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Effect of site-directed mutagenesis on conserved positions of *Drosophila* alcohol dehydrogenase

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Tyr[152] and Lys[156] may be functionally important residues in *Drosophila* ADH as they are conserved in the genus and in all short-chain dehydrogenases. In addition, unaltered Gly positions could have a crucial role in the building of the structural framework. We have modified *Drosophila* ADH and expressed the mutant forms in *E. coli*. Mutation of Tyr[152] to Glu or Gln, Lys[156] to Ile, Gly[184] to Leu, and the double mutant Gly[130] to Cys and Gly[133] to Ile, all rendered, with different substrates and at different pHs, an inactive enzyme. Results suggest that Tyr[152] and Lys[156] are involved in catalysis and that Gly[130], Gly[133] and Gly[184] contribute substantially to the structure of the active form.

Site-directed mutagenesis; Active site residue; Alcohol dehydrogenase; Short-chain dehydrogenase; *Drosophila*

1. INTRODUCTION

Drosophila alcohol dehydrogenase (ADH; alcohol NAD^+ oxidoreductase; EC 1.1.1.1) is a well-known member of the short-chain dehydrogenase family [1], although its function is homologous to that of medium-chain ADHs in mammalian liver [2]. Some secondary structure predictions have been described for the *D. melanogaster* enzyme [3,4], but, as its three-dimensional structure has not yet been elucidated, only indirect evidence is available concerning the residues involved in the active domains. Recently, the first crystallographic analysis of a short-chain dehydrogenase has been described [5]. In the meantime, a valid approach to the identification of protein sites which are critical for the catalytic function of the enzyme lies in the comparison of conserved residues among all short-chain dehydrogenases [6], and between these and all known *Drosophila* ADHs [7]. Comparisons have led to the identification of boxes of highly conserved residues. First alignment of limited regions between alcohol dehydrogenases allowed to identify the coenzyme binding region of *Drosophila* ADH [1] and afterwards, site-directed mutagenesis in Gly[14], Gly[16], Gly[19] and Asp[38] confirmed the predicted role of these amino acids in building a correct Rossman fold NAD^+-binding domain [8,9]. Another analyzed region, spanning positions 139–158, comprises two amino acids conserved in all short chain dehydrogenases, Tyr[152] and Lys[156], which are presumably involved in the substrate interactive domain [1]. Confirming evidence provided by biochemical data [10], chemical modification [11,12] and site-directed mutagenesis of the related enzymes human-placental-15-hydroxyprostaglandin dehydrogenase [13] and 11-β-hydroxysteroid dehydrogenase [14], showed that the hydroxyl group of Tyr[152] must be involved in the catalytic reaction of *Drosophila* ADH, since substitution by SDM of this Tyr for Phe totally abolishes the enzymatic activity [15]. We have now engineered new ADH mutant enzymes in the same and other conserved boxes. In ADH-Glu[152], ADH-Gln[152] and ADH-Ile[156] we have tested the effect of potentially charged groups as well as the requirement of a positively charged group at positions 152 and 156, respectively. Glycine appears to be conserved above average in the ADH polypeptide. We have produced three different substitutions affecting some Gly residues enclosed in two very conserved regions of the molecule. Gly[184] is part of a block of seven residues which appear unaltered in all *Drosophila* alcohol dehydrogenases [7] and which has been changed to Ile. Gly[130] and Gly[133] have been concomitantly altered. Gly[133] has been substituted by Ile and Gly[130] by Cys. Through this double substitution we have tried to evaluate the effect of introducing a conservative change in Gly[133] and at the same time a drastic charge change in a nearby region of the molecule, which is probably located at the outermost part in close contact with the medium. An *E. coli* JM105/pKK223-3 expression system has been designed to overcome the poor yield of yeast hosts synthesizing inactive ADH mutants [15,16]. Recombinant *Drosophila* ADH has been purified by a single step FPLC chromatographic protocol, consisting of two columns of Superose 12 and Superose 6 in series, adapted for bacterial homogenates. Both crude *E. coli* extracts and

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pure enzyme preparations have been assayed for protein content and ADH activity at different pHs and with different substrates.

2. MATERIALS AND METHODS

2.1. *Materials*

All the restriction enzymes and T4 DNA ligase were obtained from Boehringer-Mannheim. Taq polymerase and Vent polymerase were from Promega and New England Biolabs, respectively, and the DNA Sequencing Kit from Pharmacia. [α-^{35}S]dATP and Hybond-C nitrocellulose membrane were purchased from Amersham. PCR primers were synthesized by Oligos Etc. Inc. Other reported chemicals and reagents were from Sigma or Merck, and culture reagents from Difco.

2.2. *Organisms and plasmids*

The intronless coding sequence of *D. melanogaster Adh* originally cloned in plasmid p3008 [16] was transferred to the expression vector pKK223-3 (Pharmacia-LKB Biotechnology) to obtain high level induction in *E. coli* JM105 [17].

2.3. *Site-directed mutagenesis by PCR amplification*

Site-directed mutagenesis was performed following a cassette replacement method which allows two coupled PCR reactions [18], taking advantage of strategically located restriction sites, one *Bam*HI site within the coding sequence, and two flanking sites, one *Hin*dIII and one *Xba*I, upstream and downstream, respectively, of the coding sequence [16] (Table I). For each mutant, the first PCR reaction was carried out using the cloned wild-type gene as template and the mutagenic and one non-mutagenic oligonucleotide as primers, both chosen to allow for the presence of a *Bam*HI site in the amplified product (Table I). The second PCR reaction was carried out on the same template, using the entire purified product of the first PCR and a suitable primer to obtain one flanking restriction site in the amplified product. First PCR reactions were performed in a final volume of 100 μl, containing 10 ng of template (p3008-Adh), 100 pmol of each primer, 200 μM of dNTPs and 2.5 u of either Taq or Vent polymerase. Reactions were supplemented with 2.5 mM of $MgCl_2$ when using Taq polymerase or with 10 ng/μl of BSA when using Vent polymerase. Samples were first kept at 94°C for 120 s to ensure initial denaturation and then, 30 cycles of 60 s at 94°C (denaturation), 60 s at 55°C (annealing) and 60 s at 72°C (elongation), and finally, 5 min at 72°C. The desired products were purified after electrophoresis in 1.5% agarose gels. The conditions for the second PCR reaction were similar, except that Taq polymerase was always used and the following modified cycles: 45°C for the annealing step and 180 s instead of 60 s for the elongation step. The products of the second PCR were precipitated, suitably digested and cloned in p3008-*Adh*, thus replacing the equivalent wild-type region. The resulting plasmids were sequenced to verify the introduced substitutions and to confirm the absence of additional changes in the coding sequence. Sequencing of double-stranded DNA was carried out using [α-^{35}S]dATP as radioactive precursor and all DNA data were analyzed using the Software Package of the GCG of the University of Wisconsin [19].

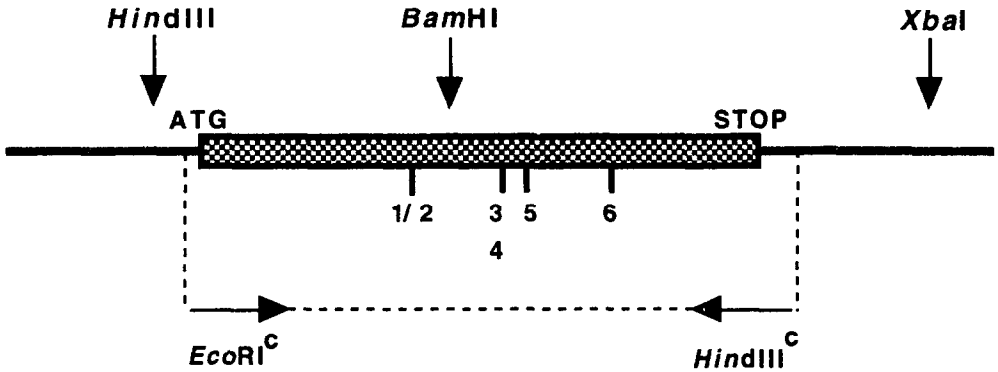
2.4. *Cloning and expression of* Adh *in* E. coli

A further PCR reaction was performed to introduce suitable restriction sites at the boundaries of the wild-type and mutant sequences which would allow cloning downstrean the *tac* promoter of the pKK223-3 vector. The primers contained an upstream *Eco*RI site

Table I

Primers for the PCR mutagenic reactions and cloning sites used to construct the expression vectors

Mutation	Mutagenic oligonucleotide[a] (position)	Mutated codon	Length 1st PCR	Restriction[b] enzymes
$Gly^{130} \rightarrow Cys$ (1)	5'TGTCCCGGTATTATCATCTGC 3' (upstream)	GGT → TGT	259 nt	*Hin*dIII and *Bam*HI
$Gly^{133} \rightarrow Ile$ (2)		GGT → ATT		
$Tyr^{152} \rightarrow Glu$ (3)	5' GGTGCCGGACTCGACGGGCA 3' (downstream)	TAC → GAG	114 nt	*Bam*HI and *Xba*I
$Tyr^{152} \rightarrow Gln$ (4)	5' GGTGCCGGACTGGACGGGCA 3' (downstream)	TAC → CAG	114 nt	*Bam*HI and *Xba*I
$Lys^{155} \rightarrow Ile$ (5)	5'CACGGCGGCTATGGTGCCGG 3' (downstream)	AAG → ATA	122 nt	*Bam*HI and *Xba*I
$Gly^{184} \rightarrow Leu$ (6)	5' GTGCGGGTGATGAGGGGGTT 3' (downstream)	GGC → CTC	225 nt	*Bam*HI and *Xba*I



[a]The mutated nucleotides are underlined and the position of the primer in the first PCR is also indicated.
[b]Restriction enzymes used for the cassette replacement in the wild-type gene.
[c]Flanking restriction sites created by PCR amplification to subclone the coding region into the pKK expression vector.

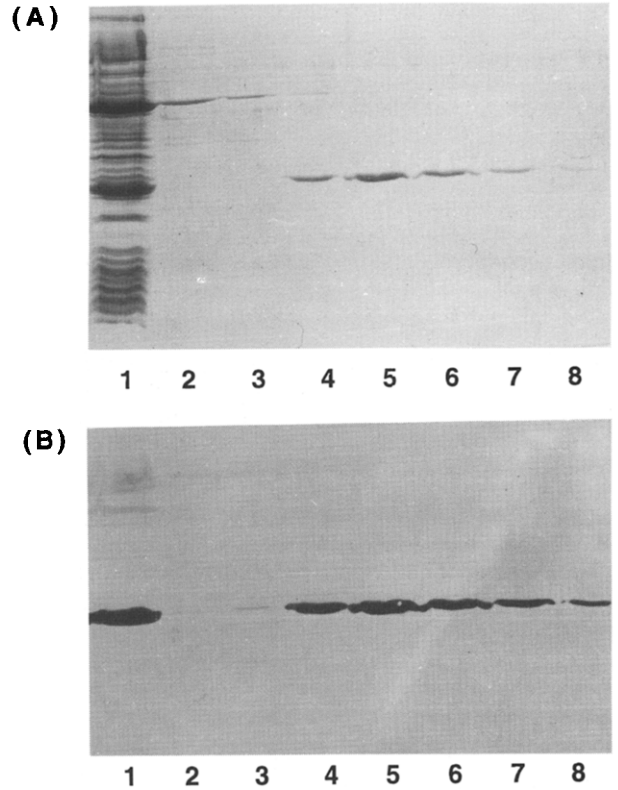


Fig. 1. (A) SDS-PAGE of the Superose (12 + 6) fractions showing ADH activity, stained for total protein with Coomassie blue. (1) 10 μl of total *E. coli* extract; (2)–(8) 10 μl aliquots of fractions 17 to 24, respectively. Each fraction had a volume of 800 μl. (B) Western blotting of an exact replica of the previous gel, showing that the Coomassie blue stained bands corresponded to the bands detected by a *Drosophila* ADH monoclonal antibody.

flanking the ATG start codon and a downstream *Hin*dIII site after the stop codon (Table I). Overnight cultures of 3 ml of LB-ampicillin were diluted to 1/6 with 30 ml fresh LB-ampicilin and were grown for 1 h (OD 0.6). IPTG was then added, to a final concentration of 1 mM and the cultures were incubated at 30°C for 3 h. Cells were harvested, washed twice in 20 mM Tris-HCl, pH 8.6, resuspended in 0.5 ml of 20 mM Tris-HCl, pH 8.6 supplemented with 5% 2-propanol and 1 mM DTT, sonicated twice for 15 s at 30 W and centrifuged in a microfuge for 15 min. These manipulations were all performed between 0°C and 4°C. The crude supernatant was used for activity assays, SDS-PAGE, immunoblotting and further purification.

2.5. *Purification of ADH*

An aliquot of 200 μl of the crude supernatant was injected to an FPLC gel filtration system (Pharmacia) with two Superose columns, 12 and 6, connected in series [15]. The equilibration and elution buffer was 20 mM Tris-HCl, pH 8.6, supplemented with 5% 2-propanol and 1 mM DTT. Samples were run at 0.3 ml/min and protein content was recorded by the absorbance at 280 nm. Fractions were further tested by SDS electrophoresis and immunoblotting, and protein was quantified by the method of Bradford [20].

2.6. Drosophila *ADH activity assay*

ADH activity was measured spectrophotometrically by the increase of absorbance at 340 nm, using different alcohols as substrate and NAD^+ as cofactor, following Juan et al. [21]. A range of pHs from 7.0 to 10.0 was assayed in the 2-propanol oxidation reaction.

2.7. *Electrophoresis and immunoblotting*

SDS-PAGE was performed in 15% acrylamide gels and proteins were visualized by Coomassie-blue staining. Western blotting was performed using a monoclonal antibody against *Drosophila* ADH (LLBE8), following the procedures reported in Visa et al. [22].

3. RESULTS

3.1. *Expression and purification of the wild-type and mutant* Drosophila Adh *from* E. coli

After different induction, sonication and recovery conditions tested the best procedure was established as follows: 30°C for the 3 h induction step and 20 mM Tris-HCl, pH 8.6, supplemented with 5% 2-propanol and 1 mM DTT as the buffer used for cell washing and sonication. The 30 ml O/N culture produced 1.7 mg of total protein, 450 μg of this corresponded to pure recombinant ADH, then overall yield amounted to 26% of the total *E. coli* protein.

The purification protocol was first set up for the wild-type enzyme, whose activity could be easily followed. Fig. 1A shows the SDS-PAGE analysis of the Superose fractions showing ADH activity for the wild-type enzyme. Proteins were electrotransferred to nitrocellulose filters and immunodetected with an ADH monoclonal antibody, as illustrated in Fig. 1B. Our data show that the only band observed in the SDS-resolving gels was recognised by a specific anti-ADH MAb. The same protocol was used to purify mutant ADH forms. Fig. 2A shows an SDS-PAGE of all the mutant enzymes, and Fig. 2B the corresponding immunodetection of an aliquot of the same samples.

3.2. *Enzymatic activity of the* ADH *mutants*

Protein recovery and enzymatic activity for wild-type ADH and mutants is shown in Table II. No activity was detected for any of the mutant forms at the standard assay conditions, that is using 2-propanol as substrate at pH 8.6. In addition to 2-propanol, ethanol and 1-butanol were also assayed at 0.12 M final concentration and ADH activity was also determined at pHs 7.0, 8.0, 9.0 and 10.0 with each substrate. Results clearly showed that none of the mutant forms oxidized any of the alcoholic substrates. Moreover, no enzymatic activity was recovered at any of the pHs assayed. Then, mutations affecting Tyr^{152}, Lys^{156}, Gly^{130}, Gly^{133} and Gly^{184} completely invalidated the catalytic function of the molecule.

DISCUSSION

Drosophila alcohol dehydrogenase is the most widely studied member of the family of short-chain dehydrogenases. The active enzyme is a homodimer composed of subunits of 255 amino acids. In the absence of a known three-dimensional structure, determination of catalyti-

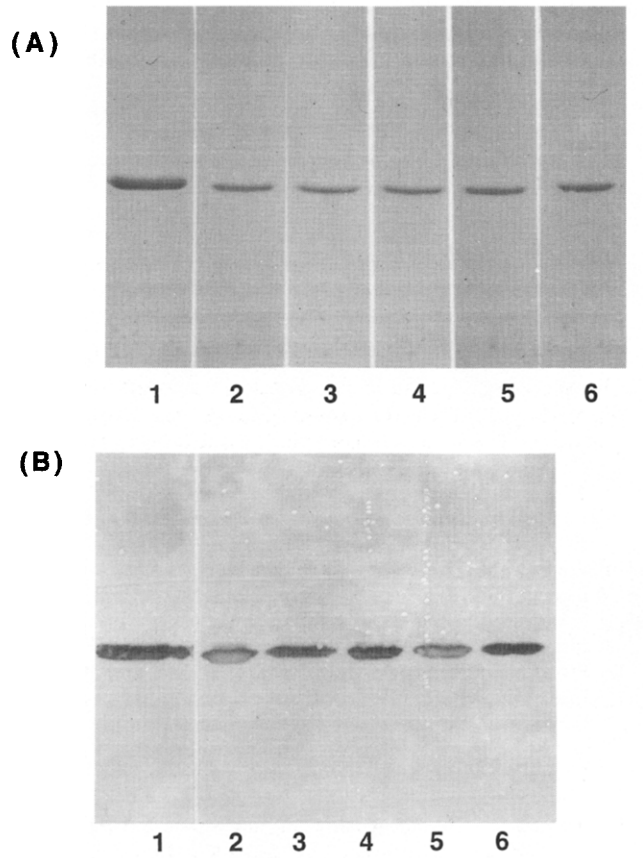


Fig. 2. (A) SDS-PAGE of purified *Drosophila* ADH enzymes, stained with Coomassie blue. (1) 10 μl of wild-type *Drosophila* ADH purified preparation from *E. coli* extracts. (2) 10 μl of mutant ADH $Gly^{130}Cys/Gly^{133}Ile$ preparation; (3) 10 μl of mutant ADH $Tyr^{152}Glu$ preparation; (4) 10 μl of mutant ADH$Tyr^{152}Gln$ preparation; (5) 10 μl of mutant ADH$Lys^{156}Ile$ preparation; (6) 10 μl of mutant ADH $Gly^{184}Leu$ preparation. All enzymes had been purified from *E. coli* extracts. (B) Western blot analysis of purified *Drosophila* ADH enzymes. (1) 10 μl of wild-type *Drosophila* ADH purified from *E. coli* extracts. (2)–(6) 10 μl of mutant ADH $Gly^{130}Cys/Gly^{133}Ile$, $Tyr^{152}Glu$, $Tyr^{152}Gln$, $Lys^{156}Ile$ and $Gly^{184}Leu$, respectively, purified from *E. coli* extracts.

cally essential residues has been approached through structural analysis and determination of conserved sites among the different family members [6,7], kinetic analysis [10] and chemical modification [11,12]. In addition, we have recently shown that the substitution of Tyr^{152} by Phe rendered a totally inactive enzyme [15], thus supporting the involvement of the hydroxyl group of Tyr in catalysis. Site-directed mutagenesis and expression in a suitable host has been used to obtain mutant forms of enzymes belonging to this complex dehydrogenase family: Tyr^{151} was substituted by Ala in human placental 15-hydroxyprostaglandin dehydrogenase [13] and Tyr^{179} by Phe or Ala in 11β-hydroxysteroid dehydrogenase [14]. In all cases the mutant enzymes were totally inactive. All these data suggest the predicted participation of this residue in the hydride ion transfer from the hydroxyl group of alcohols or steroids. Now, more data is presented on the modification of Tyr^{152}. Mutant ADHs have been engineered which contain Glu or Gln at this same position. Again, these substitutions seem to alter the functionality of the protein dramatically, since no enzyme activity was detected with the different alcoholic substrates and at any of the pHs tested. If deprotonation of the Tyr side chain promotes the formation of the hypothesized alcoholate ions in the ternary complexes at physiological pHs, replacement by Glu should facilitate the reaction. As this is not the case, we assume that Glu alters the spatial configuration of the active site during the transition state and that it is necessary to accomplish the modification of the substrate. A search of sequence database revealed another very conserved residue close to Tyr^{152}. Lys^{156} is invariably present in ADH of 36 species of *Drosophila* and also in all analyzed members of the short-chain family. The high degree of conservation, the proximity to the catalytic Tyr and the chemical features of its side chain clearly suggested that this Lys could well be enclosed in the catalytic cleft [5], providing an alkaline environment for catalysis. Its ε-NH_2 group, if suitably located with respect to Tyr, could have the effect of lowering the apparent pK_a of the phenolic group, thus facilitating the hydride transfer reaction at much lower, physiological pH. Substitution of Lys^{156} by Ile, an amino acid with an uncharged side chain, produced an inactive enzyme and supports Lys involvement in the catalytic reaction. Secondary structure predictions show that Gly^{130} and Gly^{133} are located in a turn at the end of the longest α-helix domain just preceeding a β-sheet [3]. Following the three-dimensional structure of the homologous region in the 3α,20β-hydroxysteroid dehydrogenase, these two glycines are clearly positioned on the outer surface of the molecule, at the hinge of an α–β connecting segment [5]. According to these data, both could be crucial for the spatial configuration of the enzyme. We have

Table II

Expression of *Drosophila* ADH in *E. coli*

ADH mutant	Total ADH protein (μg/μl)[a]	ADH activity (mU/μl)[b]	Specific ADH activity (mU/μg)[c]
ADH-wild type	0.188	0.924	4.800
$Gly^{130}Cys/Gly^{133}Ile$	0.112	n.d.	n.d.
$Tyr^{152}Glu$	0.150	n.d.	n.d.
$Tyr^{152}Gln$	0.175	n.d.	n.d.
$Lys^{156}Ile$	0.140	n.d.	n.d.
$Gly^{184}Leu$	0.110	n.d.	n.d.

[a]Concentration of ADH in the purified preparation.
[b]ADH activity per minute at 25°C measured using 2-propanol as substrate, at pH 8.6, per μl of purified enzyme
[c]Specific ADH activity.
n.d. = not detectable.

engineered a double mutant, Gly^{130} Cys/Gly^{133} Leu, and expressed it in *E. coli*, as previously described. The purified mutant form has been assayed with various substrates and at different pHs. No activity was recovered in any case, thus supporting the predicted contribution of both residues to the correct folding of the enzyme. The crucial role that Gly residues play in the correct folding of the enzymes is widely accepted. The primary structure analyses of *Drosophila* ADH and of the short-chain dehydrogenases support this assumption, as glycine ranks the fourth most conserved position (72%) [6,7]. We assume that some of these Gly are involved in the conformational changes coupled to substrate displacement towards the catalytic site and so directly involved in enzyme activity. The two most frequent allelic forms of *D. melanogaster* ADH only differ in a single amino acid replacement: ADH^F has a Thr and ADH^S has a Lys at position 192 [3], and this difference must be responsible for the kinetic differences reported. We decided to test the functional significance of this region by substituting Gly^{184}, the closest conserved Gly residue, by Leu. To our surprise, the engineered enzyme was as inactive as other mutant forms in which catalytical residues had been altered. We will probably not be in a position to understand the functional implications and spatial organization of the altered positions presented in this paper until the three-dimensional structure of ADH is fully elucidated.

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