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

The effect of restoring the consensus sequence in two positions

Ricard Albalat, Sílvia Atrian, Roser Gonzàlez-Duarte*

Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 645, E-08028 Barcelona, Spain

Received 5 January 1994; revised version received 9 February 1994

**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, Ala⁴Gly and Asn⁵Thr. 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 β1-α2-β2 motif of the Rossmann Fold, found in all NAD⁺/FAD⁺ binding enzymes [12]. The catalytic behaviour of mutants in positions Gly¹⁴, Gly¹⁶, Gly¹⁸ and Asp³⁸ 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, Tyr¹⁵² and Lys¹⁵⁶ [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
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$^{42}$ 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 Gyl 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 were obtained from Boehringer-Mannheim. Taq DNA polymerase was from Promega. PCR primers were synthesized by Oligos Etc. Inc. Hybond-C nitrocellulose filters, [α-$^{32}$P]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 MolMUV reverse transcriptase (BRL), in a final reaction volume of 20 μl, containing 1–2 μg of total RNA, 100 pmol of the oligonucleotide SH5 as downstream primer (Table 1), 1 mM of dNTPs, 25 units of RNase inhibitor (Boehringer) and 3 mM MgCl₂. 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 0.05 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 EcoRI and HindIII restriction sites. The presence of the desired mutation and the absence of additional changes was always verified by sequencing the PCR fragments with [α-$^{32}$P]dATP, using the Pharmacia Sequencing Kit. Positive clones were used to subclone the EcoRI/HindIII 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 μM 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 2 M NaCl. Fractions containing ADH activity were pooled and concen-

### Table 1

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Mutation</th>
<th>Sequence</th>
<th>Length 1st PCR</th>
<th>Mutation codon</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH1</td>
<td>–</td>
<td>CGGAATTCATGCTGATTTGACCAACAGG</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(upstream)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SH2</td>
<td>Ala$^{13}$ → Gly</td>
<td>ACCGCCCAGACCGGCAACGA</td>
<td>54 nt</td>
<td>GCT → GAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(downstream)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SH3</td>
<td>Asn$^{56}$ → Thr</td>
<td>ACCGCCCAGACCGGCAACGA</td>
<td>182 nt</td>
<td>AAC → ACC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(downstream)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SH5</td>
<td>–</td>
<td>ACCGCCCAGACCGGCAACGA</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(downstream)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a,b Start and Stop codons are shown in bold. Sequences used to generate flanking restriction sites EcoRI in SH1 and HindIII in SH5, are underlined. c,d Mutated nucleotides are in bold.*
treated to a final volume of 0.5 ml, using Centricon 10 Microconcentra-
tors (10,000 MW cut-off). The sample was then fractionated in Supero-
cose 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 per-
formed 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 deter-
minations, total protein extract from the bacterial cultures was incu-
bated with or without mAb LLBE8 [14] for 1 h at 37°C before measur-
ing 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
protein 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+. Kmax and km 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 con-
tained 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 Ala13Gly and Asn11Thr mutants were also sequenced. In all cases, we confirmed the presence of the
mutagenized codon and the absence of unwanted substi-
tutions.

Drosophila ADH activity was detected spectropho-
tometrically in crude protein extracts prepared from bacte-
rial 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 re-
combinant D. lebanonensis ADH retained the expected
differential antigenic features for the wild-type enzyme
with respect to that of D. melanogaster ADH*. LLBE8
specifically inhibits D. lebanonensis ADH, while D.
melanogaster ADH* activity reamins unaltered [14].
When LLBE8 was added to the recombinant ADH pro-
etin 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 Ala13Gly 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
Asn11Thr mutant unexpectedly showed irreversible de-
naturation 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, EcoRI, H, HindIII. SH1 and SH5 are the oligonucleotides used in
Reverse-PCR reactions (see Table 1).
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\textsuperscript{56}Thr mutant. 500 μl of \textit{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 \textit{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 \textit{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\textsuperscript{13}Gly and Asn\textsuperscript{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, $K_n$ and $k_{cat}$, were calculated for the recombinant wild-type and both mutant enzymes with NAD\textsuperscript{+} as coenzyme and propan-2-ol as substrate (Table 2). In spite of the fact that the positions studied affect the putative NAD\textsuperscript{+}-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 $K_n$ values. However, significant if not spectacular differences arose from the comp-
parison of the \( k_{\text{cat}} \) 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 \( k_{\text{cat}}/K_m \) 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

\( k_{\text{cat}} \) values of \( D. \) lebanonensis ADH are lower than those of \( D. \) melanogaster, while \( K_m \)\(^{\text{alcohol}} \) and \( K_m^{\text{NAD}^+} \) are comparable to those of other species [22]. The exclusive amino acid substitutions in \( D. \) lebanonensis ADH, Gly\(^{13}\)Ala and Thr\(^{46}\)Asn involve the putative \( \text{NAD}^+ \) 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 is homologous to the medium-chain ADH box \([G^{199} xx G x G]\). 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 \( A. \) 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 \( \text{NAD}^+ \) binding (\( K_m^{\text{NAD}^+} \) increased 3-fold) and to a reduction in catalytic efficiency (\( k_{\text{cat}} \) 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 \( K_m^{\text{NAD}^+} \) whereas the \( k_{\text{cat}} \) 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 \( k_{\text{cat}} \) 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 \( k_{\text{cat}}/K_m \) increase, again in agreement with the \( D. \) melanogaster direct mutant.

Position 56 is threonine in all \( D. \)rosophila 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 \( \beta \)-sheet of the \( \text{NAD}^+ \) 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 \( k_{\text{cat}} \) value increases \( \times \)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 (\( k_{\text{cat}} \) ADH\(^{\text{Gly}13} \)\( \times \)1.7), while \( r^* \) for Asn (6.4 Å) is only 1.38% greater than that of Thr (6.3 Å) (\( k_{\text{cat}} \) ADH\(^{\text{Asn}56} \)\( \times \)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

<table>
<thead>
<tr>
<th></th>
<th>wild-type</th>
<th>SD-mutant</th>
<th>wild-type</th>
<th>SD-mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>( D. ) melanogaster</td>
<td>Gly14 ( \overset{a}{=} K_m )</td>
<td>Ala14 ( \overset{a}{x} )</td>
<td>Gly14 ( \overset{a}{k_{\text{cat}}} )</td>
<td>Ala14 ( \overset{x}{1.5} )</td>
</tr>
<tr>
<td></td>
<td>( b K_m )</td>
<td>( x3 )</td>
<td>( b k_{\text{cat}} )</td>
<td>( x1.5 )</td>
</tr>
<tr>
<td>( D. ) lebanonensis</td>
<td>Ala13 ( \overset{c}{=} K_m )</td>
<td>Gly13 ( \overset{a}{=} )</td>
<td>Ala13 ( \overset{a}{x} )</td>
<td>Gly13 ( \overset{c}{=} )</td>
</tr>
</tbody>
</table>

---

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].

Acknowledgements: We are grateful to Dr. J. Fibla who kindly provided the monoclonal antibodies used in this work. We thank R. Rycroft for revising the English version of this manuscript. This work was supported by a grant from the CICYT (Plan Nacional I+D, Ministerio de Educación y Ciencia, España), BIO92-0591-C02-02.

References