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REVIEW

The catalytic reaction mechanism of drosophilid alcohol dehydrogenases ☆

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Abstract
The present review describes the current knowledge about the reaction mechanism of drosophilid alcohol dehydrogenases (*D*ADH), a member of the short chain dehydrogenase/reductase (SDR) superfamily. Included is the binding order of the substrates to the enzyme, rate limiting steps, stereochemistry of the reaction, active site topology, role of important amino acids and water molecules in the reaction and pH dependence of kinetic coefficients. We focus on the contribution from steady state kinetics where alternative substrates, dead end and product inhibitors, isotopes and mutated DADHs have been used as well as on the contributions from X-ray crystallography, NMR and theoretical calculations. Furthermore, we also raise some open questions in order to fully understand the reaction mechanism of this enzyme.

Contents

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Introduction

Drosophila alcohol dehydrogenase (*D*ADH; EC1.1.1.1) converts alcohol to aldehyde/ketone using the coenzyme NAD^+ (Eq. (1)).

$$\text{Alcohol} + NAD^+ \leftrightarrow \text{aldehyde/ketone} + NADH + H^+ \quad (1)$$

Studies of ADHs in fruit flies started in the beginning of 1960s. These studies were focused on evolutionary biology where the main interest was to investigate neutral versus selective evolution. The reason to use this enzyme system was due to its simple detection, the large amount of enzyme produced by the flies and the occurrence of allelic variants of the enzyme (Grell et al., 1965, 1968; Johnson and Denniston, 1964; Ursprung and Leone, 1965). It was possible to detect the ADH in a single fly. Several reviews have focused on this and the metabolic role of *D*ADH (Ashburner, 1998; Chambers, 1988, 1991; Geer et al., 1990, 1993; Heinstra, 1993; Sofer and Martin, 1987). In order to understand the role of ADH in fruit flies, biochemical studies of the enzyme were also started in the middle of the sixties. It was shown that the enzyme is a homodimer with a subunit molecular size of approximately 27 kDa which lacks metal ions and is not inhibited or only weakly inhibited by metal chelators such as EDTA (Borack and Sofer, 1971; Pitt-Rivers and Impiombato, 1968; Reynolds and Tanford, 1970; Schwartz and Jornvall, 1976; Sofer and Ursprung, 1968; Winberg et al., 1982b). Furthermore, it was shown that it was possible to convert one isoform to another using NAD^+ and acetone (Jacobson, 1968; Jacobson et al., 1970, 1972, Jacobson and Pfuderer, 1970; Knopp and Jacobson, 1972; Schwartz et al., 1979; Schwartz and Sofer, 1976; Ursprung and Carlin, 1968). The first partial primary sequence of *D*ADH was obtained in the middle of the 70s (Schwartz and Jornvall, 1976; Thatcher, 1977), and the first full protein and cDNA sequence in 1980 (Benyajati et al., 1980; Thatcher, 1980). Interest in this enzyme gained momentum in the late 70s and early 80s. Jörnvall and colleagues started to classify dehydrogenases and showed that *D*ADH and a bacterial ribitol dehydrogenase (RDH) belong to another class than horse liver and yeast ADH (HLADH and YADH) (Jornvall et al., 1981). The fruit fly ADH was classified as a short chain dehydrogenase/reductase (SDR) which lacks bound metal ions, while the zinc-bound enzymes HLADH and YADH were classified as long chain dehydrogenases/reductases (LDR). However, later these metallo-dehydrogenases were re-classified as medium chain dehydrogenases/reductases (MDR) (Persson et al., 1994, 2008). Thereafter, fruit fly ADHs have been characterized in more detail, using steady state kinetic methods along with X-ray crystallography, site directed mutagenesis, NMR and theoretical calculations (Benach et al., 1998, 1999, 2005; Brendskag et al., 1999; Chen et al., 1993; Cols et al., 1993, 1997; Hovik et al., 1984; Koumanov et al., 2003; Winberg et al., 1982a, 1986, 1999; Winberg and McKinley-McKee, 1988, 1994; Wuxiuer et al., 2014). Today, the SDR superfamily includes more than 140,000 members, and SDR enzymes are found in three different enzyme classes:

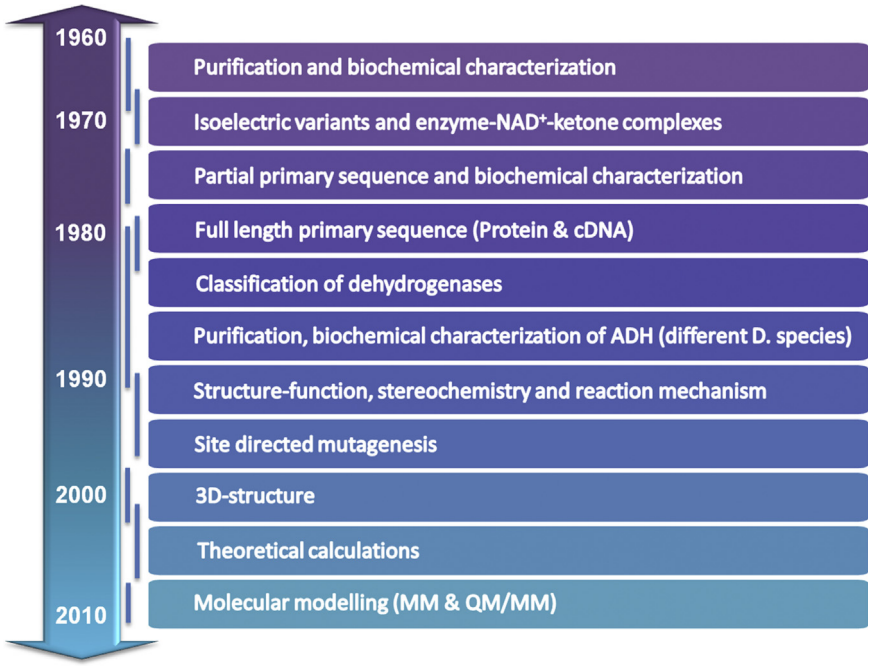


Figure 1 Milestones in studies and characterization of drosophilid alcohol dehydrogenases. The marks indicate when a certain type of characterization started and references to these studies are in the main text.

oxidoreductases, lyases and isomerases (Jornvall et al., 1995, 2010, 2013; Persson et al., 2009). Figure 1 summarizes the milestones in the biochemical characterization of *D*ADHs.

3D structure of the binary *D*ADH-NAD$^+$ complex

The first X-ray structures of a fruit fly ADH appeared in the end of 1990s when Ladenstein et al. presented the 3D structure of *Scaptodrosophila lebanonensis* ADH (*Sl*ADH) as a free enzyme, as well as binary *Sl*ADH-NAD$^+$ and ternary *Sl*ADH-NAD$^+$-ketone complexes (Benach et al., 1998, 1999). In addition, a couple of years later the same group described the structure of the ternary *Sl*ADH-NAD$^+$-2,2,2-trifluoroethanol complex (PDB ID: 1SBY) as well as the structure of another fruit fly ADH, the slow allelic variant of *Drosophila melanogaster* ADH (*Dm*ADH^S) in complex with NADH and acetate (Benach et al., 2005). Here we will focus on some of the conserved amino acids in the fruit fly ADHs and other SDR enzymes that take part in the catalytic reaction. In addition an eight-membered water chain that connects the catalytic site with the bulk water at the interconnection between the two identical *D*ADH subunits will also be discussed. A 3D model of the binary *Sl*ADH-NAD$^+$ complex is shown in Figure 2 where the coenzyme occupies both subunits. In Figure 3, the catalytic site of a single *D*ADH

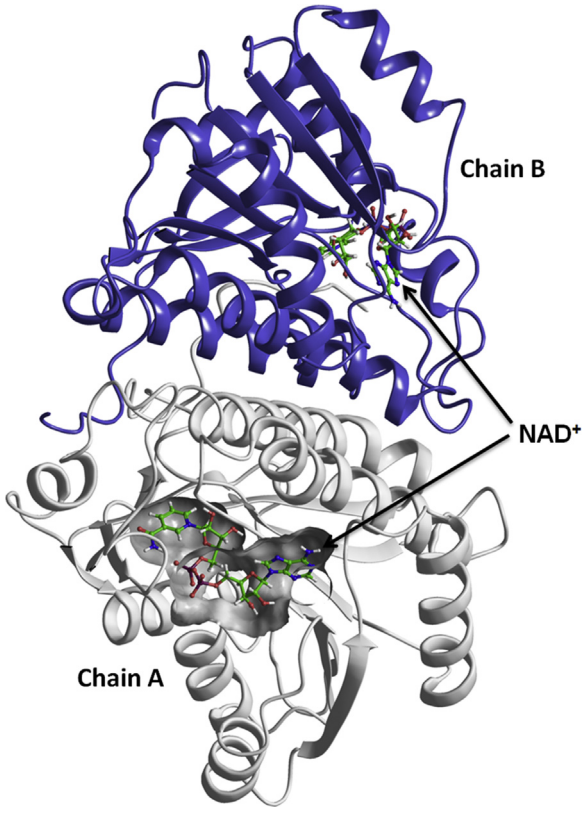


Figure 2 Structure of the *Sl*ADH homodimer with bound NAD$^+$ in both subunits, based on the X-ray structure (PDB ID: 1SBY). The NAD$^+$ molecule is represented as balls and sticks inside the active site of *Sl*ADH. The grey skin mesh indicates the NAD$^+$ binding pocket in chain A.

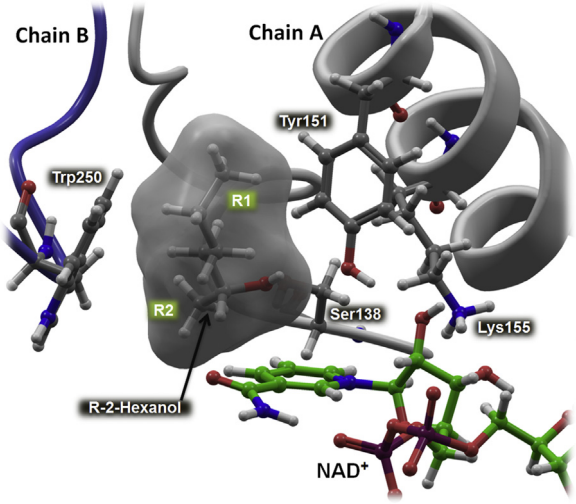


Figure 3 Model of the alcohol binding site of *Sl*ADH with R-(−)-hexan-2-ol docked into the active site of *Sl*ADH-NAD$^+$. The semi-transparent grey skin mesh represents the R_1 and R_2 parts of the bifurcated alcohol binding site in chain A. Trp250 (side chain in sticks and balls) in the C-terminal domain of chain B contributes to the R_2 part of the alcohol binding site in chain A. Shown in sticks and balls is also R-(−)-Hexan-2-ol and the nicotinamide-ribose-phosphate part of NAD$^+$ as well as the side chains of Ser138, Tyr151 and Lys155 in chain A. This model is based on the ternary *Sl*ADH-NAD$^+$-trifluoroethanol complex (PDB ID: 1SBY), where the trifluoroethanol was replaced by R-(−)-hexan-2-ol and the best docking conformation was used.

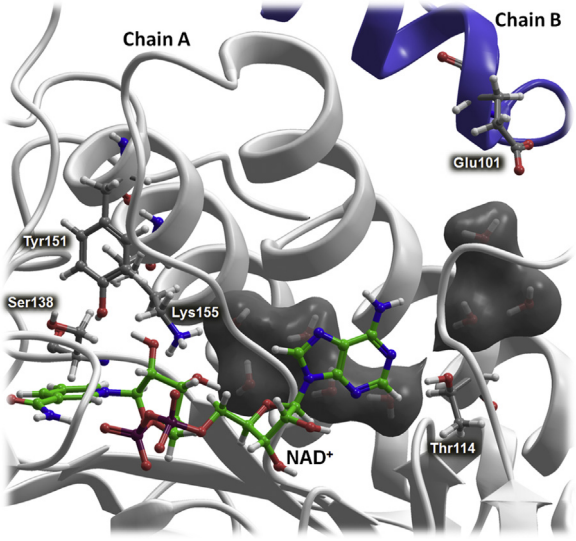


Figure 4 The eight-membered water chain in *Sl*ADH is based on the X-ray structure (PDB ID: 1SBY). The surface of the inner and outer part of the water chain is shown by the semi-transparent grey skin mesh and the side chain hydroxyl group of Thr114 links the two parts of the water chain. Shown in sticks and balls is NAD$^+$ and the side chains of Thr114, Ser138, Tyr151 and Lys155 in chain A and Glu101 in chain B.

subunit is shown in detail with the three conserved amino acids in the active site, Tyr151, Lys155 and Ser138 labelled. The side chain amino group of Lys155 binds to the O2′ and O3′ ribose hydroxyl groups of NAD^+ whereas the ribose O2′ hydroxyl group also interacts with the hydroxyl group in the side chain of Tyr151. Figure 4 shows the eight-membered water chain which connects Lys155 directly to the bulk water between the two subunits. The end of the water-chain in one subunit is linked to the carboxyl group in the side chain of Glu101 in the other subunit. Furthermore, the C-terminal end of one subunit is a part of the alcohol binding site in the other subunit as shown in Figure 3. An additional characteristic trait is the hydroxyl group in the alcohol substrate bound between the hydroxyl groups on the side chains of Ser138 and Tyr151 (Figure 3).

Binding order of substrates and active site interactions

In this section we will describe the binding order of the substrates in the inter-conversion of alcohols to aldehydes and ketones using NAD^+ and NADH as well as in the oxidation of aldehydes to acids. We will also focus on the substrate and stereo specificity of *D*ADH and the binding of alcohols and aldehydes to the enzyme active site.

Binding order and rate limiting steps in the inter-conversion of alcohols and aldehydes

Fruit fly ADHs has broad substrate specificity and convert both primary and secondary alcohols to their corresponding aldehydes and ketones (Eisses et al., 1994; Hovik et al., 1984; Winberg et al., 1982a, 1986; Winberg and McKinley-McKee, 1992). The binding order has been studied by steady-state kinetics using alternative substrates, dead end and product inhibition as well as kinetic isotope effects (Hovik et al., 1984; Winberg et al., 1982a, 1982b, 1986, Winberg and McKinley-McKee, 1994). Eqs. (2) and (3) describe the kinetic coefficients for the oxidation of alcohols to aldehydes/ketones using NAD^+. Eq. (2) uses the Dalziel nomenclature (Dalziel, 1957) and Eq. (3) uses k_{cat} and K_m in a similar way as Cleland (Cleland, 1963). By comparing these two equations it is easy to relate the φ coefficients to k_{cat} and K_m.

$$\frac{e}{v} = \varphi_0 + \frac{\varphi_1}{[NAD^+]} + \frac{\varphi_2}{[Alcohol]} + \frac{\varphi_{12}}{[NAD^+][Alcohol]} \quad (2)$$

$$\frac{e}{v} = \frac{1}{k_{cat}} + \frac{K_{m1}}{k_{cat}[NAD^+]} + \frac{K_{m2}}{k_{cat}[Alcohol]} + \frac{K_{ia}K_{m2}}{k_{cat}[NAD^+][Alcohol]} \quad (3)$$

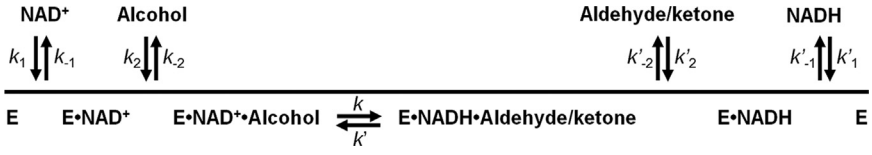
In Eqs. (2) and (3), e is the concentration of enzyme active sites, K_{ia} is the dissociation constant of the enzyme-NAD^+ complex, K_{m1} and K_{m2} are the Michaelis-Menten constants for NAD^+ and alcohol. By comparing Eqs. (2) and (3) one can see that φ_0 equals $1/k_{cat}$ and φ_1 equals K_{m1}/k_{cat} and so on. For the back reaction of aldehydes to alcohols using NADH, primed coefficients can be used such as φ'_0 or K'_{m1}. The steady-state kinetic studies revealed that the binding order of the alcohol/aldehyde substrate and the coenzyme to *D*ADH was consistent with a compulsory ordered reaction mechanism where the coenzymes bind to the free enzyme and alcohols and aldehydes bind the corresponding binary enzyme-NAD^+ and enzyme-NADH complexes (Scheme 1) (Hovik et al., 1984, Winberg et al., 1982a, 1982b, 1986, Winberg and McKinley-McKee, 1994).

The reaction rate was much faster for most of the secondary alcohols tested compared to primary alcohols. In addition, at an infinite alcohol concentration the reaction rate (k_{cat}) varied for the primary alcohols while it was constant for most of the secondary alcohols. It was shown that the rate limiting step for primary alcohols is the chemical step, i.e. the hydride transfer step (k), while for most of the secondary alcohols it is the release of the coenzyme NADH (k'_{-1}) from the binary enzyme-NADH product complex (see Scheme 1) (Hovik et al., 1984; Winberg et al., 1982a, 1982b, 1986, Winberg and McKinley-McKee, 1994).

Topology of the alcohol binding site

The various ADH alleloenzymes and ADH variants in different fruit flies have very broad substrate specificity and secondary alcohols are better than primary alcohols (for review see (Winberg and McKinley-McKee, 1992)). In all cases, they prefer small hydrophobic alcohols. The use of both R- and S-stereospecific secondary, branched primary and secondary as well as cyclic and bi-cyclic alcohols revealed a bifurcated active site where the part that interacted with the R_1-part of a secondary alcohol was longer and narrower than the part that interacted with the R_2-part of the secondary alcohol. This bifurcated hydrophobic alcohol binding site in *D*ADH was later described in detail when the 3-D structures of the free enzyme, the binary enzyme-NAD^+ and ternary enzyme-NAD^+-ketone/2,2,2-trifluoroethanol complexes were determined. Figure 3 shows a model of the ternary *Dl*ADH-NAD^+-R-(−)-hexan-2-ol complex based on *D*ADH X-ray structures.

Furthermore, kinetic and isotope effect studies revealed that the pro-S hydrogen in ethanol and other primary alcohols was transferred to the pro-S side of the nicotinamide moiety of NAD^+, showing that the alkyl chain in ethanol and other primary alcohols interacted with the R_1 part



Scheme 1 Compulsory ordered ternary complex mechanism describing the kinetics of drosophilid ADH catalysis. **E** represents the enzyme, k and k' the different rate constants with un-primed constants for the forward and primed constants for the back reaction.

of the alcohol binding site in *D*ADH (Allemann et al., 1988; Benner et al., 1985; Winberg et al., 1993). As shown in Figure 3, the C-terminal end of one subunit forms a part of the R_2 binding pocket of the other subunit. The deletion of the C-terminal amino acids 1 to 11 affected the activity of *Sl*ADH, and k_{cat}/K_m was much more reduced for primary than for secondary alcohols (Albalat et al., 1995). Although the alkyl chain of primary alcohols binds in the R_1 part of the alcohol binding pocket in the enzyme, a less hydrophobic active site environment in the mutant enzymes seems to be more critical for primary than for secondary alcohols.

Binding order and rate limiting steps in the oxidation of aldehydes

In addition to the oxidation of primary alcohols to aldehydes, *D*ADH can also oxidize aldehydes to their corresponding acids (Eisses, 1989; Eisses et al., 1985; Heinstra et al., 1983; Henehan et al., 1995; Moxon et al., 1985; Winberg and McKinley-McKee, 1998). Metabolic studies revealed that this oxidation is a part of the fly metabolism where ethanol is converted to lipids (Freriksen et al., 1991; Heinstra and Geer, 1991). The rate of the conversion of alcohols to acids by *D*ADH suggested that the formed aldehyde never leaves the active site, but that it is NADH that leaves the ternary complex (Heinstra et al., 1983). This did not fit with the kinetic studies of the inter-conversion between alcohols and aldehydes which followed a strict compulsory ordered mechanism where it was the aldehyde that left the ternary complex (Hovik et al., 1984; Winberg et al., 1982a, 1982b, 1986, Winberg and McKinley-McKee, 1994). Even though oxidation of aldehydes to acids was accompanied by a dismutation reaction (Henehan et al., 1995), i.e. aldehyde also reacted with the formed enzyme-NADH complex and formed an alcohol (Eqs. (4)-(6)), it was possible to use steady state kinetics to study the reaction mechanism for the oxidation of aldehydes to acid at basic pH using a sensitive fluorimeter (Winberg and McKinley-McKee, 1998). The oxidation of aldehydes to acids is essentially an irreversible process (Eisses, 1989; Eisses et al., 1985; Heinstra et al., 1983; Henehan et al., 1995; Moxon et al., 1985; Winberg and McKinley-McKee, 1998).

$$\text{Aldehyde} + H_2O + NAD^+ \rightarrow \text{carboxylate}^- + NADH + 2H^+ \quad (4)$$

$$\text{Aldehyde} + NADH + H^+ \leftrightarrow \text{alcohol} + NAD^+ \quad (5)$$

$$2\ \text{Aldehyde} + H_2O \rightarrow \text{carboxylate}^- + \text{alcohol} + H^+ \quad (6)$$

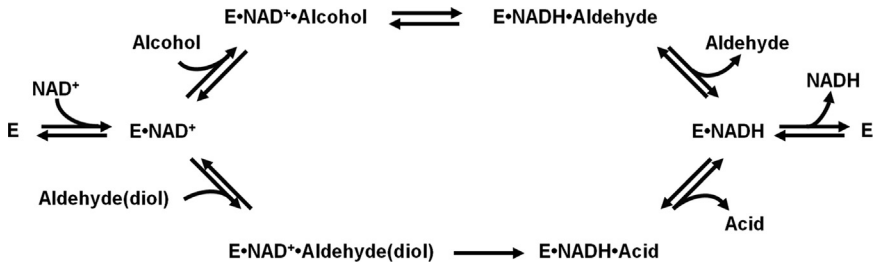
The steady-state kinetic studies involved alternative aldehyde substrates, kinetic isotope effects, dead-end and product inhibition kinetics (Winberg and McKinley-McKee, 1998). These studies revealed that the oxidation of aldehydes to acids is consistent with a strict compulsory ordered reaction mechanism, where NAD^+ is bound to the free enzyme and the aldehyde is bound to the binary enzyme-NAD^+ complex (Scheme 2). Furthermore, the rate limiting step is the release of the coenzyme product, NADH (Winberg and McKinley-McKee, 1998). In the oxidation of aldehydes, the diol form of the aldehydes was shown to bind to the enzyme-NAD^+ complex and generates the reactive ternary complex (Eisses, 1989).

Structure of the ternary enzyme-NAD^+-aldehyde complex

The X-ray structure of the ternary *Dm*ADH^S-NADH-acetate complex revealed that the methyl group of the acetate was bound to the R_2-part of the active site whilst one oxygen of the acetate was bound to the R_1-part of the binding site and the other oxygen to the catalytic residues Tyr151 and Ser138 (*Sl*ADH numbering) (Benach et al., 2005). Thus, the alkyl chain in the aldehyde bound to different parts of the bifurcated alcohol/aldehyde/ketone binding region of the enzyme depending on whether the aldehyde was oxidized to an acid (binds to *D*ADH-NAD^+) or if it was reduced to an alcohol (binds to *D*ADH-NADH). This also supported the kinetic results suggesting that in the oxidation of alcohols to an aldehyde and further to an acid, the formed aldehyde must leave the active site before the coenzyme and then be hydrolysed to a diol prior to binding to the binary enzyme-NAD^+ complex (Benach et al., 2005; Winberg and McKinley-McKee, 1998). However, even though the formed aldehyde leaves the active site before NADH, it cannot be excluded that it is still bound to the opening of the alcohol binding site as X-ray crystallography showed that a second cyclohexanone molecule was bound to the loop region (amino acids 186-191) in the ternary enzyme-NAD^+-cyclohexanone complex (Benach et al., 1999). Scheme 2 shows the mechanism for the dismutation reaction.

The pH dependence of the kinetic coefficients in the oxidation of alcohols and in the reduction of aldehydes

The pH dependence of the kinetic coefficients has been determined for two fruit fly ADHs, the slow alleloenzyme of *D. melanogaster* (*Dm*ADH^S) and *S. lebanonensis* ADH (*Sl*ADH), using steady state kinetics (Brendskag et al., 1999; Winberg et al., 1999; Winberg and McKinley-McKee, 1988). In these experiments both ethanol and propan-2-ol



Scheme 2 Compulsory ordered mechanism for the oxidation of ethanol to acetate and the dismutation of aldehydes. **E** represents the enzyme.

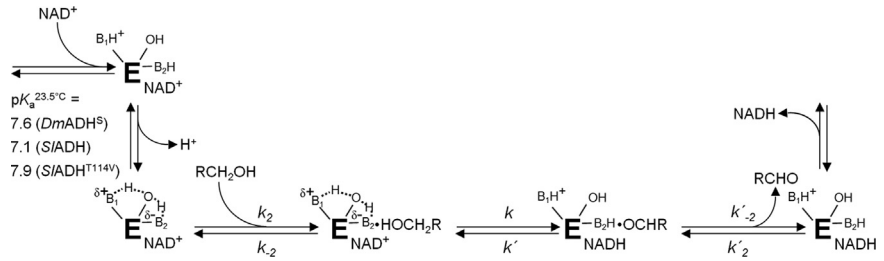
were used together with NAD^+, and acetaldehyde together with NADH. The pH dependence of the alcohol competitive inhibitors pyrazole and 2.2.2-trifluoroethanol was also studied. These results revealed that only one kinetic coefficient did not vary with pH in the forward direction, φ_0 ($1/k_{cat}$). This was the case for both ethanol and propan-2-ol which showed that neither the hydride transfer step nor the release of NADH from the binary enzyme-NADH complex was pH dependent. For the backward reaction using acetaldehyde and NADH, only φ'_2 was independent of pH. The pH dependence of φ_2 in the oxidation of alcohols and $K_{EO.I}$ for alcohol competitive inhibitors revealed that the loss of a single proton in the binary enzyme-NAD^+ complex regulated the k_{on} velocity of both alcohols (k_2) and alcohol competitive inhibitors as shown for alcohols in Scheme 3. Eq. (7) shows the relation of the rate constants (defined in Schemes 1 and 3) that build up the φ_2 coefficient. In the case of φ'_2, the un-primed and primed rate constants change place.

$$\varphi_2 = \frac{1}{k_2}\left(1+\frac{k_{-2}}{k}\left(1+\frac{k'}{k'_{-2}}\right)\right) \quad (7)$$

X-ray crystallography has shown that the hydroxyl group of an alcohol binds between two of the three conserved catalytic residues in *D*ADH (PDB ID: 1SBY) and other SDR enzymes, i.e. the hydroxyl group in the side chain of Tyr151 and Ser138 (*Sl*ADH numbering) (Benach et al., 1999; Hulsmeyer et al., 1998; Sawicki et al., 1999; Tanabe et al., 1998; Tanaka et al., 1996). Site directed mutagenesis revealed that Tyr151, Ser138 and Lys155 are essential for enzyme activity (Chen et al., 1993; Cols et al., 1993, 1997). It has been suggested that the proton lost in the binary enzyme-NAD^+ complex is the proton on the hydroxyl of the side chain in Tyr151 (Gani et al., 2008; McKinley-McKee et al., 1991). However, the ionization energy of the proton release is much larger than that of the side chain hydroxyl group of a tyrosine (Winberg et al., 1999) and theoretical calculations suggested that the proton release is not from a single amino acid, but from a coupled irregular ionization of the active site groups Tyr151 and Lys155 (Koumanov et al., 2003). These calculations suggested that it was equally possible that a proton was released from the hydroxyl of Tyr151 or from the side chain amine of Lys155. The proton from the former group was assumed to be released to the bulk solvent outside the protein core through internal water molecules in the active site. The remaining proton on the $LysNH_3^+$ side chain may then be shared with the negatively charged $TyrO^-$ through the ribose O2′ hydroxyl group of the NAD^+, which acts as a proton switch (Scheme 3). A similar sharing of a proton through this proton relay is assumed to occur if the proton at the side chain amine of Lys155 is released to the bulk solution outside the protein core through the eight-membered water-chain.

The eight-membered water-chain Is essential for enzyme activity

X-ray crystallography of *Sl*ADH showed that the hydroxyl-group of the side-chain on Thr114 connects the inner and outer part of the water-chain (Figure 4) and is believed to act as a switch during proton transfer from Lys155 to the bulk water between the two ADH subunits. In order to determine the role of the eight-membered water-chain, site directed mutagenesis, X-ray crystallography, steady state kinetics and molecular dynamics studies were performed on a mutant of *Sl*ADH with Thr144 mutated to a Val (*Sl*ADH^{T114V}) (Wuxiuer et al., 2012, 2014). As shown by X-ray crystallography, there was no connection between the outer and the inner part of the water-chain in the mutant due to the lack of a side-chain hydroxyl-group at position 114. Steady state kinetics and pH dependence of the kinetic coefficients revealed that all φ coefficients were larger for the mutant than for the wild-type enzyme except for φ_0 ($1/k_{cat}$) for propan-2-ol, i.e. the NADH release from the binary enzyme-NADH product complex (Brendskag et al., 1999; Wuxiuer et al., 2012). The hydride transfer in the ternary enzyme-NAD^+-alcohol complex was approximately four times slower than that of the wild-type enzyme and the coenzyme binding was much weaker. Furthermore, the pK_a value of the Tyr151/Lys155 couple in the binary enzyme-NAD^+ complex was approximately 1 pH unit larger than for the wild-type enzyme. The binding order of coenzyme and alcohol/aldehyde was consistent with a compulsory ordered reaction mechanism. As for the wild-type enzyme the coenzymes bind to the free enzyme while alcohols and aldehydes bind to the formed enzyme-coenzyme complexes (Scheme 1). This showed that an intact water-chain is essential for optimal enzyme activity. The studies with the mutant showed that the broken water-chain affects several events involved in the catalytic reaction such as the hydride



Scheme 3 Mechanism proposed for *Drosophila* ADH catalysis. **E** denotes the enzyme, OH the O2′ ribose hydroxyl group of NAD^+/NADH, B_1H^+ and B_2H represent the protonated form of Lys155 and Tyr151, respectively. The rate constants (*k*s) are as in Scheme 1. The proton release from the binary enzyme-NAD^+ complex results in a linked ionization of Tyr151 and Lys155, where both residues are half protonated ($\delta-$ and $\delta+$) and the O2′ ribose hydroxyl group is localized between the two groups and acts as a switch. The pK_a values at 23.5 °C for *Dm*ADH^S, *Sl*ADH and *Sl*ADH^{T114V} mutant are also shown.

transfer step, the proton transfer step from the OH group of the alcohol to the $TyrO^-$ group in the catalytic site, the proton relay and the release of protons from the binary enzyme-NAD^+ complex.

Molecular dynamics studies of *Sl*ADH and *Sl*ADH^{T114V} indicated an intact water-chain is important for the structural dynamics of the enzyme in both the free enzyme as well as in the binary enzyme-NAD^+ and the ternary enzyme-NAD^+-alcohol complexes (Wuxiuer et al., 2014). The inner part of the water-chain lines and interacts with residues involved in the binding of the adenine and the adenine-ribose as well as the nicotinamide-ribose part of the coenzyme. The lack of a side-chain OH-group at position 114 in the *Sl*ADH^{T114V} mutant resulted in a time resolved break in the hydrogen bond between Asp63 and the adenine part of the coenzyme, which also may explain the weaker coenzyme binding. Differences in molecular dynamical behaviour were also seen in the loop which was important for binding the nicotinamide part of NAD^+. Thus, the alcohol substrate had more freedom for conformational changes in the active site of the wild type enzyme. These differences in molecular dynamical behaviour may at least partially explain the faster hydride transfer in the wild-type enzyme compared to the mutant enzyme.

Unresolved mechanistic issues

The role of the catalytic triad in *D*ADH has been compared to the role of the catalytic zinc in HLADH. This is obviously not a good comparison. When the alcohol binds to the active site in HLADH, the OH-group of the alcohol binds the positively charged zinc atom, which results in a lowering of the pK_a of the alcohol and a proton release from the OH-group of the alcohol, giving a bound alcoholate (Kvassman and Pettersson, 1980). It was suggested that this proton release from the alcohol OH-group facilitates the hydride transfer step in the oxidation of alcohol to aldehyde/ketone. In a similar way, it has been proposed that the partially negatively charged Tyr151 in the catalytic triad in *D*ADH abstracts the proton from the bound alcohol and generates an alchoholate as seen for the HLADH (Brendskag et al., 1999; McKinley-McKee et al., 1991). This was questioned in a recent review (Ladenstein et al., 2008) as it does not seem likely that a $TyrO^-$ group with a pK_a around 7 should be able to abstract a proton from an alcohol with a pK_a value much higher than that of the tyrosine. Therefore, it remains to be resolved at which step in the oxidation of an alcohol the proton on the OH-group of the alcohol is transferred to the partly negatively charged tyrosine. One would assume that it is the hydride transfer step that starts and at a certain distance between the hydride and the COH carbon of the alcohol, during the formation of an aldehyde, the pK_a of the alcohol hydroxyl would be reduced to a lower value than that of the hydroxyl of Tyr151. At that point it is likely that the proton is transferred to the $TyrO^-$ group. Furthermore, it is not known how this proton transfer is affected by the water-chain and the carboxyl-group of the Glu101 residue in the other subunit. In addition, will a prevention or delay of the putative proton relay affect the rate of the hydride transfer step? These are questions still remain to be answered.

Conclusion

The reaction mechanism of *D*ADH is now well established, although some minor parts of the mechanism still need to be solved. An interesting question appears, and that is do other members of the SDR family follow the same reaction mechanism as *D*ADH? If so, one can anticipate that knowledge of the detailed reaction mechanism of *D*ADH will help in design compounds that are mechanism based inhibitors of therapeutic important SDR enzymes. Such inhibitors could be used in the development of pharmaceutical compounds used in treatment of different cancer forms, high blood pressure and various neurodegenerative disorders like Alzheimer.

Conflict of interest

The authors declare that there is no conflict of interest.

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