Modular exchange of substrate-binding loops alters both substrate and cofactor specificity in a member of the aldo-keto reductase superfamily

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Substrate specificity in the aldo-keto reductase (AKR) superfamily is determined by three mobile loops positioned at the top of the canonical (α/β)8-barrel structure. These loops have previously been demonstrated to be modular in a well-studied class of AKRs, in that exchanging loops between two similar hydroxysteroid dehydrogenases resulted in a complete alteration of substrate specificity (Ma,H. and Penning,T.M. (1999) Proc. Natl Acad. Sci. USA, 96, 11161–11166). Here, we further examine the modularity of these loops by grafting those from human aldose reductase (hAR) into the hyperthermostable AKR, alcohol dehydrogenase D (AdhD), from Pyrococcus furiosus. Replacement of Loops A and B was sufficient to impart hAR activity into AdhD, and the resulting chimera retained the thermostability of the parent enzyme. However, no active chimeras were observed when the hAR loops were grafted into a previously engineered cofactor specificity mutant of AdhD, which displayed similar kinetics to hAR with the model substrate di-glyceraldehyde. The non-additivity of these mutations suggests that efficient turnover is more dependent on the relative positioning of the cofactor and substrate in the active site than on binding of the individual species. The ability to impart the substrate specificities of mesostable AKRs into a thermostable scaffold will be useful in a variety of applications including immobilized enzyme systems for bioelectrocatalysis and fine chemical synthesis. Keywords: aldo-keto reductase/ enzyme catalysis/ oxido-reductases/substrate specificity/protein stability

Introduction

Aldo-keto reductases (AKRs) comprise a large, diverse family of oxidoreductase enzymes and are found in nearly every species (Jez et al., 1997; Jez and Penning, 2001). They share a common (α/β)8-barrel structure and catalytic mechanism, but some members of the superfamily share <30% sequence homology. These enzymes bind a nicotinamide cofactor in an extended conformation along a cleft that runs through the C-terminal face of the barrel, in contrast to the Rossman-fold motif common in other dehydrogenases. Three mobile loops on the same face form the substrate binding pocket (Fig. 1). The physiological role of many of these enzymes is unknown, but they are generally thought to fall into one of three classes. The most studied members of this family are mammalian AKRs involved in steroid and prostaglandin metabolism (Askonas et al., 1991; Penning, 1999; Cooper et al., 2007). These enzymes often have long substrate-binding loops and are highly specific for their substrates. Another well-studied class of AKRs, the aldose reductases (ARs), are involved in the interconversion of glucose to sorbitol and have been investigated as drug targets to prevent complications from diabetes (Bohren et al., 1991; Kubiseski et al., 1992). A third class of AKRs, which have been identified in a wide range of species, have no known function (Ellis, 2002). The substrate-binding loops in these enzymes are often truncated, imparting them with fairly broad substrate specificity. Additionally, they have been shown to be upregulated in response to stress, leading to the hypothesis that their physiological role is of general detoxification, metabolizing various aldehydes and ketones to less toxic species (Scoble et al., 2010).

The advent of high-throughput sequencing has allowed the complete genomes of several species to be elucidated, and the sequence data have yielded several putative members of the AKR superfamily (Di Luccio et al., 2006; Machielsen et al., 2006). One of these enzymes, alcohol dehydrogenase D (AdhD), was identified in the hyperthermophilic archaea Pyrococcus furiosus, and has been characterized by our group and others. The enzyme has a strong preference for NAD(H) as a cofactor, and oxidizes a range of sugars and alcohols (Campbell et al., 2010; Machielsen et al., 2006). The substrate binding loops in this enzyme are significantly truncated compared to AKRs identified from other organisms, with the C-terminal loop (Loop C), completely absent. This likely contributes to the broad substrate specificity and extreme thermostability of the enzyme.

The importance of the mobile loops in substrate binding and specificity was elegantly demonstrated through the creation of several chimeric hydroxysteroid dehydrogenases (HSD) where the substrate-binding loops from a 20α-HSD were grafted into a 3α-HSD enzyme scaffold (Ma and Penning, 1999). These two human enzymes share a 67% amino sequence identity. Replacement of only Loop A resulted in an enzyme with novel 17β-HSD activity, while swapping all three substrate-binding loops resulted in a complete alteration of substrate specificity, with an increase in catalytic efficiency for the 20α-HSD reaction of 1011 compared with the wild-type 3α-HSD enzyme.

Based on this impressive work, we decided to investigate a similar strategy to rationally alter the substrate specificity of AdhD. In an attempt to improve the activity of AdhD with sugars, we created several loop chimeras inserting the substrate-binding loops from hAR, which displays activity...
with glucose (Bohren et al., 1991; Tarle et al., 1993; Grimshaw et al., 1995; Kubiseski and Flynn, 1995; Crosas et al., 2003). These loop chimeras are also compared with and combined with a cofactor specificity double mutant of AdhD (K249G/H255R) that exhibits broadened cofactor specificity and improved activity compared with the wild-type enzyme (Campbell et al., 2010). A summary of loop chimera constructs appears in Table I.

Whereas the previous work exchanged substrate-binding loops between similar human hydroxysteroid dehydrogenases, the present work investigates exchanging loops between two distinct AKRs which share <30% sequence homology and exhibit significantly different functional activities. AdhD is an extremely thermostable archaeal dehydrogenase with broad substrate specificity and low activity at room temperature (its activity increases with increasing temperature up to 100°C) (Machielsen et al., 2006). Human hAR is a meso-stable mammalian reductase with a specialized function (leading to narrow substrate specificity). In addition, AdhD has a strong preference for NAD(H) as a cofactor, while hAR has a strong preference for NADP(H). Thus, in addition to the change in substrate specificity expected due to changing the substrate-binding loops, it will also be interesting to observe the effects on cofactor specificity and thermostability of the resultant mutants.

### Materials and methods

dl-Glyceraldehyde, 2,3-butanediol (mixture of dl and meso), 3-hydroxy-2-butanol, all cofactors, media and buffer components were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise noted and used without modification.

In order to design the AdhD/hAR loop chimeras, sequence and structural alignments were performed. Sequence alignments of AdhD (GenBank1469842) and hAR (GenBankAAA51713) were performed using the CLUSTALW tool, and structural

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**Table I. AdhD/hAR loop chimera constructs**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Enzyme loops</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>wt Loop A</td>
</tr>
<tr>
<td>B</td>
<td>DM Loop A</td>
</tr>
<tr>
<td>C</td>
<td>wt Loops AB</td>
</tr>
<tr>
<td>D</td>
<td>DM Loops AB</td>
</tr>
<tr>
<td>E</td>
<td>wt Loops ABC</td>
</tr>
<tr>
<td>F</td>
<td>DM Loops ABC</td>
</tr>
<tr>
<td>G</td>
<td>wt Loops ABC + Δ182–184</td>
</tr>
<tr>
<td>H</td>
<td>DM Loops ABC + Δ182–184</td>
</tr>
</tbody>
</table>

*wt refers to wild-type AdhD from *Pyrococcus furiosus*, while DM refers to the previously described cofactor specificity double-mutant K249G/H255R AdhD.*
alignments of AdhD and hAR (PDB 2ACQ) were performed with Yasara. DNA oligos corresponding to the hAR substrate-binding loops were obtained from IDT DNA, Inc. (Coralville, IA, USA) and assembled into the AdhD gene using overlap extension polymerase chain reaction (OE-PCR) (see Supplementary materials). PCR fragments were doubly digested with NcoI and HindIII and cloned into a similarly digested pET-24d vector. All constructs were verified by DNA sequencing.

AdhD/hAR Loop chimeras were initially expressed in 50-ml cultures and purified by heating of the cell extracts (80°C for 1 h), as described previously (Campbell et al., 2010). Two constructs, A (wt AdhD with Loop A) and D (DM AdhD with Loops AB), were found to be poorly expressed despite efforts to optimize the expression and purification of these samples. Thus, only enough enzyme was produced to run the initial screening assay with these constructs. Relatively pure protein (estimated >90% pure by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)) was obtained in the heat-stable extract, and was used without further purification for initial studies.

Large-scale expression and purification of wild-type AdhD and mutants followed a previously described protocol (Campbell et al., 2010). Typical yields were of the order of 300–1200 mg l⁻¹ of culture, and samples were estimated to be >98% pure by SDS-PAGE.

The hAR gene was amplified from human placenta QUICK-clone cDNA (Clontech, Mountain View, CA, USA) using forward primer 5’-GGTCTGGGAGCGCACGACG-3’ and reverse primer 5’-TTCGAAAGTCTTCAAAACTCTTCA-TGGAAGGGGTAATCCTT-3’. The reverse primer inserted a unique HindIII restriction site (underlined). The purified PCR fragment was doubly digested with NcoI and HindIII and ligated into a similarly digested pET-24d vector containing an N-terminal RGSHis tag for purification. Ligated plasmids were transformed into electrocompetent BLR Escherichia coli (Novagen, Gibbstown, NJ, USA) and plated on LB-Kan selection plates. Individual colonies were picked and grown overnight in Luria–Bertani (LB) medium containing 1% glucose and grown overnight in Luria–Bertani (LB) medium supplemented with 50 μg ml⁻¹ kanamycin and stored as glycerol stocks. Proper insertion of the hAR gene was verified by DNA sequencing.

Expression and purification of hAR followed a different protocol, as the enzyme is not highly thermostable. One liter stocks. Proper insertion of the hAR gene was verified by DNA sequencing.

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Cofactor binding energies in the ground-state ($\Delta \Delta G_b$) and transition-state ($\Delta \Delta G_b^{\ddagger}$) were obtained from Eqs. 1 and 2, (Fersht, 1985) utilizing the steady-state kinetic parameters.

$$\Delta \Delta G_b = -RT \ln \left( \frac{[K_i]_{\text{construct}}}{[K_i]_{\text{wt AdhD}}} \right)$$  \hspace{1cm} (1)

$$\Delta \Delta G_b^{\ddagger} = RT \ln \left( \frac{[k_{\text{cat}}/K_A]_{\text{construct}}}{[k_{\text{cat}}/K_A]_{\text{wt AdhD}}} \right)$$  \hspace{1cm} (2)

**Results**

A sequence and structural alignment of hAR (PDB ID 2ACQ) with a previously generated homology model of AdhD (Campbell et al., 2010) guided the insertion of the hAR substrate-binding loops into AdhD (Fig. 1). The structural alignment also identified a short loop in AdhD (corresponding to residues 182–184) not present in hAR, which could potentially sterically interfere with Loop C and prevent it from properly folding over the top of the barrel. Thus, additional mutants were generated with this short loop removed (denoted $\Delta 182$–184) to increase the likelihood of Loop C adopting its native conformation.

Loops were inserted at the genetic level through a series of oligonucleotide primers, which were used to amplify fragments of the gene containing the desired loops (see Supplementary materials). These fragments were then reassembled into a full-length gene using overlap-extension PCR, and cloned into a vector for expression.

A concern when grafting in the large substrate-binding loops from hAR was a decrease in the thermostability of the AdhD scaffold. However, a thermal purification step was still possible with the mutant enzymes, and they were further characterized by CD spectroscopy and thermal denaturation experiments. Surprisingly, the impact of these loops on enzyme stability was minimal. No change in CD signal was observed in the chimeric enzymes from 25 to 90°C, and apparent $T_m$’s determined in 6 M guanidine HCl were not statistically different from the wt AdhD (data not shown).

Loop mutants were initially screened in a plate assay after undergoing a partial purification by heat treatment (see Materials and methods). Mutants were tested for their ability to reduce D,L-glyceraldehyde with NADPH, the model substrates for hAR, and in the oxidation and reduction of 2,3-butanediol and 3-hydroxy-2-butanone, respectively, the model substrates for AdhD, using both NADH (and NADP(H) cofactors). Plates were illuminated by UV to visualize the reduced cofactor and photographed at regular intervals. A representative image of the plate after 45 min of incubation is shown in Fig. 2. At this time point, only hAR showed appreciable activity with D,L-glyceraldehyde, and clearly had a preference for NADPH over NADH. The AdhD double mutant showed the highest activity with 2,3-butanediol and 3-hydroxy-2-butanone, with little difference apparent between the NAD(H) and NADP(H) cofactors. The activity of wt AdhD was lower with these substrates, and a marked preference for NAD+ was observed with 2,3-butanediol. hAR also demonstrated activity with both 2,3-butanediol and 3-hydroxy-2-butanone, and had a slight preference for its preferred cofactor NADP(H) with these model AdhD substrates. Interestingly, constructs C and E also retained significant activity with these substrates, but only when NADP(H) was the cofactor. At longer time points, NADPH/DL-glyceraldehyde activity was also observed in these loop chimeras. For the apparently inactive mutants, longer time points were used to attempt to detect minute activity, but these constructs were not different from background, and were not investigated further.

Enzymes that were identified as active in the plate assay were grown in large-scale expression cultures and purified to homogeneity as described. A full kinetic analysis was performed with these samples to allow for fitting to the ordered bi-bi rate equation. Kinetic parameters are summarized in Table II. While the wt AdhD exhibited very little detectable activity with NADPH and D,L-glyceraldehyde, the cofactor specificity double mutant (DM AdhD) had a turnover rate $>50\%$ faster than hAR (46 vs $30 \text{ s}^{-1}$). This was offset by one to two order(s) of magnitude increases in the dissociation constant and Michaelis constant for NADPH, however, leading to a lower catalytic efficiency. The two-loop chimeras, C and E, identified as active in the plate assay demonstrated reasonable turnover with NADPH and D,L-glyceraldehyde (10–20% of hAR), but again the Michaelis constants were two to three orders of magnitude larger than those for hAR or the DM AdhD. With the model AdhD substrate 2,3-butanediol, an interesting effect is observed with the loop chimeras. Both constructs C and E demonstrated increased activity with this substrate, but had a strict requirement for NADP+ as a cofactor, in contrast to the NADP+ preference exhibited by wt AdhD. hAR, surprisingly, was found to have the highest catalytic rates with this substrate with both NAD+ and NADP+ ($k_{\text{cat}}$ of 76 and $26 \text{ s}^{-1}$, respectively); however, the catalytic efficiency was much higher with its preferred cofactor NADP+. The combination of relatively low turnover numbers and high Michaelis constants impeded the accurate determination of the full kinetic parameters for the two-loop chimeras.
order to enable fitting to the ordered bi-bi rate equation, the $K_{ia}$ term was set equal to the dissociation constant measured by fluorescence titration (Segel, 1993). As proper saturating conditions were not achieved with these mutants, the resulting kinetic parameters are given as apparent parameters.

**Table II.** Full kinetic parameters for hAR and AdhD

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cofactor</th>
<th>Substrate</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_{ia}$ (µM)</th>
<th>$K_A$ (µM)</th>
<th>$K_B$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt AdhD</td>
<td>NADPH</td>
<td>α-Glyceraldehyde</td>
<td>$&lt;0.01$</td>
<td>$43 \pm 2^a$</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>DM AdhD</td>
<td>NADPH</td>
<td>α-Glyceraldehyde</td>
<td>$46 \pm 6$</td>
<td>$35 \pm 2^a$</td>
<td>$180 \pm 40$</td>
<td>$11 \pm 4$</td>
</tr>
<tr>
<td>wt Loops AB</td>
<td>NADPH</td>
<td>α-Glyceraldehyde</td>
<td>$5.2 \pm 1.3$</td>
<td>$4.9 \pm 0.7$</td>
<td>$460 \pm 120$</td>
<td>$450 \pm 130$</td>
</tr>
<tr>
<td>wt Loops ABC</td>
<td>NADPH</td>
<td>α-Glyceraldehyde</td>
<td>$2.8 \pm 0.6$</td>
<td>$280 \pm 30$</td>
<td>$1200 \pm 300$</td>
<td>$250 \pm 70$</td>
</tr>
<tr>
<td>hAR</td>
<td>NADPH</td>
<td>α-Glyceraldehyde</td>
<td>$30 \pm 1$</td>
<td>$0.45 \pm 0.08$</td>
<td>$20 \pm 2$</td>
<td>$24 \pm 1$</td>
</tr>
<tr>
<td>wtAdhD$^b$</td>
<td>NADP$^+$</td>
<td>2,3-Butanediol</td>
<td>$1.0 \pm 0.1$</td>
<td>$37 \pm 2$</td>
<td>$63 \pm 2$</td>
<td>$29 \pm 1$</td>
</tr>
<tr>
<td>wtAdhD$^b$</td>
<td>NADP$^+$</td>
<td>2,3-Butanediol</td>
<td>$0.03 \pm 0.02$</td>
<td>$20 \pm 8$</td>
<td>$5.1 \pm 0.2$</td>
<td>$13 \pm 0.1$</td>
</tr>
<tr>
<td>DMAdhD$^b$</td>
<td>NAD$^+$</td>
<td>2,3-Butanediol</td>
<td>$15 \pm 2$</td>
<td>$11 \pm 1$</td>
<td>$460 \pm 60$</td>
<td>$690 \pm 80$</td>
</tr>
<tr>
<td>DMAdhD$^b$</td>
<td>NAD$^+$</td>
<td>2,3-Butanediol</td>
<td>$4.7 \pm 2$</td>
<td>$1.2 \pm 0.2$</td>
<td>$78 \pm 5$</td>
<td>$200 \pm 9$</td>
</tr>
<tr>
<td>wt Loops AB</td>
<td>NAD$^+$</td>
<td>2,3-Butanediol</td>
<td>ND</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>wt Loops ABC</td>
<td>NAD$^+$</td>
<td>2,3-Butanediol</td>
<td>$4.5 \pm 0.8^c$</td>
<td>$161^c$</td>
<td>$750^o$</td>
<td>$160 \pm 40^c$</td>
</tr>
<tr>
<td>wt Loops ABC</td>
<td>NAD$^+$</td>
<td>2,3-Butanediol</td>
<td>ND</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>hAR</td>
<td>NAD$^+$</td>
<td>2,3-Butanediol</td>
<td>$16 \pm 5^c$</td>
<td>$450 \pm 25^c$</td>
<td>$3800 \pm 1200^o$</td>
<td>$470 \pm 160^c$</td>
</tr>
<tr>
<td>hAR</td>
<td>NADP$^+$</td>
<td>2,3-Butanediol</td>
<td>$76 \pm 30$</td>
<td>$350 \pm 40$</td>
<td>$750 \pm 40$</td>
<td>$350 \pm 200$</td>
</tr>
<tr>
<td>hAR</td>
<td>NADP$^+$</td>
<td>2,3-Butanediol</td>
<td>$26 \pm 2$</td>
<td>$0.63 \pm 0.2$</td>
<td>$5.4 \pm 1.6$</td>
<td>$180 \pm 20$</td>
</tr>
</tbody>
</table>

$K_{ia}$ is the dissociation constant of the enzyme cofactor complex. $K_A$ and $K_B$ are the Michaelis constants of the cofactor and substrate, respectively. Experiments were performed under standard conditions as reported in the Materials and methods and (Campbell et al., 2010).

$^a$Measured by fluorescence titration.

$^b$Data from (Campbell et al., 2010).

$^c$Apparent parameters.

**Discussion**

The modular nature of the AKR substrate-binding loops has been confirmed in this work, as the AdhD scaffold was successfully imparted with hAR activity through a loop-grafting approach. Whereas a complete reversal of substrate specificity was previously shown to require the exchange of all three substrate binding loops (Ma and Penning, 1999), here it appears that only two loops are necessary for activity. Additionally, the chimeric mutants studied here maintained the high thermostability and resistance to chemical denaturants of the parent enzyme, suggesting that this technique can be used to rapidly stabilize other mesophilic AKRs.

Comparisons of the catalytic efficiencies of the various enzyme constructs are difficult to interpret due to the large difference in Michaelis constants between enzymes. Thus, activities were examined under saturating conditions for the wild-type enzyme by looking at the turnover rate, $k_{cat}$. The catalytic rate of wt AdhD is much lower than that of hAR with NAD(P)(H), both in the oxidation of the model AdhD substrate 2,3-butanediol (0.03 vs 26 s$^{-1}$) and the reduction of the model hAR substrate DL-glyceraldehyde (<0.01 vs 30 s$^{-1}$). The active loop chimeras fall in between, with construct C having similar activities with both substrates ($k_{cat}$ of 4.5 s$^{-1}$ with 2,3-butanediol vs 5.2 s$^{-1}$ with DL-glyceraldehyde) while construct E has a much faster turnover rate with 2,3-butanediol (16 vs 2.8 s$^{-1}$ with DL-glyceraldehyde). Interestingly, the DM AdhD has a much higher turnover rate with DL-glyceraldehyde than even hAR, but is comparable with the construct C with 2,3-butanediol (Fig. 3A). The steady-state kinetic parameters can also be used to calculate changes in the cofactor binding energies relative to the wt AdhD enzyme, (Fersht, 1985; Banta et al., 2002) which may shed light onto the kinetic results. In the ground state, the loop chimeras destabilized the binding of NAD$^+$ by 1–2 kcal/mol, while hAR and the previously engineered DM AdhD had ~2 kcal/mol more favorable binding energies. All constructs except for E also demonstrated a decreased free energy of binding with NADPH relative to the wt AdhD, which partially explains the improvement in activity observed in these constructs (Fig. 3B). The effect of the loop chimeras is most apparent when comparing the transition-state binding energies. Here, both constructs C and E have a significantly lower transition-state binding energy with DL-glyceraldehyde/NADPH compared with the wt AdhD (by ~3–4 kcal/mol), while those with 2,3-butanediol/NADP$^+$ remain relatively unaffected. (Fig. 3C) Thus, the loop chimeras increase the affinity of the enzyme for the NADPH/DL-glyceraldehyde transition state, but without loss of affinity for 2,3-butanediol.

In contrast to the previous work, the active loop chimeras retained activity with their native substrate. However, the strict reversal in cofactor specificity from NAD$^+$ to NADP$^+$ was unexpected. As hAR has been shown to prefer NAD(P)H, this suggests the substrate-binding loops can also impact cofactor specificity. In fact, Loop B of some AKRs was unexpected. As hAR has been shown to prefer the pyrophosphate backbone of the cofactor, thereby locking it into the binding pocket (Sanli et al., 2010). The crystal structure of hAR indicates that residue Asp216 on Loop B forms a salt-bridge with Lys262 (equivalent to Lys249 in AdhD) to form the canonical AKR ‘seat-belt’ over Lys249 in AdhD) to form the canonical AKR ‘seat-belt’ over Lys249 in AdhD (Sanli et al., 2003; Sanli and Blaber, 2001). This motif is likely absent in the wt AdhD, as Loop B is significantly truncated and lacks the large positively charged residue required to form an electrostatic interaction (Fig. 1). Grafting Loop B from hAR into AdhD may therefore reconstitute the ‘seat-belt’, and promote binding and proper orientation of the cofactor in the binding pocket. Interestingly, construct C, with both Loops A and B has the second lowest dissociation constant for NADPH, behind only hAR.
AdhD requires at least Loop B of hAR for activity with hAR-like activity, results in an inactive enzyme. As the wt into the double-mutant AdhD scaffold, which itself possesses AdhD scaffold, it is interesting that the same loops grafted energya of constructs with NADPH and NADP

Fig. 3. Effect of Loop Insertions on activity and binding energy. (A) Comparison of turnover rate for AdhD, hAR and active loop chimeras with α-glyceraldehyde/NADPH and 2,3-butanediol/NADP+ as substrates. The value of $k_{cat}$ was obtained by fits of kinetic data to the ordered bi-bi rate equation. (B) Change in the ground-state cofactor binding energy of constructs with NADPH and NADP+ relative to wt AdhD. (C) Change in the transition-state binding energies of constructs with α-glyceraldehyde/NADPH and 2,3-butanediol/NADP+ relative to wt AdhD.

\[ \Delta G_0 = -RT \ln \left( \frac{K_a}{K_d} \right) \]

\[ \Delta G^* = RT \ln \left( \frac{k_{cat}}{k_{cat}} \right) \]

Given the success in grafting the hAR loops into the AdhD scaffold, it is interesting that the same loops grafted into the double-mutant AdhD scaffold, which itself possesses hAR-like activity, results in an inactive enzyme. As the wt AdhD requires at least Loop B of hAR for activity with α-glyceraldehyde, this supports the hypothesis that cofactor binding and orientation provided by the ‘seat-belt’ mechanism is important for catalysis, and the introduction of the ‘seat-belt’ will need to be explored structurally, as was done with α-HSD (Ma and Penning, 1999). One of the mutations in the double mutant, K249G, removes the lysine residue that interacts with the aspartic acid of Loop B (Campbell et al., 2010).

Thus, the formation of a ‘seat-belt’ in the DM AdhD loop constructs is unlikely, and if this has a detrimental effect on cofactor binding, could explain the lack of activity observed in these chimeras.

Supplementary data

Supplementary data are available at PEDS online.

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References


