Articles

Site-Directed Mutagenesis of Glycine-14 and Two "Critical" Cysteinyl Residues in *Drosophila* Alcohol Dehydrogenase†

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ABSTRACT: Three amino acid residues (glycine-14, cysteine-135, and cysteine-218) previously speculated to be important for the structure and function of *Drosophila melanogaster* alcohol dehydrogenase have been investigated by using site-directed mutagenesis followed by kinetic analysis and chemical modification. Mutating glycine-14 to valine (G14V) virtually inactivates *Drosophila* ADH, and substitution of alanine at this position (G14A) causes a 31% decrease in activity. Thermal denaturation and kinetic and inhibition studies further demonstrate that replacing glycine-14 with either alanine or valine leads to structural changes in the NAD binding domain. These results provide direct evidence for the role played by glycine-14 in maintaining the correct conformation in the NAD binding domain. On the other hand, changing of cysteine-135, -218, or both to alanine (C135A, C218A, and C135A/C218A) causes no decrease in the catalytic activity of the enzyme, indicating that neither of the cysteinyl residues is essential for catalysis. C135A and wild-type enzyme are both inactivated by DTNB. In contrast, C218A and C135A/C218A are unaffected by DTNB treatment. DTNB modification of cysteine-218 can be prevented by the substrates NAD and 2-propanol, suggesting that cysteine-218 may be in the vicinity of the active site. Cysteine-135 which is normally insensitive to DTNB becomes accessible in the presence of 2-propanol and/or NAD, suggesting a conformational change induced by binding of these substrates.

Alcohol dehydrogenase (ADH)[1] (alcohol:NAD oxidoreductase, EC 1.1.1.1) from *Drosophilia melanogaster* is unique among alcohol dehydrogenases. It prefers secondary alcohols as substrates (Sofer & Ursprung, 1968; Winberg et al., 1982), requires no metal cofactors (Chambers, 1984), and stereochemically shows 4-*pro-S* hydride transfer (Benner et al., 1985). Horse liver and yeast ADHs, in contrast, are more specific for primary alcohols (Dalziel & Dickinson, 1966), require zinc ions as cofactors (Dunn & Hutchinson, 1973), and show that 4-*pro-R* stereospecificity (You, 1982). Regulation of ADH expression in *Drosophila* has been studied extensively (Sofer & Martin, 1987; Fischer & Maniatis, 1988), but ADH structure is known only at the amino acid sequence level (Thatcher, 1980; Chambers et al., 1984).

Results from previous studies on the structure and function of *Drosophila* ADH were speculative and, in certain cases, ambiguous. On the basis of primary sequence data, Benyajati et al. (1981) predicted a putative *Drosophila* ADH secondary structure. The N-terminal half consists of alternating α-helices and β-sheets, a structural feature which is common to the NAD binding domains of many dehydrogenases (Rossmann, 1983). Although *Drosophila* ADH and horse liver ADH share little overall sequence homology, a secondary structure comparison of the NAD binding domain reveals a similar structural pattern. The N-terminal region of the NAD binding fold contains the sequence $GlyXGlyX_2Gly$ (X = any residue). This "conserved sequence" (Argos & Leberman, 1985) characteristically marks the tight turn following the first β-sheet of the NAD binding domain in many NAD-dependent dehydrogenases (Table I). In *Drosophila* ADH, the first Gly residue in the "conserved sequence" is Gly-14. ADH isolated from the *Drosophila* ADH^{n11} mutant generated by ethyl methanesulfonate (EMS) treatment contains an aspartic acid substitution at position 14 (Thatcher, 1980). This mutant shows no ADH activity and has a low affinity for NAD (Place et al., 1979), indicating that Gly-14 is important for ADH activity. It has been suggested that aspartic acid substitution disturbs the tight turn in the putative NAD binding domain. However, it is unclear whether the inactivation is due to the size or the negative charge of aspartic acid.

Table I: Alignment of "Conserved Sequence" in NAD-Dependent Dehydrogenase[a]

protein	residues	sequence
GAPDH	1-20	SKIGIDGFGRIGRLVLRAAL
LDH	21-40	NKITVVGVGAVGMACAISIL
LADH	193-212	STCAVFGLGGVGSVIMGCK
DADH	8-27	NVIFVAGLGGIGLDTSKELL

[a] Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; LADH, liver alcohol dehydrogenase; DADH, *Drosophila* alcohol dehydrogenase.

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[1] Abbreviations: ADH, alcohol dehydrogenase; *Adh*, alcohol dehydrogenase gene; G14A, G14V, C135A, and C218A, *Drosophila* ADH mutated at glycine-14 to alanine, glycine-14 to valine, cysteine-135 to alanine, and cysteine-218 to alanine, respectively; C135A/C218A, double mutation of cysteine-135 and -218 to alanine; $K_{m(app)NAD}$, apparent Michaelis-Menten constant for NAD; $K_{m(app)Alc}$, apparent Michaelis-Menten constant for alcohol; K_i, inhibition constant; k_{cat}, turnover number in Michaelis-Menten model; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EMS, ethyl methanesulfonate; DTNB, 5,5'-dithiobis(2-nitrobenzoate); TNB, 5-thio-2-nitrobenzoate; NEM, *N*-ethylmaleimide; RF, replicative form; PCR, polymerase chain reaction; M1 and M2, mutagenic primers; LB and LR, primers containing, respectively, *Bam*HI and *Eco*RI linkers.

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A second uncertainty in the enzymology of *Drosophila* ADH concerns the catalytic sulfhydryl residues. It has long been thought that the two cysteinyl residues at positions 135 and 218 in *Drosophila* ADH are required for catalytic function (Chambers, 1984). Chemical modification has been used to identify the essential sulfhydryl group in this enzyme (Thatcher, 1981; Chambers et al., 1981). However, results have been ambiguous and contradictory, and the function of these sulfhydryl groups remains obscure.

In order to determine the exact function of Gly-14 and the two cysteinyl residues, we have constructed a full-length cDNA of *Drosophila Adh* and subcloned it under the control of the λ PL promoter. Following successful expression of wild-type enzyme in an *Escherichia coli* host, we created mutants in which Gly-14 is replaced by Ala or Val (G14A and G14V, respectively) and mutants containing Ala substitution at Cys-135 (C135A), Cys-218 (C218A), or both Cys-135 and Cys-218 (C135A/C218A) by site-directed mutagenesis to investigate systematically the functions of these residues.

This paper describes the construction of a full-length cDNA of *Drosophila Adh*, its expression and mutation, and the enzymatic properties of mutated and wild-type *Drosophila* ADHs.

Experimental Procedures

Vectors, Bacterial Strains, and Phage. Plasmid containing *Drosophila melanogaster Adh* (psAC1) was a gift from W. Sofer (Goldberg, 1980). *E. coli* defective in dUTPase and uracil glycosylase genes (BW313: dut^- and ung^-) was received from the *E. coli* Genetic Stock Center, School of Medicine, Yale University. Wild-type strain (CSH50: dut^+ and ung^+) was described by Miller (1972) and provided by Kunkel (1985). *E. coli* host LC137 defective in protease activity (lon^-) was characterized by Goff et al. (1984). Expression vector with the λ PL promoter (pPL2), plasmid with a cI857ts gene (pCI857), and *E. coli* M5219 harboring a cI857ts defective prophage were described by Greer (1975).

Enzymes, Chemicals, and Antibody. All restriction endonucleases, DNA ligase, DNA kinase, mung bean nuclease, T4 DNA polymerase, and Klenow DNA polymerase were purchased from Bethesda Research Laboratories, New England Biolabs, Inc., or Promega Corp. *Taq* DNA polymerase kits were purchased from Perkin-Elmer Cetus. Sequenase kits were obtained from United States Biochemicals Corp. NAD, NADP, DTNB, Cibacron blue 3GA, and Sephadex G-100 were purchased from Sigma Chemical Co. Deoxynucleoside triphosphates labeled at the α position with ^{32}P or ^{35}S were products of Du Pont Co.–NEN or ICN Biomedicals, Inc. Oligodeoxynucleotides for site-directed mutagenesis were synthesized by an automated DNA synthesizer (Applied Biosystems, Model 380A). ADH isolated from *Drosophila* and rabbit antibodies against the fly protein were prepared by Batzer et al. (1988).

Construction of the Full-Length cDNA. A 3.2-kilobase *Xba*I fragment containing *Drosophila Adh* was excised from psAC1 and subcloned in M13mp18. Single-stranded M13/*Adh* containing uracil was prepared in an *E. coli* host defective in dUTPase and uracil glycosylase (BW313) as described by Kunkel (1985). This single-stranded template was hybridized with synthetic oligodeoxynucleotides containing the base sequence corresponding to seven codons in the carboxyl end of one exon and seven codons in the amino terminus of the adjacent exon. The complementary strand lacking uracil was synthesized in vitro along the M13 template using Klenow fragment of *E. coli* DNA polymerase I in the presence of all four deoxynucleoside triphosphates. Transformation of a dut^+, ung^+ *E. coli* host (CSH50) selects against the uracil-containing strand, so that progeny phage should contain predominantly *Drosophila Adh* without introns. This prediction was confirmed by DNA sequence analysis.

Construction of the Expression Vector. One further modification to M13/*Adh* cDNA was made to facilitate subcloning into pPL2. An *Eco*RI site was introduced by Kunkel's method of site-directed mutagenesis immediately upstream of the ATG start codon. Since there is a unique *Bal*I site 244 bp downstream from the TAA termination codon, the new *Eco*RI site enabeled us to excise the entire ADH coding sequence. After mung bean nuclease digestion to create a blunt end from the cohesive termini produced by *Eco*RI digestion, *Adh* cDNA was inserted into pPL2 at 12 bp downstream from the Shine–Dalgarno sequence (Guarente et al., 1980) while preserving the correct translation reading frame. This plasmid construct (pPL2/*Adh*) produces wild-type ADH.

Site-Directed Mutagenesis. To produce point mutations by Kunkel's method (1985), a wild-type *Adh* cDNA was excised from pPL2/*Adh* and subcloned into M13mp19. Point mutations were introduced, using mutagenic primers containing base sequences coding for the substitutions. The scheme of the mutagenesis experiments was the same as described for intron deletion. The entire length of mutated *Drosophila Adh* cDNAs was sequenced to confirm that no other mutations had occurred.

Polymerase Chain Reaction. Several attempts at constructing the C218A mutant by Kunkel's method were unsuccessful. We then used the polymerase chain reaction (PCR) (Higuchi et al., 1988) to generate the C218A mutation. Four synthetic primers were prepared in order to produce a 655 bp partial *Drosophila Adh* cDNA with a Cys to Ala mutation at position 218 (primers M1 and M2). A *Bam*HI site at the 5′ end (primer LB) and an *Eco*RI site at the 3′ end (primer LR) matched the *Bam*HI site in *Adh* cDNA and the *Eco*RI site in the pPL2 vector. The first step was to make a 270 bp fragment primed by LB and M1 and a 416 bp fragment primed by M2 and LR using PCR conditions described by Higuchi (1988) and the protocol of the "GeneAmp DNA Amplification Reagent Kit" (Perkin-Elmer Cetus). These two fragments were complementary for 31 bp around the C218A mutation locus. A mixture of 0.3 pmol of each DNA fragment was extended for 20 cycles of PCR in the presence of 50 pmol of each primer LB and LR. The 655 bp DNA fragment produced was joined at the *Bam*HI and *Eco*RI sites of pPL2 vector containing wild-type *Drosophila Adh* cDNA by DNA ligase. This procedure constructed a pPL2 derivative containing a C218A mutation. To generate the C135A/C218A double mutant, the 655 bp PCR fragment was inserted in the same sites of C135A cDNA.

Expression of Wild-Type and Mutated ADH in E. coli. E. coli M5219 which carries the cI857 thermosensitive λ repressor gene was transformed with pPL2 plasmids containing *Adh* cDNA. Colonies grown in the presence of ampicillin were screened for *Adh* cDNA by colony hybridization (Maniatis et al., 1982). Liquid cultures of positive colonies were grown to mid-log phase at 28 °C, heat shocked for 30 min at 42°C, and incubated at 37 °C for 3 h. Cells from the cultures were harvested and sonicated. *Drosophila* ADH was identified by an enzyme activity assay (Lee, 1982) and by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The results of SDS–PAGE were analyzed by densitometric scanning (Bio-Rad, VD-620).

In order to express protease-sensitive mutants of *Drosophila* ADH, protease-defective *E. coli* LC137 was cotransformed

with the pPL2 vector containing the *Adh* cDNA insert and plasmid pCI857. Colonies were grown in the presence of ampicillin and kanamycin. Liquid cultures of transformants were grown to mid-log phase at 30 °C, heat-shocked for 15 min at 42 °C, and incubated at 37 °C for 4 h.

Purification of Drosophila ADH from E. coli. Large-scale cultures of transformed cells at late-log phase were lysed by sonication. The protein precipitated between 40% and 60% saturation of ammonium sulfate was dissolved in column buffer (50 mM Tris-HCl and 12 mM EDTA, pH 8.3) and applied to a Sephadex G-100 column (2.5 × 120 cm). Eluted fractions containing the majority of ADH activity were pooled and applied to a Cibacron Blue 3GA–agarose column (2.5 × 20 cm) equilibrated with the same column buffer. Protein was eluted by a 0–2.0 M NaCl gradient. *Drosophila* ADH was eluted at approximately 1.5 M NaCl, concentrated in an Amicon ultrafiltration cell, and stored at −20 °C. Aliquots from each step of purification were analyzed for protein concentration (Bio-Rad Protein Assay) and ADH activity.

Western Blot of Drosophila ADH. Identification of cloned proteins on SDS–PAGE using an antibody against *Drosophila* ADH was described in Batzer et al. (1988). Samples for SDS–PAGE were prepared from crude extracts of *E. coli* M5219 transformed with the expression vector containing or lacking *Adh* cDNA.

Thermal Denaturation Study. Crude extracts of *E. coli* LC137 containing wild-type or mutant *Adh* cDNA were incubated at 40 °C. Aliquots of 100 μL from each sample were taken even 6 min and assayed for ADH activity (Lee, 1982). All assays were performed in duplicate.

Determination of Kinetic Parameters and Inhibition Studies. ADH activity was determined spectrophotometrically at 340 nm in the presence of two substrates, 2-propanol and NAD, in 100 mM Tris-HCl buffer (pH 8.7 unless otherwise specified) at 25 °C. Reactions were initiated by adding ADH. One unit of activity is defined as a change in absorbance at 340 nm of 0.001/min (Sofer & Ursprung, 1968). Enzyme concentrations used for kinetic studies were adjusted so that absorbance changes at 340 nm were linear for at least 1 min. Concentrations of NAD and alcohol were specified as follows: the $K_{m(app)NAD}$ was determined for NAD between 0.1 and 1 mM with a constant alcohol concentration of 10 mM, and the $K_{m(app)Alc}$ for alcohol at concentrations from 2 to 10 mM with a constant NAD concentration of 1 mM. All K_m and V_{max} values were calculated from duplicate initial velocities determined at five substrate concentrations using the ENZFITTER program (Elsevier–BIOSOFT) on a personal computer. The K_i values were calculated from K_m values determined as above at four concentrations of the inhibitor (NADP) with the NAD concentration from 0.06 to 1 mM at a constant alcohol concentration.

DTNB Modification of Wild-Type and Mutated Drosophila ADH. Drosophila ADH (10 nmol) was mixed with DTNB (100 nmol) in 1 mL of 0.2 M Tris-HCl buffer (pH 8.0) at 25 °C in the presence or absence of 1 mM NAD and 10 mM 2-propanol. The absorbance at 412 nm, the λ_{max} of 5-thio-2-nitrobenzoate (TNB), was recorded at 0.25-min intervals on a spectrophotometer (Gilford-Response II).

DTNB Inactivation of Wild-Type and Mutated Drosophila ADH. Drosophila ADH (4 μg) was incubated in 200 μL of 100 μM DTNB and 0.2 M Tris-HCl buffer (pH 8.0) at 25 °C. Twenty-microliter aliquots from each sample were taken every 3 min and assayed for ADH activity in duplicate.

RESULTS

Construction of Full-Length cDNA and Site-Directed Mutagenesis. The *Drosophila* ADH gene contains two introns. In order to express this ADH in *E. coli*, it was necessary to remove them. This was accomplished by site-directed deletion as described under Experimental Procedures. Sequence analysis of the cDNA construct showed that both introns of *Drosophila Adh* were deleted and that all the three exons were fused correctly, preserving in-frame translation. We achieved a minimum in vitro mutation efficiency of 30% using the method developed by Kunkel (1985) to delete introns and produce specific mutations of *Drosophila Adh. Mutagenesis* efficiency of 14% was observed by using the PCR method. All mutated *Adh* cDNAs were completely sequenced to confirm that unintentional mutations had not been introduced.

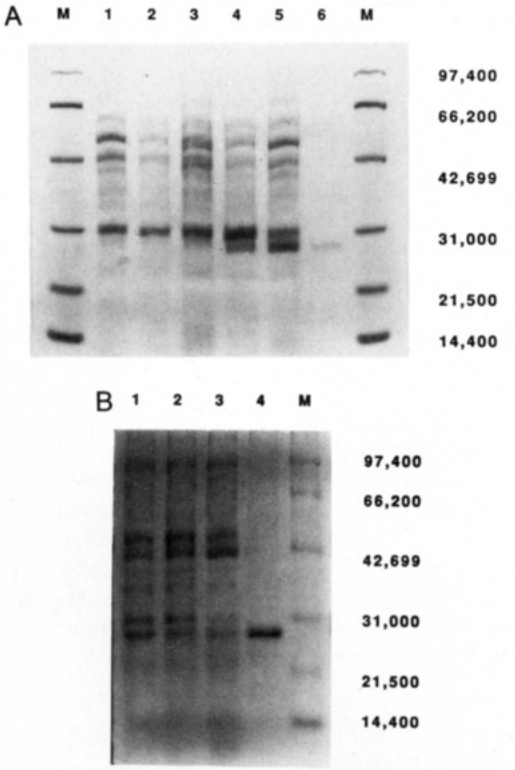

FIGURE 1: Expression of *Drosophila* ADH in *E. coli* cells. Coomassie blue stained SDS–PAGE of crude extracts from (A) *E. coli* M5219 cells: (1) nontransformed control; transformed by (2) pPL2; (3) pPL2/*Adh* cDNA in which *Adh* cDNA is inserted in an opposite orientation; (4) pPL2/*Adh* cDNA without heat shock; (5) pPL2/*Adh* cDNA with heat shock; (6) ADH purified from *Drosophila melanogaster*. (B) *E. coli* LC137 cells transformed by: (1) pPL2/*Adh* cDNA; (2) pPL2/G14A; (3) pPL2/G14V; (4) purified wild-type ADH. M = molecular weight size marker.

Expression and Purification of Drosophila ADH from E. coli Host. Lysates of *E. coli* M5219 cells transformed with pPL2/*Adh* cDNA were analyzed for ADH activity and protein bands. SDS–PAGE of the lysates (Figure 1A) shows that the transformed cells produce ADH protein and the expressions depend on the orientation of *Adh* cDNA and heat inactivation of λ repressor. The SDS–PAGE pattern also shows that *Drosophila* ADH produced in *E. coli* is the same size as the authentic protein (27 kDa) (Figure 1A, lane 6). On the basis of ADH activity and SDS–PAGE, expression of G14A, C135A, C218A, and C135A/C218A in M5219 is adequate for the purification of these ADH proteins, whereas ADH activity in the pPL2/G14V-transformed M5219 culture is undetectable. In order to ensure the expression of this ADH mutant, Western blot analysis was performed. The result (Figure 2) shows that the transformants of G14A and G14V in M5219 produce ADH.

The level of expression of G14V can be improved in a

Table II: $K_{m(app)}$ and k_{cat} Values for Cloned Wild-Type ADH and G14A[a]

enzyme form	$K_{m(app)}$ (mM) NAD	$K_{m(app)}$ (mM) 2-propanol	k_{cat} (s^{-1})	$k_{cat}/K_{m(app)}$ (s^{-1} mM^{-1}) NAD	$k_{cat}/K_{m(app)}$ (s^{-1} mM^{-1}) 2-propanol
wild type (authentic)[b]	0.19	0.69			
wild type (recombinant)	0.12 ± 0.02	0.61 ± 0.09	2.8 ± 0.02	23.3	4.6
G14A	0.42 ± 0.03	0.56 ± 0.02	1.9 ± 0.01	4.5	3.4

[a] Enzymes were assayed as described under Experimental Procedures. [b] This value is from Chambers (1984).

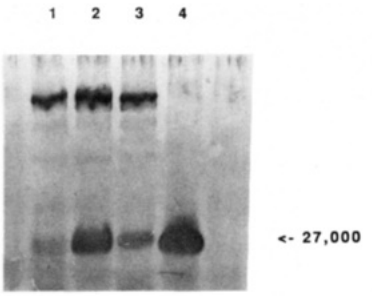

FIGURE 2: Western blot of *Drosophila* ADH expressed in *E. coli* M5219. Lysates of *E. coli* M5219 transformed by (1) pPL2, (2) pPL2/G14A, and (3) pPL2/G14V were analyzed by SDS–PAGE and Western blot using rabbit polyclonal antibody against *Drosophila* ADH. Purified wild-type *Drosophila* ADH (4) was the positive control.

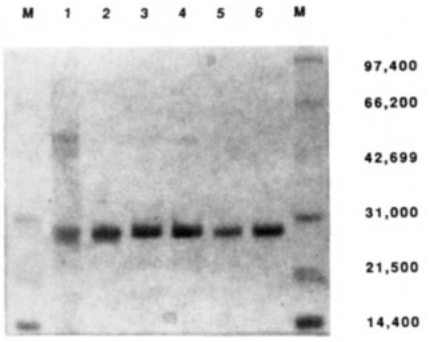

FIGURE 3: Coomassie blue stained SDS–PAGE of purified *Drosophila* ADH and mutants. Lane 1 is crude extract control; lanes 2–6 are ADHs purified from *E. coli* M5219 transformed by (2) pPL2/*Adh* cDNA, (3) pPL2/G14A, (4) pPL2/C135A, (5) pPL2/C218A, and (6) pPL2/C135A/C218A. M = molecular weight size marker.

protease-defective host (LC137) (Goff et al., 1984) as shown in Figure 1B. However, the specific activity of ADH in the crude extracts of G14V-transformed *E. coli* LC137 is less than 1% of wild-type, making purification extremely difficult. With the exception of G14V, all the ADH proteins have been purified to homogeneity (Figure 3).

Thermal Denaturation Studies. Chambers et al. (1984) showed that *Drosophila* ADH can be denatured by heating at 40 °C. We carried out thermal denaturation on wild-type, G14A, and G14V. For experimental consistency, these three enzymes were expressed in *E. coli* LC137. Our results (Figure 4) show that wild type loses 30% of its activity after 1 h at 40 °C, whereas G14A loses 90% of its original activity under identical conditions. Since the host strain, *E. coli* LC137, used for this experiment is deficient in proteases, it is unlikely that the thermal inactivation of G14A is caused by protease cleavage, although we cannot rule this out completely. The specific activity of G14V was too low to determine the effect of heat treatment.

Kinetic Analysis of Wild-Type Drosophila ADH and G14A. We find that the kinetic behavior and substrate specificity of cloned *Drosophila* ADH are essentially identical with those of ADH isolated from the fruit fly (Table II). Comparisons between cloned wild-type *Drosophila* ADH and its mutants can be extrapolated, therefore, to ADH isolated from *Drosophila*. G14V mutant has almost no ADH activity, while G14A mutant shows decreased activity (69% of wild type) (Table II). Replacing Gly-14 with alanine increases the K_m for NAD approximately 3-fold with little effect on the K_m for 2-propanol. Further, the $k_{cat}/K_{m(app)NAD}$ is decreased 5-fold in G14A, while the $k_{cat}/K_{m(app)Alc}$ shows no significant differences between wild type and G14A mutant. These results suggest that the mutation in G14A affects the binding of NAD but not of 2-propanol.

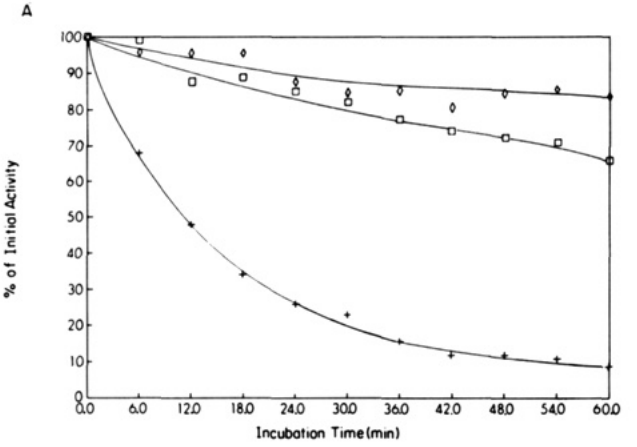

FIGURE 4: Thermal denaturation of wild-type and mutant *Drosophila* ADH. Crude extracts (1 mL) of *E. coli* LC137 transformed with *Adh* cDNA and mutants were incubated in sonication buffer at 40 °C. Aliquots of 100 μL were assayed for ADH activity at 6-min intervals. Relative ADH activity is shown as a percentage of initial velocity without heat treatment. (□) WT; (+) G14A; (◇) G14V.

Table III: Activities of *Drosophila* ADH and Mutants

enzyme	amino acid changes	rel act.[a]
WT	wild type	1.00
G14A	$Gly^{14} \rightarrow Ala^{14}$	0.69
C135A	$Cys^{135} \rightarrow Ala^{135}$	1.17
C218A	$Cys^{218} \rightarrow Ala^{218}$	1.08
C135A/C218A	$Cys^{135} \rightarrow Ala^{135}$, $Cys^{218} \rightarrow Ala^{218}$	1.45

[a] Calculated from the k_{cat} value of each enzyme. The maximum standard error is 0.08.

Inhibition Studies. Initial velocities of wild-type *Drosophila* ADH and G14A mutant were analyzed at alcohol saturation in the absence or presence of NADP at concentrations ranging from 100 to 1000 μM. The Lineweaver–Burk plot of $1/v$ versus $1/[NAD]$ shows a single $1/v$ intercept for all NADP concentrations indicating that NADP inhibits *Drosophila* ADH competitively. The slopes of these lines for wild-type ADH are 1.07, 1.17, 1.53, and 2.07 at zero, 100, 500, and 1000 μM NADP, respectively. However, differences among slopes for G14A are insignificant. The K_i values of NADP calculated from these data are 0.944 mM for wild-type ADH and 11.9 mM for G14A. These results indicate that the structure of the NAD binding domain in G14A has been changed such that NADP cannot compete efficiently with NAD for binding to the active site.

Activity of C135A, C218A, and the Double-Mutated ADH. Table III shows the k_{cat} of purified C135A, C218A, and

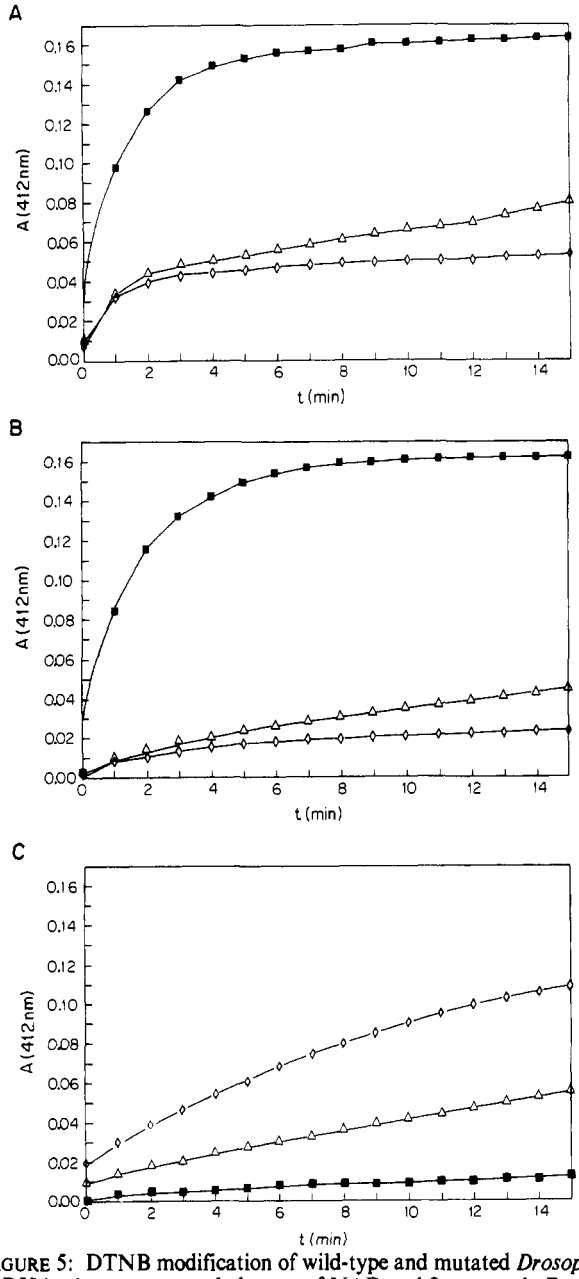

FIGURE 5: DTNB modification of wild-type and mutated *Drosophila* ADH in the presence and absence of NAD and 2-propanol. Purified ADH (10 nmol) was mixed with DTNB (100 nmol) in 1 mL of 0.2 M Tris-HCl buffer (pH 8.0) at 25 °C. Absorbance at 412 nm was recorded. (A) Wild type; (B) C135A; (C) C218A. The double mutant (C135A/C218A) showed no DTNB modification. (■) Absence of substrates; (△) presence of 1 mM NAD; (◇) presence of 1 mM NAD and 10 mM 2-propanol.

double-mutant ADHs relative to wild type. Replacement of either cysteine residue, 135 or 218, with alanine causes no decrease in ADH activity. Interestingly, the double mutant in which both cysteine residues are replaced by alanine (C135A/C218A) results in a 45% increase in activity over the wild type.

DTNB Modification and Inactivation of Drosophila ADH. Monitoring the absorbance at 412 nm after DTNB treatment shows that the C135A mutant has approximately the same sensitivity to DTNB modification as wild type but C218A is essential insensitive to DTNB (Figure 5). The double mutant (C135A/C218A) treated by DTNB serves as the control. It shows to be unreactive with DTNB since a plot of absorbance against time appears practically parallel to the abscissa in both the absence and presence of NAD and 2-propanol. Calculations from the absorbance coefficient of TNB reveal that 1.2

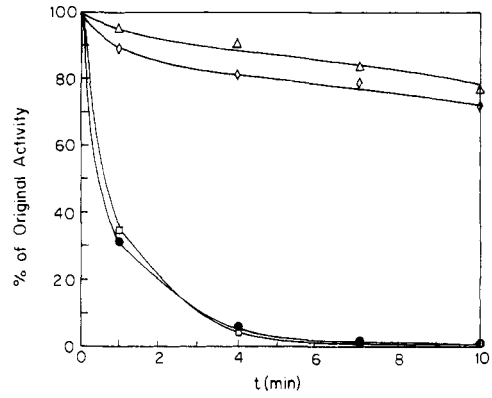

FIGURE 6: DTNB inactivation of wild-type and mutated *Drosophila* ADH. Purified ADH (4 μg) was incubated in 200 μL of 100 μM DTNB and 0.2 M Tris-HCl buffer (pH 8.0) at 25 °C. Aliquots of 20 μL from the sample were taken every 3 min and assayed for ADH activity. (●) Wild type; (□) C135A; (◇) C218A; and (△) C135A/C218A.

mol of DTNB is converted into TNB per mole of wild-type ADH monomer. This means that only one of the two cysteine residues in each monomer is modified. This result is consistent with previous observations (Thatcher, 1981). In the presence of DTNB, the wild-type enzyme and C135A, in which only 218 is cysteine, are inactivated rapidly, whereas C218A, in which only residue 135 is cysteine, and the double mutant are essentially unaffected (Figure 6). These results clearly indicate that the cysteinyl residue at position 218 reacts with DTNB and this leads to inactivation of the enzyme. Since mutagenesis (Table III) shows that this cysteinyl residue is not required for ADH activity, the DTNB-induced inactivation may result from steric interference at the substrate binding domain. Figure 5 shows that DTNB modification of Cys-218 can be prevented by the substrates, 2-propanol and/or NAD, indicating that Cys-218 is probably in or near the active site. On the other hand, Cys-135, which does not normally react with DTNB, becomes partially reactive in the presence of NAD and 2-propanol.

Discussion

Drosophila ADH differs from horse liver ADH in metal ion requirement, substrate preference, and stereospecificity of hydride transfer. When compared to ADH from other species, *Drosophila* ADH has little sequence homology, but the predicted secondary structure in the NAD binding domain bears a strong resemblance to that of horse liver ADH (Benyajati et al., 1981). Crystallographic studies of numerous NAD-dependent dehydrogenases reveal a common structure of six parallel β-sheets with five intervening α-helices (Rossmann, 1983). The first three β-sheets and two α-helices comprise the AMP binding unit (Rossmann et al., 1975). A "conserved sequence" of $GlyXGlyX_2Gly$ is located at the sharp turn between the first β-sheet and the α-helix in the nucleotide binding domain of many dehydrogenases (Argos & Leberman, 1985), kinases (Walker et al., 1982), and oncoproteins (Sternberg & Taylor, 1984). Any disturbance of this tight turn would concomitantly diminish nucleotide binding.

The glycyl residue at position 14 of *Drosophila* ADH is the first residue in the "conserved sequence" and thus represents an appropriate site for studying the structural role of the "conserved sequence" in NAD-dependent dehydrogenases, in general, and Drosophila ADH, in specific. We systematically substituted glycine-14 in *Drosophila* ADH with amino acids having progressively larger side chains, i.e., alanine and valine. Our results show that G14A is partially active while G14V

is almost inactive. G14A is less stable than wild-type ADH at elevated temperature. Consistent with the suggestion of Benyajati and her colleagues (Benyajati et al., 1981), our results prove that the size of residue 14 must be minimal in order to maintain the tight turn. Replacing glycine-14 in *Drosophila* ADH with a larger residue disturbs the tight turn of the "conserved sequence", thus interfering with NAD binding. This view is further supported by our results comparing the kinetic parameters of wild type and the G14A mutant of *Drosophila* ADH.

The k_{cat}/K_m is an apparent second-order rate constant that refers to the properties of the free enzyme and free substrate and the activation energy of forming the enzyme–substrate complex (Fersht, 1985). Since *Drosophila* ADH follows the Theorell–Chance mechanism with NAD binding first (Winberg et al., 1982), $k_{cat}/K_{m(app)}$ for NAD in this case reflects the degree of complementarity of enzyme–NAD binding. The $k_{cat}/K_{m(app)}$ value for NAD in G14A is 5-fold lower than that of wild type; however, the $k_{cat}/K_{m(app)}$ value for 2-propanol is essentially unaltered. These results suggest that enzyme–NAD binding is interrupted by Ala substitution; no effect on enzyme–alcohol binding is observed. Increased binding energy affects two factors: increased energy level for enzyme–NAD binding revealed by a higher K_m and increased transition state binding energy revealed by a lower k_{cat}. Increased binding energy should result from structural changes in the NAD binding domain since no charged residue has been introduced. Because of the structural change, some noncovalent bonds, such as hydrogen bonds, ionic interaction, and hydrophobic interaction, may be weakened, thus, interrupting the correct binding of NAD. Inhibition studies support this point. Noting that the only difference between NAD and NADP is an extra phosphate group on the ribose moiety of the adenine mononucleotide portion of the molecule, a 13-fold increase of the K_i for NADP to inhibit G14A competitively indicates that for this mutation NADP fits poorly in the NAD binding domain.

In general, cysteinyl residues are considered potential catalytic sites in enzymes because of the nucleophilic nature of the sulfhydryl group. Previous studies on *Drosophila* ADH (Thatcher, 1981; Chambers et al., 1981) have fond that this dimeric enzyme contains two cysteins per monomer, Cys-135 and Cys-218. In the native enzyme, one cysteine reacts rapidly with DTNB with concomitant loss of ADH activity. The other is almost unreactive toward DTNB. However, which cysteinyl residue was the reactive residue was not determined in these studies.

In order to investigate the role of these cysteines, we constructed mutants of *Drosophila* ADH in which the cysteinyl residues at positions 135, 218, or both are replaced by alanine. All mutants show identical or slightly elevated ADH activity relative to wild-type enzyme (Table III). These results strongly suggest that neither of the two cysteines is required for the catalytic activity of *Drosophila* ADH. To further clarify DTNB reactivity, we treated wild-type *Drosophila* ADH and the three mutants, C135A, C218A and C135A/C218A, with DTNB. Both chemical modification and enzyme inactivation were monitored. The results of these studies show that the mutant C218A is insensitive to DTNB modification and treated protein remains enzymatically active while an identical experiment carried out on C135A shows rapid DTNB reactivity and concomitant loss of ADH activity. A straightforward interpretation of these observations is that Cys-218 is exposed to DTNB modification while Cys-135 is environmentally inaccessible to DTNB. These results are different from previous observations (Chambers et al., 1981) which suggested that Cys-135 was the DTNB-reactive residue and found that, after chemical modification by iodoacetamide, most of Cys-218 was recovered as unmodified cysteine. However, Cys-135 was never recovered from modified enzyme for a conclusive identification because this residue is located in a large hydrophobic region. On the other hand, Thatcher (1981) indicated that *Drosophila* ADH was resistant to alkylation with iodoacetic acid or iodoacetamide. Therefore, chemical modification alone is insufficient to determine which is the reactive residue. In contrast, site-directed mutagenesis, coupled with chemical modification, provides an unequivocal identification of the DTNB-accessible cysteinyl residue in *Drosophila* ADH.

Inactivation of an enzyme by a chemical reagent which modifies certain residues is classically interpreted as evidence that the modified residue is critical for activity. This can be proven only by replacement of the suspect residue with other appropriate residues, for example, replacing cysteine by alanine or serine. In papain, an active-site sulfhydryl group has been strongly supported by the loss of activity upon replacement with glycine or serine (Clark & Lowe, 1978). In our case, replacing each cysteinyl residue with an uncharged alanine residue does not alter enzymatic activity while chemical modification totally destroys the activity, suggesting that neither cysteine is required for catalytic function. Similar to our observation, Profy and Schimmel (1986) reported that none of the three cysteinyl residues in the β-subunit of glycyl-tRNA synthetase was required for catalysis although enzyme activity was sensitive to *N*-ethylmaleimide (NEM) modification. They suggested that alkylation by NEM introduced a steric effect that led to the inactivation of the enzyme. This probably is an appropriate explanation for our results.

The DTNB modification of Cys-218 can be prevented by the substrates, NAD and 2-propanol, confirming that Cys-218 is in or near the active site. We suggest that this cysteine in *Drosophila* ADH may play a structural or conformational role rather than a catlaytic role. To our surprise, Cys-135 becomes accessible to DTNB in the presence of NAD and/or 2-propanol. It is conceivable that these substrates induce a conformational change which is followed by the exposure of Cys-135 to DTNB modification.

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Registry No. ADH, 9031-72-5; NAD, 53-84-9; 2-propanol, 67-63-0; glycine, 56-40-6; L-cysteine, 52-90-4.

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Activation of Human Factor V by Factor Xa and Thrombin†

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ABSTRACT: The activation of human factor V by factor Xa and thrombin was studied by functional assessment of cofactor activity and sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by either autoradiography of ^{125}I-labeled factor V activation products or Western blot analyses of unlabeled factor V activation products. Cofactor activity was measured by the ability of the factor V/Va peptides to support the activation of prothrombin. The factor Xa catalyzed cleavage of factor V was observed to be time, phospholipid, and calcium ion dependent, yielding a cofactor with activity equal to that of thrombin-activated factor V (factor Va). The cleavage pattern differed markedly from the one observed in the bovine system. The factor Xa activated factor V subunits expressing cofactor activity were isolated and found to consist of peptides of M_r 220 000 and 105 000. Although thrombin cleaved the M_r 220 000 peptide to yield peptides previously shown to be products of thrombin activation, cofactor activity did not increase. N-Terminal sequence analysis confirmed that both factor Xa and thrombin cleave factor V at the same bond to generate the M_r 220 000 peptide. The factor Xa dependent functional assessment of ^{125}I-labeled factor V coupled with densitometric analyses of the cleavage products indicated that the cofactor activity of factor Xa activated factor V closely paralleled the appearance of the M_r 220 000 peptide. This observation facilitated the study of the kinetics of factor V activation by allowing the activation of factor V to be monitored by the appearance of the M_r 220 000 peptide (factor Xa activation) or the M_r 105 000 peptide (thrombin activation). Factor Xa catalyzed activation of factor V obeyed Michaelis-Menten kinetics and was characterized by a K_m of 10.4 nM, a k_{cat} of 2.6 min^{-1}, and a catalytic efficiency (k_{cat}/K_m) of 4.14 × 10^6 M^{-1} s^{-1}. The thrombin-catalyzed activation of factor V was characterized by a K_m of 71.7 nM, a k_{cat} of 14.0 min^{-1}, and a catalytic efficiency of 3.26 × 10^6 M^{-1} s^{-1}. This indicates that factor Xa is as efficient an enzyme toward factor V as thrombin.

Factor V circulates in plasma as a high molecular weight single-chain protein (Nesheim et al., 1979a; Esmon, 1979; Dahlback, 1980; Katzmann et al., 1981; Kane & Majerus, 1981; Mann et al., 1981) which is proteolytically cleaved to yield the coagulation cofactor factor Va (Nesheim & Mann, 1979; Esmon, 1979; Suzuki et al., 1982). Factor Va is an essential, nonenzymatic cofactor of the coagulation complex prothrombinase, the catalyst which converts prothrombin to thrombin (Owren, 1947a,b; Ware & Seegers, 1948; Murphy & Seegers, 1948; Heldebrandt et al., 1973; Suttie & Jackson, 1977). The prothrombinase complex consists of the cofactor factor Va, the serine protease factor Xa, calcium ions, and an appropriate cell membrane (Tracy et al., 1981, 1983b; Kane

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