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Enzymic and Structural Studies on *Drosophila* Alcohol Dehydrogenase and Other Short-Chain Dehydrogenases/Reductases

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Abstract. Enzymic and structural studies on *Drosophila* alcohol dehydrogenases and other short-chain dehydrogenases/reductases (SDRs) are presented. Like alcohol dehydrogenases from other *Drosophila* species, the enzyme from *D. simulans* is more active on secondary than on primary alcohols, although ethanol is its only known physiological substrate. Several secondary alcohols were used to determine the kinetic parameters k_{cat} and K_m. The results of these experiments indicate that the substrate-binding region of the enzyme allows optimal binding of a short ethyl side-chain in a small binding pocket, and of a propyl or butyl side-chain in large binding pocket, with stereospecificity for R(−) alcohols. At a high concentration of R(−) alcohols substrate activation occurs. The k_{cat} and K_m values determined under these conditions are about two-fold, and two orders of magnitude, respectively, higher than those at low substrate concentrations.

Sequence alignment of several SDRs of known, and unknown three-dimensional structures, indicate the presence of several conserved residues in addition to those involved in the catalyzed reactions. Structural roles of these conserved residues could be derived from observations made on superpositioned structures of several SDRs with known structures. Several residues are conserved in tetrameric SDRs, but not in dimeric ones. Two halohydrin-halide-lyases show significant homology with SDRs in the catalytic domains of these enzymes, but they do not have the structural features required for binding NAD^+. Probably these lyases descend from an SDR, which has lost the capability to bind NAD^+, but the enzyme reaction mechanisms may still be similar.

Key words: Alcohol dehydrogenase — *Drosophila simulans* — Halohydrin-halide-lyase — Secondary alcohols — Short-chain dehydrogenases/reductases — Substrate activation

Introduction

The enzymes alcohol dehydrogenase (ADH; alcohol: NAD^+ oxido-reductase; EC 1.1.1.1) from *Drosophila melanogaster*, and its sibling species, *Drosophila simulans*, have been the subject of intense study with regard to their evolutionary biology (Chambers 1988), population genetics (Van Delden 1982), and structural features (Benach et al. 1999).

Drosophila ADH is the best characterized member of the short-chain dehydrogenase/reductase (SDR) family of structurally related enzymes with substrate specificities, ranging from simple alcohols to compounds like steroids, prostaglandins, etc., identified in many prokaryotes and eukaryotes, and which also includes several epimerases (Jörnvall et al. 1995). The ubiquitous distribution of short-chain type dehydrogenases is also evident from the fact that 80 sequences coding for members of this family have been identified in the genome of *Caenorhabditis elegans* (*C. elegans* sequencing consortium 1998). The other well-known family of medium-chain alcohol dehydrogenases, to which the enzymes from horse liver and yeast belong, is more restricted in sub-

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strate specificities, and its members generally have preferences for simple primary alcohols (Jörnvall 1994).

Several modeling studies were performed of *Drosophila* ADH before its X-ray structure was determined (Chenevert et al. 1995; Smilda et al., 1998b). Conclusions derived from these studies have been confirmed by the recently determined X-ray structure of the enzyme from *D. lebanonensis* (Benach et al. 1998) and its binary and ternary complexes with NAD^+ and ketones (Benach et al. 1999), including the closure of a substrate-binding loop after binding NAD^+ and substrate, and the prediction of residues involved in the formation of two short helices in this loop (Smilda et al. 1998b). ADHs from *D. lebanonensis* and *D. melanogaster* differ at 18% of the amino acid positions and have identical polypeptide chain lengths, except for an additional N-terminal residue in the latter. This means that polypeptide folds will be reasonable superimposable (Chothia and Lesk 1986) and that the structure-function model developed for the former may be extrapolated to the latter one.

D. melanogaster alleloenzyme ADH-S differs from ADH-F in the replacement of threonine by a positively charged lysine at position 192 at a rather central position in this loop. This explains a stronger binding of NAD^+ by ADH-S and may influence selection of either of the two alleloenzymes in natural populations (Smilda et al. 1998b; Benach et al. 1999). ADH of the sibling species, *D. simulans*, differs at positions 1 and 82 from ADH-S of *D. melanogaster*. Glu-82 is replaced by lysine, which may interact with NAD^+.

Here we present several studies on *Drosophila* ADH and other SDRs in order to obtain more insight in the relation between structure and properties of these enzymes. Enzymic properties of *D. simulans* ADH were determined, which confirm that the enzyme is more active on secondary than on primary alcohols, with a strong stereospecificity for R(−) alcohols. The fact that *Drosophila* species use an enzyme which has higher specific activities for secondary alcohols for the conversion and detoxification of the primary alcohol ethanol is very puzzling. This feature warrants a comprehensive study of the enzymic and structural properties of *Drosophila* alcohol dehydrogenase in relation to those of other SDRs.

We also investigated a phenomenon, called substrate activation, in which increased k_{cat} values are measured when high substrate concentrations are used, together with a high concentration NAD^+. Substrate activation has been described earlier for both liver and *Drosophila* ADHs using high concentrations of several primary and secondary alcohols (Dalziel and Dickinson 1966; Sofer and Ursprung 1968; Hovik et al. 1984; Winberg et al. 1982, 1986). As these studies are rather fragmentary we have performed a more systematic study with different substrates and their enantiomorphs, for a better understanding of this phenomenon from a structural perspective.

As dissociation of the product NADH plays a predominant role in the reaction of *Drosophila* ADH with secondary alcohols, which is promoted at the high alcohol concentrations at which substrate activation occurs, and as *D. simulans* ADH has a higher affinity for NAD^+ and its analogues than the *D. melanogaster* variants near their pH optimum of 9.5 (Smilda et al. 1998a), we have used the enzyme from *D. simulans* for the kinetic experiments.

Several additional X-ray structures and sequences of SDRs have been published since our previous modelling study (Smilda et al. 1998b). This larger collection of structures allows the recognition of additional conserved features which may be of importance for structure and function in this class of enzymes. In addition, in this comparison we include the sequences of two homologous bacterial halohydrin hydrogen-halide-lyases, which share the catalytic domain with the SDRs, but have not the structural features essential for binding NAD^+.

Materials and Methods

Enzymes

A homozygous strain of *Drosophila simulans* was kindly provided by Dr. W. van Delden (Department of Genetics, University of Groningen). Alcohol dehydrogease was isolated as described earlier using affinity chromatography and gel filtration (Smilda et al. 1998a). This procedure resulted in pure and stable homogeneous enzyme preparations. Only preparations (Smilda et al. 1998a) with identical specific activities on ethanol, relative to protein concentrations determined by the method of Bradford (1976), or amino acid analysis were used for the kinetic experiments.

Amino acid sequences of short-chain dehydrogenases/reductases and a bacterial dehalogenase were aligned using the program CLUSTALW (Thompson et al. 1994). The structural alignments were performed using the program O (Jones et al. 1991). The secondary structure assignments of proteins with known structures were performed with PROCHECK (Laskowski et al. 1993).

Chemicals

NAD^+ was purchased from Boehringer (Mannheim, Germany). Cyclopentanol, cyclohexanol and S(+) 2-butanol were from Janssen Chimica (Tilburg, The Netherlands). Glycine, R(−) 2-butanol, R(−) 2-pentanol, S(+) 2-pentanol, R(−) 2-hexanol, S(+) 2-hexanol, R(−) 2-octanol, S(+) 2-octanol were from Fluka Biochemika (Buchs, Switzerland). R(−) 2-heptanol and S(+) 2-heptanol were from Sigma-Aldrich (Milwaukee, USA). All other reagents and biochemicals were from Merck (Darmstadt, Germany).

Kinetic measurements

All reactions were started by adding enzyme preparations with a known concentration, determined as described above, to a total reaction volume of 3 ml and the initial rate of NADH formation at 23°C was measured at 340 nm with a Kontron Uvikon 930 UV/VIS spectrophotometer.

To determine the rate constant, k_{cat} or the Michaelis constant, K_m

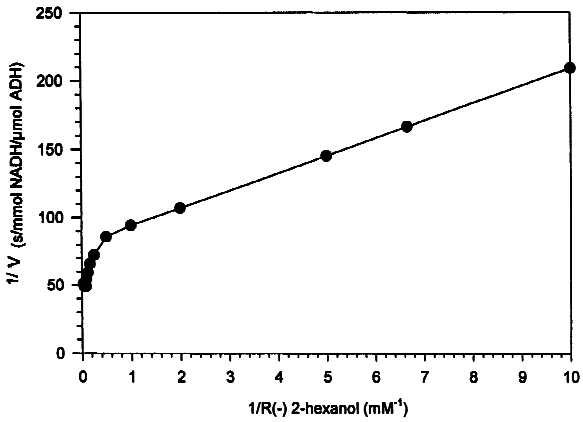

Fig. 1. Lineweaver–Burk plot for *Drosophila simulans* alcohol dehydrogenase with data obtained with R(−)2-hexanol as substrate.

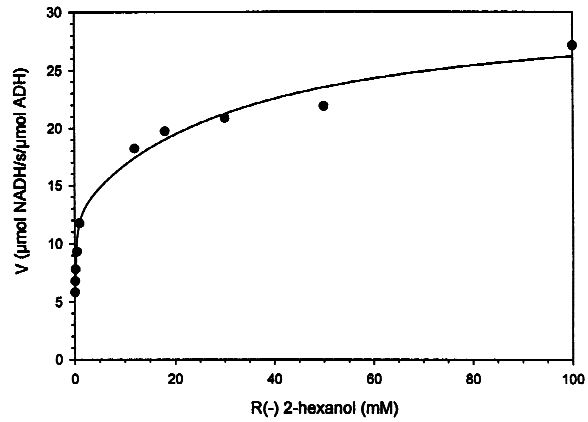

Fig. 2. Computer-calculated least squares fit of velocity versus R(−)2-hexanol concentrations for *Drosophila simulans* alcohol dehydrogenase using a modified form of the Michaelis–Menten sequation: $v = V_1[S]/(K'_m + [S]) + V_2[S]/(K''_m + [S])$.

for primary and secondary alcohols, a concentration of 0.5 mM NAD^+ in 0.1 M glycine-NaOH buffer pH 9.5 was used. This is about 20 times the K_m for NAD^+ for *D. simulans* (Smilda et al. 1998a). Alcohol concentrations in the range of 0.1–50 mM or 0.1–100 mM were used. The kinetic parameters were calculated from Lineweaver–Burk plots (Dixon and Webb, 1979), or a modified Michaelis–Menten equation in the case of substrate activation, and a best fit to the data computed by the method of least squares (Dubrow and Pizer 1977) using the program Scientist (Micromath Scientific Software, Salt Lake City, USA).

Results and Discussion

Substrate Specificity and Stereospecificity

The kinetic parameters, k_{cat} and K_m, were determined at fixed NAD^+ concentrations and varying alcohol concentrations. Alcohol concentrations of 0.1–100 mM were used except for R(−) 2-heptanol and S(+) 2-heptanol. In this case alcohol concentrations of 0.1–50 mM were used. The upper limit of 50 mM was set by the low solubility of these alcohols. In most cases the double-reciprocal plots were linear over all substrate concentrations. However, for a number of substrates, no straight line was observed. Figure 1 shows in a double reciprocal plot with R(−) 2-hexanol as substrate that the data could only be fitted by drawing two lines through the experimental data, one at low substrate concentrations, and the other at high ones, indicating substrate activation. The two distinct V and K_m values, which could not be determined accurately from these double reciprocal plots, or from Eadie–Hofstee plots, were calculated from a modified form of the Michaelis–Menten equation:

$$v = V_1[S]/(K'_m + (S)) + V_2[S]/(K''_m + (S)) \quad (1)$$

in which $V_1 = k'_{cat}[E]$, $V_2 = (k''_{cat} - k'_{cat})[E]$, where (S) is the concentration of alcohol, V_1 and K'_m are the maximum velocity and Michaelis–Menten constant at low substrate concentrations, respectively, and $(V_1 + V_2)$ and K''_m are the maximum velocity and Michaelis–Menten constant at high substrate concentrations, respectively. The best fits for the velocity versus the substrate curves were calculated by the method of least squares and is shown in Fig. 2 for R(−) 2-hexanol. Table 1 shows the rate constants, k_{cat}, the Michaelis constants, K_m and the substrate specificity constants (k_{cat}/K_m) of secondary alcohols for *D. simulans* alcohol dehydrogenase. A number of substrates show the two sets of values, indicated by k'_{cat}, K'_m and k'_{cat}/K'_m (low substrate concentrations) and k''_{cat}, K''_m and k''_{cat}/K''_m (high substrate concentrations). For the other substrates, an equally good best-fit could be obtained if the second term in equation 1 was neglected, indicating that no substrate activation occurs (Table 1).

The kinetic parameters presented in Table 1 confirm that *Drosophila* ADH has a strong preference for secondary alcohols. It has been suggested that the rate-limiting step in the conversion of secondary alcohols is the release of NADH from the binary enzyme-NADH complex and that k_{cat} does not depend on the nature of the alcohol. Our data, however, show that there are differences between the measured k'_{cat} values, although differences in K'_m values also have a large influence on the spread of substrate specificity constants (k'_{cat}/K'_m).

Before the X-ray structure was known, the alcohol-binding region of *Drosophila* alcohol dehydrogenase was already predicted to contain one small and one large hydrophobic binding site, which determine the stereospecificity of the enzyme for R(−) alcohols (Winberg, McKinley-McKee 1992; Winberg et al. 1982, 1986; Hovik et al. 1984). The X-ray study of Benach et al. (1999) confirms this prediction, and also describes the two subcavities for substrate binding: a larger one which can accomodate a total of four aliphatic carbon atoms (but which also binds the methyl group if ethanol is substrate), and a smaller one for three aliphatic carbon atoms. Our data show that R(−) 2-pentanol is a better substrate than R(−) 2-hexanol, indicating that a chain of

Table 1. Kinetic parameters for *Drosophila simulans* alcohol dehydrogenase obtained at conditions of low (k'_{cat}, K'_m, and k'_{cat}/K'_m) and high (k''_{cat}, K''_m and, k''_{cat}/K''_m) substrate concentrations. Substrate activation occurs at high concentrations

Substrate	k'_{cat} (s^{-1})	k''_{cat} (s^{-1})	K'_m (mM)	K''_m (mM)	k'_{cat}/K'_m ($s^{-1} \cdot mM^{-1}$)	k''_{cat}/K''_m ($s^{-1} \cdot mM^{-1}$)
ethanol	3.3 ± 1.6		1.9 ± 0.9		1.7	
2-propanol	16.4 ± 6		0.4 ± 0.1		41	
R(−) 2-butanol	22.2 ± 4	28.0 ± 5	0.10 ± 0.01	25.0 ± 5	222	1.1
S(+) 2-butanol	20.8 ± 3	34.8 ± 5	0.09 ± 0.02	34.2 ± 4	231	1.0
R(−) 2-pentanol	39.7 ± 6	77.3 ± 2	0.09 ± 0.01	65.3 ± 6	441	1.2
S(+) 2-pentanol	16.5 ± 5		1.3 ± 0.3		12.7	
R(−) 2-hexanol	12.8 ± 3	28.0 ± 4	0.14 ± 0.02	32.9 ± 4	91	0.9
S(+) 2-hexanol	4.9 ± 1		1.1 ± 0.4		4.5	
R(−) 2-heptanol	10.5 ± 3	17.4 ± 2	0.46 ± 0.08	15.2 ± 2	23	1.1
S(+) 2-heptanol	5.2 ± 1		1.2 ± 0.3		3.7	
cyclopentanol	9.1 ± 2	10.7 ± 3	0.13 ± 0.03	16.0 ± 2	70	0.7
cyclohexanol	8.4 ± 3	24.4 ± 5	0.28 ± 0.05	87.7 ± 7	30	0.3

three rather than one of four aliphatic carbon atoms is preferred in the larger sub-cavity. The smaller sub-cavity is wider than the larger sub-cavity, which shows that S(+) 3-methyl-2-butanol is a better substrate than the R(−) enantiomer of this alcohol (Hovik et al. 1984). But alkyl chains longer than two C-atoms cannot bind anymore in the smaller sub-cavity, as shown by the large increase of K'_m in going from S(+) 2-butanol to S(+) 2-pentanol. The smaller sub-cavity, however, binds strongly aliphatic ethyl chains, as is evident from very similar kinetic parameters for R(−) 2-butanol and S(+) 2-butanol (Table 1) and the fact that inactive ternary complexes of ADH with NAD^+ and 3-ketones (like 3-pentanone, 3-hexanone, etc.) are much more stable than those with 2-ketones (Smilda et al. 1998c). Kinetic experiments of ADH with R(−) 3-alcohols have not yet been performed, but higher reaction rates may be expected compared with those obtained with R(−) 2-alcohols.

That *Drosophila* species use an enzyme with high specific activities for secondary alcohols for conversion and detoxification of the primary alcohol ethanol is very puzzling. It was proposed that *Drosophila* ADH may be involved in other metabolic processes because of its high activity with secondary alcohols (Winberg et al. 1986), but no clear evidence for this has yet been found. *Drosophila* ADH is related evolutionarily to other SDRs with specificity for secondary alcohols with very complex structures, and its higher activity with secondary alcohols may suggest evolutionarily descent from enzymes specific to substrates with more complex structures than ethanol. But the reaction of *Drosophila* ADH with secondary alcohols is dangerous even for the enzyme, as it may form an abortive ternary complex with the oxidized substrates NAD^+ and ketone. These compounds form a covalent reaction product and cause irreversible enzyme inhibition, not only under experimental conditions, but also in vivo (Schwartz and Sofer 1976; Smilda et al. 1998c).

Substrate Activation

We also investigated a phenomenon, called substrate activation, in which increased k_{cat} values are measured at a high concentration NAD^+ when high substrate concentrations are used. The k''_{cat}, K''_m, and k''_{cat}/K''_m values in Table 1 are the kinetic parameters for *D. simulans* alcohol dehydrogenase as a result of this phenomenon. *D. simulans* alcohol dehydrogenase exhibits substrate activation with the secondary alcohols R(−) 2-butanol, S(+) 2-butanol, R(−) 2-pentanol, R(−) 2-hexanol, R(−) 2-heptanol, cyclopentanol, and cyclohexanol (Table 1), but not with primary alcohols. For other secondary alcohols, the size of the substrate-binding pockets probably does not allow substrate binding at the investigated concentrations with sufficient affinity to attain substrate activation in vivo.

Benach et al. (1999) explain substrate activation from their observation that in the crystal structure of the ternary complex with a cyclohexanone-NAD^+ adduct an extra cyclohexanone molecule was bound to the active site of *Drosophila* ADH. However, our observation that substrate activation only occurs with secondary alcohols which bind with high specificity to the active site (Table 1) suggests that non-specific binding of a second substrate molecule at the active site may not be the explanation for substrate activation, and that it probably results from an acceleration of dissociation of the produced NADH, from the enzyme, at high substrate concentrations (Winberg et al. 1986). The high K''_m values for substrate activation indicate that the affinity for secondary alcohols to the *D. simulans* ADH-NADH complex are about two orders of magnitude lower than to the *D. simulans* ADH-NAD^+ complex. Physiological concentrations of secondary alcohols probably will not be so high as to enable substrate activation. However, investigation of this feature adds to insight about substrate-binding properties of the enzyme.

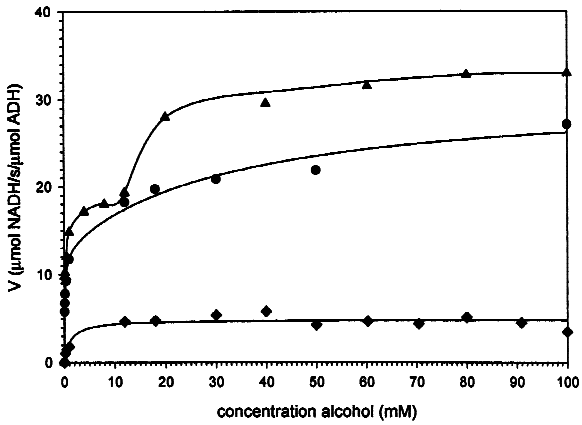

Fig. 3. Reaction velocity of *Drosophila simulans* alcohol dehydrogenase versus alcohol concentration. ◆, S(+)2-hexanol; ●, R(−)2-hexanol; ▲, (R)-2-hexanol in the presence of 10 mM S(+)2-hexanol.

Inhibition experiments showed that *D. simulans* ADH not only has a higher affinity than the *D. melanogaster* alleloenzymes for NAD^+, but also for NADH (Heinstra et al. 1988). This means that substrate activation is more prominent with *D. simulans* ADH, as the enzyme has a higher affinity for NAD^+ and its analogues than the *D. melanogaster* variants. The lower k'_{cat} values without substrate activation for secondary alcohols for *D. simulans* alcohol dehydrogenase, as compared to other *Drosophila* alcohol dehydrogenases, may also be caused by its higher affinity for NADH.

As S(+) alcohols will bind to the same site as R(−) alcohols, they could act at high concentrations as competitive inhibitors of the conversion of the latter ones. We have investigated the reaction rates with increasing R(−) 2-hexanol concentrations at several fixed S(+) 2-hexanol concentrations (10–100 mM). To our surprise, we found no inhibition, but an additional sudden activation in the 15–20 mM range of added R(−) 2-hexanol (Fig. 3), which is about half the K''_m value of substrate activation by this alcohol (Table 1). We do not yet have an explanation for this observation, but it may indicate that binding of S(+) 2-hexanol to one subunit may activate the other subunit in the dimer.

Subunit Interactions

One of the most surprising features of the recently determined X-ray structure of *D. lebanonensis* ADH is that the C-terminal residues of one subunit contribute to the substrate-binding site of the other one. In such cases one would expect cooperative behavior. However, this has never been observed in the many kinetic studies of *Drosophila* ADH. We have been able to isolate hybrid dimers of *D. melanogaster* ADH-FS (Smilda et al. 1998a), which do not show Michaelis–Menten kinetics. Negative cooperativity was observed, which could be explained by the presence of binding sites with different substrate affinities so that the stronger binding site is occupied first (Fersht 1985). But the physical and enzymic properties of *D. melanogaster* ADH-FS are not just an average of those of *D. melanogaster* ADH-FF and ADH-SS. This will influence environmental selection according to the ratio of ADH-F and ADH-S alleles in natural populations (Smilda et al. 1998a). These properties of hybrid ADH-FS molecules, and the additional activation by S(+) 2-hexanol on the conversion of R(−) 2-hexanol at high substrate concentrations (Fig. 3) indicate that the two substrate-binding sites in an DADH dimer indeed are not independent from each other, since it is possible that contributions of both dimer subunits to each substrate-binding site are responsible.

Model Building Studies

Twelve sequences of SDRs, and the sequence of two bacterial halohydrin hydrogen-halide-lyases (Yu et al. 1994) have been aligned (Fig. 4). Accession numbers and abbreviations of these proteins are summarized in Table 2. The 3-D structure of nine of these have been published, including the coordinates: alcohol dehydrogenase from *Drosophila lebanonensis* (1A4U), dihydropteridine reductase (1DHR), carbonyl reductase (1CYD), 7α-hydroxysteroid dehydrogenase (1AHH), 3α,20β-hydroxysteroid dehydrogenase (2HSD), trihydroxynaphthalene dehydrogenase (1YBV), cis-biphenyl-2,3-dihydrodiol-2,3 dehydrogenase (1BDB), tropinone reductases (1AE1 and 2AE1). Five sequences of enzymes with unknown 3-D structures have been included in the alignment: glucose-1 dehydrogenase (DHG1), acetoin diacetyl reductase (BUDC), prostaglandin dehydrogenase (PGDH) and two halohydrin hydrogen-halide-lyases from *Corynebacterium* sp. strain N-1074 (HHEA and HHEB).

Our previous model-building study (Smilda et al. 1998b) included only five SDRs. This number has now been increased to fourteen which allows to make a better discrimination between conserved and varied parts of the structure. The nine SDRs with known 3-D structures share a common folding topology, where each subunit forms a single domain structure, consisting of a sheet of seven parallel β-strands (A–F and H), and six parallel α-helices (B–G). However, 1DHR is lacking helix αC, 1A4U has an additional strand (βG) (Benach et al. 1998), and 2HSD has an additional helix (αH) at the C-terminal end, indicating that small structural differences are possible (Fig. 4). Fig. 5 shows a ribbon representation of the *D. lebanonensis* ADH monomer with labelled secondary structural elements (Benach et al. 1998).

The three SDRs with unknown structures probably also share these secondary structure elements. The alignment shows that five amino acids are conserved among

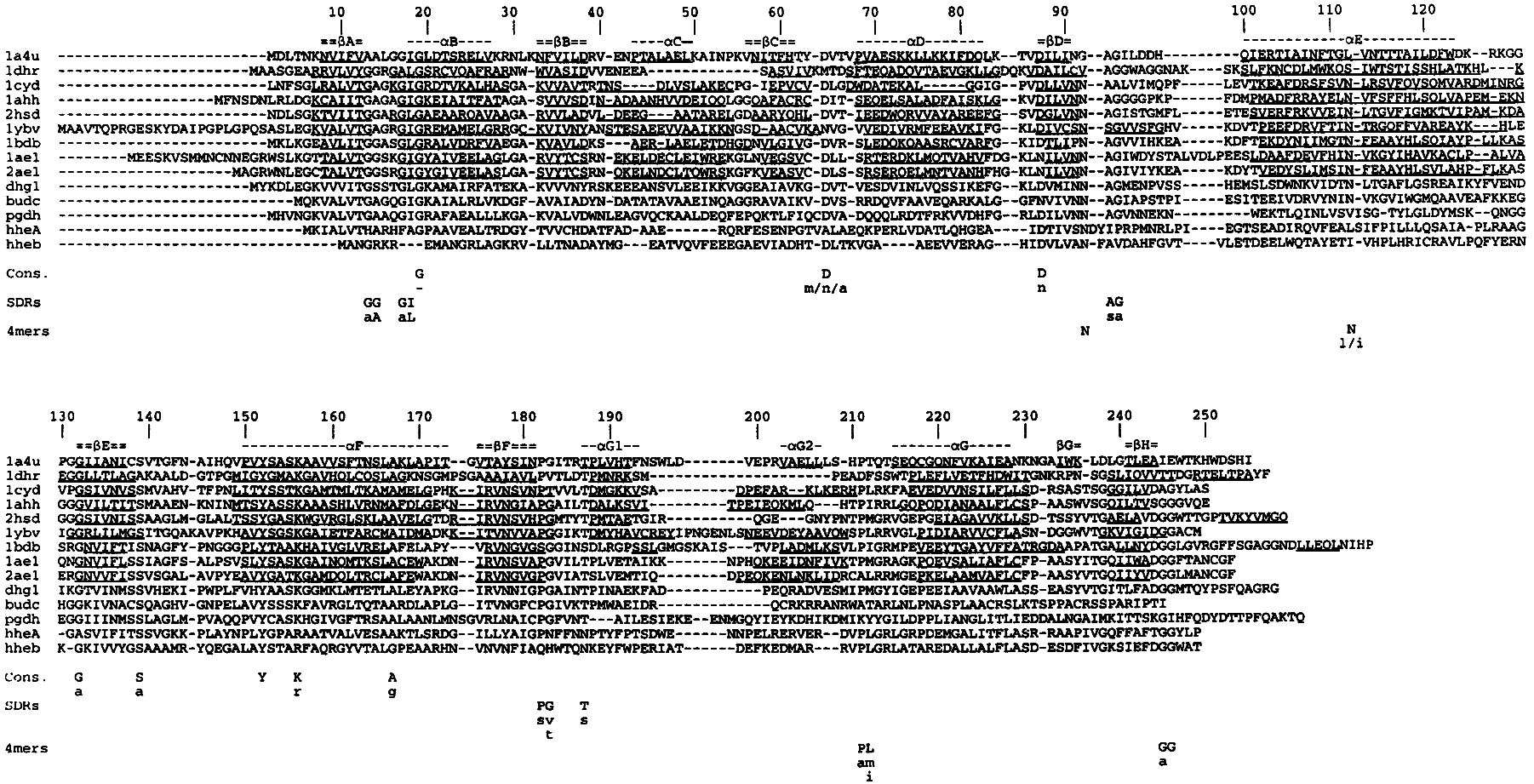

Fig. 4. Multiple sequence alignment of 12 short-chain dehydrogenases/reductases and two halohydrin hydrogen-halide lyases. On top is shown the numbering according to *D. melanogaster* ADH. In the second line, secondary structure elements are presented as identified in 1a4u. Corresponding secondary structure elements are underlined in the sequence of SDRs with known structure. Helix αG1 is present in ternary complexes of 1a4u with NAD^+ and ketones, but not in the apoenzyme or its binary complex with NAD^+. Abbreviations and other data of the enzymes are presented in the text and Table 2. On the lower lines conserved residues are indicated in capitals (and rare replacements in small face). Cons, in all sequences; SDRs, in NAD^+ binding sites; 4mers, in tetrameric enzymes.

Table 2. Substrate, type of cofactor used, and subunit composition of several short-chain dehydrogenases/reductases, and two halohydrin hydrogen-halide lyases

	Substrate	Cofactor	Subunit	Accession number
1a4u	primary/secondary alcohols	NAD	2	p10807
1dhr	dihydropteridin	NAD	2	p11348
1cyd	2-propanol	NADP	4/1	np031647
1ahh	7α-hydroxysteroid	NAD	4	p25529
2hsd	3α,20β-hydroxysteroid	NAD	4	640224
1ybv	trihydroxynaphthalen	NADP	4	2624733
1bdb	cis-biphenyl-2,3-dihydrodiol	NAD	4	p47227
1ae1	tropin	NADP	4	p50162
2ae1	tropin	NADP	4	p50163
dhg1	β-D-glucose	NADP	4	p39482
budc	acetoin	NAD	4	q04520
pgdh	15-hydroxyprostaglandin	NAD	2	p15428
hhea	1,3-dichloro-2-propanol	—	4	jc2292
hheb	1,3-dichloro-2-propanol	—	4	jc2293

Webpage for the 3-D structures: www.rcsb.org/pdb/

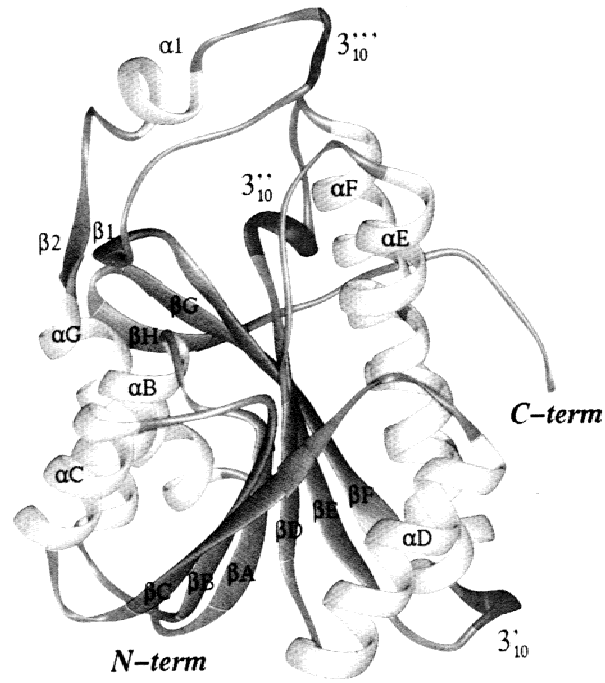

Fig. 5. Ribbons representation of the *D. lebanonensis* ADH monomer with secondary structural elements (Fig. 3 in Benach et al. 1998). Reproduced with permission from the author and printer. α1, β1, and β2 are secondary structural features in the substrate-binding region, discussed more extensively by Benach et al. (1998, 1999).

the 14 sequences with a few exceptions: Gly19, Gly132, Tyr152, Lys156, and Ala167. Gly19 is located in αB and is responsible for cofactor binding. Gly132 is part of βE, but the function of this amino acid has not been established. Tyr152, Lys156, and Ala167 are part of αF, in which Tyr152 and Lys156 are part of the catalytic "triad," Ser139-Tyr152-Lys156 (Tanaka et al. 1996b). The conservation of Ala167 has not yet been described.

The SDR enzymes use NAD(H) or NADP(H) as cofactor and differ in subunit composition. Table 2 shows the subunit composition, substrate, and cofactor used in well characterized short-chain dehydrogenases/reductases, most of them with known 3-D structures. The majority of investigated SDRs are tetramers. Only few are dimers. The tetrameric enzymes have conserved features not observed in the dimers, which may indicate that they are of importance for the tetrameric structures. Two of these will be discussed below.

Aspartate at position 64, located between βC and αD, is well conserved. Tanaka et al. (1996a) showed that this residue binds to the NH_2 group of the cofactor's adenine moiety and that in the 3-D structures of 1CYD, 2HSD and 1AHH, it has equivalent positions. Although this amino acid is almost conserved, it is replaced by a methionine residue in 1DHR and an asparagine residue in 1YBV. An aspartate at position 66 in 1DHR (DADH numbering) cannot bind to the same position of the cofactor because it is located in the bend between βC and αD, directed outwards from the enzyme (Fig. 6). Instead, a glutamine at position 71 (DADH numbering) binds to the same position of the cofactor, as does Asp 64 in the others (Fig. 6). Although Asp64 is not completely conserved, its main function is probably stabilizing the cofactor.

In nine out of twelve SDRs aspartate is found at position 87, with Asn in the other three enzymes. This amino acid is located at the beginning of the short β-strand βD and interacts with the conserved glycine at position 132. This amino acid is located in βE, a strand located between the helices αE and αF. These two helices are important for subunit/subunit interaction in all SDRs. The interaction of Asp87 and Gly132 might be important for the overall enzyme stability and structure. At the other side of this strand, two conserved amino acids, Ala93 and Gly94, are present (Fig. 7) (Ala94 in 1CYD and Ser93 in 1YBV). These might be important in positioning the cofactor. Since these are small amino acids they can fit better in the "bend" of the cofactor (Fig. 7).

A conserved alanine residue at position 167, located at the end of helix αF, is present in all SDRs (Fig. 4), but no attention has been given to this conserved amino acid yet. Superpositioning of this alanine residue shows that it interacts with two amino acids located at position 238 and 239 in the loop between βG and βH.

The two most important helices in all SDRs are helices αE and αF, which play an important role in subunit interaction and enzyme catalysis. Sequence alignment of helix αE (Fig. 4) shows that asparagine at position 112 is conserved in nine enzymes, but not in DADH, 1DHR and PGDH. Superpositioning of structures of SDRs with known 3-D structures showed that Asn112 interacts with the residue at position 155 (Thr, Ala or Ser) located in αF (results not shown). This might indicate that interaction of these two residues is necessary for bringing αE closer

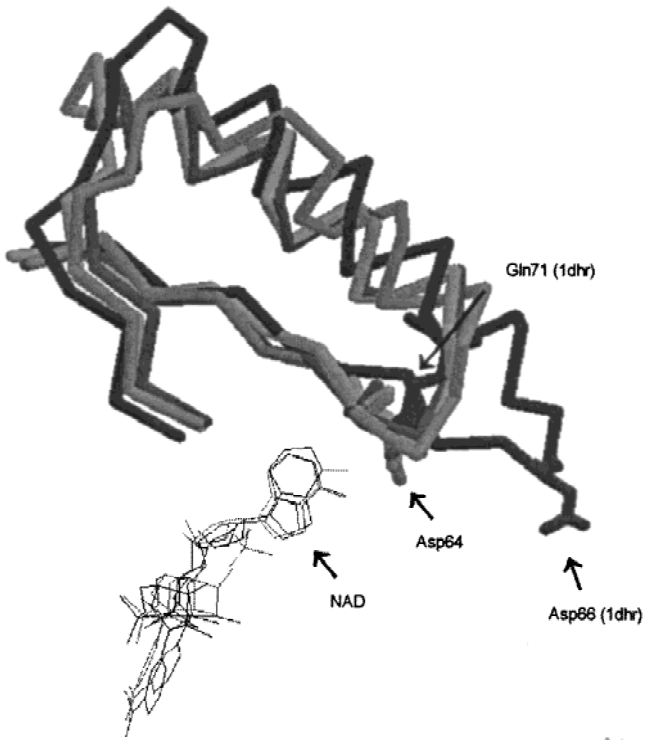

Fig. 6. Representation of βC, αD, and the region between both secondary structure elements, including Asp64, of four short-chain dehydrogenases/reductases. The enzymes shown are dihydropteridine reductase (1DHR), carbonyl reductase (1CYD), 7α-hydroxysteroid dehydrogenase (1AHH), and *Drosophila* alcohol dehydrogenase (1A4U). The positions of Asp64, Asp66 (1DHR), Gln71 (1DHR), and bound NAD^+ molecules in the complexes are shown. The structures were superimposed with the program O (Jones et al. 1991) and created using the program RASMOL.

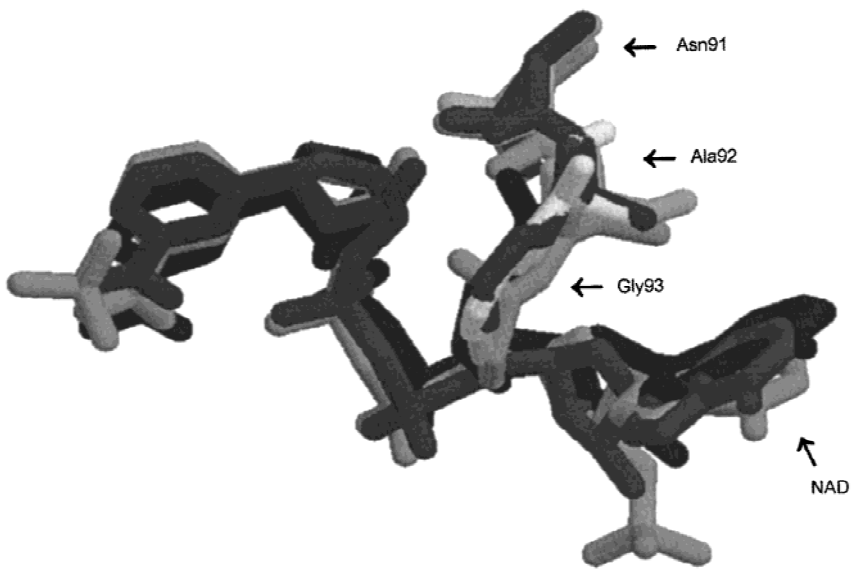

Fig. 7. Representation of the highly-conserved residues Asn91, Ala93, and Gly94, located at the end of βD, and their interaction with the cofactor. The enzymes shown are dihydropteridine reductase (1DHR), carbonyl reductase (1CYD), 7α-hydroxysteroid dehydrogenase (1AHH), trihydroxynaphtalene dehydrogenase (1YBV), and *Drosophila* alcohol dehydrogenase (1A4U). The positions of Asn91, Ala93, and Gly94, and bound NAD^+ molecules in the complexes are shown. The structures were superimposed with the program O (Jones et al. 1991) are created using the program RASMOL.

to αF, resulting in better subunit/subunit interactions. The three enzymes without this Asn are dimeric enzymes, while all the others are tetramers (Table 2). The folding of four subunits into an active enzyme is more restricted in tetramers than in dimers. This could be why Asn112 is conserved in the tetramers.

Most of the aligned enzymes have glycines at position 245 and 246 (Fig. 4), but they are not present in 1A4U (Ile and Glu), 1DHR (Thr and Thr), 1CYD (Ala and Gly), and PGDH (Gly and Ile). They are also not present in BUDC, as this enzyme has a much shorter C-terminus. In many enzymes both glycine residues are preceded by an aspartate. When present, the glycine at position 245 interacts with Pro211 at the C-terminal side of the substrate-binding loop. The glycine residue at position 246 interacts with the amino acid at position 212 (Tanaka et al. 1996b). The two conserved glycine residues, in combination with amino acids located at position 211 and 212, have not been discussed before. It is striking that this structural feature is, again, only present in enzymes forming tetramers and not in dimeric ones.

The last line of Fig. 4 shows the amino acid sequences of two halohydrin hydrogen-halide-lyase (HHEA and HHEB) (Yu et al. 1994), which are dehalogenases and not short-chain dehydrogenases/reductases. Yu et al. (1994) have published the sequences of these two en-

zymes, which have a low, but significant, sequence similarity, except for the N-terminal regions. These authors also observed significant homology of these two sequences with those of several members of the SDRs in the carboxyl terminal region (that of secondary structural elements αG, βG and βH if Fig. 4). It is obvious from our alignment that this similarity extends over a larger region of the structure, including secondary structure regions βD, βE, αF, and βF. The presence of the catalytic "triad" residues of SDRs is very striking: Ser139, Tyr152, and a basic arginine, instead of Lys156, in the HHEA and HHEB enzymes. The region of αE shows little similarity, but helical structures are still predicted.

Several of the other conserved SDRs features discussed earlier are also present in HHEA and HHEB, such as Asp87, Gly132 (Ala in HHEA), and Ala167 (Gly in HHEB). Yu et al. (1994) present evidence that HHEA and HHEB may be tetrameric enzymes. Typical residues for tetrameric SDRs, like Asn92 and the two glycines at position 245 and 246, are also present in HHEA and HHEB, while also proline at position 211 with glycine 245 and an aliphatic hydrophobic residue at position 212, with which glycine 246 interact, are conserved. But Asn 112, located in αE and conserved in tetrameric SRDs, is replaced by Leu and Ile in HHEA and HHEB.

As already mentioned by Yu et al. (1994), HHEA and HHEB do not show sequence similarity in the first 80 residues. However, there are many identities between HHEA and SDRs in the NAD^+ binding Rossman fold (βA, αB and βB). NAD^+ binding in dehydrogenases/reductases requires the presence of several residues with short-side chains, preferably Gly or Ala, in order to have room for accommodating the adenine moiety of NAD^+ with the consensus sequence Gly/Ala-Gly/Ala-X-X-Gly/Ala-X-Gly at positions 13–19. The sequence of HHEA at these positions is His-Ala-Arg-His-Phe-Ala-Gly. Model building shows that the large side-chains of these amino acid residues fill the room available for binding the adenine moiety in SDRs rather completely. Also the conserved Ala-Gly sequence (93–94), which is important for positioning the cofactor has been replaced by residues with larger side chains in HHEA and HHEB. Residues at positions 182, 183, and 187, which are also involved in binding the cofactor, are not conserved (Chenevert et al. 1995; Smilda et al. 1998b; Benach et al. 1999). These observations lead to the hypothesis that HHEA and HHEB have descended from a short-chain dehydrogenase/reductase, but have lost the capability to bind NAD^+. The enzymic reaction mechanism may have similarities with those of the SDRs.

Conclusions

These studies show that for a better understanding of the influence of selection on alcohol dehydrogenase variants in natural *Drosophila* populations it is important to know more about the properties and structural features of the enzyme. The recent elucidation of the X-ray structures of the enzyme and its binary and ternary complexes (Benach et al. 1998, 1999) is the essential first step for attaining this goal. But, it is also evident that a comprehensive overview is still lacking. The fact that all SDRs are either dimers or tetramers indicate that the quaternary structure of the enzyme is of functional importance, although kinetic studies do not show cooperativity. The observation that heterotetramers of *Drosophila* alcohol dehydrogenase differ in properties from homotetramers (Smilda et al. 1998a) also indicate the necessity of separate functional studies on alleloenzymes in heterozygotes in population genetics.

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