The Catalytic Reaction and Inhibition Mechanism of Drosophila Alcohol Dehydrogenase: Observation of an Enzyme-bound NAD-ketone Adduct at 1.4 Å Resolution by X-ray Crystallography

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Drosophila alcohol dehydrogenase (DADH) is an NAD⁺-dependent enzyme that catalyzes the oxidation of alcohols to aldehydes/ketones. DADH is the member of the short-chain dehydrogenases/reductases family (SDR) for which the largest amount of biochemical data has been gathered during the last three decades. The crystal structures of one binary form (NAD⁺) and three ternary complexes with NAD⁺-acetone, NAD⁺-3-pentanone and NAD⁺-cyclohexanone were solved at 2.4, 2.2, 1.4 and 1.6 Å resolution, respectively. From the molecular interactions observed, the reaction mechanism could be inferred. The structure of DADH undergoes a conformational change in order to bind the coenzyme. Furthermore, upon binding of the ketone, a region that was disordered in the apo form (186-191) gets stabilized and closes the active site cavity by creating either a small helix (NAD⁺-acetone, NAD⁺-3-pentanone) or an ordered loop (NAD⁺-cyclohexanone). The active site pocket comprises a hydrophobic bifurcated cavity which explains why the enzyme is more efficient in oxidizing secondary aliphatic alcohols (preferably R form) than primary ones. Difference Fourier maps showed that the ketone inhibitor molecule has undergone a covalent reaction with the coenzyme in all three ternary complexes. Due to the presence of the positively charged ring of the coenzyme (NAD⁺) and the residue Lys155, the amino acid Tyr151 is in its deprotonated (tyrosinate) state at physiological pH. Tyr151 can subtract a proton from the enolic form of the ketone and catalyze a nucleophilic attack of the Cα atom to the C4 position of the coenzyme creating an NAD-ketone adduct. The binding of these NAD-ketone adducts to DADH accounts for the inactivation of the enzyme. The catalytic reaction proceeds in a similar way, involving the same amino acids as in the formation of the NAD-ketone adduct. The pKₐ value of 9-9.5 obtained by kinetic measurements on apo DADH can be assigned to a protonated Tyr151 which is converted to an unprotonated tyrosinate (pKₐ 7.6) by the influence of the positively charged nicotinamide ring in the binary enzyme-NAD⁺ form. pH independence during the release of NADH from the binary complex enzyme-NADH can be explained by either a lack of electrostatic interaction between the coenzyme and Tyr151 or an apparent pKₐ value for this residue higher than 10.0.

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Keywords: Drosophila alcohol dehydrogenase; crystal structure; NAD-ketone adduct; inhibition; catalytic reaction mechanism

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Abbreviations used: NC, non-crystallographic; SDR, short-chain dehydrogenases/reductases; Vₘ, maximum velocity; rms, root-mean-square; ADH, alcohol dehydrogenase; DADH, Drosophila alcohol dehydrogenase; 3α,20β-HSDH, Streptomyces hydrogenans 3α,20β-hydroxysteroid dehydrogenase; 7α-HSDH, Escherichia coli 7α-hydroxysteroid dehydrogenase; DHPR, rat liver dihydropyridine reductase; mSr, sepiapterin reductase; BIN, DADH binary complex with NAD⁺; ADA, DADH ternary complex with NAD⁺ and acetone; ADO, DADH ternary complex with NAD⁺ and 3-pentanone; ADC, DADH ternary complex with NAD⁺ and cyclohexanone; DTT, dithiothreitol.

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Introduction

Drosophila alcohol dehydrogenase (EC 1.1.1.1) is an NAD(H)-dependent oxidoreductase that oxidizes alcohols to aldehydes/ketones, both for detoxification and metabolic purposes. The active enzyme is a dimer of two identical subunits, each with a molecular mass of 27,400 Da (Thatcher, 1980; Benyajati et al., 1981; Chenevert et al., 1995). DADH belongs to the short-chain dehydrogenases/reductases (SDR) family of enzymes (Persson et al., 1991; Jörnvall et al., 1995) with its typical α/β single domain (Ghosh et al., 1991, 1994a) comprising seven parallel β-strands with three α-helices on each side and a loop region that gives specificity to the enzyme by forming the active site pocket. Drosophila ADH is the only SDR structure in which the C terminus is involved in subunit interactions and active site closure (Benach et al., 1998). Three well conserved residues among SDR enzymes (the triad Ser138, Tyr151 and Lys155 in DADH) are known to be involved in the catalytic reaction, based on mutagenesis (Albalat et al., 1992; Chen et al., 1993; Swanson & Frey, 1993; Cols et al., 1993, 1997; Oppermann et al., 1997; Nakajin et al., 1998) and X-ray analysis and are involved in the catalytic reaction (Ghosh et al., 1994a,b; Jörnvall et al., 1995; Tanaka et al., 1996a,b; Auerbach et al., 1997; Hülsmeyer et al., 1998; Tanabe et al., 1998). Tyr151 is the most conserved residue in SDR and probably acts as a general base in the catalytic reaction. Lys155 has a dual role: fixation of the nicotinamide ring of the coenzyme and lowering of the pKₐ value of Tyr151. Finally, Ser138 is also conserved in the SDR family (only in DHPR, Ala129 aligns with Ser138) and assists the proper positioning of the hydroxyl or carbonyl group of the substrate.

A very exhaustive biochemical work and analysis of kinetic data has been performed on DADH during the last years (Winberg et al., 1982a,b, 1983, 1985, 1986, 1993; Hovik et al., 1984; Winberg & McKinley-McKee, 1988a,b, 1992, 1994, 1998; McKinley-McKee et al., 1991; Winberg, 1989). In contrast to horse liver ADH (Dalziel & Dickinson, 1966a,b; Tsai, 1968; Eklund et al., 1976a,b), Drosophila ADH shows preference for secondary rather than primary alcohols. For the first ones, the reaction mechanism follows a compulsory ordered pathway with the coenzyme as leading substrate. The rate-limiting step is the release of NADH from the binary enzyme-NADH complex and Vₘ does not depend on the nature of alcohol. DADH has higher preference for the R enantiomeric alcohols rather than the S forms. Branched, cyclic and bicyclic alcohols are also good substrates, but multisubstituted, too large or rigid compounds like sugars, prostaglandins or steroids are not. The oxidation of primary alcohols is controlled by a rate-limiting step that involves at least ternary complex interconversion and NADH release with Vₘ depending on the type of alcohol. It has been shown that primary alcohols including ethanol bind to one specific region of the active site cavity of the enzyme. Substrate activation with secondary alcohols has been observed in DADH (Hovik et al., 1984; Winberg et al., 1982a, 1986; T. Smilda et al., unpublished results). As in horse liver alcohol dehydrogenase, this might occur via formation of an alcohol-NADH-enzyme complex that dissociates faster than the NADH-enzyme complex (Dalziel & Dickinson, 1966b). Kinetic studies have proposed pKₐ values for enzyme-linked groups in the different steps of the reaction. In the apo form, two groups (one with a pKₐ of 6.5 and another with pKₐ 9.0) influence the rate constant for NAD⁺ binding, whereas only a single group affects the binding of the alcohol substrate or competitive inhibitors with an apparent pKₐ of 7.6. Release of NADH does not depend on the pH (Winberg & McKinley-McKee, 1988b). Concerning the three isoforms already described for DADH, Adh-5 (the native form) can be transformed to the more electronegative and less active Adh-3 and Adh-1 isoforms by exposing the enzyme to NAD⁺ and a ketone. These are supposed to contain different amounts of a ketone-NAD⁺ adduct that blocks the entrance of the active site and therefore inhibits the enzyme in solution (Day et al., 1974; Elliott & Knopp, 1975; Heinstra et al., 1986; Winberg & McKinley-McKee, 1988a), and in vivo (González-Duarte & Atrian, 1986).

From the crystallographic analysis of four ADH complexes, we propose a reaction mechanism for Drosophila lebanonensis ADH, which can be extended to the other species of Drosophila, since all the essential residues for function reported for this species appear fully conserved throughout the genus (Atrian et al., 1998).

Results and Discussion

Overall structure and quality of the models

D. lebanonensis alcohol dehydrogenase exists as a dimer in solution and in the crystal. We have previously reported (Benach et al., 1998) that the subunit shows the typical fold characteristic for the short-chain dehydrogenases/reductases family (Persson et al., 1991; Krook, 1993; Jörnvall et al., 1995) and is similar to those whose three-dimensional structure have been solved so far (Ghosh et al., 1991, 1994a,b; Bauer et al., 1992; Varughese et al., 1992; Su et al., 1993; Azzi et al., 1996; Breton et al., 1996; Tanaka et al., 1996a,b; Thoden et al., 1996; Andersson et al., 1996; Auerbach et al., 1997; Hülsmeyer et al., 1998; Mazza et al., 1998; Nakajima et al., 1998). As shown in Figure 1, the subunit of DADH has an α/β single domain structure with a characteristic dinucleotide binding motif called a Rossmann fold (βα-βα-ββ-α-β-βα-β-α-β). It consists of a central β-sheet flanked on each side by three parallel α-helices. The motif βD-αE-βE-αF-βF-αL-αD-αG-βG, together with the adjoining loops is responsible for quaternary association and substrate binding.
The crystal structure of the binary form (E·NAD$^+$) (BIN) and three ternary forms of DADH (E·NAD$^+$·acetone (ADA), E·NAD$^+$·3-pentanone (ADO) and E·NAD$^+$·cyclohexanone (ADC)) were solved by molecular replacement at a resolution of 2.4, 2.2, 1.4 and 1.6 Å, respectively. The refined crystal structure of the apo form of Drosophila alcohol dehydrogenase at 1.9 Å (Benach et al., 1998) was used as a search model. Tables 1 and 2 summarize data collection and the results of crystallographic refinement.

The crystal asymmetric unit contains a homodimer and the two subunits are related by a local non-crystallographic symmetry in those cases where the protein crystals belong to space group $P_{21}$ (BIN, ADA, ADO). The ternary complex formed with cyclohexanone crystallized in $P_{32}2_1$ and a typical DADH dimer is formed by a crystallographic 2-fold axis (parallel with the $x$-axis and at a height of 1/6 relative to the $z$-axis). Models were refined to crystallographic $R$-factors of 21.3 % (BIN), 19.8 % (ADA), 18.4 % (ADO) and 19.0 % (ADC) and final free $R$-factors were: 24.3 % (BIN), 25.8 % (ADA), 21.3 % (ADO), 21.2 % (ADC). The rms deviations of the models from ideal geometry range from 0.013 to 0.008 Å for bond lengths and about 2$^\circ$ for bond angles (see Table 2). Ramachandran plots of the main-chain conformational angles show that about 90 % of the residues lie within the most favored regions and about 10 % in additionally allowed regions. Like in the apo form of DADH, only one residue, Lys83, lies in the disallowed region. The averaged temperature factor of the refined models agrees well with the statistically estimated $B$-factor obtained from a Wilson plot (Wilson, 1949). The rms deviations between the aligned residues in the two subunits are 0.05 Å (BIN), 0.16 Å (ADA), 0.32 Å (ADO) for $C_a$ and 0.08 Å (BIN), 0.31 Å (ADA), 0.55 Å (ADO) for all atoms. The mean errors in the atomic coordinates, estimated from a Luzzati plot by using the

Table 1. X-ray data collection statistics for binary complex and ternary complex data sets

<table>
<thead>
<tr>
<th>Name/code coenzyme/inhibitor</th>
<th>Binary BIN NAD$^+$</th>
<th>Ternary ADA acetone NAD$^+$</th>
<th>Ternary ADO 3-pentanone NAD$^+$</th>
<th>Ternary ADC cyclohexanone NAD$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell parameters: $a$ (Å)</td>
<td>65.6</td>
<td>66.7</td>
<td>66.8</td>
<td>54.1</td>
</tr>
<tr>
<td>$b$ (Å)</td>
<td>55.4</td>
<td>53.4</td>
<td>53.0</td>
<td>54.1</td>
</tr>
<tr>
<td>$c$ (Å)</td>
<td>70.0</td>
<td>70.4</td>
<td>70.5</td>
<td>168.8</td>
</tr>
<tr>
<td>$\beta$ (deg.)</td>
<td>107.0</td>
<td>107.1</td>
<td>107.2</td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>$P_2_1$</td>
<td>$P_2_1$</td>
<td>$P_2_1$</td>
<td>$P_3_{21}$</td>
</tr>
<tr>
<td>Total reflections</td>
<td>98,390</td>
<td>108,862</td>
<td>103,695</td>
<td>565,296</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>18,139</td>
<td>25,664</td>
<td>103,695</td>
<td>40,649</td>
</tr>
<tr>
<td>Completeness: % (range, Å)</td>
<td>99 (25-2.4)</td>
<td>100 (99-2.16)</td>
<td>93.8 (15-1.32)$^b$</td>
<td>99 (20-1.57)</td>
</tr>
<tr>
<td>last shell</td>
<td>98 (2.49-2.40)</td>
<td>100 (2.24-2.16)</td>
<td>93.7 (1.43-1.40)</td>
<td>91 (1.63-1.57)</td>
</tr>
<tr>
<td>$R$-merge: % (range, Å)</td>
<td>10.6 (25-2.4)</td>
<td>8.6 (99-2.16)</td>
<td>6.0 (15-1.32)</td>
<td>5.7 (20-1.57)</td>
</tr>
<tr>
<td>last shell</td>
<td>27.0 (2.49-2.40)</td>
<td>26.5 (2.24-2.16)</td>
<td>40 (1.43-1.40)</td>
<td>11.3 (1.63-1.57)</td>
</tr>
<tr>
<td>$I/\sigma &gt; 3$: % (range, Å)</td>
<td>83 (25-2.4)</td>
<td>78 (99-2.16)</td>
<td>70 (15-1.32)</td>
<td>97 (20-1.57)</td>
</tr>
<tr>
<td>last shell</td>
<td>66 (2.49-2.40)</td>
<td>57 (2.24-2.16)</td>
<td>42 (1.43-1.40)</td>
<td>93 (1.63-1.57)</td>
</tr>
<tr>
<td>Redundancy $&gt; 3$: % (range, Å)</td>
<td>96 (25-2.4)</td>
<td>96 (99-2.16)</td>
<td>67 (15-1.32)</td>
<td>97 (20-1.57)</td>
</tr>
<tr>
<td>last shell</td>
<td>95 (2.49-2.40)</td>
<td>93 (2.24-2.16)</td>
<td>63 (1.35-1.32)</td>
<td>99 (1.63-1.57)</td>
</tr>
<tr>
<td>Source</td>
<td>CuK$_\alpha$</td>
<td>CuK$_\alpha$</td>
<td>X31 EMBL, Hamburg</td>
<td>711 Max-lab, Lund</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.5418</td>
<td>1.5418</td>
<td>0.9511</td>
<td>0.9960</td>
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<tr>
<td>Temperature (K)</td>
<td>290</td>
<td>290</td>
<td>100</td>
<td>100</td>
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</table>

Cell parameters of DADH apo form are: $a = 65.3$ Å, $b = 55.8$ Å, $c = 70.0$ Å, $\beta = 107.1^\circ$ (Benach et al., 1998).

$^a$ $R$-merge = $\Sigma_i|\langle T_i \rangle - \langle T_i \rangle|/\Sigma_i \langle T_i \rangle$, where $\langle T_i \rangle$ is the mean intensity of the $i$th observation and $\langle T_i \rangle$ is the mean intensity for that reflection.

$^b$ Only data to 1.4 Å were used.
working set of reflections (Luzzati, 1952) are 0.25 Å (BIN, ADA), 0.15 Å (ADO), 0.16 Å (ADC).

The main peptide chain was generally very well defined in all four models. Coenzyme (NAD\(^+\)) and inhibitor molecules were easily fitted into the electron density maps. In the apo form of DADH the region from Thr186 to Thr191 has not been modeled because it was disordered and the loop region ranging from Gly15 to Leu17 had high B-factors. The binary form of DADH has a better-defined structure for the glycine-rich loop (Gly15 to Leu17) and adopts a slightly different conformation when compared to the apo form, due to the interaction with the pyrophosphate group of the coenzyme. In the binary complex the loop from Thr186 to Thr191 is disordered, allowing the substrate to enter the active site cavity. The overall B-factor of the NAD\(^+\) molecule in the binary form is high (50 Å\(^2\)) but the omit \(F_o - F_c\) maps (using phases from the refined model of the binary complex) calculated at 2.4 Å enabled us to unambiguously model the coenzyme molecule. The less ordered part of the coenzyme molecule is the nicotinamide ring. In the ternary complexes the \(2F_o - F_c\) electron density maps are usually of good quality and exhibit no breaks in the peptide chain. Electron density for the NAD\(^+\) and ketone molecules can be clearly seen and modeled in \(F_o - F_c\) maps. The NAD\(^+\) molecules have an overall B-factor of: 21.9 (ADA), 12.0 (ADO), 10.2 Å\(^2\) (ADC). Due to the presence of the ketone and coenzyme the region from Thr186 to Thr191 gets stabilized as a small helix (ADA or ADO) or an ordered loop (ADC) that covers the active site. The following residues were found to adopt alternative conformations in ADO at 1.4 Å resolution: Lys27(A), Glu40(B), Thr58(B), Glu70(B),

<table>
<thead>
<tr>
<th>Name/code</th>
<th>Binary BIN</th>
<th>Ternary ADA</th>
<th>Ternary ADO</th>
<th>Ternary ADC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bin/Code</td>
<td>NAD(^+)</td>
<td>NAD(H)-acetone</td>
<td>NAD(H)-3-pentanone</td>
<td>NAD(H)-cyclohexanone</td>
</tr>
<tr>
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<td>8.0-2.2</td>
<td>8.0-1.4</td>
<td>8.0-1.6</td>
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<tr>
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<td>3962(2 × 1963)</td>
<td>1002(2 × 50)</td>
<td>1046(2 × 50)</td>
</tr>
<tr>
<td>Coenzyme/adduct</td>
<td>53</td>
<td>131</td>
<td>485</td>
<td>233</td>
</tr>
<tr>
<td>Solvent</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>Dithiothreitol</td>
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<td>-</td>
<td>-</td>
<td>7</td>
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<tr>
<td>Cyclohexanone</td>
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<td>-</td>
<td>-</td>
<td>0.5</td>
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<tr>
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<td>-</td>
<td>0.05</td>
<td>0.5</td>
</tr>
<tr>
<td>NCs restraints (kcal mol(^{-1})Å(^{-2}))</td>
<td>300 (main-chain)</td>
<td>300 (main-chain)</td>
<td>300 (main-chain)</td>
<td>-</td>
</tr>
<tr>
<td>(X-PLOR)</td>
<td>250 (side-chain)</td>
<td>250 (side-chain)</td>
<td>250 (side-chain)</td>
<td>-</td>
</tr>
<tr>
<td>0.00 (resid 38:43)</td>
<td>0.00 (resid 40)</td>
<td>0.00 (resid 50)</td>
<td>-</td>
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<tr>
<td>(REFMAC)</td>
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<td>0.05 (main-chain)</td>
<td>0.05 (all atoms)</td>
<td>-</td>
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<td>R-factor (%)(^a)</td>
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<td>19.8</td>
<td>18.4</td>
<td>19.0</td>
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<td>Free R-factor (%)(^b)</td>
<td>24.3</td>
<td>25.8</td>
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<td>21.2</td>
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<td>rmsd from ideal values:</td>
<td>Bond lengths (Å)</td>
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<td>0.010</td>
<td>0.009</td>
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<td>Bond angles (°)</td>
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<td>1.85</td>
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<td>23.55</td>
<td>23.7</td>
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<td>Improper dihedrals (°)</td>
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<td>2.11</td>
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<td>1.94</td>
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<td>Ramachandran(%)</td>
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<td>89.2</td>
<td>89.6</td>
<td>90.5</td>
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<td>Allowed (disallowed)</td>
<td>10.0(0.4(^d))</td>
<td>9.7(0.4(^d))</td>
<td>9.1(0.4(^d))</td>
<td>10.2(0.4(^d))</td>
</tr>
<tr>
<td>Luzzati coordinate errors (Å)(^i)</td>
<td>0.25(0.30)</td>
<td>0.24(0.23)</td>
<td>0.15(0.17)</td>
<td>0.16(0.18)</td>
</tr>
<tr>
<td>Mean B-values (Å(^2))</td>
<td>Wilson(^h)</td>
<td>18.8</td>
<td>16.5</td>
<td>13.2</td>
</tr>
<tr>
<td>All atoms</td>
<td>26.4(54.6(^i))</td>
<td>24.9</td>
<td>13.2(^i)</td>
<td>11.3(^i)</td>
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<td>10.3</td>
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<tr>
<td>Subunit B</td>
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<td>25.1</td>
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<td>Solvent</td>
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<td>Coenzyme/adduct</td>
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<td>Dithiothreitol</td>
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<td>Ca(^2+)</td>
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<td>-</td>
<td>7.4</td>
</tr>
</tbody>
</table>

\(^a\) R-factor = Σ|F\(_o\) - F\(_c\)| / Σ|F\(_o\)|.
\(^b\) 10% of reflection data. (Brünger, 1992b)
\(^c\) Residues are: 39-42, 56-56, 76-81, 188-190, 255-255.
\(^d\) Residue K83 in both subunits.
\(^e\) Diffraction data were measured at 100 K.
\(^f\) Calcium ion was found lying on a crystallographic 2-fold axis between two symmetry-related molecules not involved in dimer interaction. Octahedral coordination is achieved by Asp2, Thr4, Wat25, Wat6 and crystallographic mates.
\(^g\) Calculated from 3 Å to maximal resolution.
\(^h\) Calculated from 5 Å to maximal resolution.
\(^i\) N-terminal Met1 is carboxylated.

Table 2. Refinement statistics for the binary and ternary complexes of Drosophila lebanonensis ADH

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Reaction Mechanism of Drosophila Alcohol Dehydrogenase
Arg102(B), Leu119(A), Lys167(A), Arg185(A,B), Lys235(A,B).

Architecture of the active site cavity

In short-chain dehydrogenases/reductases (SDR), the region comprising residues from βF to αG (from Thr186 to Pro210, in DADH) is usually responsible for the specificity of the individual enzymes. This results in reduced sequence homology and a certain degree of conformational flexibility. This region is generally formed by one or several helices or/and loop regions of variable length. It is often partly disordered or has higher B-factors than the rest of the structure (Ghosh et al., 1994a,b, 1995) and changes its conformation to accommodate the substrate (Tanaka et al., 1996a,b; Andersson et al., 1996, 1997).

In the apo form of DADH the active site pocket is formed by helix α2 (residues 202 to 206), the C terminus of the other subunit (250–254) and a disordered part (186-191). In the ternary complex formed with NAD⁺ and acetone or 3-pentanone, the loop region from Thr186 to Thr191 acts as a gate (disordered when the enzyme is in its apo and binary form) and closes the active site by forming a small helix (α1) and a non-polar cavity (see Figures 1 and 2(a)). Drosophila alcohol dehydrogenase in contrast to horse or yeast ADH shows higher affinity with secondary alcohols than with primary ones. During the past years a large amount of kinetic data using different kinds of enantiomeric alcohols has precisely suggested a topology for the Drosophila ADH active site cavity (Hovik et al., 1984; Winberg et al., 1982a, 1986, 1993; Smilda, 1997; T. Smilda et al., unpublished results). By inspecting the three-dimensional crystal structures of the DADH complexes with acetone or 3-pentanone and NAD⁺ (at 2.2 and 1.4 Å, respectively) the active site cavity can be divided in two regions of different size, which we have called R1 and R2 (see Figure 3). The region R1 is extended (the longest distance in this cavity is about 10 Å) and can be further divided into two smaller sub-regions R1(a) and R1(b); R1(a) is formed in the space between the side-chains of residues Leu206, Leu95, Phe192, Ile145, Val148 and Val202. The smaller cavity R1(b) is near the coen-

![Figure 2](image_url). Arrangement of amino acid side-chains at the active site entrance (Thr186:Phe192) in the ternary complex with NAD⁺/3-pentanone (a) and NAD⁺/cyclohexanone (b). Note that residues 187-191 form a one-turn helix in the ternary complex shown in (a). RIBBONS (Carson, 1987) was used to create this Figure.
zyme (O7N), the side-chain of residues Ile183 and Val189. The R2 region is smaller but slightly wider than R1 (longest distance is 3 Å) and is formed by the side-chain of residues Val139 (CG2), Thr140 (CG2), Pro181(O), Gly182 (O, CA), Ser138 (OG), Ile183 (N), NAD (C5N) and Trp250(CH2) (from the other subunit). A dividing wall is formed between R1 and R2 by the side-chains of residues Ile145 and Ile183. Approximate volumes of the sub-cavities R1(R1a, R1b) and R2 are 115 Å³ (96 Å³, 19 Å³) and 85 Å³, respectively. Truncation of the C terminus by site-directed mutagenesis (like: Ile254Stop, His249Stop, Ile244Stop, Ala243-Stop) affects the enzymatic activity (Albalat et al., 1995). The latter experimental data have also shown that the reaction with primary alcohols is more markedly affected than with secondary ones.

In the ternary complex formed with NAD⁺ and cyclohexanone (ADC) solved at 1.6 Å resolution, the disordered loop (residues 186-191) does not form a helix but instead an ordered loop that only partially covers the active site (see Figure 2(b)). In this complex we found that a second molecule of cyclohexanone was trapped between this loop and residues Gly93 and Leu95. The aliphatic region of this cyclohexanone molecule points to the interior of the active site cavity and the side-chain of residue His190. The carbonyl group interacts with the atom O₂⁺ of the nicotinamide ribose moiety of the coenzyme. On the other side of this loop we found a cyclic molecule of oxidized DTT (a concentration of 10 mM was added to the crystallization solution to ensure a reducing medium), the hydroxyl groups of the DTT molecule interact with residues His190 (ND1) and Thr184 (O) and the two sulfur atoms are close to the side-chains of residues Leu206 and Ile183. Both cyclohexanone molecules are in the chair conformation. The hydrophobic active site cavity in this case is similar to the other ternary complexes studied. Side-chains of the residues Leu188 and Val189 are substituted by the aliphatic groups of the extra molecules of cyclohexanone and DTT, respectively. The very high-resolution data obtained at 1.4 Å for the ternary complex of NAD⁺ and 3-pentanone (ADO) shows that the ketone has reacted covalently with the coenzyme. From high resolution $F_o - F_c$ omit maps a distance of approximately 1.5 Å was found between the C² atom of the ketone and the C-4 atom of the nicotinamide ring of the coenzyme (see Figure 4). We observed also a distortion in the planarity of the nicotinamide ring. A similar situation was found in the active sites of the ternary complexes formed with NAD⁺ and acetone or cyclohexanone (ADA, ADC; see Figure 5). The latter results and already published data (Di Sabato, 1970; Winberg & McKinley-McKee, 1988a; T. Smilda et al., unpublished results) suggested that the ketone molecule has reacted with the coenzyme in the active site of DADH and has formed a covalent ketone-NAD adduct, which represents the first case of coenzyme-inhibitor adduct formation in SDR enzymes studied by protein crystallography (see Inhibition mechanism).

By studying the 2.4 Å omit maps of the binary form of DADH we have observed that the active site loop (186-191) is still disordered, only the residue Thr186 gets partially stabilized by the presence of the coenzyme (distance OG1 to O1N: 2.6 Å) and the aromatic ring of Phe192 changes its position by twisting the ring towards the active site. The average B-factor for this region is 54.6 Å². In the interior of the active site cavity we found a bound water molecule in between the hydroxyl groups of Tyr151 and Ser138 (distances: 2.6 and 3.4 Å, respectively) and the nicotinamide moiety of the

![Figure 3. Stereoview of the active site cavity of DADH; main residues lining the two different active site sub-cavities (R1a, R1b, R2) are plotted. The accessible surface was calculated with a 1.4 Å probe (dotted surface). Figure produced with RIBBONS (Carson, 1987).](image-url)
Figure 4. (a) Stereoview of the initial difference Fourier omit map ($F_o - F_c$) at 1.4 Å resolution and contoured at a $1\sigma$ level. (b) Enlarged view of the same map showing the relative positions of the NAD and 3-pentanone moieties of the NAD$^+$/3-pentanone adduct. Picture created with RIBBONS (Carson, 1987).
Coenzyme binding mode

The NAD\(^+\) molecule in the binary form of DADH and the NAD part of the ketone-NAD adduct complex bind to the enzyme in a similar way. The omit difference maps calculated at the highest resolution always gave a very clear and continuous density for the coenzyme and coenzyme adducts (see Figure 4(a)). The nicotinamide part of NAD\(^+\) is buried in the active site cavity having the side-chain of Leu188 as an anchor in between the ribose moiety and the nicotinamide ring. The adenine moiety of NAD\(^+\) is bound sandwiched between the side-chains of Arg38, Asp63, Val64, Ala92 and Ile106. The bound NAD\(^+\) molecule is in an extended conformation with the adenine ring in the anti and the nicotinamide ring in the syn conformation, similar to other SDR crystal structures. The distance between C6 of the adenine and C2 of the nicotinamide is within 13.7 Å (binary form) and 14.0 Å (ternary forms) close to the values of 14.2 Å for mSr, 14.4 Å for 7α-HSDH, 14.6 Å for 3α,20β-HSDH. The syn conformation of the nicotinamide ring suggests a B-face 4-pro-S hydride transfer reaction in contrast to liver alcohol dehydrogenase where the hydride is transferred from the alcohol to the coenzyme through the A-face. Both ribose rings reveal a C2\(^\text{endo}\) puckering.

Interactions between the coenzyme/coenzyme adduct and DADH are shown in Figure 6 and Table 3. The region ranging from Gly15 to Leu17, that was slightly disordered in the apo form, changes its conformation and gets stabilized upon coenzyme binding; structural changes are more pronounced in the ternary complexes than in the binary. This conformational change possibly improves the interaction between the pyrophosphate moiety of the NAD\(^+\)/NAD-adducts and the main-chain nitrogen atoms of the glycine loop (residues Gly15 to Ile17). The carbonyl group of Gly15 changes its conformation from forming a H-bond with the nitrogen atom of residue Gly18 (apo) to residue Leu19 (binary and ternary complexes). In *Drosophila* ADH, this glycine-rich loop shows a certain degree of flexibility to interact with the pyrophosphate moiety of the coenzyme; mutations like Gly13Val or Gly13Asn inactivate the enzyme (Thatcher, 1980; Chen *et al.*, 1990) and mutant Gly13Ala retains only 69% of wild-type activity (Chen *et al.*, 1990). On the other hand, restoring the glycyl residue in *D. lebanonensis* at position 13 (mutant Ala13Gly) increases the activity by 168% (Albalat *et al.*, 1994).

Residue Asp37 forms H-bonds with the O2\(^−\) and the O3\(^−\) hydroxyl groups of the adenine ribose moiety of the coenzyme. This position in the enzyme has been assumed to regulate the specificity of SDR enzymes towards NAD\(^+\) or NADP\(^+\) (Tanaka *et al.*, 1996a). In *Drosophila* ADH an aspartate at position 37 favors the use of NAD\(^+\) during the enzymatic reaction. Chen *et al.* (1991) showed that changing the charged residue Asp in position 37...
by a polar residue like Asn enables the enzyme to use either NAD$^+$ or NADP$^+$ for the oxidation of alcohols. Inclusion of a relatively large hydrophobic residue (Asp37Ile) resulted in an inactive enzyme. Less direct mutations like replacement of Ala44 by a positively charged residue (e.g. Arg) increases cofactor specificity for NADP$^+$ (Chen et al., 1994). It is observed for the first time in short-chain dehydrogenases/reductases, that substitution of an amino acid that is not directly in contact with the coenzyme affects the affinity via coulombic attraction/repulsion. See Figure 6 for a description of the amino acids involved in the binding of the coenzyme.

Several water molecules are found between the NAD$^+$ molecule and the enzyme; all of them are present also in the ternary forms suggesting that they might be of structural importance. Wat49 in ADO forms hydrogen bonds with the atom O2N of the coenzyme molecule and N Gly18 (distances 2.7 and 2.8 Å, respectively). Wat23 is 2.8 Å away from O Gly91, 2.8 Å from O Ala12 and 2.7 Å from the previous water. Wat63 interacts with O Gly93 and O1A NAD$^+$ (2.9 and 2.7 Å, respectively; see Table 3).

Inhibition mechanism

By adding either secondary alcohols and ketones to the flies’ food/agar or incubating pure or crude enzyme extracts with high concentrations of NAD$^+$ and a ketone, the native form Adh-5 can be con-

Table 3. Distances between the 3-pentanone-NAD adduct and protein residues in ADO

<table>
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<tr>
<th>NAD-adduct</th>
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<th>Atom</th>
<th>$d$(Å)</th>
<th>NAD-adduct</th>
<th>Residue</th>
<th>Atom</th>
<th>$d$(Å)</th>
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<td>OG1</td>
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</table>

Distances were found by using the program XPLOR (Brünger, 1992a); only oxygen or nitrogen atoms are printed. Some NAD-adduct distances referred in the text are in bold.

ADO; ternary complex formed with 3-pentanone-NAD adduct and the enzyme at 1.4 Å resolution.
verted into more electro-negative and inactive isoforms: Adh-3 and Adh-1 (Day et al., 1974; Elliott & Knopp, 1975; Heinstra et al., 1986; González-Duarte & Atrian, 1986; Winberg & McKinley-McKee, 1988a). Flies subjected to prolonged starvation also exhibit Adh-isozyme conversion (Heinstra et al., 1986). The conversion is specific for NAD, since no conversion is observed with NADH, NADP or NADPH. It has been shown that neither pure NAD nor acetone alone could convert Adh-5 into Adh-3 or Adh-1 (Winberg et al., 1983). The appearance of these electrophoretically different DADH isoforms has been postulated to be caused by conformational changes induced by the coenzyme/ketone or the formation of a complex between enzyme and a ketone-NAD adduct (Winberg & McKinley-McKee, 1988a; T. Smilda et al., unpublished results). DADH-isozyme formation and enzyme inactivation might be regarded as a buffering system to bind and detoxify low levels of potent toxic keto-compounds (Heinstra et al., 1986).

The mechanism of conversion has been described (Winberg & McKinley-McKee, 1988a) as follows: after formation of a reversible ternary enzyme-NAD-ketone complex (Adh-3) an irreversible process occurs and the enzyme-NAD-ketone complex (Adh-1) appears. A difference in between the absorption peaks in UV spectra measured for the mixtures of NAD-enzyme (340 nm) and NAD-acetone-enzyme (228 nm) indicates that the nicotinamide moiety of the enzyme no longer shares the same environment (Winberg et al., 1983, 1986; Winberg & McKinley-McKee, 1988a). The appearance of these electrophoretically different DADH isoforms has been postulated to be caused by conformational changes induced by the coenzyme/ketone or the formation of a complex between enzyme and a ketone-NAD adduct (Winberg & McKinley-McKee, 1988a; T. Smilda et al., unpublished results). DADH-isozyme formation and enzyme inactivation might be regarded as a buffering system to bind and detoxify low levels of potent toxic keto-compounds (Heinstra et al., 1986). The mechanism of conversion has been described (Winberg & McKinley-McKee, 1988a) as follows: after formation of a reversible ternary enzyme-NAD-ketone complex (Adh-3) an irreversible process occurs and the enzyme-NAD-ketone complex (Adh-1) appears. A difference in between the absorption peaks in UV spectra measured for the mixtures of NAD-enzyme (340 nm) and NAD-acetone-enzyme (228 nm) indicates that the nicotinamide moiety of the enzyme no longer shares the same environment (Winberg et al., 1983, 1986; Winberg & McKinley-McKee, 1988a). The difference might be due to a covalently bound ketone or/and by a non-polar surrounding.

The high-resolution data available for the three ternary complexes (ADA, ADO, ADC) indicated that such NAD-ketone adducts were indeed formed in the active site of DADH (see Figures 4 and 5). Aldehydes and ketones exist in solution as equilibrium mixtures of two isomeric forms, the keto and the enol-form. The concentration of the enolic form is usually very small (acetone, $K_{eq} = 1.5 \times 10^{-7}$; cyclohexanone, $K_{eq} = 5 \times 10^{-5}$). When the enolic form of a ketone enters the active site of the DADH-NAD complex, residues Ser138 and Tyr151 probably orient the hydroxyl group of the enol. The bifurcated active site pocket of DADH optimally positions the secondary enol molecule and a proton transfer from the enol to the deprotonated hydroxyl group of Tyr151 (see Catalytic mechanism) generates an enolate anion. The enolate ion can be regarded as two resonant structures with a negative charge being delocalized between the oxygen atom and the C atom. One of the resonant structures has a lone electron pair on the C atom that might nucleophilically attack the C-4 position of the NAD nicotinamide ring forming a covalent bond (see Figure 7). A ketone-NAD adduct is then generated in the active site of Drosophila ADH (see Figure 4(b)). The keto-enol equilibrium might be driven towards higher enol concentration due to the formation of this irreversibly bound coenzyme adduct. Ketone/pyruvate-NAD adducts have been studied by Di Sabato (1970) and $^{13}$C-NMR has been used to investigate adduct formation in urocanase (Schubert et al., 1995). Pyruvate adduct reaction catalyzed by lactate dehydrogenase has been characterized by Everse et al. (1971) and Burgner & Ray (1984).

The two aliphatic groups of the ketone moiety of the 3-pentanone-NAD adduct (ADO) interact with the bifurcated active site cavity. The methyl group nearest to the C atom bound to the coenzyme is in the R2 region (nearest residues Pro181, Ser138, Val139 and Ile183) and the ethyl group in R1 (nearest residues Ile145, Leu188, Val189). The oxygen atom of the ketone is in between the hydroxyl...
groups of Ser138 and Tyr151, with a distance of 2.6 and 2.5 Å, respectively. An NAD-acetone adduct was also found in the complex formed with acetone, NAD$^+$ and DADH (ADA). The acetone molecule forms a covalent bond with NAD$^+$ in a similar way to that of the 3-pentanone-NAD(H) adduct DADH complex (see Figure 5). When compared to the other ketone-NAD adduct complexes, the cyclohexanone-NAD DADH complex shows a more pronounced distortion of the planarity of the nicotinamide ring, possibly due to steric interactions in between the two rings. The cyclohexanone ring is in the chair conformation and its aliphatic part interacts with residues Ile145, Thr140, Ile183, Phe192, with a molecule of DTT and another bound cyclohexanone. Surprisingly, the oxygen atom of the ketone moiety of the cyclohexanone-NAD adduct is found in between the side-chain atoms of Ser138 (OG) and Thr140 (CG2) (distances 2.7 and 3.6 Å, respectively). A rearrangement of the cyclohexanone-NAD adduct after its formation in the active site cavity of DADH is likely and explains this peculiar result. Another possibility is that Ser138 or another residue is involved in the formation of the NAD-ketone adduct.

The formation of such adducts in DADH (ADA, ADO) tightly blocks the active site cavity and results in a dead-end enzyme or abortive complex. Winberg & McKinley-McKee (1988a) have proposed that an additional covalent bond formation reaction might occur between the enzyme and the NAD-adduct and therefore explain why such abortive complexes are irreversibly inactive. Our crystallographic data do not, however, favor any further covalent bond between the adduct molecule and the enzyme in any of the crystal structures solved at high resolution (ADA, ADO, ADC). The interaction of the ketone-NAD-adduct with the hydrophobic cavity of the enzyme seems to be strong enough to inactivate the enzyme in solution. Nevertheless, activity of these abortive complexes is regained after separation of the adduct from the enzyme by

---

**Figure 8.** $C^\alpha$ representations (stereo) of the compared structures after least-squares superposition. (a) The apo form of DADH is shown in green and the binary complex in blue, the rms distance is 0.70 Å for 508 $C^\alpha$ atoms; (b) the binary form in blue and the ternary complex (NAD-3-pentanone) in red, the rms distance is 0.39 Å for 508 $C^\alpha$ atoms. O (Jones et al., 1991) was used to create this picture.
native polyacrylamide gel electrophoresis, but still depends on the chemical nature of the ketone. With 3-pentanone, 3-hexanone, 3-heptanone and 3-octanone only little activity is regained (Smilda, 1997; T. Smilda et al., unpublished results) indicating that the interaction with the size of the ketones’ aliphatic moiety is crucial for the inhibition reaction and formation of the abortive complex.

DADH is inhibited by pyrazoles in a competitive fashion, pyrazole ($K_i \approx 4.4 \mu M$), 4-bromo-pyrazole ($K_i \approx 0.8 \mu M$), 4-iodo-pyrazole ($K_i \approx 1.6 \mu M$) (Winberg et al., 1982b). 4-Bromo-pyrazole might be the most effective inhibitor due to the similar van der Waals radius of the bromine group compared to a methyl group. In the metalloenzyme horse liver ADH, pyrazole forms a bridge between the active site Zn$^{2+}$ and C-4 of the positively charged pyridine ring of NAD$^+$ (Theorell & Yonetani, 1963; Eklund et al., 1982). In addition, binding of NADH to the horse liver enzyme is accompanied by a 15 nm blue shift of the 340 nm absorption maximum of the coenzyme (Theorell & Yonetani, 1963). No such shift is observed with the binary NADH complex of Drosophila ADH (Winberg et al., 1982b).

### Catalytic reaction mechanism of Drosophila alcohol dehydrogenase

The formalism of the Theorell-Chance mechanism describes the predominating pathway for the conversion of a wide range of primary and also some secondary alcohols by horse liver alcohol dehydrogenase (Theorell & Chance, 1951; Dalziel & Dickinson, 1966a,b). The same mechanism is also valid for the reaction of Drosophila alcohol dehydrogenase with secondary alcohols (Winberg et al., 1982a, 1986). The Theorell-Chance mechanism represents a compulsory ordered pathway where the ternary complexes are not kinetically significant:

$$E + NAD^+ \leftrightarrow E \cdot NAD^+ + R - OH$$

$$\leftrightarrow E \cdot NAD^+ \cdot R - OH$$

$$\leftrightarrow E \cdot NADH \cdot R = O$$

$$\leftrightarrow R = O + E \cdot NADH$$

$$\leftrightarrow E + NADH$$

In DADH, the coenzyme is the leading substrate and the dissociation of the binary enzyme-NADH complex is the rate-limiting step. For alcohols following such kinetics $V_m$ is constant and independent of the alcohol used. However, with primary alcohols $V_m$ is much lower, varies with the type of alcohol and the rate-limiting step is the interconversion of the ternary complexes, i.e. the catalytic step in between alcohol and aldehyde formation (Hovik et al., 1984; Winberg et al., 1982b; 1986).

By examining the high-resolution crystal structures of the apo form (Benach et al., 1998), binary and ternary complexes of DADH (BIN, ADA, ADO, ADC), the considerable and extensive amount of biochemical data gathered for many ADHs from various Drosophila species (Winberg et al., 1982a,b, 1983, 1985, 1986, 1993; Hovik et al., 1984; Heinstra et al., 1986, 1988, 1989; Winberg & McKinley-McKee, 1988a,b; McKinley-McKee et al., 1991; Albatal et al., 1992; Chen et al., 1993; Cols et al., 1993, 1997) and the available crystallographic data on other members of the short-chain dehydrogenase/reductases family (Ghosh et al., 1994a,b; Jörnvall et al., 1995; Tanaka et al., 1996a,b; Auerbach et al., 1997; Hülsmeyer et al., 1998;
Figure 10. Proposed catalytic reaction mechanism of Drosophila alcohol dehydrogenase.
Tanabe et al., 1998) we can propose a reaction mechanism for Drosophila ADH.

When the binary complex of DADH is formed, the structure undergoes a conformational change (mostly affecting the relative position of helices zB, zC, zD and zG) and at least three water molecules are replaced by binding of the coenzyme (see Figure 8(a)). The coenzyme forms about 11 hydrogen bonds directly with the enzyme and three are mediated by water molecules. The latter conformational change could be related to considerable protection against unfolding that the enzyme experiences in the presence of NAD⁺ (Ribas de Pouplana et al., 1991). The entrance gate to the active site is the region from 186 to 191, disordered in the apo and partially ordered in the binary form. When the alcohol molecule binds to the active site, a water molecule (Wat12) is involved in a hydrogen bond with the Tyr151 residue and the NAD⁺ (Ribas de Pouplana et al., 1991). The entrance gate to the active site is the region from 186 to 191, disordered in the apo and partially ordered in the binary form. When the alcohol molecule binds to the active site, a water molecule (Wat12) is involved in a hydrogen bond with the Tyr151 residue and the NAD⁺ (Ribas de Pouplana et al., 1991). The entrance gate to the active site is the region from 186 to 191, disordered in the apo and partially ordered in the binary form. When the alcohol molecule binds to the active site, a water molecule (Wat12) is involved in a hydrogen bond with the Tyr151 residue and the NAD⁺ (Ribas de Pouplana et al., 1991). The entrance gate to the active site is the region from 186 to 191, disordered in the apo and partially ordered in the binary form. When the alcohol molecule binds to the active site, a water molecule (Wat12) is involved in a hydrogen bond with the Tyr151 residue and the NAD⁺ (Ribas de Pouplana et al., 1991). The entrance gate to the active site is the region from 186 to 191, disordered in the apo and partially ordered in the binary form.

The aliphatic chains of the alcohol interact with the bifurcated and non-polar active site of DADH. The position of the ketone-adducts in the ternary complexes of DADH (ADA, ADO) show that the so-called triad of residues, Ser138, Tyr151 and Lys155, are directly involved in the reaction mechanism (Albalat et al., 1992; Chen et al., 1993; see Figure 9). Once the catalytic cavity has oriented and fixed the alcohol, its hydroxyl group is placed in between the oxygen atoms of the side-chains of Ser138 and Tyr151. The reaction occurs under exclusion of external water molecules, because the active site cavity is closed to the bulk solvent (see Figure 3). A proton can then be easily transferred from the substrate to the enzyme (see Figure 10). Mutations like Ser138Ala or Ser138Cys (Cols et al., 1997) resulted in totally inactive enzymes. Ser138 either orients the alcohol substrate or stabilizes the transition reaction intermediate during the oxidation process, or both. Lys155 has possibly two roles: first, to assist in the proper orientation of the coenzyme by forming hydrogen bonds with the oxygen atoms of the nicotinamide-ribose moiety and second, to lower the pKₐ of the Tyr151 residue through electrostatic interactions. The pKₐ of Tyr151 seems to be strongly influenced also by the positively charged nicotine ring of the NAD⁺ molecule in the binary complex.

**Substrate and stereospecificity**

The bifurcated active site cavity of DADH favors a more efficient oxidation of secondary alcohols in comparison to primary ones. In horse liver alcohol dehydrogenase, the alcohol binding region is a mono-cavity and the enzyme shows higher affinity with primary alcohols (increasing with the length of the aliphatic chain) rather than with secondary ones (Dalziel & Dickinson, 1966a,b; Tsai, 1968; Eklund et al., 1976a,b). The non-polar character of the DADH active site cavity lowers the specificity for multisubstituted alcohols that contain hydroxyl groups or other polar groups in other positions of the molecule like sugars or polyols. The relative rigidity and size of the active site cavity is probably unfavorable for the binding of too large or rigid molecules such as prostaglandins or steroids. Methanol is not a substrate of DADH, its small size allowing many different orientations in the active site cavity may hamper the catalytic reaction. The enzyme shows high affinity towards branched and bicyclic secondary alcohols. This might imply that the enzyme is also involved in other metabolic processes such as monoterpene or phenol metabolism (Winberg et al., 1982a, 1986; Winberg & McKinley-McKee, 1992). However, ethanol is the most abundant alcohol in the natural habitats where the fruit-fly Drosophila is usually found. The higher specificity towards secondary alcohols as compared to ethanol seems surprising. Baker (1996) has proposed that the affinity towards secondary alcohols might be important for Drosophila to catalyze the oxidation of acetaldehyde (structurally similar to secondary alcohols as a gem-diol) to acetate (Henehan et al., 1995; Winberg & McKinley-McKee, 1998). Acetate is later condensed with CoA to form acetyl-CoA. In this way Drosophila can convert ethanol to acetyl-CoA, a compound that is used in many important metabolic processes (Heinstra et al., 1989).

DADH shows higher specificity towards the R enantiomeric secondary alcohols. This is a result of the asymmetric shape of the bifurcated cavity in relation to the position of the catalyzing residues (Tyr151) and the NAD⁺ molecule (see Figure 3). In DADH, the active site cavity can roughly accommodate a total of four aliphatic carbon atoms in sub-cavity R1 and three aliphatic carbon atoms in sub-cavity R2. Biochemical data are in complete agreement with our structural description of the DADH active site, because it shows that the binding strength should be highest with R-(−)-2-hexanol. A decrease in binding energy appears when adding one more methyl group (R-(−)-2-heptanol). A branch at the C3 carbon atom (3-methyl-2-butanol) causes a decrease of the binding strength in relation to the unbranched alcohol (R-(−)-2-butanol) in the R enantiomer, but not in the S form, indicating that the R2 sub-cavity (slightly wider than the R1 sub-cavity) can accept an extra methyl group without affecting the binding of the alcohol (Winberg et al., 1986; Winberg & McKinley-McKee, 1992).

For ethanol and other primary alcohols the rate-limiting step is complex and involves at least hydride transfer, ternary complex dissociation and release of NADH from the binary enzyme-NADH complex. Vₘₕ usually increases with the carbon chain length of the primary alcohol (Winberg et al., 1986; Winberg & McKinley-McKee, 1992).
A secondary alcohol might be more easily positioned in the active site cavity, because the crucial orientation of the C1-OH moiety is fixed by the wall region (residues Ile145 and Ile183) and the two active site sub-cavities (R1, R2; see Active site architecture). Due to a higher degree of orientational freedom it might be more time-consuming for the primary alcohol to assume the proper orientation and position when binding to the active site cavity. This process obviously slows the ternary complex interconversion and becomes the rate-limiting step together with the release of NADH. Biochemical data have shown that primary alcohols preferably bind to the larger sub-cavity R1 (Winberg et al., 1982a, 1986; Hovik et al., 1984). Sub-cavity R1 is more hydrophobic and is slightly narrower than R2, thus facilitating a stronger interaction with the aliphatic chain of the primary alcohol. An identical primary isotope effect of 2.5 for the two differently deuterated ethanol molecules: 1S-[2H1]ethanol and 1R-[2H1]ethanol, showed that deuteron was transferred to the coenzyme in the 1S-[2H1] form (Winberg et al., 1993). Therefore, the ethanol methyl group must interact with the larger sub-cavity R1. By modeling the two enantiomeric deuterodehydrogenase R1-S-[2H1]ethanol and R1-R-[2H1]ethanol into the active site cavity of DADH it is easily recognized that the methyl group is better accommodated and has tighter interactions with the sub-cavity R1(b) than with R2. The sub-cavity R2 might be too wide for the ethanolic methyl group to bind and get properly oriented.

In D. lebanonensis ADH, mutations like Ile254Stop, His249Stop, Ile244Stop, Ala243Stop reduce the enzymatic catalytic efficiency (Albalat et al., 1995). Kinetic data have shown that the catalytic decrease is not the same for all alcohols. The C-terminal truncations affected more markedly primary alcohols than secondary ones. As shown by the description of the architecture of the active site of DADH, Trp250 is directly involved in the closure of the active site (sub-cavity R2). Primary alcohols bind to sub-cavity R1, whereas secondary ones bind to both sub-cavities (R1, R2). Truncations that remove the wall formed by the side-chain of Trp250 might alter considerably the apolar character of the catalytic pocket, especially of the sub-cavity R2. If the substrate is a secondary alcohol, both alkyl chains bind to R1 and R2 and preserve the hydrophobic environment during ternary complex interconversion. However, primary alcohols that only bind to the sub-cavity R1 in an enzyme where the C terminus has been removed, suggest that the R2 sub-cavity might not properly exclude water molecules from the active site and decrease the enzymatic efficiency.

Substrate activation

Inspecting the crystal structure of the ternary complex with the cyclohexanone-NAD adduct (ADC) at 1.6 Å resolution we observed that an extra cyclohexanone molecule and an oxidized cyclohexanone molecule were bound to the active site of DADH. Instead of a small covering helix, the region ranging from 186 to 191 formed in this complex an ordered loop (see Figure 2(b)). These extra bound molecules seem to indicate an intermediate step during the substrate binding process or product/coenzyme release. Substrate activation has been observed for D. melanogaster ADH-F, with 2-butanol and cyclohexanol (Hovik et al., 1984), for D. melanogaster ADH-S with R(−)-2-octanol; for D. lebanonensis ADH with 1-butanol, 2-propanol and 2-butanol (Winberg et al., 1982a, 1986). For D. simulans alcohol dehydrogenase no substrate activation with primary alcohols but with R(−)-2-butanol, S(+)-2-butanol, R(−)-2-pentanol, R(−)-2-hexanol, cyclopentanol and cyclohexanol has been observed (T. Smilda et al., unpublished results). In horse liver alcohol dehydrogenase, substrate activation occurs via formation of an alcohol-NADH-enzyme complex that dissociates easier than the NADH-enzyme complex (Dalziel & Dickinson, 1966b). In DADH, high alcohol concentrations can facilitate the formation of an E-NAD+.alcohol(N) non-reacting complex. In this complex, the loop region (186-191) might adopt a conformation more similar to the ternary complex with cyclohexanone (extended loop) as compared to the complexes with acetone or 3-pentanone (small helix). This ternary complex can bind another alcohol molecule and form a productive complex E-NAD+.alcohol(N).alcohol(S) followed by oxidation of the substrate. The aliphatic side-chains of secondary alcohols, in contrast to primary alcohols (that bind to R1), probably exclude water from the active site cavity by binding to both sub-cavities R1 and R2. After product release an E-NADH-alcohol(N) complex is formed. The structure of the E-NAD-cyclohexanone(adduct) (ADC) shows that the side-chains of residues Leu188 and Val189 are exposed to the solvent and that a smaller number of direct interactions are formed between the enzyme and the coenzyme molecule. The latter might destabilize the complex E-NADH-alcohol(N) in relation to the enzyme-NADH complex resulting in a faster total reaction.

On the pH dependence of the reaction mechanism

The analysis of kinetic data obtained from D. melanogaster ADH-S alleloenzyme has shown that the formation of the E-NAD+ complex is regulated by two enzyme-linked groups with apparent pKa values of 6.0-7.0 and 9.0-9.5 (Winberg & McKinley-McKee, 1988b). We suggest that the second pKa reflects Tyr151 (pK, of free tyrosine in water is 9.7). In the apo form, Tyr151 has a slightly lower pKa than fully solvated tyrosine due to the proximity of the charged residue Lys155 (distance 4.5 Å), at the same time a hydrophobic environment destabilizes its unprotonated state. At pH > 9.5 the unprotonated Tyr-OH form of Tyr151...
facilitates the binding of the NAD$^+$ molecule to the apoenzyme, caused by a favorable electrostatic interaction between the tyrosinate group and the positively charged nicotinamide ring of the coenzyme (distance between NAD$^+$-N1N and Tyr151-OH is 3.7 Å and 3.8 Å, in BIN and ADO, respectively). His190 seems to be responsible for the increase in the formation rate of the E-NAD$^+$ complex observed in the pH range from 6.0 to 7.0. The following factors are in support of this assumption: (a) the $pK_a$ value of a fully solvated histidine residue is about 6.5; (b) His190 is in a region near the active site cavity that is strongly affected by coenzyme/substrate binding; and (c) its protonated state inhibits binding of the NAD$^+$ molecule by electrostatic repulsion.

The release of the NAD$^+$ molecule from the E-NAD$^+$ complex is controlled by only a single group with an apparent $pK_a$ of 7.6 that can again be assigned to Tyr151 (Winberg & McKinley-McKee, 1988b; McKinley-McKee et al., 1991). By binding of NAD$^+$ to the apoenzyme the positively charged nicotinamide ring of the coenzyme (distance 3.7 Å) is likely to decrease further the $pK_a$ of Tyr151 from about 9.0-9.5 (APO) to 7.6 (BIN). In the binary E-NAD$^+$ complex, His190 does not affect the release of the coenzyme. A $pK_a$ for His190 lower than 6.0 could be caused by a partial ordering of the active site loop (186 to 191) and subsequent burying of the imidazole group. In agreement with our crystal structures showing that the active site is excluded from the solvent, ternary complex interconversion does not involve proton release to the bulk solvent. A low $pK_a$ value of Tyr151 enables and facilitates the abstraction of the hydroxyl proton from the alcohol at physiological pH in the manner of general base catalysis.

Binding of pyrazole, 2-propanol or ethanol to the E-NAD$^+$ complex is regulated by a group with an apparent $pK_a$ of about 7.6 (Winberg & McKinley-McKee, 1988b) that can also be assigned to Tyr151. Kinetic data have shown that neither binding of acetaldehyde nor release of NADH from the binary complex E-NADH are pH-dependent in the range $6.0 \leq pH \leq 10.0$ (Winberg & McKinley-McKee, 1988b). This finding can be explained in two different ways: (1) the $pK_a$ value of Tyr151 could be higher than 10.0 due to a neutral nicotinamide ring in the reduced form (instead of a positively charged one in NAD$^+$) and the $pK_a$ for His190 is lower than 6.0 due to partial ordering of the loop (186-191); (2) the release of NADH or binding of acetaldehyde is not affected by the protonation state of Tyr151 or any other group (see Figure 10).

The residue Ser138 could in principle play the same role as Tyr151. That is, it could alternatively serve as the general base that subtracts the proton from the alcohol substrate. Thus its conservation among SDR could be explained. Its $pK_a$ should then be lowered from about 16 (fully solvated Ser residue) to the kinetically observed $pK_a$ 7.6 of the unprotonated group in the binary E-NAD$^+$ complex. The $pK_a$ of Tyr151 should then be lower than 6.0 and therefore not observed by kinetic measurements. In this case two negatively charged side-chains would co-exist during catalysis close to each other ($d = 4.3 \text{ Å}$) and would make this deprotonation pathway highly energetic. Electrostatic $pK_a$ calculations (our unpublished data) have, however, not shown any tendency for a decrease of the Ser138 $pK_{a_{\text{pr}}}$ whereas a clear tendency for a reduction of the Tyr151 $pK_a$ was indicated.

### Materials and Methods

#### Crystallization of DADH binary and ternary complexes

*D. lebanonensis* wild-type was grown in conventional medium, harvested from large population cages and purified from 100 g wet weight batches as previously described (Juan & González-Duarte, 1980; Ribas de Pouplana et al., 1991). Two new chromatographic gels, Blue-Sepharose Fast Flow and HR-Sephacyrl S-200 replaced the conventional matrices. The purity of the enzyme preparation was exhaustively assessed by SDS-PAGE and amino acid analysis. Contaminants were kept under 1%.

The purified protein was concentrated to about 5 mg/ml and kept in 20 mM Tris-HCl (pH 7.5), 0.1 mM dithiothreitol, 0.01% sodium azide. The concentration of the protein was determined by a calculated extinction coefficient at 280 nm using the total number of tyrosine, tryptophan and cysteine residues (Gill & Himmel, 1989).

Binary (enzyme-coenzyme) and ternary (enzyme-coenzyme-inhibitor) complexes were prepared by co-crystallization. They were crystallized by the sitting drop vapor-diffusion method as published previously (Ladenstein et al., 1995). The coenzyme (10 mM NAD$^+$ for the binary complex (BIN), 1 mM NAD$^+$ for the ternary complexes) was dissolved in the protein solution and 1% of ketone (acetone (ADA), 3-pentanone (ADO) or cyclohexanone (ADC)) was added to the reservoir solution before the droplets (5-10 μl) were set. The droplets were made by adding 5μl protein solution (5 mg/ml) to 5μl reservoir solution. Crystallization conditions were similar to those of the apo form of DADH (Benach et al., 1998). Usually only changes in the pH and concentration of the precipitant (PEG) were required. Crystals suitable for X-ray analysis were obtained after several weeks from 28% (w/v) PEG 2000 (BIN, ADA, ADO)/20% PEG 2000 (ADC), 0.2 M CaCl$_2$, 0.1 M Tris-HCl pH 7.0 (BIN)/pH 7.5 (ADA, ADO)/pH 8.0 (ADC) at 4 °C.

#### X-ray data collection and processing

Four X-ray diffraction data sets were collected. Data sets to 2.4 Å of the binary form of DADH and the ternary complex formed with acetone (2.2 Å) were collected with a Mar Research imaging plate detector installed on a MAC Science/Siemens rotating anode generator (CuK$_x$). Crystals of the ternary complex formed with 3-pentanone were measured at the X31 beam line at the DORIS storage ring, DESY, Hamburg (Germany) and diffracted beyond 1.4 Å ($λ = 0.9511$ Å, 1° frames). The ternary complex formed with cyclohexanone diffracted beyond 1.6 Å and was measured at the 711 beam line at the Max-lab, Lund (Sweden) ($λ = 0.9960$ Å, 1° frames). Both crystals of the 3-pentanone and cyclohexanone complex were mounted into a fiber loop. The crystals were flash-cooled in a 100 K nitrogen beam in the pre-
sence of 15% (v/v) glycerol as cryoprotectant. The raw data were indexed and evaluated using DENOZO (Otwinowski & Minor, 1997). SCALEPACK (Otwinowski & Minor, 1997) was used for merging and scaling of the data. Data collection statistics are shown in Table 1.

The binary (BIN) and two ternary complexes (ADA, ADO) crystallized in P21, the same space group as apo DADH, cell parameters vary in the range of 1.2-Å and a similar β value was observed. The ternary complex formed with cyclohexanone, despite using crystallization conditions very similar to the conditions for the other ternary complexes, crystallized in a trigonal space group. Plotting simulated precession pictures of the raw intensity data evaluated in P1 with the program PATTERN (Lu, 1998) showed a -3m1 Laue symmetry. According to the systematic extinctions following l = 3n, the space group could be assigned to P321 or P321. These two space groups accommodate six asymmetric units. Assuming one monomer of 27 kDa/a.u. results in a theoretical packing density of 2.7 Å³/kDa, a typical density for protein crystals (Matthews, 1968).

Molecular replacement

The crystal structures of the four complexes were solved by molecular replacement searches (Rossmann & Blow, 1962) with AMoRe (Navaza, 1994). The three-dimensional crystal structure of the dimeric molecule of apo DADH (Benach et al., 1998) was used as a search model for the binary (BIN) and two ternary complexes (ADA, ADO). Only one subunit was required to solve the third ternary complex (ADC).

Cross-rotation and translation functions were calculated using the program AMoRe (Navaza, 1994) implemented in the CCP4 suite (CCP4, 1994). Prominent peaks were obtained in both cross-rotation and translation functions at a resolution range of 10.0-3.5 Å. Properly oriented and translated models were subjected to a rigid body refinement using each dimer subunit as a sub-group at a resolution range of 10.0-3.0 Å. In the case of the ternary complex formed with cyclohexanone and NAD⁺ (ADC), examination of the translation function in P321 and P321 allowed us to assign P321 as the correct space group. In the ternary complex formed with cyclohexanone the monomers within the typical DADH dimer are related by a crystallographic 2-fold symmetry axis.

Model building and crystallographic refinement

The molecular models were gradually improved by several cycles of manual model building and crystallographic refinement in XPLOR (Brünger, 1992a) and REFMAC (Murshudov et al., 1997) accompanied by electron density averaging. Model building was carried out using the program O (Jones et al., 1991). An average of ten macro-cycles was necessary to fully refine the crystal structures. To avoid model bias the same starting model of DADH apo form was used for all four independent (BIN, ADA, ADO, ADC) crystallographic refinements.

The initial weighted Fo - Fc maps were calculated between 8.0 and 2.4-1.4 Å resolution (CCP4) and were usually improved by electron density averaging using RAVE (Jones, 1992; Kleywegt & Jones, 1994b). Regions that changed their conformation in relation to the apo form of DADH were easily seen and adjusted in the electron density map. The model was refined in XPLOR using geometric parameters obtained from small-molecule data by Engh & Huber (1991). One refinement cycle consisted of: conventional positional refinement, molecular dynamics refinement and torsional simulated annealing at a starting temperature of 5000 K (Brünger, 1988; Rice & Brünger, 1994), followed by grouped B-factor refinement under strict NC symmetry.

As the refinement proceeded, the corresponding ligands were built into the resulting difference electron density maps and were included for final refinement. In the binary form of DADH (BIN) we found a bound molecule of NAD⁺. Inspection of the high resolution Fo - Fc maps in the active site cavity of the ternary complexes showed that the ketone molecules present in the crystallization solution formed a covalent bond with the coenzyme (NAD⁺) resulting in three different NAD-ketone adducts: acetone-NAD, 3-pentanone-NAD and cyclohexanone-NAD as already proposed by Winberg & McKinley-McKee (1988a). Moreover, in the ternary complex formed with cyclohexanone we identified three more bound ligands: an extra molecule of cyclohexanone (not covalently bound to NAD⁺), a reduced molecule of dithiothreitol and a Ca²⁺. Bond and angle parameters for the NAD⁺ region of the NAD-adduct were taken from PROTIN (CCP4, 1994). Structures of the different ketones and dithiothreitol were taken from the Brookhaven Protein Data Bank. For the NAD-adducts a hybridization orbital state of type sp³ was assumed for carbon atoms C4N and N1N in the nicotinamide ring, thus affecting the planarity of the ring. A distance of a C-C single bond (1.54 Å) between the C4N and the C° atom of the ketone was considered and confirmed by Fo - Fc electron density maps at 1.4 Å resolution. Once the refinement of the 3-pentanone-NAD DADH complex (ADO) was completed, the atomic structure of its NAD-adduct was used to generate structural parameters for the NAD-adducts present in the other ternary complexes (ADA and ADC).

REFMAC was used in the last steps of the crystal structure refinement of all four complexes. NCS constraints were relaxed to restraints and individual isotropic B-factor refinement was used instead of group β-factors. Well-coordinated water molecules having peaks above 2.5σ in the (Fo - Fc)2 maps were found with the help of XPLOR (Brünger, 1992a) and included in the model. See Table 1 for a complete description of the refinement statistics of the binary and ternary models of DADH.

Throughout the model building and refinement process, the quality of the model was assessed by a number of criteria, including Ramachandran plots with the program PROCHECK (Laskowski et al., 1993), rotamer analysis (RSC_fit), peptide orientation analysis (Pep_flip) and residue real-space electron density fit analysis (RS_fit) using the program O (Jones et al., 1991). To calculate protein surfaces and volumes, VOIDOO (Kleywegt & Jones, 1994a) and XPLOR (Brünger, 1992a), respectively, were used. WHATIF (Vriend, 1990) was used to check the best orientation of O° and N° atoms in residues like Asn, Arg, Asp, Gln and His to optimize its hydrogen bonding with their surrounding and to find hydrogen bonds present in the molecule. The program RIBBONS (Carson, 1987) was used to produce ternary and quaternary representations of DADH.

PDB accession codes

The refined coordinates have been deposited in the Brookhaven Protein Data Bank. Binary form (access code 1B14), ternary form with NAD-acetone (1B15), ternary
form with NAD-3-pentanone (1B16), ternary form with NAD-cyclohexanone (1B2L).

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