Site-Specific Mutagenesis of *Drosophila* Alcohol Dehydrogenase: Evidence for Involvement of Tyrosine-152 and Lysine-156 in Catalysis†

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ABSTRACT: Amino acid sequence comparisons reveal that tyrosine-152 and lysine-156 of *Drosophila* alcohol dehydrogenase (ADH) are conserved in homologous dehydrogenases, suggesting that these residues are important in catalysis. To test this hypothesis, we used site-directed mutagenesis to substitute tyrosine-152 with phenylalanine, histidine, or glutamic acid or to substitute lysine-156 with isoleucine. All of these mutants are catalytically inactive. Two mutants were active: A cysteine mutation of tyrosine-152 has 0.25% of wild-type ADH activity, while an arginine substitution of lysine-156 retains 2.2% of wild-type ADH activity. Kinetic analysis shows that the cysteine mutant increases $K_{m(ethanol)}$ 56-fold and $K_{m(propan-2-ol)}$ 100-fold, while $K_{m(NAD)}$ values are essentially unaltered. The arginine mutant also shows the significant enlargement of $K_{m(ethanol)}$, but not of $K_{m(NAD)}$. Furthermore, the cysteine mutant and arginine mutant have different substrate specificity and behave differently on competitive inhibition than wild-type ADH. These results suggest that both tyrosine-152 and lysine-156 have essential roles in catalysis by *Drosophila* ADH.

Alcohol dehydrogenase (ADH)[1] (alcohol:NAD oxidoreductase, EC.1.1.1.1) of *Drosophila melanogaster* differs from other ADHs in substrate preference (Sofer & Ursprung, 1968; Winberg et al., 1982), stereospecificity of hydride transfer (Benner et al., 1985), and metal ion requirement (Chambers, 1984). Moreover, it has little similarity in amino acid sequence to other ADHs (Benyajati et al., 1981). Interestingly, *Drosophila* ADH is homologous to the mammalian 11β- and 17β-hydroxysteroid dehydrodenases, the bacterial 3β- and 3α,20β-hydroxysteroid dehydrogenases, and human 15-hydroxyprostaglandin dehydrogenases, as well as various other dehydrogenases (Baker 1990a,b, 1991; Baron et al., 1991; Persson et al., 1991; Tannin et al., 1991). This similarity to enzymes that regulate steroid and prostaglandin levels increases the importance of understanding the mechanism of action of ADH.

A salient feature of sequence comparisons of ADH with its homologs is the conservation of tyrosine-152 and lysine-156 of ADH in the other members of this protein superfamily (Figure 1). To determine the function of these two residues in *Drosophila* ADH, we have created mutants in which tyrosine-152 has been replaced by phenylalanine (Y152F), cysteine (Y152C), histidine (Y152H), and glutamic acid (Y152E). Also, lysine-156 was mutated into isoleucine (K156I) and arginine (K156R). This paper describes the enzymatic properties of these mutants and their comparison with wild-type *Drosophila* ADH.

EXPERIMENTAL PROCEDURES

Materials. The vectors, bacterial strains, and phage used for expression and mutagenesis of *Drosophila* ADH were as described previously (Chen et al., 1990). "Altered Site System" was purchased from Promega Corp. All restriction endonucleases, DNA ligase, DNA kinase, and Klenow fragment of *Escherichia 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. NAD^+, pyrazole, 2,2,2-trifluoroethanol (TFE), Cibacron Blue 3GA, Sephadex G-100, nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), phenazine methosulfate (PMS), and goat anti-rabbit IgG–alkaline phosphatase conjugate were purchased from Sigma Chemical Co. or Aldrich Chemical Co., Inc. Deoxynucleoside triphosphates labeled at the α position with ^{32}P or ^{35}S were products of Du Pont-NEN Co. or ICN Biomedicals, Inc. Oligodeoxynucleotides for site-directed mutagenesis were synthesized using an automatic DNA synthesizer (Applied Biosystem, Model 380A). Rabbit antisera against *Drosophila* ADH were prepared by Batzer et al. (1988).

Expression and Purification of the Wild-Type and Mutated Drosophila ADH. Construction of the full-length cDNA and expression vector was described in a previous paper (Chen et al., 1990). *E. coli* strain M5219 which carried the cI857 thermosensitive-repressor gene served as an expression host. Both Kunkel's method (1985) and the Altered Sites System developed by Promega Corp. (Titus, 1991) were used to produce point mutations. The wild-type ADH and its mutants expressed were identified by Western blot and purified using a procedure developed previously in our laboratory (Chen et al., 1990).

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[1] Abbreviations: ADH, alcohol dehydrogenase; *Adh*, alcohol dehydrogenase gene; Y152C, Y152E, Y152F, Y152H, K156I, and K156R, *Drosophila* ADH mutated at tyrosine-152 to cysteine, tyrosine-152 to glutamic acid, tyrosine-152 to phenylalanine, tyrosine-152 to histidine, lysine-156 to isoleucine, and lysine-156 to arginine, respectively; K_s, dissociation constant; TFE, 2,2,2-trifluoroethanol; NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; PMS, phenazine methosulfate; PBS, phosphate-buffered saline.

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D. melanogaster **Alcohol Dehydrogenase**	151	Val	Tyr	Ser	Gly	Thr	Lys	Ala	Ala	158
Human 17β-OH Steroid Dehydrogenase	154	Val	Tyr	Cys	Ala	Ser	Lys	Phe	Ala	161
Rat 11β-OH Steroid Dehydrogenase	178	Ser	Tyr	Ser	Ala	Ser	Lys	Phe	Ala	185
S. hydrogenans **3α,20β-OH Steroid Dehydrogenase**	151	Ser	Tyr	Gly	Ala	Ser	Lys	Trp	Gly	158
Pseudomonas **3β-OH Steroid Dehydrogenase**	150	Gly	Tyr	Ser	Ala	Ser	Lys	Ala	Ala	157
Human 15-OH Prostaglandin Dehydrogenase	150	Val	Tyr	Cys	Ala	Ser	Lys	His	Gly	157
Eubacterium **Bile Acid 7-Dehydroxylase-genes 1,3**	156	Gly	Tyr	Pro	Thr	Ser	Lys	Ala	Gly	163
Eubacterium **Bile Acid 7-Dehydroxylase-gene 2**	156	Gly	Tyr	Pro	Ala	Ser	Lys	Ala	Ser	163
K. aerogenes **Ribitol Dehydrogenase**	167	Val	Tyr	Thr	Ala	Ser	Lys	Phe	Ala	174
R. meliloti **NodG**	153	Asn	Tyr	Cys	Ala	Ser	Lys	Ala	Gly	160

FIGURE 1: Comparison of the *Drosophila* alcohol dehydrogenase segment containing tyrosine-152 and lysine-156 with that in homologous dehydrogenases.

Table I: Kinetic Parameters[a] for the Oxidation of Ethanol and Propan-2-ol by Wild-Type ADH and Mutants Y152 and K156

enzymes	k_{cat} (s^{-1})	$K_{s(NAD)}$ (mM)	$K_{m(NAD)}$ (mM)	$K_{m(Alc)}$ (mM)	$k_{cat}/K_{m(NAD)}$ ($mM^{-1}\cdot s^{-1}$)	$k_{cat}/K_{m(Alc)}$ ($mM^{-1}\cdot s^{-1}$)
			Ethanol			
wild-type	3.6	0.041	0.021	1.75	170	2.1
Y152C	8.7×10^{-3}	0.083	0.047	102	0.19	8.5×10^{-5}
K156R	0.079	0.096	0.010	5.7	8.3	0.014
			Propan-2-ol			
wild-type	10.2	0.018	0.044	0.63	230	16
Y152C	0.12	0.13	0.14	65	0.83	1.8×10^{-3}
K156R	1.1	0.10	0.019	1.3	55	0.83

[a] The standard error of each value is less than 10%.

Determination of Kinetic Parameters. ADH activity was determined spectrophotometrically at 340 nm in 0.1 M glycine–NaOH buffer (pH 9.8) at 25 °C. The concentrations of NAD^+ and alcohol for the determination of the kinetic parameters were specified as follows: NAD^+, 0.02–2 mM; ethanol, 1–125 mM; and propan-2-ol, 1–100 mM. For experiments studying the effect of pH on the catalytic efficiency of wild-type and mutated ADH, the buffers were as follows: pH 6.5–7.5, 0.1 M MOPS–NaOH; pH 8.0–8.5, 0.1 M Tris-HCl; and pH 9.0–10.5, 0.1 M glycine–NaOH. The activities of wild-type ADH and Y152C were determined at different pH values with varying concentrations of propan-2-ol and a saturating concentration of NAD^+. The resulting enzyme velocities were used to calculate K_m and k_{cat} using the Enzfitter program (Elsevier-BIOSOFT) in an IBM PC computer.

Substrate Specificity Studies. Nine primary or secondary alcohols were tested for substrate specificity of both wild-type ADH and mutants. $K_{m(app)Alc}$ and k_{cat} were determined for each alcohol substrate with the concentration varying from 1 to 100 mM and the NAD^+ concentration held constant at 1 mM. The values of the catalytic efficiency k_{cat}/K_m for different alcohols were calculated and plotted vs the hydrophobicity constant π of the alcohol substrates (Winberg & McKinley-McKee, 1992; Leo et al., 1971).

Inhibition Studies. Inhibition experiments were performed at a constant NAD^+ concentration of 1 mM and a propan-2-ol concentration that varied from 1 to 100 mM. K_i values of two ADH competitive inhibitors were calculated from the K_m values determined at concentrations of 0, 2.5, and 5 mM of 2,2,2-trifluoroethanol or 0, 2.5, and 5 μM of pyrazole.

Nondenaturing Agarose Gel Electrophoresis and Western Blots. Methods of electrophoresis, ADH activity staining, and Western analysis were that described by Jiang et al. (1992). Agarose (1%) was prepared in 54 mM Tris and 82.5 mM boric acid buffer. Since wild-type ADH and Y152C mutant can be purified, pure protein samples (24 μg of wild-type ADH and 58 μg of Y152C) were analyzed by activity staining. Mutants without enzyme activity can be analyzed only by Western blot. Each well was loaded with 10–15 μL of protein sample. After electrophoresis for 3.5 h at 10 mA and 4 °C, the gel lanes were analyzed for ADH protein either by activity staining or by Western blot. Staining was carried out for 1 h at 37 °C on a shaker in a light-sealed chamber containing 100 mL of 0.05 M Tris-HCl, pH 8.5, 40 mg of NAD^+, 8 mg of PMS, 20 mg of NBT, and 2 mL of propan-2-ol. For Western analysis, the proteins were electrophoretically transferred to Biotrans membrane. The membrane was then incubated in blocking solution (Dulbecco's PBS/5% nonfat milk) at 4 °C for 12 h, and then in 1:10 (antibody/blocking solution) dilution of primary *Drosophila* ADH antibody for 24 h at 4 °C. The membrane was washed for 3 h in six changes of Dulbecco's PBS and then immersed in 1:500 dilution of goat anti-rabbit IgG–alkaline phosphatase conjugate for 24 h at 4 °C. The membrane was washed as before, and the bound antibody was visualized by reacting the alkaline phosphatase conjugate with NBT and BCIP followed by several times washing in water.

RESULTS

Expression and Purification of Wild-Type and Mutated Drosophila ADH from Transformed E. coli Cultures. Our specific mutations of *Drosophila* ADH were achieved by using both Kunkel's method (1985) and the protocol developed from Promega Corp. (Titus, 1991). All mutated ADH cDNA were completely sequenced to confirm the presence of correct codon substitution for Y152C, Y152E, Y152F, Y152H, K156I, and K156R without other alterations.

Lysates of *E. coli* M5219 cells, transformed with wild-type or mutated ADH cDNA, were used for ADH activity and Western blot analysis. Our results shows that the transformed *E. coli* strain M5219, which is a wild-type in protease efficiency, produces all of the ADH proteins, indicating that all these mutated ADH proteins are stable in the bacterial host cells. However, Y152E, Y152F, Y152H, and K156I reveal no detectable ADH activity (data not shown). Y152C and K156R are enzymatically active. They were then purified to homogeneity as determined by electrophoresis on polyacrylamide gel containing sodium dodecyl sulfate (data not shown).

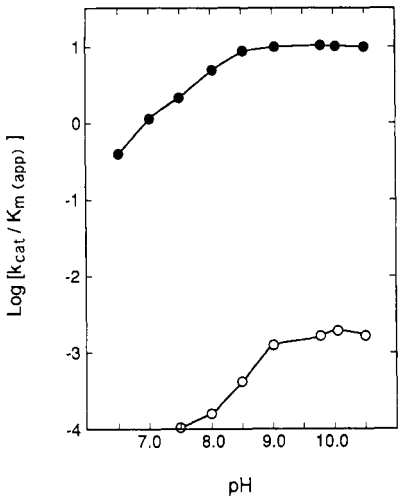

FIGURE 2: Effect of pH on the activity of wild-type *Drosophila* ADH and Y152C mutant. Values of k_{cat} and K_m using propan-2-ol as substrate were determined for wild-type ADH (●) and Y152C (○) (see Experimental Procedures) and plotted as $\log(k_{cat}/K_m)$ at the pH values indicated.

Kinetic Analysis of Wild-Type and Mutated Drosophila ADH. The activity of ADH was determined using conditions which were previously described (Chen et al., 1990). Table I lists the kinetic parameters including K_m, k_{cat}, $K_{s(NAD)}$, and catalytic efficiences (k_{cat}/K_m) for both ethanol and propan-2-ol. As seen in Table I, mutation of tyrosine-152 to cysteine lowers k_{cat} for ethanol by 1/400 and k_{cat} for propan-2-ol by 1/90 when compared with wild-type ADH. Also, $K_{m(ethanol)}$ increases 56-fold and $K_{m(propan-2-ol)}$ 100-fold, while $K_{m(NAD)}$ values are increased only 2- or 3-fold. Mutation of lysine-156 to arginine also significantly reduces k_{cat} and increases $K_{m(ethanol)}$, with little changes in $K_{m(NAD)}$. The dissociation constant $K_{s(NAD)}$ for the Y152C and K156R mutants is independent of alcohol substrate; a slight variation with alcohol substrate exists for the wild-type ADH. These results are consistent with the proposed mechanism of an ordered reaction with NAD^+ binding first (Winberg & McKinley-McKee, 1988). For both the Y152C and K156R mutants k_{cat}/K_m values for ethanol and propan-2-ol have been decreased substantially, suggesting that tyrosine-152 and lysine-156 are involved in substrate binding.

Effect of pH on the Activity of Wild-Type ADH and Y152C Mutant. In order to evaluate the pK_a values for the wild-type ADH and Y152C mutant, the K_m and k_{cat} values of these two ADHs were determined at different pH values ranging from pH 6.5 to 10.5 using propan-2-ol as substrate. The results of these experiments are shown in Figure 2. The wild-type ADH has a pK_a of 7.5, in agreement with that found by Winberg and McKinley-McKee (1992). Interestingly, the Y152C mutant has a pK_a at pH 8.5.

Substrate Specificity Studies. The active site of *Drosophila* ADH can accommodate a variety of alcohols different in structures. Therefore, specificity toward these substrates reflects the topology of the substrate binding domain in *Drosophila* ADH (Winberg et al., 1982, 1986; Hovik et al., 1984). The catalytic efficiency, k_{cat}/K_m, is plotted vs the hydrophobicity constant π for primary and secondary alcohols with two- to six-carbon chains (Figure 3). Wild-type ADH has essentially the same k_{cat}/K_m for all the primary alcohols and secondary alcohols. In contrast, the k_{cat}/K_m values of Y152C and K156R depend on the hydrophobicity of the alcohol substrate, suggesting that these mutants have an altered topology in the substrate binding domain.

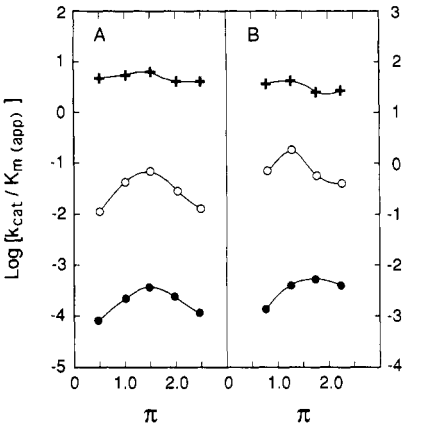

FIGURE 3: Catalytic efficiency and hydrophobicity relationship of wild-type ADH, Y152C, and K156R. Catalytic efficiencies, k_{cat}/K_m, were determined as described in the Experimental Procedures. Primary alcohols with three- to six-carbon chains and secondary alcohols with three- to six-carbon chains were used as substrates. The hydrophobicity constants, π, for the individual alcohol substrate used for the experiment are as follows: ethanol, 0.5; propan-1-ol, 1.0; butan-1-ol, 1.5; pentan-1-ol, 2.0; hexan-1-ol, 2.5; propan-2-ol, 0.8; butan-2-ol, 1.3; pentan-2-ol, 1.8; hexan-2-ol, 2.3 (Winberg & McKinley-McKee, 1992). Wild-type (+); Y152C (○); and K156R (●). (A) Primary alcohols; (B) secondary alcohols.

Table II: Inhibition Constants for ADH Inhibitors: Pyrazole and 2,2,2-Trifluoroethanol[a]

K_i	wild-type	Y152C	K156R
pyrazole (μM)	2.6	2.1	11.4
TFE (mM)	1.2	5.7	1.9

[a] The inhibition experiments were performed as described in the Experimental Procedures. The K_i values were calculated from K_m values determined at various concentrations of inhibitors using the Enzfitter program (Elsevier-BIOSOFT) in an IBM PC. All the Lineweaver–Burk profiles showed competitive inhibition patterns.

Inhibition Studies. It has been reported that pyrazole and 2,2,2-trifluoroethanol inhibit *Drosophila* ADH by competing with alcohol (Winberg & McKinley-McKee, 1988). We find that both pyrazole and TFE are alcohol competitive inhibitors of the Y152C and K156R mutants (Table II), although there are differences in their binding specificities. Pyrazole has a similar affinity for wild-type ADH and Y152C, but only 25% of the affinity for K156R. In contrast, the affinity of TFE for Y152C and K156R is 20% and 60%, respectively, of that for wild-type ADH.

Structural Integrity of Drosophila ADH Mutants. Wild-type ADH is a dimer. The loss of catalytic activity in the various mutants could be due to either their dissociation or aggregation. To investigate this possibility, we analyzed the structural integrity of the different mutants by nondenaturing agarose gel electrophoresis followed by activity staining and Western analysis (Jiang et al., 1992). As standards, we used ADH-S and ADH-F, the slow and fast migrating isoforms of *Drosophila* ADH (Thatcher, 1980). As shown in Figure 4A, ADH-S, ADH-F, the wild-type ADH, and Y152C are dimers. The intensity of the lane with Y152C is much lighter than that of the standards and wild-type ADH, reflecting the low enzymatic activity observed for this mutant. The Western blot (Figure 4B) shows that all the mutants have a mobility within that of the two standards, suggesting that they are dimers in spite of their low or null activity. The signal for Y152F is weak, which may be due to the structural changes in this mutant that affect its affinity for the antibody to wild-type ADH.

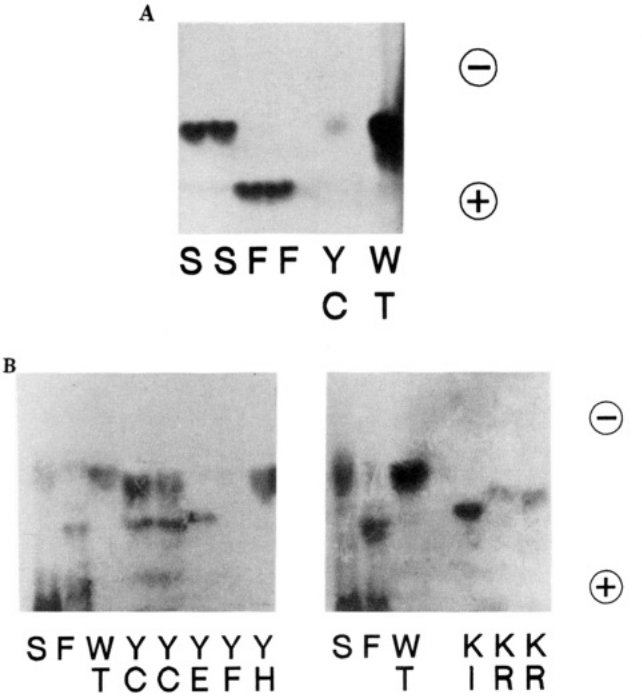

FIGURE 4: Nondenaturing gel electrophoresis and Western analysis of wild-type ADH and its mutants. Purified wild-type ADH (WT) and Y152C (TC) and lysates of *E. coli* cells transformed by plasmid pPL2 carrying wild-type ADH (WT), Y152C (YC), Y152E (YE), Y152F (YF), K156I (KI), and K156R (KR) were analyzed by electrophoresis on nondenaturing agarose gels. ADH-S (S) and ADH-F (F) were standard markers. (A) ADH activity staining; (B) Western analysis. (+) and (–) indicate cathode and anode, respectively.

Recombinant ADH migrates like ADH-S, consistent with the fact that the cDNA was constructed from the allele of ADH-S (Chen et al., 1990). The mobilities of Y152E and K156I are shifted to that like ADH-F. This is consistent with the increase in negative charge (Y152E) or decrease in positive charge (K156I) of these mutants. Mutant Y152C gave two bands, a major band like ADH-S and a minor band like ADH-F. Enzymatic activity resides in the major band (Figure 4A).

DISCUSSION

A variety of methods have been used to elucidate the mechanism of action of *Drosophila* ADH. These include the following: chemical modification of specific amino acids, analysis of kinetics as a function of pH, mutagenesis of specific residues, and comparison of the ADH amino acid sequence with that of other dehydrogenases. In some instances these studies have yielded consistent answers; in others they have not. An example of the former comes from mutagenesis studies showing that glycine-14 and aspartic acid-38 of ADH are important in binding the NAD^+ cofactor (Chen et al., 1990, 1991). This is consistent with the recently reported tertiary structure of the *Streptomyces hydrogenans* 3α,20β-hydroxysteroid dehydrogenase–NADH binary complex (Ghosh et al., 1991) and earlier analyses of the NAD^+ binding site of dehydrogenases (Wierenga et al., 1985). On the other hand, mutagenesis studies showed that neither cysteine-135 nor cysteine-218 of ADH is essential (Chen et al., 1990), despite other reports that chemical modification of cysteine inhibits enzyme activity. In the latter case, it appears that inhibition of activity by chemical modification of cysteine residues is due to steric effects instead of alteration of a residue that is essential for enzymic oxidation or reduction of the substrate.

The strong conservation of tyrosine-152 and lysine-156 of ADH in homologous enzymes that act on steroids, prostaglandin, sugars, and other compounds suggests that these two amino acids have an important functional role in catalysis by ADH. To test the roles of tyrosine-152 and lysine-156, we constructed a series of mutants that would have different chemical properties. First, we substituted tyrosine with phenylalanine, which lacks a phenolic group and is otherwise structurally similar to tyrosine. The Y152F mutant is inactive. Interestingly, we find that cysteine can substitute for tyrosine (Table I), although the Y152C mutant has about 0.25% the k_{cat} of the wild-type enzyme, indicating that a thiol group can perform the function of the phenolic group. Although this suggests the catalysis by ADH requires a deprotonable residue, the ADH^{null} phenotype showed by Y152E and Y152H mutants indicates that more than a deprotonated side chain is required for catalysis. Reduced or null activity displayed by the mutated ADH proteins could be due to structural changes that alter ADH from its native dimer state or to more subtle conformational changes that affect, for example, substrate binding to the enzyme. Western analysis (Figure 4B) of the mutants shows that they are dimers, indicating that major structural changes did not occur. The other possibility, a subtle conformational change in the mutant enzymes, is likely, although as described below, we consider the chemical interaction between tyrosine-152 and the substrate to be importnat in catalysis.

Our finding that tyrosine-152 in *Drosophila* ADH has an essential role in catalysis is in agreement with that of human 15-hydroxyprostaglandin dehydrogenase (Ensor & Tai, 1991)

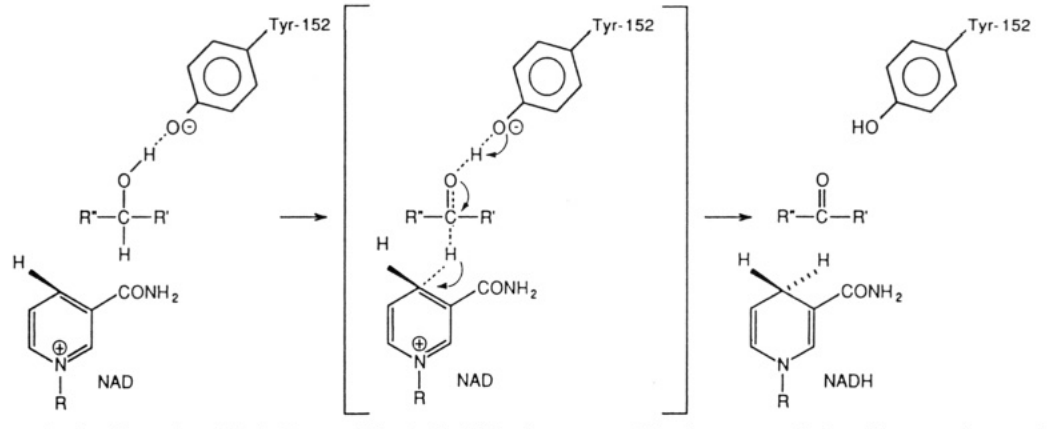

FIGURE 5: Proposed role of tyrosine-152 in *Drosophila* alcohol dehydrogenase. The deprotonated phenolic group in tyrosine-152 forms a hydrogen bond with the hydroxyl group of the alcohol substrate. Tyrosine-152 then serves as a catalytic base to facilitate electron transfer from the phenolic hydroxyl to a hydrogen attached to the carbonyl group. NAD^+ takes the hydride ion produced to complete the reaction.

and rat 11β-hydroxysteroid dehydrogenase (Obeid & White, 1992). In the former enzyme, conversion of its homologous tyrosine to alanine results in a catalytically inactive protein (Ensor & Tai, 1991). In the latter enzyme conversion of its homologous tyrosine to either phenylalanine or serine results in a catalytically inactive enzyme (Obeid & White, 1992).

We also investigated the role of lysine-156, which is conserved in ADH and its homologs. Mutating lysine to isoleucine, which has an uncharged side chain, led to inactive protein. However, a K156R mutant was active (Table I), albeit with only 2.2% the k_{cat} compared to the wild-type enzyme, indicating that a basic residue is important at this position. Mutation of the homologous lysine in 11β-hydroxysteroid dehydrogenase to arginine results in a catalytically inactive enzyme (Obeid & White, 1992).

The Y152C and K156R mutants have similar K_m values for NAD^+ as the wild-type ADH. This and the observed increase in K_m for alcohol substrates in both mutants indicate that the principal effect of these mutations is best explained in the interaction of the substrate with the enzyme and not in the binding of the cofactor to the enzyme.

Two other experiments reported here also indicate the importance of tyrosine-152 and lysine-156 in ADH catalysis. In contrast to wild-type ADH which has little variation of k_{cat}/K_m for the substrates tested, the activity of the Y152C and K156R mutants depends on the length of the carbon chain of both primary and secondary alcohols (Figure 3). Moreover, the competitive inhibitors pyrazole and TFE bind differently to the two mutants (Table II). Mutations at tyrosine-152 and lysine-156 appear to disturb the topology of the substrate binding domain, altering substrate specificity. Pyrazole inhibits liver ADH by forming ternary complexes with catalytic zinc and C-4 of the nicotinamide moiety of NAD^+ (Theorell & Yonetani, 1963). Surprisingly, our results showed that pyrazole is an efficient competitive inhibitor for *Drosophila* ADH (Table II), an enzyme which does not require zinc for activity.

Our finding that substitution of tyrosine-152 with phenylalanine leads to an inactive enzyme, while substitution with cysteine, which has a deprotonatable side chain, leads to a partially active enzyme, suggests that the chemistry of tyrosine side chains is important in ADH catalysis. It is interesting that the Y152C mutant has a higher pK_a than the wild-type ADH. Indeed, the pK_a of Y152C mutant is 8.5, which is similar to that of cysteine (p$K_a \sim 8.5$). In contrast, the wild-type ADH has a pK_a of 7.5, which is over 2 orders of magnitude lower than that of the phenolic of tyrosine (p$K_a \sim 10.0$). If tyrosine-152 is deprotonated in wild-type ADH, then its local environment is likely to contain residues which lower the pK_a of tyrosine. Lysine-156 may be one of these residues, especially in view of our finding that a positively charged residue is important at this position. Figure 5 shows how tyrosine could be involved in ADH catalysis: the deprotonated phenolic group abstracts a proton from the alcohol side chain, facilitating subsequent transfer of hydride ion to NAD^+.

Gordon et al. (1992) have recently published the preliminary X-ray crystallographic data on alcohol dehydrogenase from *Drosophila lebanonensis* which has 82% sequence identity to the ADH of *Drosophila melanogaster* (Villarroya et al., 1989). Elucidation of the tertiary structure of *D. lebanonensis* ADH will clarify the role of tyrosine-152 and lysine-156 in ADH catalysis, as well as provide important insights into the enzymatic mechanisms of homologs such as 11β-hydroxysteroid dehydrogenase and 15-hydroxyprostaglandin dehydrogenase.

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