEN		
								269	272	279

Fig. 1. (A) $NADP^+$-binding residues of AKR1C5 crystal structure (taken from PDB: 1Q13). The side-chains of the residues (white) form hydrogen bond interactions (dotted lines) with $NADP^+$ (sky-blue), except that Y216 stacks with the nicotinamide ring of the coenzyme. The AKR1C33 model structure was superimposed with the AKR1C5 structure, and its F217 and N272 targeted for mutation are shown in green. (B) Comparison of the coenzyme-binding residues among rabbit AKRs (1C5, 1C29–1C32, and 1C33). Since AKR1C33 lacks a residue corresponding to P225 of the other enzymes, its residue numbers of 269–279 are indicated below its sequence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Materials and methods

Mutagenesis and purification of recombinant enzymes

Mutagenesis was performed using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and templates (the pGEX-2T and pCold I expression plasmids harboring the cDNAs for AKR1C33 and AKR1C32, respectively [24]) according to the protocol described by the manufacturer. The mutations were carried out using each set of forward and reverse mutagenic oligonucleotides (Supplementary Table S1). The complete coding regions of the cDNAs in the expression plasmids were sequenced by using a Beckman CEQ8000XL DNA sequencer to confirm the presence of the desired mutation and to ensure that no other mutation had occurred.

The expression constructs were transformed into *Escherichia coli* BL21 (DE3) pLysS cells (Invitrogen, Carlsbad, CA). The recombinant wild-type and mutant forms of AKR1C33 and AKR1C32 were expressed in the *E. coli* cells and purified by the methods for purification of AKR1C1 [25] and AKR1B19 [26], respectively. The purified enzyme fractions were concentrated by ultrafiltration and dialyzed against 10-mM Tris–HCl, pH 8.0, containing 5-mM 2-mercaptoethanol, 0.5-mM EDTA and 20% (v/v) glycerol. Purity was confirmed by SDS–polyacrylamide gel electrophoresis. Typical yields for all proteins were 3–10 mg/L of culture, as measured by the method of Bradford [27] using bovine serum albumin as the standard.

Assay of enzyme activity and kinetic analysis

The reductase and dehydrogenase activities of the enzymes were assayed at 25 °C by measuring the rate of change in NAD(P)H absorbance at 340 nm, except that an absorbance at 366 nm was monitored in the assay with high concentrations of NAD(P)H [24]. In the assay of low dehydrogenase activity, the rate of formation in NAD(P)H fluorescence (at 455 nm with an excitation wavelength of 340 nm) was monitored. The standard reaction mixture for the reductase activity consisted of 0.1 M potassium phosphate, pH 7.4, 0.1 mM NADPH, 4 mM pyridine-3-aldehyde (P3A) and enzyme, in a total volume of 2.0 mL. In the standard reaction mixture for the dehydrogenase activity, 0.25 mM $NADP^+$ and 20 μM 5α-androstane-3α,17β-diol (5αAD) for AKR1C33 or 0.4 mM β-ionol for AKR1C32 were used as the coenzyme and substrate, respectively. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation and oxidation of 1 μmol NAD(P)H per min.

The K_m and k_{cat} values were determined with five different concentrations of substrate or coenzyme in the presence of saturated concentrations of the corresponding coenzyme or substrate by fitting the initial velocities to the Michaelis–Menten equation. The kinetic studies in the presence of inhibitors were carried out in a similar manner. The inhibition patterns were judged from the Lineweaver–Burk plots, and inhibition constants, K_{is} (slope effect) and K_{ii} (intercept effect), were determined from replots of the slopes and intercepts, respectively, versus inhibitor concentration [28]. All kinetic measurements were performed at least three times, and the constants were expressed as the means ± SD. Steroids used as substrates and inhibitors were obtained from