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Drosophila lebanonensis ADH: analysis of recombinant wild-type enzyme and site-directed mutants

The effect of restoring the consensus sequence in two positions

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Abstract

Unique amino acid substitutions occur in *D. lebanonensis* ADH. They are found within the putative NAD^+-binding domain and affect residues that are otherwise highly conserved in all other species of the genus. To restore the consensus amino acids, we have constructed an expression system for this enzyme in *E. coli*, and engineered two mutants, $Ala^{13}Gly$ and $Asn^{56}Thr$. The biochemical and kinetic features of these *retromutants* are consistent with increased catalytic efficiency and thermal stability. Thus, results show that wild-type *D. lebanonensis* ADH can be improved by site-directed mutagenesis.

Key words: Short-chain dehydrogenase; Alcohol dehydrogenase; *Drosophila lebanonensis*; Site-directed mutagenesis; Catalytic efficiency

1. Introduction

Alcohols are oxidized in *Drosophila* by a member of the short-chain dehydrogenase family, in contrast to most eukaryotes, which have developed a medium-chain enzyme [1]. *Drosophila* alcohol dehydrogenase (ADH) is a non-metalloenzyme, active as a dimer of two subunits of 253–255 amino acids, which shares no homology with the Zn-containing medium-chain proteins [2]. The three-dimensional structure of the medium-chain horse liver ADH [3] allowed the identification of functional residues involved in coenzyme and substrate binding, hydride transfer, metal coordination and monomer surface interaction. However, no tertiary structure is yet known for *Drosophila* ADH and therefore the structure/function relationships must be approached through different strategies. Putative critical residues for enzyme architecture and catalytic properties, highlighted as conserved positions among all short-chain dehydrogenases and all known *Drosophila* ADHs [2,4,5], have been analyzed by either chemical modification [6] or site-directed mutagenesis [7–11]. Fragment 9–39 of the *Drosophila* polypeptide is the only segment alignable with the medium-chain ADHs [2], in which the *homologous* fragment (194–224) forms the $\beta1$–$\alpha2$–$\beta2$ motif of the Rossmann Fold, found in all NAD^+/FAD^+ binding enzymes [12]. The catalytic behaviour of mutants in positions Gly^{14}, Gly^{16}, Gly^{19} and Asp^{38} of the *D. melanogaster* enzyme is in agreement with the predicted involvement of this region in the binding of the cofactor and its preference for NAD^+ versus $NADP^+$ [7]. Sequence alignments have also allowed the prediction of two key residues for substrate interaction, Tyr^{152} and Lys^{156} [2], whose substitution in *D. melanogaster* ADH yields inactive or poorly active enzymes [9–11].

Until now, all site-directed mutants have been engineered with *D. melanogaster* ADH, by far the best known species of the genus at all levels [13]. Nevertheless, valuable information could be obtained from other species whose ADH has been characterized [5]. The fact that 110 out of 255 positions of the subunit polypeptide are not conserved in *Drosophila* species provides an excellent source of evolutionary tested enzyme variants and, among these, *D. lebanonensis* appears to be an excellent candidate for function/structure analysis. Specific activity of *D. lebanonensis* ADH is lower than that of other *Drosophila* ADHs, but, paradoxically these flies exploit alcohol-rich environments, and eventually outgrow *D. melanogaster* in number, probably because they

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Numbering of *D. lebanonensis* ADH positions is (−1) in relation to *D. melanogaster*, due to differences in the amino acid number at the N terminus.

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accumulate larger amounts of the enzyme [14]. The amino acid sequence of *D. lebanonensis* ADH has been determined [15], the biochemical features of the enzyme have been described [16] and the ADH-coding gene has been isolated and analyzed [17,18]. Four unique amino acid substitutions with respect to all other *Drosophila* ADHs: Ala^{13}, Phe^{33}, Leu^{45} and His^{60} make this enzyme particularly interesting. These changes, as well as others present in only one additional species of all known *Drosophila* ADHs (Thr^{43}, Asn^{56} and Thr^{61}), are in the putative NAD^+-binding pocket and may therefore be considered responsible for the different catalytic properties of the enzyme.

The purpose of the present study is to analyze Ala^{13} and Asn^{56} in *D. lebanonensis* ADH. In order to establish a heterologous expression system, an intronless *Adh* gene of *D. lebanonensis* was constructed using reverse-PCR, and subsequently cloned in *E. coli*. Site-directed mutagenesis was then performed on this construct to change Ala^{13} to Gly and Asn^{56} to Thr, to reconstruct evolutionary events and restore the consensus ADH residues. Recombinant wild-type ADH and Ala^{13}Gly and Asn^{56}Thr mutant enzymes were purified by FPLC from crude bacteria homogenates and the catalytic effects of each substitution were evaluated.

2. Materials and methods

2.1. Materials, organisms and plasmids

Restriction enzymes werer obtained from Boehringer-Mannheim. Taq DNA polymerase was from Promega. PCR primers were synthesized by Oligos Etc. Inc. Hybond-C nitrocellulose filters, [α-^{35}S]dATP and Ligation Kit were purchased from Amersham. Other chemicals and reagents were from Sigma and Merck, and culture media reagents were from Difco. Plasmid pBluescript was from Stratagene. *E. coli* JM105 and plasmid pKK223-3, used to express the *Adh* gene, were from Pharmacia-LKB Biotechnology. *D. lebanonensis* flies were from a natural population caught in Gandesa, Tarragona (Spain) and maintained in our laboratory under standard conditions for several years.

2.2. RNA preparation and reverse-PCR

Total RNA of *D. lebanonensis* was purified from larvae according to Jowett [19]. cDNA was synthesized using 200 units of MoMuLV reverse transcriptase (BRL), in a final reaction volume of 20 μl, containing 1–2 μg of total RNA, 100 pmols of the oligonucleotide SH5 as downstream primer (Table 1), 1 mM of dNTPs, 25 units of RNase inhibitor (Boehringer) and 3 mM $MgCl_2$. Samples were then incubated for 10 min at 23°C, 45 min at 42°C and 5 min at 94°C to ensure initial hybrid denaturation. For the PCR reaction, 100 pmol of the upstream primer SH1 (Table 1), 2.5 units of Taq DNA polymerase and 8 μl of 10 × PCR buffer were added to a final volume of 100 μl. A 30-cycle amplification was carried out at 94°C/30 s, 50°C/60 s and 72°C/60 s. Finally, samples were kept at 72°C for 10 min before fragment purification.

2.3. Cloning and expression of D. lebanonensis Adh in E. coli

The PCR product was initially subcloned in the plasmid pBluescript for restriction analysis and sequencing. In order to produce the recombinant enzyme, the *Adh* coding region of *D. lebanonensis* was cloned in pKK223-3 and the recombinant plasmid was used to transform *E. coli* JM105 (Fig. 1). Overnight cultures in 50 ml of LB-ampicillin were diluted to 500 ml of fresh LB-ampicillin and grown for 1.5 h. IPTG was then added to a final concentration of 1 mM and cultures were incubated at 30°C for 3 h. Cells were harvested, washed twice in 20 mM Tris-HCl pH 8.6, resuspended in 2 ml of the same buffer, sonicated three times for 15 s at 30 W and centrifuged in a microfuge for 15 min. Manipulations were performed at 4°C. Crude supernatant was used for activity assays, SDS-PAGE, immunoblotting and further purification.

2.4. Site-directed mutagenesis by PCR

Mutagenic PCR amplifications were successfully carried out by the method described in [9], with primers SH1 and SH2 (first PCR) and SH5 (second PCR) to obtain the Ala^{13}Gly mutant, and primers SH1 and SH3 (first PCR) and SH5 (second PCR) for the Asn^{56}Thr mutant (Table 1). Thus, in both cases, the final PCR product was the mutated coding region flanked by *Eco*RI and *Hin*dIII restriction sites. The presence of the desired mutation and the absence of additional changes was always verified by sequencing the PCR fragments with [α-^{35}S]dATP, using the Pharmacia Sequencing Kit. Positive clones were used to subclone the *Eco*RI–*Hin*dIII segment in the expression vector pKK223-3.

2.5. Purification of recombinant ADH

The following purification protocol was followed to obtain pure recombinant *D. lebanonensis* ADH. *E. coli* total protein extract in 20 mM Tris-HCl pH 8.6, supplemented with 10^{-4} M DTT, was injected in a Blue-Sepharose Hi-Trap/5 column adapted to an FPLC System (Pharmacia). After washing with the same buffer, ADH was eluted with 1 M NaCl. Fractions containing ADH activity were pooled and concen-

Table 1
Primers for the reverse transcriptase and PCR reactions

Oligo	Mutation	Sequence	Length 1st PCR	Mutation codon
SH1	–	CCGAATTC**ATG**GATTTGACCAACAAG[a] (upstream)	–	–
SH2	Ala^{13} → Gly	ACCGCCCAGA**C**CGGCAACGA[b] (downstream)	54 nt	GCT → GGT
SH3	Asn^{56} → Thr	GGAAGGTGATG**G**TCACCTTG[c] (downstream)	182 nt	AAC → ACC
SH5	–	GGAAGCTTGC**TTA**GATGTGCGAGCT[d] (downstream)	–	–

[a,d]Start and Stop codons are shown in bold. Sequences used to generate flanking restriction sites *Eco*RI in SH1 and *Hin*dIII in SH5, are underlined.
[b,c]Mutated nucleotides are in bold.

trated to a final volume of 0.5 ml, using Centricon 10 Microconcentrators (10,000 MW cut-off). The sample was then fractionated in Superose 12 and pure ADH was recovered in 2 fractions. Protein content was recorded following the absorbance at 280 nm. Fractions were further tested by SDS-PAGE and immunoblotting, and quantified by the method of Bradford (Bio-Rad). Mono Q 5/5 HR equilibrated with the same buffer was used for ion exchange chromatography.

2.6. Protein determinations: electrophoresis, immunoblotting and antibody recognition

SDS-PAGE was performed in 12.5% acrylamide gels and proteins were visualized by Coomassie-blue staining. Western blotting was performed with a monoclonal antibody against *D. lebanonensis* ADH (LLBE8), following [20].

2.7. Enzymatic determinations: activity inhibition, kinetic parameters, pH profiles and thermal stability

ADH activity was measured spectrophotometrically by the increase in absorbance at 340 nm, using propan-2-ol as substrate and NAD^+ as cofactor in 20 mM Tris-HCl pH 8.6 [16]. For activity inhibition determinations, total protein extract from the bacterial cultures was incubated with or without mAb LLBE8 [14] for 1 h at 37°C before measuring activity. Kinetic constants for NAD^+ were determined using 0.0625, 0.125, 0.250, 0.5, 1.0, 1.5, 2.0, 2.5 and 4.0 mM NAD^+ with a constant alcohol concentration of 120 mM. Kinetic constants for propan-2-ol were measured using 2.5, 5.0, 10, 15, 20, 40 and 120 mM alcohol with 2.0 mM of NAD^+. K_m and k_{cat} values were calculated with the program ENZFITTER on a Personal Computer. ADH activity for wild-type and mutants was also determined at pH 7.0, 8.0, 9.0 and 10.0 with 120 mM propan-2-ol and 2 mM NAD^+. To evaluate the thermal stability of the enzyme, samples of purified wild-type ADH and mutant forms were incubated at 40°C. Aliquots from each sample were taken at 0 min, 10 min, 25 min, 40 min and 60 min, and assayed for ADH activity. Molarities of NAD^+ and alcohols refer to final concentrations. All activity tests were performed at least twice.

2.8. Sequence analysis

Cloning and sequencing were designed using the Sequence Analysis Software Package of Genetics Computer Group of the University of Wisconsin (GCG) [21]. This was also used for the analysis of DNA restriction patterns and protein comparison tables.

3. Results

3.1. Construction and expression of the D. lebanonensis *wild-type and mutant* Adh *genes*

The reverse-PCR protocol provided an intronless *Adh* gene suitable for an *E. coli* expression system (Fig. 1). Sequencing of the cloned fragment revealed that it contained the correct coding region of the *D. lebanonensis Adh* gene [17], so reverse transcription of mRNA and PCR amplification had introduced no artefactual changes. The $Ala^{13}Gly$ and $Asn^{56}Thr$ mutants were also sequenced. In all cases, we confirmed the presence of the mutagenized codon and the absence of unwanted substitutions.

Drosophila ADH activity was detected spectrophotometrically in crude protein extracts prepared from bacterial cultures induced by IPTG. No activity was found in untransformed cells, nor in cells transformed with the pKK223-3 vector without insert. An antibody inhibition test was carried out in order to determine whether recombinant *D. lebanonensis* ADH retained the expected differential antigenic features for the wild-type enzyme with respect to that of *D. melanogaster* ADH^s. LLBE8 specifically inhibits *D. lebanonensis* ADH, while *D. melanogaster* ADH^s activity remains unaltered [14]. When LLBE8 was added to the recombinant ADH protein extract, enzymatic activity fell to 18% of the control samples (protein extracts without antibody). This was in agreement with the expected antigenic behaviour for the non-recombinant enzyme.

3.2. Purification of recombinant D. lebanonensis *wt and mutant enzymes*

To obtain wild-type ADH and the $Ala^{13}Gly$ mutant of *D. lebanonensis*, we followed the purification protocol described in section 2. Calculated yield was 1 mg of pure protein per 500 ml of bacterial culture. However, the $Asn^{56}Thr$ mutant unexpectedly showed irreversible denaturation in the presence of 1 M NaCl. Although SDS-PAGE revealed ADH-containing fractions after the Blue-Sepharose step, neither exhaustive dialysis nor gel filtration restored activity. This denaturing effect was

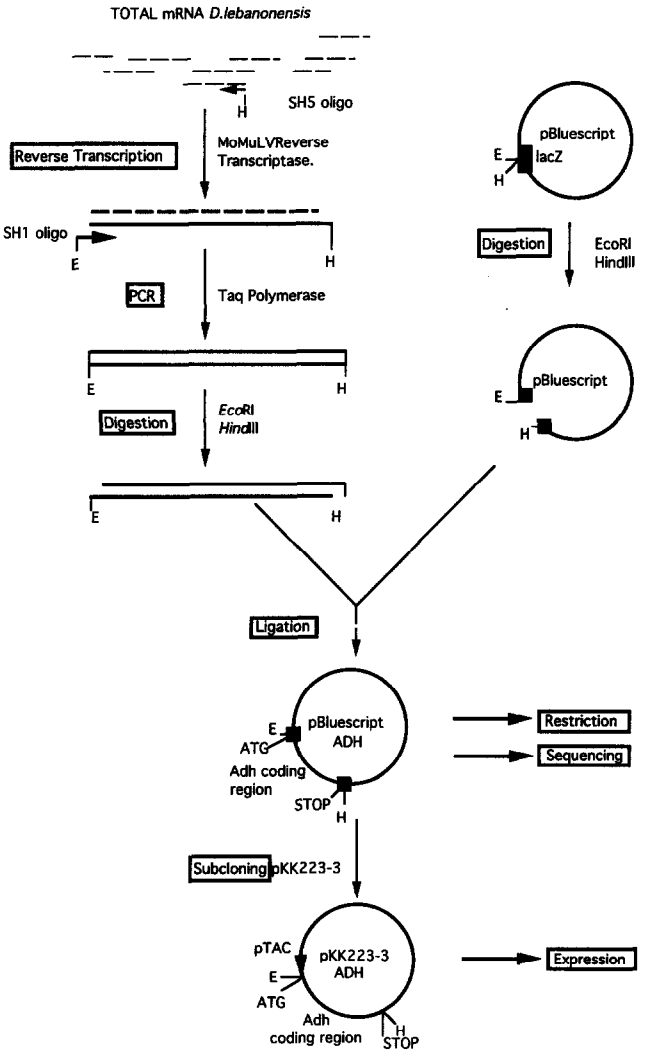


Fig. 1. Design of the expression plasmids for *D. lebanonensis* ADH. E, *Eco*RI, H, *Hin*dIII. SH1 and SH5 are the oligonucleotides used in Reverse-PCR reactions (see Table 1).

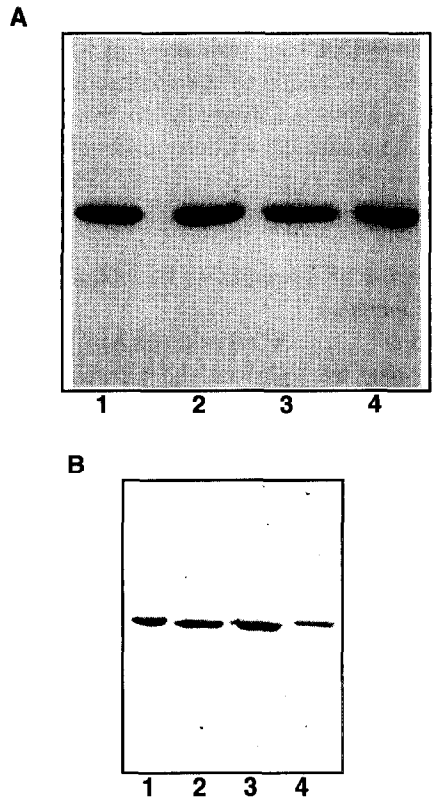


Fig. 2. (A) Coomassie blue-stained SDS-PAGE of purified *Drosophila* ADH and mutants. (1) 10 μg of wild-type ADH from adult flies; 10 μl of recombinant ADH purified from *E. coli* JM105 transformed with (2) pKK223-wild-type ADH; (3) pKK223-Ala[13]Gly mutant and (4) pKK223-Asn[56]Thr mutant. (B) Western blot analysis of purified ADH using mAb LLBE8 against *D. lebanonensis* ADH. Lanes correspond to the same samples as displayed in (A).

confirmed after incubation of crude protein extracts from bacteria expressing this mutant in NaCl. A 75% decrease in ADH activity was obtained after 3 min incubation at 4°C with 1 M NaCl; even 0.1 M NaCl led to a decrease of 65% in enzymatic activity. In view of these results an alternative purification procedure was used for the ADH Asn[56]Thr mutant. 500 μl of *E. coli* protein extract in 20 mM Tris-HCl pH 8.6 was injected in a MonoQ FPLC column equilibrated with the same buffer. Eluent contained most of the initial ADH activity, whereas a high proportion of *E. coli* proteins remained bound to the gel matrix. The ADH-containing fractions were pooled, concentrated and then fractionated in Superose 12. Overall calculated yield was 600 μg of pure ADH from 500 ml of culture. When recombinant wild-type ADH was purified according to this protocol it showed a kinetic behaviour which was comparable to that obtained following the affinity chromatography procedure described above.

Fig. 2A shows the SDS-PAGE analysis of the purified recombinant wild-type *D. lebanonensis* ADH and mutant forms, and Fig. 2B shows the results of a Western blot immunodetected with mAb LLBE8. The only band present in the fractions showing enzymatic activity was recognized by the anti-ADH specific mAb, and moreover the amount of protein synthesized was similar for the wild-type and mutant forms.

3.3. *pH profiles for ADH*

The influence of pH on wild-type and mutant ADH activities was tested at different pH from 7.0 to 10.0. Recombinant wild-type ADH showed a maximum at pH 8.0, with a slight decrease at higher pH. Ala[13]Gly and Asn[56]Thr mutants showed maximum activity at pH 10.0 and lower activity at pH 8.0 and 9.0. In all cases, however, differences never amounted to more than 20%. A decrease in activity of more than 60% appeared at pH 7.0 for wild-type and mutant enzymes, with similar pH profiles for the three proteins.

3.4. *Thermal stability of the wild-type and mutant enzymes*

Thermal denaturation tests were performed in duplicate samples obtained from independent purifications, to rule out the effect of specific batch contaminants. Results clearly showed different behaviour of the mutant enzymes versus the wild-type. Whereas wild-type ADH lost more than 50% of activity after 20 min at 40°C, and showed no detectable activity after 60 min incubation, both mutants retained 50% activity after 60 min at 40°C.

3.5. *Kinetic characterization of wild-type and mutated enzymes*

The kinetic constants, K_m and k_{cat}, were calculated for the recombinant wild-type and both mutant enzymes with NAD^+ as coenzyme and propan-2-ol as substrate (Table 2). In spite of the fact that the positions studied affect the putative NAD^+-allocating region, the coenzyme and substrate binding ability of the mutants remained essentially the same as that of the wild-type protein, as reflected by similar K_m values. However, significant if not spectacular differences arose from the com-

Table 2
K_m and k_{cat} values for wild-type and mutant enzymes

	K_m^{NAD+} (mM)	K_m propan-2-ol (mM)	k_{cat} (s^{-1})	k_{cat}/K_m^{NAD+} ($s^{-1} \cdot mM^{-1}$)	k_{cat}/K_m^{prop} ($s^{-1} \cdot mM^{-1}$)
WT enzyme	0.23 ± 0.02	2.41 ± 0.6	1.74 ± 0.02	7.57	0.72
Ala[13]Gly mutant	0.24 ± 0.02	2.57 ± 0.3	2.92 ± 0.03	12.17	1.14
Asn[56]Thr mutant	0.20 ± 0.02	2.50 ± 0.4	2.31 ± 0.03	11.55	0.92

parison of the k_{cat} parameter, as both mutants showed higher catalytic rate than the recombinant wild-type. Then, if we consider the efficiency of the enzyme, measured by the k_{cat}/K_m ratio, both substitutions led to a clear improvement of function: an increase of 1.7-fold for the Ala[13]Gly mutant and 1.3-fold for the Asn[56]Thr mutant.

4. Discussion

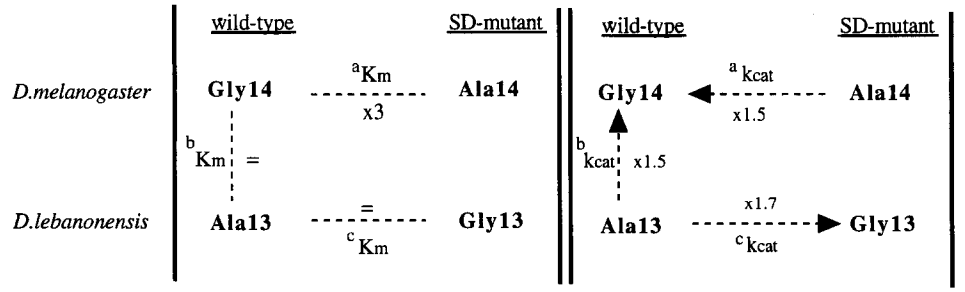
k_{cat} values of *D. lebanonensis* ADH are lower than those of *D. melanogaster*, while $K_m^{alcohol}$ and $K_m^{NAD^+}$ are comparable to those of other species [22]. The exclusive amino acid substitutions in *D. lebanonensis* ADH, Gly[13]Ala and Thr[56]Asn involve the putative NAD^+-binding region and, therefore, could partially account for the kinetic properties of this enzyme. In particular, position 13 (14 in *D. melanogaster*) is the first residue of the conserved box [G[13(14)] L G I G], representative of the short-chain dehydrogenase motif [G[14] xxx G x G] which belongs to the Rossmann Fold and it is homologous to the medium-chain ADH box [G[199] xx G G x G]. Studies on *D. melanogaster* mutants in this position show that the only substitution rendering an active enzyme is Gly/Ala: a site-directed Val[14] mutant [7] and an EMS-induced Asp[14] form [23] produce a dead enzyme. This is in agreement with alanine being the only 'natural' alternative found among short-chain DH's, Ala[13] in *D. lebanonensis* ADH, and among eukaryotic medium-chain DH's, Ala[199] in the medium-chain *Alcaligenes eutrophus* ADH [24].

The kinetic behaviour of the Gly[14]Ala mutant form of *D. melanogaster* ADH has been reported to show a 31% decrease in activity. This was attributed to a lower affinity for NAD^+ binding ($K_m^{NAD^+}$ increased 3-fold) and to a reduction in catalytic efficiency (k_{cat} decreased 1.47-fold) [7]. The authors claimed that Ala[14] interfered with coenzyme binding, and that this, in turn, affected both enzyme-substrate interaction and the ternary complex dissociation rate. We have restored glycine to position 13 of the *D. melanogaster* enzyme. Our results clearly show that this *retromutant* maintains the $K_m^{NAD^+}$ whereas the k_{cat} increases 1.7-fold. It is remarkable that the only allowed substitution in this position (Gly/Ala) decreases the efficiency of the enzyme, irrespective of its molecular background, i.e. SDM-*D. melanogaster* (Ala[14]) and wild-type *D. lebanonensis* (Ala[13]) share a similar decrease in k_{cat} with respect to wild-type *D. melanogaster* (Gly[14]) and SDM-*D. lebanonensis* (Gly[13] (Table 3). In our mutant, K_m for propan-2-ol is unaltered, with only a modest k_{cat}/K_m increase, again in agreement with the *D. melanogaster* direct mutant.

Position 56 is threonine in all *Drosophila* ADHs, except *D. lebanonensis* (Asn[56]) and *D. mayaguana* (Ile[56]). This position is not only non-conserved among short-chain dehydrogenases, but also enclosed in a hyper-variable region, which is difficult to align [4]. However, this threonine, which lies in the third β-sheet of the NAD^+ binding domain, is conserved among all animal medium-chain alcohol dehydrogenases (position 238) [2]. Thr[56/238] could have an important role in the accomodation of the coenzyme, facilitating correct interaction with small alcohols. Other short-chain dehydrogenases, as well as class III and sorbitol dehydrogenases, which utilize much larger substrates, have an Asn or Asp residue at 238. Thus, the Asn[56]Thr mutant in *D. lebanonensis* restores the ideal amino acid for a short-chain ADH. This mutant shows a similar behaviour to Ala[13]Gly; its k_{cat} value increases × 1.3. The kinetic effects of both substitutions are in good agreement with the size of the amino acids involved. In fact, $r°$ is 5.0 Å for Gly, and 5.5 Å for Ala [25], and their substitution produces a 10% increase in their packing volume (k_{cat} ADH^{Gly13} × 1.7), while $r°$ for Asn (6.4 Å) is only 1.38% greater than that of Thr (6.3 Å) (k_{cat} ADH^{Thr56} × 1.3).

Thermal denaturation has been tested in pure enzyme preparations to check the stability of the recombinant enzyme. Both *retromutants* were significantly more stable than the wild-type enzyme. After 1 hour incubation at 40°C the former retained 50% of their activity, while the latter became totally inactive. This was in agreement with data obtained with *D. melanogaster* ADH, which

Table 3
Relationship between $K_m^{NAD^+}$ and k_{cat} values of wild-type and mutants of *D. melanogaster* and *D. lebanonensis* ADH.



Data are: (a) from [24], (b) from [7] and (c) from our results.

retained 60% more activity than the Gly[14]Ala mutant under the same conditions [7]. Again, we show that the presence of a larger amino acid in position 13/14 disturbs the architecture of the molecule and lowers its stability.

In summary, when considering the consensus sequence for ADH in all analyzed *Drosophila* species, it appears that selection has fixed the most suitable amino acid in each position of the polypeptide chain. Thus, any alteration to this sequence would have a negative effect. By the same argument, restoring the consensus sequence in *D. lebanonensis* would produce a more efficient enzyme. This, according to our data, is the case. *Retromutants* are better enzymes, as our results fully support an improvement in enzyme efficiency and thermal stability. It could be argued that in *D. lebanonensis*, the wild-type enzyme, albeit kinetically unfavourable, was fixed because the large amount of ADH synthesized compensated for the reduction in its catalytic efficiency.

On the other hand, the kinetic differences between wild-type *D. lebanonensis* ADH and the engineered mutants are not as profound as those obtained when mutating *D. melanogaster* ADH. In both *D. lebanonensis* mutants, K_m values for NAD^+ and propan-2-ol remain unaltered and k_{cat} values show a slight increase. This could be due to an inherent plasticity of the *D. lebanonensis* enzyme, in contrast to the more evolved form of *D. melanogaster*, in which further substitutions would impair the catalytic function. The ancestral phylogenetic position of *D. lebanonensis* also supports this hypothesis [26].

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