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Adding a positive charge at residue 46 of *Drosophila* alcohol dehydrogenase increases cofactor specificity for NADP$^+$

Zhuo Chen[a], Igor Tsigelny[c], William R. Lee[b], Michael E. Baker[d,*], Simon H. Chang[a,*]

[a]*Department of Biochemistry, Room 322, Choppin Hall, Louisiana State University, Baton Rouge, LA 70803, USA*
[b]*Department of Zoology, Louisiana State University, Baton Rouge, LA 70803, USA*
[c]*Department of Chemistry and Biochemistry, 0654, University of California at San Diego, La Jolla, CA 92093-0623, USA*
[d]*Department of Medicine, 0623B, 9500 Gilman Drive, University of California at San Diego, La Jolla, CA 92093-0623, USA*

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Abstract We previously reported that the D39N mutant of *Drosophila* alcohol dehydrogenase (ADH), in which Asp-39 is replaced with asparagine, has a 60-fold increase in affinity for NADP$^+$ and a 1.5-fold increase in k_{cat} compared to wild-type ADH [Chen et al. (1991) Eur. J. Biochem. 202, 263–267] and proposed that this part of ADH is close to the 2′-phosphate on the ribose moiety of NADP$^+$. Here we report the effect of replacing Ala-46 with an argine residue, an A46R mutant, on binding of NADP$^+$ to ADH and its catalytic efficiency with the NADP$^+$ cofactor, and a modeling of the three-dimensional structure of the NAD$^+$-binding region of ADH. The A46R mutant has a 2.5-fold lower $K_{m(app)NADP^+}$ and a 3-fold higher k_{cat} with NADP$^+$ compared to wild-type ADH; binding of NAD$^+$ to the mutant was unchanged and k_{cat} with NAD$^+$ was lowered by about 30%. For the A46R mutant, the ratio of k_{cat}/K_m of NAD$^+$ to NADP$^+$ is 85, over ten-fold lower than that for wild-type ADH. Our model of the 3D structure of the NAD$^+$-binding region of ADH shows that Ala-46 is over 10 Å from the ribose moiety of NAD$^+$, which would suggest that there is little interaction between this residue and NAD$^+$ and explain why its mutation to arginine has little effect on NAD$^+$ binding. However, the positive charge at residue 46 can neutralize some of the coulombic repulsion between Asp-39 and the 2′-phosphate on the ribose moiety of NADP$^+$, which would increase its affinity for the A46R mutant. We also constructed a double mutant, D39N/A46R mutant, which we find has a 30-fold lower $K_{m(app)NADP^+}$ and 8-fold higher k_{cat} with NADP$^+$ as a cofactor compared to wild-type ADH; binding of NAD$^+$ to this double mutant was lowered by 5-fold and k_{cat} was increased by 1.5-fold. As a result, k_{cat}/K_m for the double mutant was the same for NAD$^+$ and NADP$^+$. The principle effect of the two mutations in ADH is to alter its affinity for the nucleotide cofactor; k_{cat} decreases slightly in A46R with NAD$^+$ and remains unchanged or increases in the other mutants.

Key words: *Drosophila* ADH; NADP$^+$ binding to ADH; Steroid and prostaglandin dehydrogenase

1. Introduction

Drosophila alcohol dehydrogenase (ADH) has a preference for secondary alcohols [1–7] and does not require a metal ion for catalysis, in contrast to the yeast and horse liver alcohol dedhydrogenases. *Drosophila* ADH shares a common ancestor [8–12] with human 11β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and 15-hydroxyprostaglandin dehydrogenase; enzymes that regulate the concentration of glucocorticoids, estrogens, and prostaglandins, respectively, in humans and are thus of importance in various endocrine-related diseases [13–15].

The mechanism of catalysis of ADH and its homologs is only beginning to be understood. The importance of the N-terminus of ADH in binding the nucleotide cofactor was first shown when Thatcher sequenced an inactive ADH mutant and found that Gly-15 was replaced by aspartic acid [16]. Site-specific mutagenesis studies with cloned *Adh* also indicate that the N-terminus is important in binding of the nucleotide cofactor [5,17]. Other studies indicate that Tyr-153 and Lys-157 are important in hydride ion transfer [18,19]. The latter is in agreement with similar studies on 11β-hydroxysteroid dehydrogenase [20] and NAD$^+$-dependent 15-hydroxyprostaglandin dehydrogenase [21].

Among the questions that are still partially answered for ADH and its homologs are the determinants for specificity for NAD$^+$ and NADP$^+$. Some progress has been made from analysis of crystal structures of two ADH homologs: *Streptomyces hydrogenans* 20β-hydroxysteroid dehydrogenase [22] co-crystallized with NAD$^+$, and rat dihydropteridine reductase [23,24] co-crystallized with NADH. Despite their low sequence similarity, their tertiary stuctures have many similarities: both show that the amino-terminus consists of a $\beta\alpha\beta$ fold, a structure that comprises the binding domain for the AMP moiety of the nucleotide cofactor in a wide range of non-homologous oxidoreductases [25–28]. The orientation, however, of the nucleotide cofactor differs in the two tertiary structures, with the orientation of NADH in dihydropteridine reductase similar to that in other oxidoreductases [25–28]. Our previous studies with ADH mutants [5,17] suggested that the 2′-OH of the ribose moiety of AMP may be close to *Drosophila* ADH's Asp-39 at the C-terminus of the second β-strand in the $\beta\alpha\beta$ fold. This orientation is similar to that found for dihydropteridine reductase and for human 15-hydroxyprostaglandin dehydrogenase [29]. The latter conclusion is based on a photoaffinity labeling study.

Like those studying glutathione reductase [28] and yeast alcohol dehydrogenase [30], we are attempting to engineer an ADH molecule that has a cofactor specificity different from that of the wild-type enzyme. Scrutton et al. [28] found that a combination of adding a negatively charged residue and removing positively charged residues close to the C-terminus of the second β-strand improved the catalytic efficiency of glutathione reductase with NADH by a factor of ~70 while lowering the catalytic efficiency with NADPH by a factor of ~250. Fan et al. [30] found that replacing an aspartate residue with a glycine residue at the C-terminus of the second β-strand in yeast alcohol dehydroge-

*Corresponding authors. S.H. Chang. Fax: (1) (508) 388-5321.
M.E. Baker: Fax, (1) (619) 534-1424. E-mail, mbaker@ucsd.edu

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nase yield an enzyme with similar catalytic efficiency for NAD^+ and $NADP^+$.

These reports suggest that adding one or more positively charged residues near the C-terminus of the second β-strand on ADH could increase the affinity for $NADP^+$. We have adopted this approach in constructing mutants in which Ala-46 in wild-type ADH and in the D39N mutant is replaced with an arginine, and determined the effect on binding of, and catalytic efficiency with, $NADP^+$ and NAD^+. As reported here, a positive charge at position 46 increases the affinity for $NADP^+$ compared to wild-type ADH. A D39N/A46R mutant has the same k_{cat}/K_m for $NADP^+$ and NAD^+. We also constructed a 3D model of ADH using 20β-hydroxysteroid dehydrogenase and dihydropteridine reductase as templates. This aaproach has been successfully used by Krook et al. to model the 3D structure of human $NADP^+$-dependent 15-hydroxyprostaglandin dehydrogenase [31] and human $NADP^+$-dependent 15-hydroxyprostaglandin dehydrogenase (carbonyl reductase) [32] using 20β-hydroxysteroid dehydrogenase as a template, despite the extensive sequence divergence among these enzymes, giving us confidence in this approach to model the structure of ADH. Our findings for ADH may be useful for studies with its steroid and prostaglanding dehydrogenase homologs, in which the determinants for cofactor specificity have not been studied.

2. Materials and methods

2.1. Materials

The vectors, bacterial strains and phage used for expression and mutagenesis of *Drosophila* ADH were described previously by Chen et al. [15,19]. Altered Site System was purchased from Promega Corp. All restriction endonucleases, DNA ligase, DNA kinase, and Klenow fragment of *E. coli* DNA polymerase I were ordered from Bethesda Research Laboratories, New England Biolabs Inc., or Promega Corp. Sequenase kits were obtained from United States Biochemicals Corp.

2.2. Expression and purification of the wild-type and mutated Drosophila ADH

A vector containing the full-length ADH cDNA in *E. coli* strain M5219, which carries the cI857 thermosensitive-repressor gene, was used to express wild-type and mutated ADH [5,19]. The Altered Sites System (Promega) and Kunkel's method were used to produce point mutations [33]. The expression of wild-type ADH and its mutants in *E. coli* cells was verified by Western blot analysis and ADH enzyme activity, as described previously [5,19].

2.3. Determination of kinetic parameters

ADH activity was determined spectrophotometrically at 340 nm in 100 mM glycine-NaOH buffer (pH 9.8) at 25°C. The concentration of NAD^+ and alcohol for the determination of the kinetic coefficients were specified as follows: NAD^+ and $NADP^+$ 0.02–2 mM, and propan-2-ol 1–100 mM. $K_{m(app)}$ was calculated from the initial-rate data measured at a constant concentration of NAD^+ or $NADP^+$ using the Enzfitter program.

2.4. 3D modeling of ADH

We used the homology program (Biosym, 1994) to model the ADH structure using as a template the reported tertiary structures of *S. hydrogenans* 20β-hydroxysteroid dehydrogenase [22] and rat dihydropteridine reductase [23,24]. The NAD^+ structure was obtained from the Brookhaven Protein Database. The ADH structure with NAD^+ was calculated by extensive energy minimization. The backbone of ADH was constrained during minimization of 2,000 iterations.

3. Results

The codon substitutions for A46R, and the double mutants D39N/A46R and D39R/A46R were confirmed by dideoxy DNA sequencing. All of the mutants could be expressed in an *E. coli* host. The D39R/A46R mutant has no activity, although it can be detected by Western analysis. Both A46R and the double mutant D39N/A46R are enzymatically active and were purified to homogeneity as verified by SDS-PAGE (data not shown).

3.1. Kinetic analysis of wild-type ADH and mutants

Table 1 summarizes the results of the analysis of wild-type ADH, A46R, D39N/A46R, and D39N. Replacing Ala-46 by arginine lowers the K_m for $NADP^+$ by 2.5 fold and increases k_{cat} by about 2.5 fold; thus, k_{cat}/K_m is 6.5 fold higher than that of the wild-type ADH. In the A46R mutant, the K_m for NAD^+ decreases slightly, while k_{cat} decreases by 30%; thus k_{cat}/K_m is about 60% of the wild-type ADH.

The double mutation, D39N/A46R, lowers the K_m of $NADP^+$ by 30-fold and increases k_{cat} 8-fold compared to wild-type ADH. Compared to D39N mutant, the double mutant has a K_m for $NADP^+$ that is 2 fold higher, while k_{cat} is about 20% lower. In D39N/A46R the catalytic efficiency, k_{cat}/K_m, with $NADP^+$ is 250 fold higher than that of wild-type ADH and 40% of D39N.

The affinity of NAD^+ for the double mutant decreased by 3.5-fold compared to the D39N mutant, while k_{cat} was unchanged; k_{cat}/K_m was 27% of that of wild-type and the D39N mutant. As a result, the D39N/A46R mutant has a similar k_{cat}/K_m for $NADP^+$ and NAD^+.

Fig. 1 shows a model of part of the region in ADH that binds NAD^+. Residues 13–18, which are part of the first turn in the $\beta\alpha\beta$ structure, form a glycine-rich hydrophobic pocket that allows close approach of the adenosine moiety. The model is

Table 1
$K_{m(app)}$ and k_{cat} values for wild-type ADH*, D39N*, A46R and D39N/A46R

Substrates	Enzymes	$K_{m(app)}$ (mM)	k_{cat} (s^{-1})	$k_{cat}/K_{m(app)}$ ($s^{-1} \cdot mM^{-1}$)	Mutant/WT (ratio of $k_{cat}/K_{m(app)}$)	WT/mutant (ratio of $k_{cat}/K_{m(app)}$ $NAD^+/NADP^+$)
NAD^+	Wild-type	0.048 ± 0.001	10.2	213	1	925
	A46R	0.057 ± 0.003	7.3	127	0.60	84.5
	D39N	0.073 ± 0.002	15.3	210	1	1.55
	D39N/A46R	0.260 ± 0.03	15.0	58	0.27	1
$NADP^+$	Wild-type	3.09 ± 0.70	0.715	0.230	1	
	A46R	1.27 ± 0.10	1.9	1.5	6.5	
	D39N	0.050 ± 0.003	6.80	136	590	
	D39N/A46R	0.100 ± 0.003	5.7	58	250	

*Data are from [5].

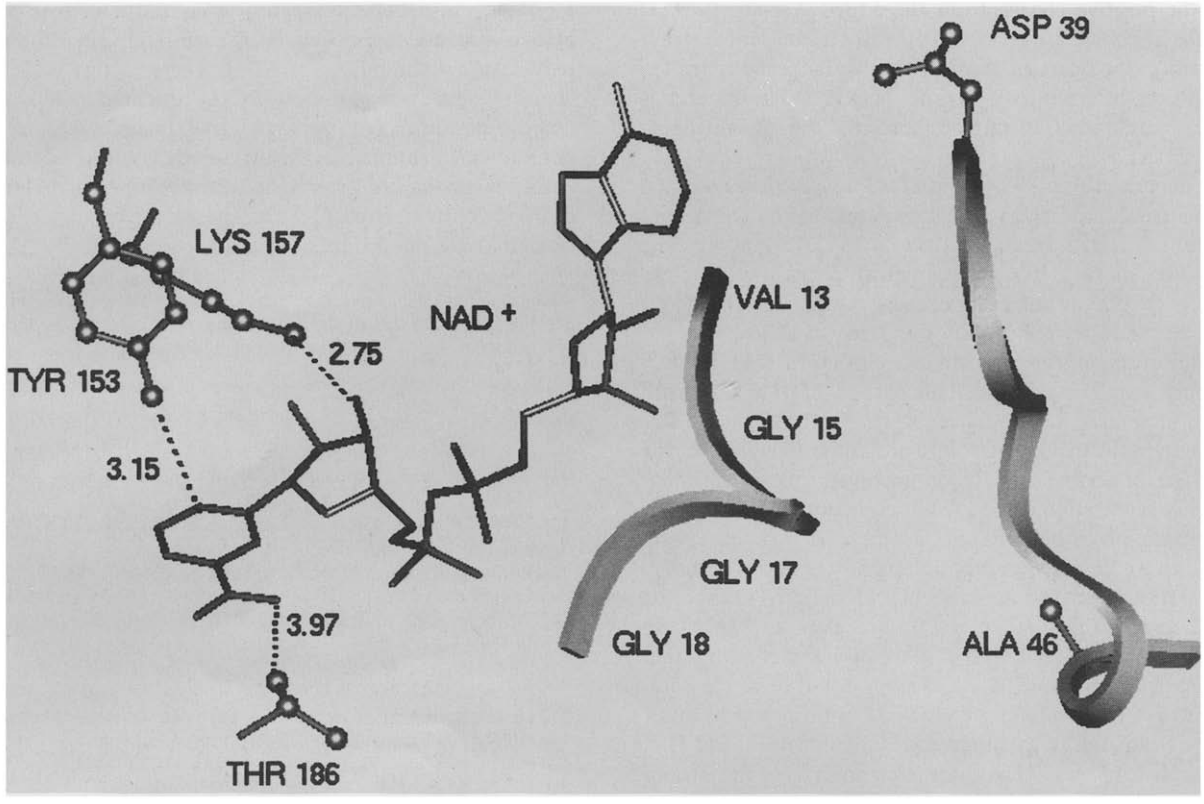

Fig. 1. A model of the interaction of *Drosophila* alcohol dehydrogenase with NAD^+. *Drosophila* ADH was modeled using 20β-hydroxysteroid dehydrogenase [22] and dihydropteridine reductase [23,24] as templates. Asp-39 and Ala-46, which have been mutated in this study, are shown. A ribbon shows Val-13 through Gly-18, which are part of the β-strand, turn, α-helix that are part of the canonical βαβ fold that binds NAD^+. Tyr-153, Lys-157, and Thr-186 are also shown. Tyr-153 is the proposed catalytic residue. The positively charge Lys-157 is proposed to lower the pK_a of tyrosine [19]; lysine's interaction with the ribose moiety may orient NAD^+ for pro-S hydride transfer [24]. The interaction of Thr-186 with the carboxamide group may stabilize the orientation of the cofactor with respect to the substrate and catalytic residue(s).

consistent with previous investigations of the effects on ADH acitivity of mutations in Gly-15. Mutation to alanine reduces ADH activity by 30% [17], presumably due to alanine's side chain interfering with adenosine binding; more disruptive mutations, in which either valine [17] or aspartic acid [16] replace Gly-15, lead to an inactive mutant.

The modeled structure reveals that Asp-39 and Ala-46 are distant from adenosine. Nevertheless, Asp-39 and Arg-46 are sufficiently close to have coulombic interactions with a 2′-phosphate on $NADP^+$. Fig. 1 also shows Tyr-153 and Lys-157, which are thought to be in the catalytic site [18,19], and Thr-186, which, unexpectedly, is close to the carboxamide moiety of NAD^+.

4. Discussion

Wild-type ADH has a marked preference for NAD^+ over $NADP^+$; k_{cat}/K_m is 925-fold higher for NAD^+ than for $NADP^+$ [5]. We found that the negative charge of Asp-39 is an important influence on cofactor specificity [5]. Indeed, a D39N mutant of ADH, in which Asp-39 is replaced with asparagine, has a 60 fold higher affinity and a 9.5 fold higher k_{cat} for $NADP^+$ compared to the wild-type ADH; binding of NAD^+ has a 35% lower affinity and k_{cat} increases by 1.5 fold. The net effect of the D39N mutation is that k_{cat}/K_m for $NADP^+$ increases by 590 fold, while k_{cat}/K_m for NAD^+ is unchanged. Thus, k_{cat}/K_m for NAD^+ is 1.5 fold higher than for $NADP^+$.

The modeled tertiary structure of ADH shown in Fig. 1 can clarify the basis for the above results and the other findings reported here. The position of Asp-39 shown in Fig. 1 indicates that there would be a strong coulombic repulsion between the 2′-phosphate on $NADP^+$ ribose and Asp-39 that would lower the affinity of $NADP^+$ for wild-type ADH. On the other hand, Asp-39 is far enough from NAD^+ so that the coulombic interaction with adenine is not a major contributor to binding energy of NAD^+. Thus, replacing Asp-39 with asparagine does not appreciably change the affinity of NAD^+ for ADH because of the similar size of the side chain. Evidently, the negative charge on Asp-39 is not essential for NAD^+ binding to ADH or the enzyme's active conformation. It may be that a role of Asp-39 is to prevent $NADP^+$ binding to ADH.

The 3D model predicts that the binding pocket can accommodate the phosphate of $NADP^+$ and that adding one or more positive charges to a nearby part of ADH will improve $NADP^+$ binding. The A46R mutation of ADH confirms this prediction. The A46R mutant has a 6.5-fold increase in k_{cat}/K_m with $NADP^+$ compared to wild-type ADH, and a 40% lower k_{cat}/K_m with NAD^+. The ratio of k_{cat}/K_m of NAD^+ to $NADP^+$ is 85, over tenfold lower than that for wild-type ADH. There are two contributions to the stabilization of $NADP^+$ binding to ADH

due to the positive charge from the added Arg-46. First, it shields the 2′-phosphate on $NADP^+$ from the coulombic repulsion of the carboxylate of Asp-39; that is, it compensates for the repulsion between Asp-39 and $NADP^+$. The second is arginine's coulombic attraction towards the 2′-phosphate group on $NADP^+$.

Replacement of Asp-39 by asparagine accomplishes the first part. As a result, adding an A46R mutation to the D39N mutant does not have a dramatic effect on $NADP^+$ binding. The D39N/A46R mutant has an equivalent K_{cat}/K_m with either $NADP^+$ or NAD^+ as cofactors because k_{cat}/K_m decreases 57% for $NADP^+$ and 72% for NAD^+ compared to D39N.

The importance of the conformation of the $\beta\alpha\beta$ fold in cofactor binding is well established. This part of ADH is higly conserved in other *sec*-alcohol dehydrogenases [10–12,34,35]. The loss of activity in the D39R/A46R mutant is likely to be due to the effect of adding two strong positively charged residues on ADH conformation, which is not compensated for by coulombic attraction to $NADP^+$.

Although we have focused on interaction of the N-terminal part of ADH and NAD^+, the molecular model shows that parts of the C-terminal domain of ADH are close to NAD^+. In particular, Tyr-153, Lys-157, and Thr-186 interact with either nicotinamide or its ribose. Tyr-153 is proposed to be the catalytically active residue [4,18,19]; Lys-157 is proposed to lower the pK_a of tyrosine to facilitate catalysis at neutral pH [19]. Another role for Lys-157 has come from analysis of the tertiary structure of dihydropteridine reductase [24]. Varughese et al. [24] proposed that the interaction of the hololog ous Lys-150 on rat dihydropteridine reductase with the 2′-hydroxy group on the nicotinamide ribose could be important in orienting NAD^+ for pro-S hydride transfer. This appears also to be true for ADH.

An unexpected finding is the interaction of Thr-186 with the carboxamide substitutent of the nicotinamide group, which is similar to that reported by Varughese et al. [24] for Asn-186 of rat dihydropteridine reductase. This was surprising because Thr-186 is not a well-conserved residue in the *sec*-alcohol dedydrogenase superfamily and would not be expected to be functionally important. Indeed, Thr-186 is in one of the most divergent segments, which is thought to be due to its importance in substrate specificity [10,11,34,36] and the fact that the substrate structures are diverse. The interaction of Thr-186 with NAD^+ may be important in stabilizing the orientation of the cofactor with the substrate and catalytic residue(s) in ADH and explain why this part of ADH and its homologs is important in substrate specificity.

Oxidation of alcohols by ADH proceeds by an ordered reaction [3,4,37] in which the binding of NAD^+ induces a conformational change in ADH that promotes substrate binding and catalysis. Fig. 1 suggests that the interactions between NAD^+ and Tyr-153, Lys-157, and Thr-186 are important in stabilizing the conformation that allows the substrate to interact with the nicotinamide group and Tyr-153 for hydride transfer [19].

Among the ADH homologs, only dihydropteridine reductase has been studied regarding cofactor specificity. Grimshaw et al. [38] found that replacing Asp-37 with isoleucine on dihydropteridine reductase reduced the affinity of both NADH and NADPH. However, the release of NADPH was slower and of NADH faster in the mutant.

Recently, Holm et al. [39] presented evidence that *E. coli* UDP-galactose-4-epimerase has structural similarity to 20β-hydroxyprostaglandin dehydrogenase and dihydropteridine reductase, two homologs of ADH. The structure of UDP-galactose-4-epimerase [40] shows NAD^+ oriented as reported for dihydropteridine reductase. UDP-4-galactose-epimerase is homologous to animal and viral 3β-hydroxysteroid dehydrogenases, *Nocardia* cholesterol dehydrogenase, and plant dihydroflavonol reductases [4]. This expands the relevance of information about the residues important in catalysis by ADH.

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