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Three-dimensional structures of MDR alcohol dehydrogenases

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Abstract. In this report we describe the main features of the initially determined alcohol dehydrogenase, that of horse liver, relate this to the human enzyme structures and review recent structural studies on mutants and new complexes of the enzymes. We further review the structure of a bacterial alcohol dehydrogenase to arrive at a coherent picture of medium-chain dehydrogenase/reductase alcohol dehydrogenases in general.

Keywords. Crystal structures, dimeric ADH, tetrameric ADH, zinc, NADH.

Introduction

Alcohol dehydrogenase from horse liver was one of the first dehydrogenases for which the structure was determined [1–3]. Much of the data concerning the general structure, coenzyme binding, substrate binding and conformational changes were produced during the 1970s and 1980s, and this work was collected and discussed in a number of reviews [4–7]. In this review, we re-examine the structural properties in view of advances in further studies on this enzyme type in order to emphasize general aspects of medium-chain dehydrogenase/reductase (MDR) alcohol dehydrogenase (ADH) structures.

General structure

The structural features were obtained from crystallographic studies of the ethanol active E-isozyme, ADH1E (for nomenclature see [8]) of horse liver alcohol dehydrogenase [3, 9–11], which will be briefly reviewed in the first part of this paper. This enzyme is a class 1 MDR-ADH and is dimeric, in contrast to corresponding fungal, bacterial and yeast ADHs, which are tetrameric. By now, about 70 coordinate sets for dimeric MDR-ADHs are available in the protein data bank, as well as several tetrameric MDR-ADHs.

General subunit structure

The ADH subunit in general is distinctly divided into two domains, the coenzyme binding domain and the catalytic domain, separated by a long deep cleft, containing the active site with its catalytic zinc atom [3] (Fig. 1). The coenzyme binding domain is built up by about 150 residues located in the C-terminal half of the enzyme, and the catalytic domain is formed by about 170 residues from the N-terminal half of the molecule and of about 50 residues from the C-terminal part.

The coenzyme binding domain

The general structure of the coenzyme binding domain is similar to the corresponding domain in other dehydrogenases. This was an exciting fact that attracted much attention when first discovered [12]. Particularly, the discovery that the coenzyme binding domain was located differently in different dehydrogenases and combined with completely distinct other domains suggested a lego-like molecular formation of these enzyme families [13], as is now well-known for many mosaic proteins. The coenzyme binding domain has the Rossmann fold [12, 13] and constitutes two mononucleotide binding halves. For ADH, the do-

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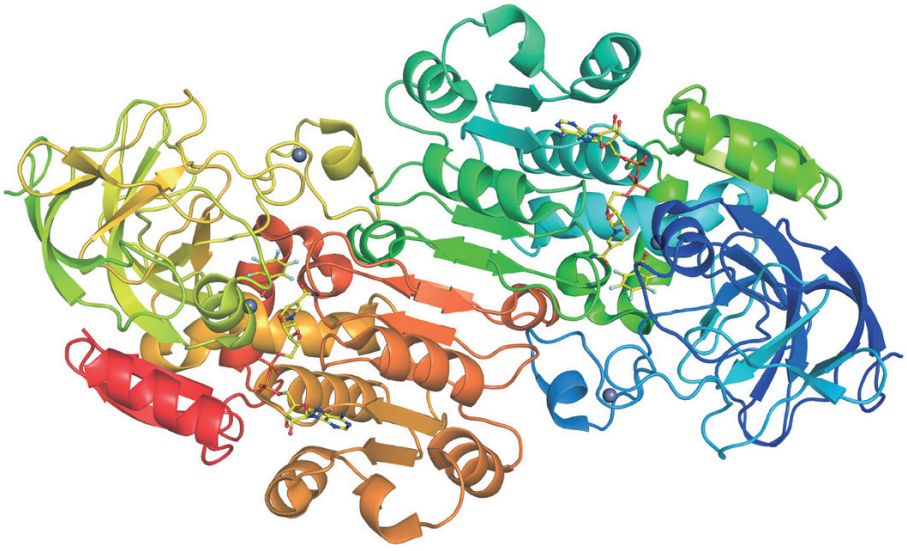

Figure 1. The horse liver ADH dimer in complex with NADH and pentafluorobenzyl alcohol. The zinc ions are dark blue spheres. The color scheme is called the Jones rainbow. The N-terminus of one subunit is blue, and it gradually changes to red at the C-terminus of the second subunit. The dimer interface is between the green and the brown strands and helices.

main is more symmetric than the corresponding domain in most other dehydrogenases and has two $(\alpha\beta)_3$ mononucleotide units forming the dinucleotide domain via an approximate twofold rotation by an axis going between strands 1 and 4. Since the helices in these $(\alpha\beta)_3$-motifs come out on different sides of the parallel sheet in each half, clefts are created on both sides of the domain suitable for binding of the coenzyme NAD(H). A feature of the domain is the characteristic sequence motif after the first strand of the first mononucleotide binding half, GxGxxG, where the first two glycine residues make room for the phosphates of the coenzyme and allow it a close approach to main chain atoms for hydrogen bonding [14].

Coenzyme binding

NAD(H) binds in an extended conformation across the C-terminal edge of the parallel β-sheet of the domain such that each half of the dinucleotide is positioned on separate sides of the sheet, the adenosine half in a cleft at the surface of the domain and the nicotinamide half deep in the protein at the active site cleft [5, 9]. The adenine base is positioned in a hydrophobic cleft with the amino group at position 6 pointing out towards the surface. The adenosine ribose is bound with double hydrogen bonds to an aspartate, Asp223, constituting a common feature of dehydrogenases with preference for NAD over NADP. The aspartate would create repulsion of the extra phosphate group of NADP due to charge and space, thus explaining the coenzyme preference of the enzyme. The adenosine ribose is also bound to the lysine side chain of Lys228. The pyrophosphate of NAD is bound to the main chain by hydrogen bonds and to two arginines, Arg47 and Arg369, one from each of the two halves of the catalytic domain. The ribose on the nicotinamide half of NAD is hydrogen-bonded to a main chain carbonyl and to Ser48 and His51. The nicotinamide is anchored to the main chain by its amino group.

The catalytic domain

The catalytic domain has a complicated arrangement that was originally described as three β-structural motifs plus a few helices [3]. The domain contains two halves, the larger N-terminal part and a smaller C-terminal part, which sequence-wise are separated by the residues corresponding to the coenzyme binding domain. The N-terminal part was later shown to have similarities with a group of structures including GroES [15]. The C-terminal part of the domain is a long helix and a βαβ-motif bound to the main sheet of the N-terminal part by its last strand.

Zinc ions

Mammalian MDR ADHs normally contain two zinc ions per subunit [16] both bound to the catalytic domain [3]. One of these is a structural ion [17] liganded by four cysteine residues [3]. This zinc ion stabilizes a long loop excursion from the sheet structure. The other zinc ion is at the active site. It is essential for catalysis and is coordinated by two cysteine residues, Cys46 and Cys174, and a histidine, His67. The fourth position coordinates the substrate or a water molecule.

The dimer

The dimer is stabilized mainly by a large flat hydrophobic surface formed from the C-terminal residues of the coenzyme binding domain [3]. This area is lined on one side by the last strand of the parallel sheet of the

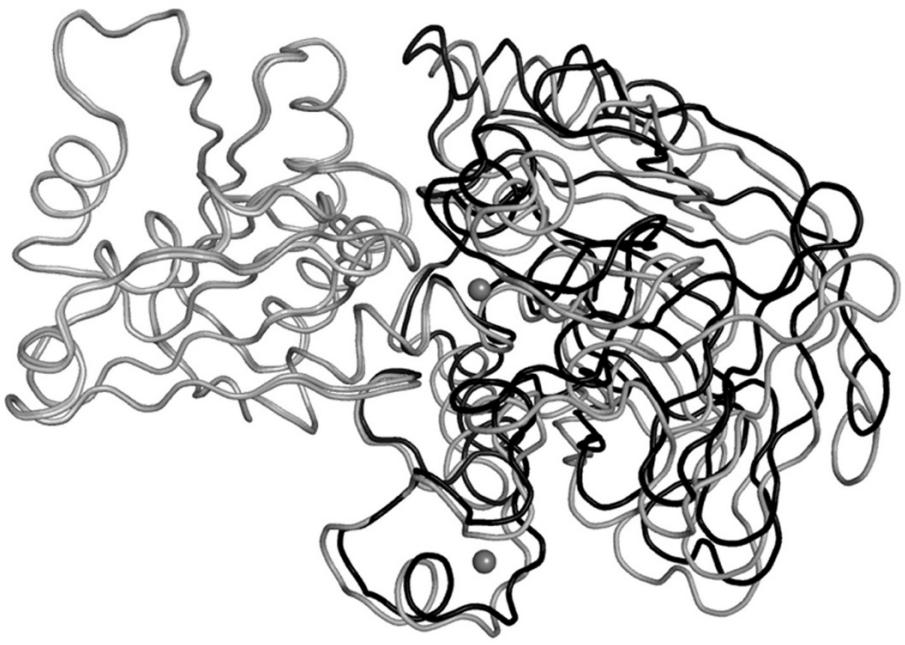

Figure 2. Conformational differences between the catalytic domain in the apoenzyme (in gray) and holoenzyme (in black) shown by a main-chain drawing. The co-enzyme binding domains are superoposed and are all in grey. The zinc ions are shown as dark balls.

domain that is connected anti-parallel to the same strand in the second subunit of the dimer. On the other side of the interaction area, a short strand from each subunit forms a small anti-parallel sheet. The catalytic domains are located on each side of this central unit of two coenzyme binding domains, thus producing an elongated dimer (Fig. 1). The central part and the catalytic domains are separated into three units by long deep clefts.

Conformational changes

The enzyme undergoes large conformational changes when the coenzyme is bound and was the first enzyme for which domain rotations were demonstrated [18]. When the coenzyme binds, the catalytic domains rotate about 10° (Fig. 2) and close the binding cleft between the domains around the coenzyme [9]. The protein is then closed around the nicotinamide ring at the active site. The holoenzyme structure with coenzyme present is generally called the closed form and the coenzyme-free apoenzyme the open form. To allow for the rotation of the catalytic domains and the closure around the coenzyme, a loop on the coenzyme binding domain, residues 293–298, has to adopt a different conformation in the holo versus the apoenzyme.

Substrate binding

Substrate binding was originally investigated by aromatic alcohols/aldehydes (Fig. 3) and substrate-like compounds like trifluoroethanol [11, 19]. The alcohol/aldehyde functionality binds to the zinc atom in hydrogen-bond distance to Ser48. The remaining parts of the substrate bind in a hydrophobic cleft with residues from both the catalytic and coenzyme binding domains and the nicotinamide ring. The inner parts of this substrate pocket close to the active site zinc ion are formed by Ser48, Phe93, Phe140 and Leu141. The middle part of the pocket, further away from the zinc ion, is lined by Leu57, Leu116, Val294 and Ile318. The outer rim of the substrate cleft is lined by Phe110, Met306 and Leu309.

Catalytic mechanism

The general events in catalysis were established early by Theorell and co-workers [20]. The structural data could then be combined with the available kinetic knowledge to a description of all catalytic events in molecular terms [6]. For most of the ADHs, the mechanism is ordered such that coenzyme binds before the substrate. This is explained by the structural data since the nicotinamide forms part of the substrate binding site. For an alcohol substrate, the positive charge of the nicotinamide ring of NAD^+ contributes to the deprotonation of the alcohol proton and promotes binding to the active site zinc ion. The substrate and nicotinamide ring are placed close to each other to facilitate hydride transfer. The ternary complex of pentafluorobenzyl alcohol with NAD^+ provides the structure of the resting state of the enzyme with the pro-R hydrogen of the substrate pointing towards the C4 atom of the NAD^+ (Fig. 3).

The steroid-active isozyme

Most crystallographic investigations of the horse liver ADH have been performed on the isozyme where

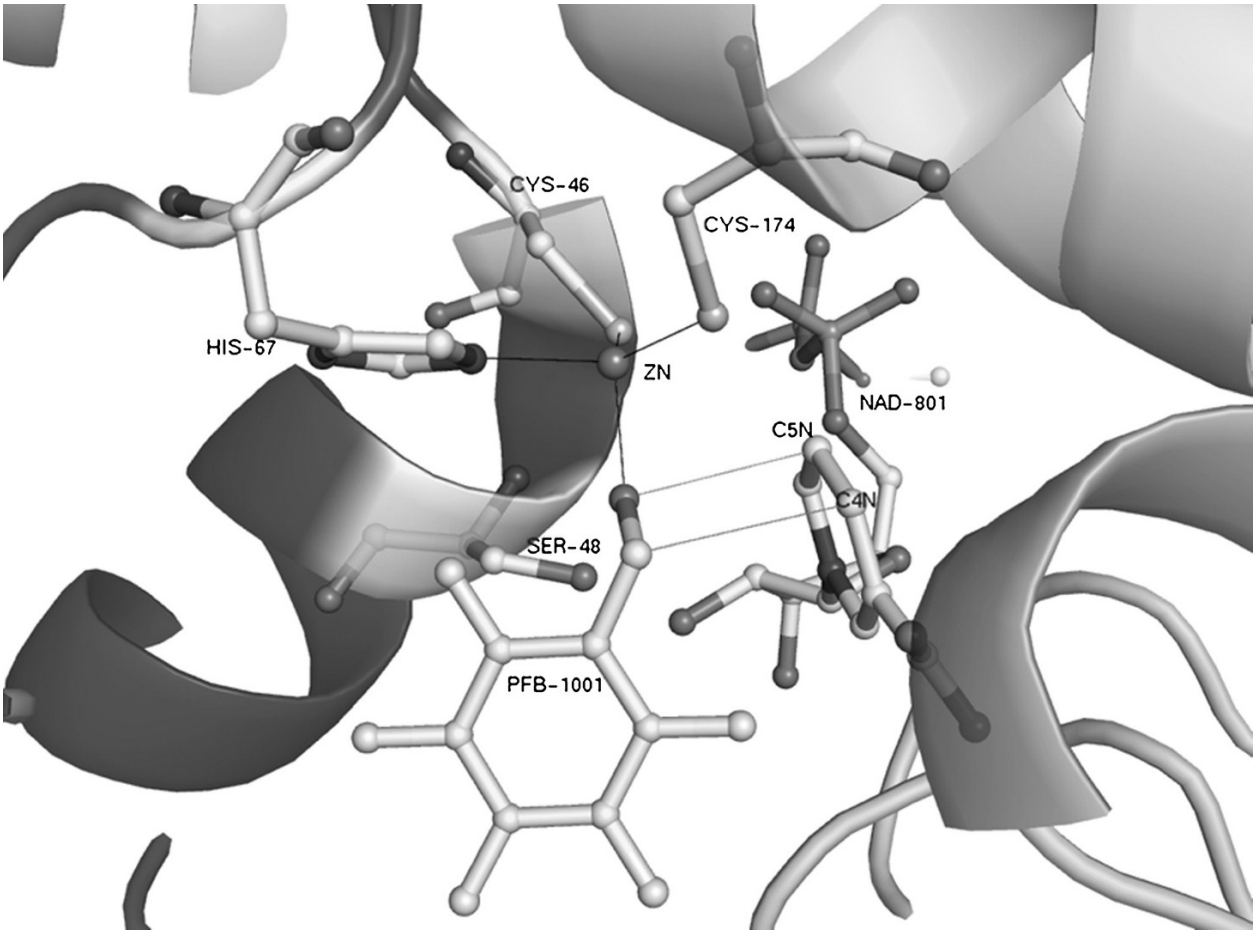

Figure 3. Binding of pentafluorobenzylalcohol and NADH at the active site of horse ADH.

both subunits are of the ethanol-active E-type. But horse liver ADH also has a steroid-active subunit, which differs from the E-type at 10 positions: nine substitutions and one deletion [21, 22]. The major structural difference of the S-subunit compared with the E-subunit is in the loop region containing the deletion [23]. A result is a widening of the substrate channel that allows the steroid to bind.

Metal-substituted enzymes

Crystallographic studies of active site metal depleted enzymes and enzymes where the active site metal has been substituted by cobalt and cadmium reveal very similar structures from the zinc containing enzyme [24–26]. The same conformational changes as for the native enzyme also occur in the absence of metal at the active site. Later studies have focused on copper and cadmium substituted enzymes [27–30].

The structure of the cadmium enzyme in complex with NADH and isobutyramide revealed the partial formation of an adduct between the isobutyramide inhibitor and NADH [30]. It provides evidence of the contribution of a shift from the keto to the enol tautomer during aldehyde reduction. NADH is puckered in a twisted boat conformation. Puckering of NAD(H) has also been observed in a number of other complexes [31, 32]. It is quite possible that the nicotinamide ring is generally present in the puckered form. Given the lower resolution of the earlier structures and the restraint used for the NAD ring, this was initially not observed.

Mutant structures

The conformational change requires the flexible loop in the coenzyme binding domain to rearrange. The structure of a double mutant where the highly conserved Gly293 and Pro295 of this loop were mutated to Ala and Thr, respectively, shows that the conformations of both free and complexed forms of the mutated enzyme are very similar to the open form of the enzyme [33]. Binding of NAD^+ and trifluoroethanol to this mutant form does not cause the conformational change to a closed form, but the nicotinamide ribose moiety and alcohol are not in fixed positions. Molecular modeling shows that the new side chains cannot be accommodated in the

closed form without steric alterations. This enzyme has a lower affinity to NAD^+, but a larger turnover number. This is consistent with the conclusion from kinetic studies [34] that domain opening and closing are the rate-limiting steps in catalysis.

His51 has been proposed to facilitate proton exchange at the active site and to participate in a proton relay system [11]. In order to reveal its role, the His51Gln mutant has been investigated [35]. The structure of the double mutant His51Gln;Lys228Arg was determined in a complex with NAD^+ and difluorobenzyl alcohols. It shows that Gln51 isosterically replaces histidine in interactions with the nicotinamide ribose of the coenzyme and that Arg-228 interacts with the adenosine monophosphate of the coenzyme without affecting the protein conformation. A conclusion from the kinetics of this mutant was that His51 participates in, but is not essential for, proton transfers in the mechanism.

The structure of the Phe93Ala mutant crystallized with NAD^+ and pentafluorobenzyl alcohol is similar to the structure of the wild-type enzyme complex except that the pentafluorobenzyl alcohol is not found in one position [36]. The substitution greatly increases the mobility of the benzyl alcohol and decreases the probability that the substrate is pre-organized for hydride transfer.

The structures of the substrate pocket Val292Thr mutant in complex with NAD^+-pyrazole and wild-type ADH-NAD^+-4-iodopyrazole ternary complexes are very similar [32]. Subtle changes in the mutant enzyme cause large changes in coenzyme binding and small changes in hydride transfer. In these complexes, one pyrazole nitrogen binds to the catalytic zinc, and the other nitrogen forms a partially covalent bond with C4 of the nicotinamide ring, which adopts a boat conformation postulated to be relevant for hydride transfer.

Inhibitor binding

In the structures of the complexes with NADH and N-cyclohexylformamide or N- formylpiperidine, the carbonyl oxygens of the inhibitors bind to the catalytic zinc and form a hydrogen bond to the hydroxyl group of Ser48 [37, 38]. The six-membered rings have hydrophobic interactions. Formamides are unreactive analogues of the aldehyde substrates [37]. The structure of NADH and methylhexylformamide resembles the expected Michaelis complex with NADH and aldehyde, and shows that the reduced nicotinamide ring of NADH is puckered, as predicted for the transition state for hydride transfer.

The ternary enzyme complexes of thiolane 1-oxides with NADH demonstrate that in stereoisomer rings bind in the same position at the substrate binding site, but the geometry of the complexes suggests that the sulfoxides are not transition-state analogs [39]. Significantly, the butyl groups of the two isomers are accommodated differently by flexible amino acid side chains adopting alternative rotameric conformations.

High-resolution structures

Today several structures of horse liver ADH have been determined to a resolution better than 2 Å. The best resolution so far is for the complex with NADH and DMSO, which was determined at 1.0 Å resolution [30]. The nicotinamide ring in this complex is puckered in a twisted boat conformation. A boat conformation has also been observed in another high-resolution structure of a NAD^+ pyrazole complex and in a complex with NADH and methylhexylformamide (Fig. 4). Such a conformation has been postulated to be relevant for hydride transfer [30, 32].

Human ADH structures, substrate specificity

Primary structure analysis and modeling highlighted early the structural and functional differences for the human enzyme [40, 41]. Later, several of these were characterized in tertiary structures. The human class I enzymes with subunits ADH1A (earlier called α), ADH1B (earlier called β) and ADH1C (earlier called γ) are most similar to the horse E-subunit. The overall structure of each isozyme is highly similar to the others and to the horse enzyme. The isozymes differ somewhat in the substrate pocket and thus in substrate specificity.

ADH1A

A major difference of the ADH1A isozyme compared to the horse enzyme is an exchange of Phe93 at the substrate pocket with an Ala that enlarges the active site near the catalytic zinc and creates the specificity for branched substrates and inhibitors, and better activity with secondary alcohols [42]. The substitution of Gly for Arg at position 47 in the ADH1A isozyme promotes a greater extent of domain closure [43].

ADH1B

There are several variants of the β subunits. The most common ADH1B1 has a Thr48 instead of Ser48 close to the active site zinc ion and has a more restricted substrate-binding site near the catalytic zinc atom [43]. Both the decreased V_{max} and the decreased rate of NADH dissociation for this enzyme compared to the horse enzyme seems not to be due to substrate-interacting amino acid substitutions but to structural rearrangements resulting from multiple sequence differences between the two enzymes.

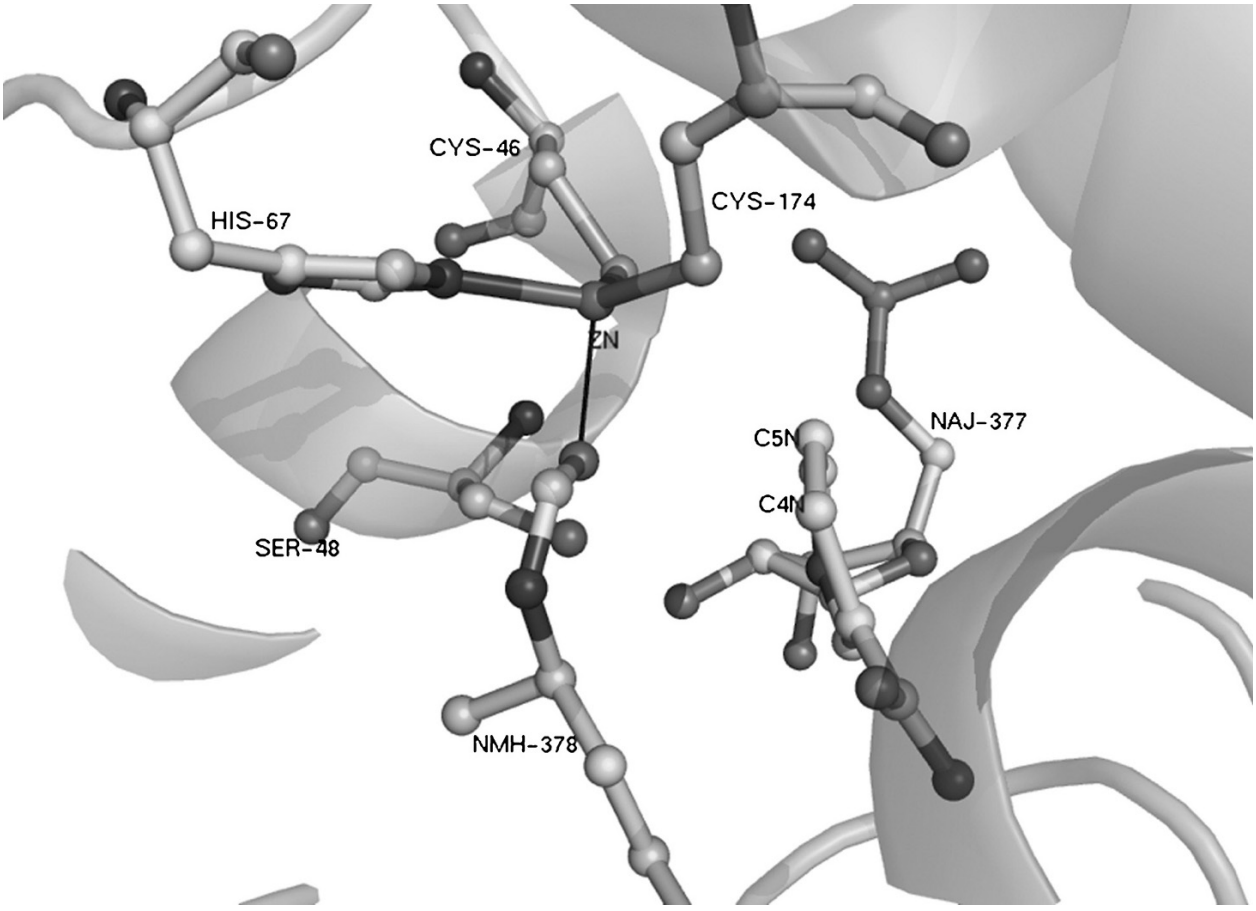

Figure 4. Puckering of coenzyme in the structure of horse liver ADH bound to R-N-1-methylhexyl formamide (pdb-code 1p1r). The puckering of the NADH ring is clearly visible.

Human ADH1B1 has an Arg at position 47. The substitution of Arg47 by Gly produces an enzyme with coenzyme binding characteristics more similar to the wild-type enzyme than to the enzyme with His at position 47, but the structure of the Gly47 variant exhibits differences in and around the coenzyme binding site [44]. These changes involve a small rigid-body rotation of the catalytic domain towards the coenzyme domain and local rearrangements of amino acid side chains, such as a 1.0 Å movement of Lys228, relative to the native enzyme. These structural alterations may compensate for the loss of coenzyme interactions contributed by Arg47 and can explain the high affinity of the Gly47 variant for the coenzyme. ADH1B2 has a His instead of a coenzyme binding Arg47 but no large changes in the enzyme structure [44]. This enzyme has a 100-fold lower affinity for coenzyme.

In the human ADH1B3, Arg369 that binds to the phosphates of the coenzyme in ADH1B1 is substituted with Cys [45]. Except at this position, its three-dimensional structure is the same as the ADH1B1 structure, and two waters occupy the same positions as the nitrogen atoms of Arg369. The loss of electrostatic interaction results in elevated dissociation constants for NAD^+ and NADH compared to ADH1B1.

ADH1C

The structure of the ADH1C2 isozyme in a binary complex with NADH has a closed conformation like that of horse ADH1E with NADH [38]. ADH1C1 and ADH1C2 isozymes are active toward steroid substrates. For ADH1C isozymes, it has been shown that Ser48 is almost exclusively responsible for this enzyme's ability to oxidize 3β-hydroxy-5β-steroids.

ADH2

The structure of human class II alcohol dehydrogenase (ADH2) has not yet been determined, but the structure of the closely related mouse enzyme has been determined in a binary complex with the coenzyme NADH and in a ternary complex with both NADH and the inhibitor N-cyclohexylformamide [46]. The subunits in the crystallographic dimer have slightly different closure of the active-site cleft, both about halfway between the open and closed

structures of horse ADH1. As a consequence of the semi-open conformation and some other structural differences, the active site cleft is more voluminous than that of ADH1 but not as open and funnel-shaped as that of ADH3.

ADH3

ADH3 catalyzes the oxidation of long-chain alcohols such as omega-hydroxy fatty acids [47] and S-hydroxymethyl-glutathione [48], an adduct between formaldehyde and glutathione. Asp57 and Arg115 contribute to glutathione binding and Arg115 contributes to binding of omega-hydroxy fatty acids.

The human glutathione-dependent formaldehyde dehydrogenase is unique among the structurally studied members of the ADH family in that it follows a random bi bi kinetic mechanism. The structures show that the apoenzyme has a semi-open domain conformation that permits random addition of alcohol or NAD(H) [49, 50]. This conformation is halfway between the open and closed conformations described for the horse enzyme. Moreover, there is no significant domain movement upon binding of the coenzyme or the substrate, 12-hydroxydodecanoic acid. The semi-open conformation and changes in elements of secondary structure provide a structural basis for the ability of ADH3 to bind S-hydroxymethyl-glutathione and 10-hydroxydecanoate.

ADH4

The ADH4 isozyme has 69 % sequence identity with ADH1B but differs at five positions in the substrate pocket, which creates a substrate pocket that is wider close to the active site zinc ion but is narrower in the middle region of the pocket [51]. This leads to different kinetic properties. The nicotinamide ring of the NAD(H) molecule is twisted relative to its position in the ADH1B isozyme. That, in combination with a wider substrate pocket in the vicinity of the catalytic zinc, can explain the high K_m for small substrates. The narrowing of the middle region of the substrate pocket may explain the decreased K_m values with long substrates and low affinity for 4-methylpyrazole, a property due to a large methionine residue in the substrate site.

The amphibian enzyme ADH8

The amphibian enzyme ADH8 (class IV-like) is a vertebrate ADH with specificity towards NADP(H). The three-dimensional structure of ADH8 shows a substrate-binding pocket with a large volume that explains both the high catalytic efficiency of ADH8 with retinoids and the high K_m for ethanol. Instead of Asp223, the Gly223-Thr224-His225 sequence of ADH8 gives a preference for NADP(H) [52].

Tetrameric ADHs

Whereas the mammalian medium-chain alcohol dehydrogenases are dimeric, several fungal and bacterial enzymes are tetramers. Most biochemical work has been done on the different yeast enzymes, but most structural studies of tetrameric MDR ADHs have been done on enzymes from thermophilic organisms. Here, we describe the tetrameric ADHs from the structure of *Escherichia coli* ADH which has properties similar to the class I enzymes. The kinetic parameters indicate that its physiological function is like that of the fermentative ADHs. The structure of a similar fermentative enzyme, ADH I from *Saccharomyces cerevisiae,* was recently determined (pdb-code: 2hcy).

***Escherichia coli* ADH**

Three-dimensional structure of an ethanol-induced, tetrameric ADH from *E. coli* has been determined in the absence and presence of NAD [53]. In relation to the class I ADHs, the major difference is that the *E. coli* ADH structure lacks 21 residues corresponding to residues 118–139 of horse liver ADH. This long stretch, which contains two β-strands, a β-turn and less-ordered residues, is replaced with a short turn that exposes a tyrosine residue at the substrate binding site. Besides this, the only differences in chain length are one-residue or two-residue differences at connections between secondary structure elements.

The ADH tetramer is arranged as a dimer of two dimeric ADHs interacting back-to-back with the active sites exposed on the sides opposite to these interactions (Fig. 5). Each dimer is similarly arranged as the dimeric ADHs with the β-sheet of the coenzyme binding domains of each subunit interacting antiparallel with each other, forming a 12-stranded sheet. The tetramer interactions between the two dimers involve contacts of each subunit in one dimer with both subunits in the other. One type of contact is with the loop containing the structural zinc ion.

The structure of the holoenzyme has the coenzyme bound in three of the four subunits, whereas one subunit surprisingly has no bound NAD. The coenzyme is bound in roughly the same boomerang-shaped conformation as in horse liver ADH. When NAD binds to the enzyme, a closed conformation of the enzyme is obtained by rotation of the catalytic domains. The magnitude of the rotation of the catalytic domain relative the coenzyme domain is as large in *E. coli* ADH as in horse ADH, but the rotation axis is differently located. When the central coenzyme binding domains of *E. coli* ADH and horse ADH are superimposed, the catalytic domains are rotated roughly along the long axis of the molecule. This is possible because of the long deletion of 60 residues in

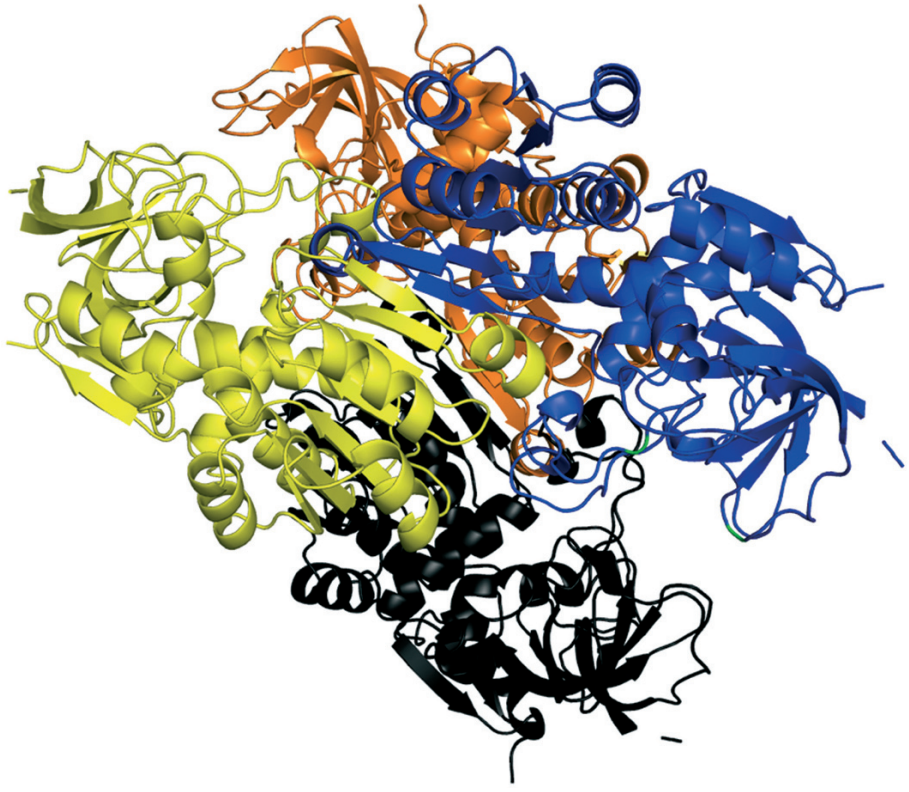

Figure 5. Tetrameric *E. coli* ADH. The four chains are colored differently, one dimer in yellow and blue and the second in black and gold.

E. coli ADH. This rotation of the catalytic domain forms a backside of the molecule that is more flat, which is advantageous for tetramer formation. In horse ADH and other dimeric ADHs, the domain rearrangement is possible only if a loop in the coenzyme binding domain drastically changes its conformation. In *E. coli* ADH, the corresponding loop is shorter and has similar conformations in both the apo- and holoenzyme.

Mesophilic and thermophilic tetrameric ADHs

The NADP(H)-dependent alcohol dehydrogenase of *Clostridiim beijerinckii* in the apo-and holoenzyme forms and of the holo-alcohol dehydrogenase of *Thermoanaerobacter brockii* have structures similar to that of the *E. coli* tetrameric ADH [54]. The tetramers are composed of two dimers, each structurally homologous to the dimer of ADHs of vertebrates. The specificity of the two bacterial ADHs toward NADP(H) is determined by residues Gly198, Ser199, Arg200 and Tyr218, with the latter three making hydrogen bonds with the 2′-phosphate oxygen atoms of the coenzyme. The thermophilic enzyme has a more hydrophilic exterior, a more hydrophobic interior, a smaller surface area, more proline and alanine residues, and fewer serine residues than the mesophilic *C. beijerinckii* enzyme, and the number of intersubunit interactions is increased [55, 56]. Mutants of the mesophilic enzyme with enhanced thermal stability have stronger interactions in the quaternary structure [55].

Other structures of the tetrameric medium chain ADHs have been determined from the hyperthermophilic archaeons *Aeropyrum pernix* [57, 58] and *Sulfolobus solfataricus* [59, 60], from *Bacillus stearothermophilus* [61], *Bacillus subtilis* protein yhfP (pdb-code:1tt7), *Thermotoga maritima* (pdb-code:1vj0) and yeast (pdb-code:2hcy).

Variable coordination of the active site zinc ion

Tetrameric *E. coli* ADH

The classical active site zinc ion in ADH has a tetrahedral coordination of two cysteines, one histidine and a water molecule. The water molecule is substituted by substrate during the catalytic action. In the apoenzyme, one subunit per tetramer has the classic coordination, while the others have a Glu-coordinated zinc [53]. The geometry in this is still roughly tetrahedral, but instead of the open binding site for water/substrate, the glutamate, corresponding to Glu68 in the horse enzyme, has bound to the metal (Fig. 6). The situation is reversed for the holoenzyme, where only the subunit that corresponds to the classic coordination in the apoenzyme has a Glu-coordinated zinc. So, three of the subunits of the apoenzyme and

one subunit in the holoenzyme have the different active site zinc coordination with Glu instead of water/substrate. The position of the zinc atom is displaced in the Glu-coordinated subunits by about 2 Å away from the substrate pocket.

No water molecule is bound to zinc ions in the Glu-coordinated subunits. Instead, water is bound further out in the substrate cleft at about a distance of 5 Å from the active zinc ion, too far away to be considered coordinating. The coordination geometry of the zinc in these sites also appears to be such that binding of a free ligand is strongly impaired for steric reasons. From this, it appears that the Glu-coordinating subunit exist in a form that is not able to bind substrate.

For *E. coli* ADH, the holoenzyme subunits with the classic zinc coordination binds coenzyme, whereas the subunits with Glu-coordinated zinc has no coenzyme bound. A possible reason can be that when Glu moves in to coordinate zinc, it loses its interactions with the arginine corresponding to Arg369, which becomes mobile and moves away from its classical position. This impairs coenzyme binding, since Arg369 binds the phosphates of the coenzyme. The present structure of *E. coli* ADH implies that a shift of coordination of the zinc ion to an active form is influenced by the binding of coenzyme. Such a transition opens the zinc ion for coordination of substrate. The advantage of having a non-productive coordination with Glu should in such a case be that the exchange of free ligand should be facilitated. Theoretical calculations also indicate that Glu could be coordinating during the reaction cycle to be able to get rid of the bound water molecule and/or the product [62, 63].

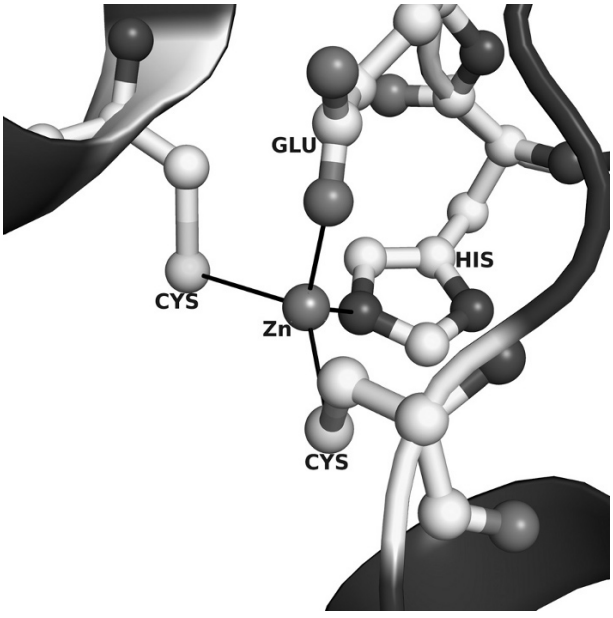

Figure 6. Changed coordination of the active site zinc ion in some subunits of *E. coli* ADH.

Glu-coordination of the active site zinc in other ADHs

There is a conserved glutamic acid close to the active site in all the known ADHs. Active site zinc coordinated by Glu has been observed also in other tetrameric ADHs, while it has not been observed in most dimeric ADH structures. For ADH from *C. bejerinckii* the apoenzyme had the active site tetracoordinated with Glu as one ligand [54]. In the holoenzyme three of the four subunits had a normal coordination without Glu and one with such coordination [54], similarly as for the structure of *E. coli* ADH. The tetrameric ADH from *S. solfataricus* also has a zinc bound Glu [59]. In this case, the apoenzyme was determined.

A study of the tetrameric NADPH-dependent ADH from *T. brockii* has also implemented Glu as a ligand but not given any role in catalysis. It only exchanges with a water molecule [64]. In their proposed reaction mechanism, a pentacoordinated zinc atom with both water and substrate bound to zinc. However, no role for the zinc-bound water is implemented in the reaction scheme; rather, involvement in deprotonation of the alcohol is indicated. The role of a pentacoordinated water molecule in close interplay with the nicotinamide ring was recently suggested based on a high-resolution structure of horse LADH [31].

The only dimeric ADH for which this type of coordination has been observed is for the human ADH3. This was first found in one of the subunits in a binary complex [49], and later also in a ternary complex with NAD and the inhibitor dodecaonic acid [50]. It shows normal zinc coordination in apoenzyme and a binary complex with 12-hydroxydodecanoic acid [50].

The role of the transient coordination of the active site zinc to Glu in the ADH3 catalytic cycle was investigated by studying enzymes in which residues corresponding to Glu68 and Arg369 were substituted with Leu. The Glu mutant resulted in impaired binding of substrates. For the substrate 12-hydroxydodecanoic acid, there was a decrease in the rate of hydride transfer. Binding of the coenzyme is not affected by the Glu substitution, but the disruption of the interaction between Glu and Arg is implicated in the displacement of active site zinc.

Hydrogen tunneling

Quantum mechanical behavior in connection with hydrogen chemistry has attracted much interest. In this context, ADH has been a model system for the experimental and theoretical investigations of the phenomenon of hydrogen tunneling [61, 65–72]. Mutations close to the active site of Phe93 to a Trp or to a Val have shown differential effects on hydrogen tunneling. This

suggests that residues not directly involved in the catalytic mechanism could contribute to protein dynamics that can influence the rate of the reaction [62].

The ability of ADH to crystallize and diffract to high resolution as well as the combination of structural changes that lead to interesting effects on catalysis has made ADHs one of the favorite models for studying enzyme-catalyzed reactions.

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