Mechanistic Implications from Structures of Yeast Alcohol Dehydrogenase Complexed with Coenzyme and an Alcohol[☆]

Bryce V. Plapp*, Henry A. Charlier, Jr.^a, and S. Ramaswamy^b

Department of Biochemistry, The University of Iowa, Iowa City, IA 52242, USA

Footnotes

Abbreviations: ADH, alcohol dehydrogenase; TFE, 2,2,2-trifluoroethanol (“ETF” in the PDB file, with the methylene carbon labeled as “C2”); rmsd, root mean square deviation.

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* Corresponding author. Phone: (319) 335-7909. Fax: (319) 335-9570 E-mail: bv-plapp@uiowa.edu.

^a Present address: Department of Chemistry and Biochemistry, 1910 University Drive, Boise State University, Boise, ID 83725. E-mail: henrycharlier@boisestate.edu

^b Present address: Institute for Stem Cell Biology and Regenerative Medicine (inSTEM), National Center for Biological Sciences, GKVK Post, Bellary Road, Bangalore 560065, India. E-mail: ramas@instem.res.in

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ABSTRACT

Yeast alcohol dehydrogenase I is a homotetramer of subunits with 347 amino acid residues, catalyzing the oxidation of alcohols using NAD^+ as coenzyme. A new X-ray structure was determined at 3.0 Å where both subunits of an asymmetric dimer bind coenzyme and trifluoroethanol. The tetramer is a pair of back-to-back dimers. Subunit A has a closed conformation and can represent a Michaelis complex with an appropriate geometry for hydride transfer between coenzyme and alcohol, with the oxygen of 2,2,2-trifluoroethanol ligated at 2.1 Å to the catalytic zinc in the classical tetrahedral coordination with Cys-43, Cys-153, and His-66. Subunit B has an open conformation, and the coenzyme interacts with amino acid residues from the coenzyme binding domain, but not with residues from the catalytic domain. Coenzyme appears to bind to and dissociate from the open conformation. The catalytic zinc in subunit B has an alternative, inverted coordination with Cys-43, Cys-153, His-66 and the carboxylate of Glu-67, while the oxygen of trifluoroethanol is 3.5 Å from the zinc. Subunit B may represent an intermediate in the mechanism after coenzyme and alcohol bind and before the conformation changes to the closed form and the alcohol oxygen binds to the zinc and displaces Glu-67.

Structures for yeast (*Saccharomyces cerevisiae*) alcohol dehydrogenase 1 (ADH1) determined at 2.4 Å (PDB ID, 4W6Z) show that the homotetramer is formed from back-to-back asymmetric dimers of subunits [1]. One subunit, in a “closed” conformation, binds the NAD coenzyme, and the substrate analog, trifluoroethanol, in an apparently “productive” mode that resembles an expected Michaelis complex, with the *pro-R* hydrogen of the methylene carbon of the alcohol directed toward C4N of the nicotinamide ring and the alcohol oxygen binding to the catalytic zinc in the classical tetrahedral coordination with Cys-43, Cys-153, and His-66. The other subunit, in an “open” conformation, has no bound coenzyme, and the catalytic zinc has an alternative, inverted, coordination where Glu-67 displaces the trifluoroethanol. (The open-to-closed conformational change involves a rotation of about 13° of the catalytic domain toward the coenzyme binding domain.) We suggested that the enzyme crystallized in the asymmetric form and that any coenzyme that might have been bound to the open subunit dissociated from the complex when the crystals were soaked for some days (for transport to the synchrotron) in a solution that did not contain the coenzyme. The asymmetry is puzzling because titrations of the active sites of the enzyme have suggested that all four subunits in a tetramer can bind coenzyme, and there is no good evidence for cooperativity in binding of NAD or in the kinetics of catalysis [1]. (See also Supporting Information in Ref. [1] for results on the stoichiometry of coenzyme binding.) Thus, it was necessary to determine if the crystallized enzyme would be asymmetric and would have coenzyme bound to both subunits if the concentration of coenzyme were maintained until the moment when the crystals were flash vitrified for data collection at 100 K.

Therefore, we examined data again for other crystals that were kept in crystallization media containing coenzyme, even if the crystals diffracted less well. From tens of data sets, from some thousands of crystallization attempts under different conditions by nine researchers, most crystals appeared to have the *P622* space group [2], but were twinned. However, two data sets processed in the *P321* space group, with a unit cell half as large as the one solved previously. The crystals in the *P321* space group were partially twinned, and structures could not be determined earlier. Now, using molecular replacement with a dimer from the structure determined at 2.4 Å (4W6Z.pdb) as the search model, we found that these crystals have a dimer in the asymmetric unit, that the subunits have different conformations, and that both subunits bind coenzyme and alcohol. The dimer forms the biologically relevant tetramer via a crystallographic axis. The structures of the enzymes in the two different crystal forms are very similar, but the subunit with

the open conformation in the new structure has small, local alterations that accommodate the coenzyme binding. The open conformation retains the alternative coordination of the catalytic zinc, and it appears that this subunit could represent an intermediate on the pathway to forming a productive ternary complex.

1. Materials and methods

1.1. Protein preparation

The gene for *Saccharomyces cerevisiae* ADH1 (*adc1*, YOL086c; UniProtKB, P00330), the constitutive, fermentative enzyme from the laboratory strain of baker's yeast was expressed from plasmid YEp13 in host yeast that did not express any of the three medium-chain, zinc-containing alcohol dehydrogenases [3–5], and the protein was purified to homogeneity as described [6].

1.2 Crystallography

The crystals were prepared by the hanging drop method at 4 °C with 10 mg/ml yeast ADH1, 100 mM sodium *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonate buffer, pH 8.4 (measured at 25 °C), 0.25 mM EDTA, 2 mM LiNAD⁺, 0.2 M 2,2,2-trifluoroethanol (TFE), 1 mM YbCl₃, and 7% MPEG5000 (Fluka polyethylene glycol 5000 monomethyl ether) over reservoirs containing ~16 % (15–19%) MPEG 5000 and 0.2 M TFE. For shipping to the synchrotron, the crystals were transferred to a solution with the same buffer and 2 mM NAD⁺ as in the original drop, except that the concentration of TFE was raised to 0.287 M and MPEG-5000 was 25%.

Data were collected May 18, 1997, on synchrotron beamline BW7A at the EMBL/DESY unit in Hamburg at 100 K, at a wavelength of 1.39 Å and a crystal to detector distance of 180 mm, with a MAR CCD 130 mm detector, and processed with Denzo, Scalepack, and Truncate [7,8]. A molecular replacement solution was found with the AB dimer of the tetrameric structure of ADH1 (4W6Z.pdb) using MolRep [8]. Further refinement used REFMAC5 and model building with the program O [8,9]. The refinement was accomplished with the twin operator (*h*, *k*, *l*, and –*h*, –*k*, *l*, with final refined twin fractions of 0.714 and 0.286) and by using 4 TLS elements, for the 2 catalytic domains, residues 1–154 and 294–347, with the associated 2 zincs and TFE, and the 2 coenzyme binding domains, residues 155–293, with the associated NAD. The structure was analyzed with PROCHECK, SFCHECK [8], MolProbity [10], and PARVATI [11]. The estimated solvent content is 55 % and the Matthews coefficient is 2.75 Å³/Da. The data and

refinement statistics are given in Table 1. The X-ray coordinates and structure factors have been deposited in the RCSB Protein Data Bank as entry 5ENV.pdb.

2. Results

2.1. Overall structure

Subunit A of the present structure is very similar to subunits A and C in the previous structures (4W6Z.pdb) in having the closed conformation, bound coenzyme, and the oxygen of trifluoroethanol ligated to the catalytic zinc. In contrast, the B subunit is more open and similar to the B and D subunits in the previous structures, but, significantly, the B subunit has bound coenzyme. (The AB and CD dimers in the previous structures are the asymmetric units, related by a translation in the unit cell, producing two similar AB:AB and CD:CD tetramers.) The α -carbons of the AB dimer in the present structure are superpositioned on the α -carbons of the AB dimer from the previous structure (4W6Z.pdb) with an rmsd of 0.58 Å, the A subunits are superpositioned with an rmsd of 0.34 Å, and the B subunits with an rmsd of 0.66 Å. The A and B subunits of the present structure superposition on one another with an rmsd of 1.8 Å, indicating conformational differences in the subunits. After residues from the coenzyme binding domains (155–293) are superpositioned (rmsd 0.56 Å), the catalytic domains superposition with an rmsd of 0.84 Å, with a relative rotation of $\sim 12^\circ$, which is similar to the rotation angle that describes the closure of the A subunit relative to the B subunit in the previous structure (Fig. 1). The conformational change closes up the active site cleft about an axis perpendicular to the plane indicated by the “X”. Several secondary structural elements in the B subunit with bound coenzyme of the present structure show small movements as compared to the previous B subunit without coenzyme, including residues 201–208, 224–226, 245–250, 269–275, and 325–341, apparently due to the binding of coenzyme, but the overall “open” states are similar.

The biologically relevant molecule is a tetramer, formed from two back-to-back AB dimers about a crystallographic axis, as discussed and shown previously [1].

The average *B* factor for residues B6–B24, B41–B63 and B317–B341 are higher than those residues in the A subunit. These regions form loops of the catalytic domain that show the largest movement when the conformation changes from open to closed (Fig. 1). The overall *B* factor of 57 Å² for the whole structure is decreased to 18 Å² when the *B* factors from the TLS refinement

are not added to the individual atomic B factors, suggesting that the four TLS elements have considerable mobility as units.

Electron density for Yb^{3+} was not observed, and an anomalous difference map showed no substantial peaks of density above background. Data for another crystal in the same space group prepared under similar conditions, but without the YbCl_3 and diffracting to 3.5 Å, yields a very similar overall structure, with NAD and trifluoroethanol binding in both subunits ($R/R_{\text{free}} = 0.21/0.28$). This suggests that YbCl_3 did not affect the crystal packing and influence the observed asymmetry.

2.2. Coenzyme binding

The subunits A and B have clear electron density for bound coenzyme, although the nicotinamide ring of the NAD^+ in the B subunit appears to have more flexibility as electron density is lower (Fig. 2). The B and D subunits in the previous structures (4W6Z.pdb) had no evidence for bound coenzyme. The state of oxidation of the NAD cannot be determined from the X-ray data, but the crystallization medium contained 0.2 M trifluoroethanol, a competitive inhibitor against alcohol oxidation with a K_i value of 2.8 mM, which should prevent reduction of the NAD^+ [3].

The binding of the coenzyme to the coenzyme binding domains is similar in subunits A and B of the present structure, where side chains of Phe-221, Ser-246, Asp-201, and Lys-206 are near the adenosine moiety, and backbone NHs of Gly-181 and Leu-182 interact with the pyrophosphate oxygens O1A and O1N, respectively (Fig. 2A,B). In the previous structure (4W6Z.pdb), the 8-iodo substitution on the adenine ring of the NAD extends into the solvent and does not interact with the protein or affect the structure. The electron density maps in Fig. 2 show that in subunit A, amino acid residues from the catalytic domain, e.g., His-44, Thr-45, His-48, and Arg-340, make hydrogen bonds with O2A, O3D, O2D, and O2N, respectively, of the coenzyme, whereas in subunit B, because the cleft is more open, these residues from the catalytic domain do not make close contacts with the coenzyme, as illustrated in more detail in Fig. 3. The differing interactions of the coenzyme with the catalytic domain show the hydrogen bonding with red, dashed lines in subunit A (atomic coloring) with His-44, Thr-45, His-48, Val-295, and Arg-340, and the corresponding residues from subunit B (in green), which do form hydrogen bonds. The carboxyamido group of the nicotinamide ring in subunit A forms hydrogen bonds

with Val-268 O, Ser-293 O and Val-295 N, but in subunit B, the interaction with Val-295 is too long because the peptide bond with Tyr-294 is flipped, as it is in subunits B and D in the structures that did not contain coenzyme (4W6Z.pdb). Thus, the coenzyme in subunit B interacts with the coenzyme binding domain, which includes residues 155–293, but not with residues from the catalytic domain, which includes residues 1–154 and 294–347.

2.3. Zinc coordination and trifluoroethanol binding

The coordination of the catalytic zincs in subunits A and B is compared in Fig. 4. (Separate views of the electron density maps at 2.4 Å were presented previously [1].) The catalytic zinc in subunit A has the classical coordination with Cys-43, His-66, Cys-153, and the oxygen of trifluoroethanol, as found in the previous structure for subunits A and C. The catalytic zinc in subunit B has the alternative (“inverted”) coordination with Cys-43, His-66, Glu-67 and Cys-153, as found previously for the B and D subunits. The inversion (analogous to a Walden inversion that changes the stereochemistry) is accompanied by a movement of ~2.6 Å of the zinc and small changes in the positions of SG of Cys-43 and the carboxyl group of Glu-67, relative to the classical coordination. Apparently, during the domain closure, the carboxylate of Glu-67 is displaced, and the zinc and trifluoroethanol move to form the coordination observed in the ternary complex in subunit A.

Subunit A appears to have NAD and trifluoroethanol bound in a geometry that resembles a Michaelis complex poised for catalytic reaction as shown in subunits A and C in the previous structure [1]. The oxygen of the alcohol is ligated to the catalytic zinc (2.1 Å) and the methylene carbon (C2) is 3.5 Å from C4N of the nicotinamide ring, with a good geometry for hydride transfer. In contrast, in subunit B, the trifluoroethanol is bound differently, similar to the position found in subunits B and D of the previous structure. The alcohol oxygen is 3.5 Å from the catalytic zinc (too far for direct ligation), and C2 is 3.7 Å from C4N. The oxygen can form hydrogen bonds with OG1 of Thr-45 and SG of Cys-153. The electron density for trifluoroethanol is moderate in subunit B, and because the occupancy is estimated to be about 50%, the K_d value would correspond to a value of 287 mM, the concentration of trifluoroethanol in the final crystallization medium. The estimation only indicates that the binding is relatively weak, as compared to the K_d for binding of trifluoroethanol to the enzyme-NAD⁺ complex of

0.28 mM as determined from competitive inhibition against ethanol [3]. There are no data for binding of trifluoroethanol to apoenzyme.

2.4. Asymmetry of the dimeric unit

The important new result of this study is that the crystallographic asymmetric unit is a dimer of subunit A with a closed conformation and subunit B with an open conformation where both subunits have bound coenzyme. In the previous structure, the subunits with the open conformation did not have observable electron density for coenzyme. Those crystals had been prepared in the presence of coenzyme (8-iodo-NAD), but were soaked in a solution that contained no coenzyme (because the supply was all used for the hanging drops), providing conditions that could allow coenzyme to dissociate before flash vitrification at 100 K for crystallography. Based on the kinetic constants for NAD^+ and trifluoroethanol binding, the active sites should have been 96% saturated under the crystallization conditions [3]. However, if the enzyme has pre-existing asymmetry or if the crystal lattice packing interactions led to asymmetry, an open conformation could have allowed coenzyme to dissociate from the enzyme in the crystal. Dissociation of coenzyme from the closed conformation is not likely because of the extensive interactions between the two domains (Fig. 2A). The present study maintained the coenzyme concentration in the crystal for data collection and shows that the open conformation can bind coenzyme, and release it, if the concentration of NAD^+ decreases as in the previous study. The results also show that the asymmetry is not caused by dissociation of coenzyme. These results support the proposal that the open conformation of the enzyme binds the coenzyme and changes to the closed conformation before hydrogen transfer.

Although the unit cell dimensions of the two forms of crystals differ, the packing of the tetramers seems to be similar, and it remains to be determined if the enzyme has cooperative active sites (intrinsic asymmetry) or if crystal lattice interactions caused the asymmetry. The difference in energies of the two conformational states of the subunits may not be very large, and one can imagine that lattice interactions could affect the conformations. It is not unusual that dimeric units of ADHs are asymmetric, but at this time there is no evidence for cooperativity in yeast ADH1, which has Michaelis-Menten kinetics and an ordered Bi Bi or preferred ordered mechanism [1,3,12,13]. The origin of the asymmetry is considered further in the following.

Because the dimers are formed with extensive interactions between the coenzyme binding domains of the subunits, it is conceivable that binding of coenzyme in one subunit could decrease the binding of coenzyme in the second subunit, a form of “negative” cooperativity, because of small structural rearrangements. Such a scenario was proposed for a yeast cinnamyl alcohol dehydrogenase [14]. In order to evaluate this possibility with yeast ADH1, we created a holodimer of A subunits by superpositioning the coenzyme binding domain of subunit A onto that of the B subunit. (See coordinates “holodimer-model.pdb” in Supplementary Material.) Comparison of the hypothetical AA dimer with the actual AB dimer shows no serious steric conflicts would be created if an AB dimer converted to the AA dimer, because there are few changes in the dimer interface, which consists of two strands of β sheet with residues 274–280 and 288–292 connected by a loop. The molecular two-fold axis of the dimer is through residues 277 and 290. This dimer interface is similar to the one in horse liver ADH, but the conformational change in that enzyme results in flipping the loop that includes residues from 294–299 so that Val-294 moves into the substrate binding site [15]. This loop movement is critical for the horse enzyme, as shown by the kinetics and structure of a doubly substituted (G293A/P295T) enzyme, which is locked in the open conformation and binds coenzyme less tightly than does the wild-type enzyme [16]. The corresponding loop in the yeast enzyme does not undergo a similar conformational change, and there seems to be no structural basis for negative cooperativity where the binding of coenzyme in one subunit would affect directly the binding of coenzyme in the second subunit of the dimer. In the tetramer, which is formed by back-to-back dimers with the active sites on the front, exposed, sides of the molecule, it appears that the conformational change would not be sterically prohibited because the catalytic domain moves away from the tetramer interface and many of the A to B and B to B contacts are retained. (See Supporting Information in Ref. [1] for listing of contacts.)

The crystal lattice contacts could also affect the conformation of the dimer. There are several contacts between the A subunit in one tetramer with the B subunit of another tetramer by a symmetry-related crystallographic axis. These include reciprocal hydrogen bonding and hydrophobic interactions among residues in regions 71–85 and 141–144, and hydrogen bonding and electrostatic interactions between residues A17 and B330, A19 and B17, A323 and B334, and A327 and B336. However, these interactions are not certain, as the *B* factors for the residues in subunit B are relatively high. In contrast, contacts between A subunits are more limited: A319

to A233 and A236 to A347. If subunit B were to adopt the conformation of subunit A, unfavorable steric clashes could occur among residues in regions 84–85 and 141–145, and unfavorable electrostatic interactions could be generated between residues 327 and 330. Of course, some of the unfavorable interactions could be alleviated by changes in local conformations or packing interactions. Nevertheless, it appears possible that crystal lattice interactions could affect the conformation of the B subunit, favoring its more open conformation. The energetics of the conformational change and the crystal lattice interactions cannot be evaluated from the information presently available. Whether or not the asymmetry is pre-existing in solution or caused by crystal-packing is not resolved by the present studies, but we think that what is observed in the crystal represents an energetically accessible state that is relevant for catalysis.

3. Discussion

3.1. Asymmetry and conformational changes in other ADHs

Asymmetry between subunit structures and heterogeneity of coenzyme binding in other ADHs is often observed, even as kinetic studies on the various ADHs provide no good evidence for or against cooperativity in binding coenzyme or in catalysis. The dimeric molecule of horse liver ADH in the apoenzyme form (e.g., 8ADH.pdb) or in a variety of binary complexes has crystallographically identical subunits with an “open” conformation, but most ternary complexes with NAD(H) and a substrate analog (e.g., 4DXH.pdb) have asymmetric dimers, although both subunits are structurally very similar and both bind coenzyme in a “closed” conformation [17]. (“Apoenzyme” is the form of enzyme that contains the catalytic zinc, whereas “holoenzyme” has NAD(P) bound also.) The open conformation is retained for apoenzyme modified on 24 of the 30 lysine residues with isonicotinimidyl groups and crystallized in a different space group with different lattice contacts (7ADH.pdb), except for a few local changes in peptide backbone conformation [18]. In these structures of horse liver ADH, the catalytic zinc has the classical coordination. Cod liver ADH1 (1CDO.pdb) crystallizes as an asymmetric dimer with coenzyme bound to both subunits, one in a closed conformation and the other in an open conformation, similar to the conformations found in yeast ADH1, and both of the catalytic zincs have the classical coordination [19]. The human ADH3 homodimers are the asymmetric units in the apoenzyme and various binary and ternary complexes (1TEH.pdb, 2FZE.pdb, 1MC5.pdb), and

the subunits have slightly different conformations and alternative modes of zinc ligation [20–23]. Although many of the complexes were formed by soaking of the apoenzyme crystals, co-crystallization with NAD^+ gave similar structures, in a different space group, suggesting that crystal lattice contacts might not affect the conformations. The dimeric ADH apoenzyme from *Arabidopsis thaliana* crystallizes with symmetric subunits, but the holoenzyme is asymmetric with one subunit bound to NAD and the other lacking NAD (4ROT.pdb, 4RQU.pdb) [24]. The conformational change on binding NAD is similar to that found for horse liver ADH1E, and the catalytic zinc has the classical coordination in both the apoenzyme and the binary complex with NAD. The subunits of the dimeric *Haloferax mediterranei* glucose dehydrogenases complexed with coenzyme (NADP^+ or NADPH) are the asymmetric units (2VWP.pdb, 2VWQ.pdb), and various conformational changes (including coordination of the catalytic zinc) occur when ternary complexes are formed by soaking the enzyme- NADP^+ complex with glucose or gluconolactone (2VWH.pdb, 2VWG.pdb), showing that crystal lattice contacts do not prevent some structural changes [25].

The tetrameric ADHs that are most similar to yeast ADH1 could offer more relevant examples for asymmetry, but various results are obtained for different enzymes. The monomer of the apoenzyme of *Sulfolobus solfataricus* ADH is the asymmetric unit (1JVB.pdb), and it has the alternative coordination of the catalytic zinc, whereas a dimer of the holoenzyme co-crystallized with NAD(H) and 2-ethoxyethanol is the asymmetric unit, although the two subunits have almost identical structures, and both subunits have the classical zinc coordination (1R37.pdb) [26]. The catalytic domain is rotated about 11° toward the coenzyme binding domain by the conformational change upon binding substrates, similar to yeast ADH1. The tetramers of the inducible *Escherichia coli* ADH crystallized with or without NAD (with a 5-His tag on the amino terminal) are the asymmetric units. Subunit A of the apoenzyme has the classical coordination for the catalytic zinc whereas subunits B, C, and D have the alternative coordination. Subunit A of the holoenzyme has the alternative zinc coordination and no bound coenzyme, and subunits B, C, and D have the classical zinc coordination and bound coenzyme [27]. The catalytic domains of subunits B, C, and D of the holoenzyme are rotated $4.5\text{--}7.5^\circ$ toward the coenzyme domain as compared to the apoenzyme. Interestingly, a different crystal form for holoenzyme with a tetramer as the asymmetric unit and all 4 subunits occupied with NAD was obtained with a version of *E. coli* ADH that has an engineered carboxyl terminal

extension with 32 amino acid residues, and the structures of all four subunits (4GKV.pdb) are very similar to the B, C, and D subunits of the holoenzyme previously determined [28]. Perhaps crystal packing does not have a large effect on the conformation of the holoenzyme as the *E. coli* proteins have different amino acid residue extensions and different crystal forms. The *Pseudomonas aeruginosa* ADH complexed with NADH and ethylene glycol has two tetramers with 8 essentially identical subunits as the asymmetric units, but possible crystallographic 222-symmetry is disrupted, only due to asymmetrical hydrogen bonding at an interface between subunits (1LLU.pdb) [29]. The catalytic zinc has the classical coordination in the ternary complexes.

The various structural results for these enzymes show that both open and closed conformations and classical and alternative coordination of the catalytic zinc can be observed in dimeric and tetrameric ADHs in apo- and holoenzyme forms. The flexibility and diversity may arise because of small differences in energetics between conformational states and lattice interactions. In some cases, the occupancies of subunits by coenzyme are low, which may arise from the conditions used for preparation and cryoprotection of the crystals, such as when the yeast ADH1 complex was soaked in the absence of coenzyme (for 4W6Z.pdb). The tetrameric enzymes (1R37.pdb, 4GKV.pdb, 1LLU.pdb) appear to have all four subunits fully occupied by coenzyme and similar “closed conformations”, suggesting that the subunits can be mechanistically independent. The oxidation state of the NAD (NAD^+ or NADH) for holoenzyme complexes is not established by spectroscopy or crystallography with the resolution of data obtained, and some complexes may contain a mixture of substrate and product. The closest analog of the present yeast ADH structure is that of dimeric human ADH3, where the apoenzyme was soaked with substrates, and one subunit binds NADH and *S*-(hydroxymethyl)glutathione in a closed conformation with classical zinc coordination, and the other subunit binds NADH in a more open form with a “bipyramidal” zinc (1MC5.pdb), which could be interpreted as dual occupancy for the zinc in classical and alternative coordinations [22,23].

3.2. Mechanistic implications of the open conformation and alternative zinc coordination

The new results for yeast ADH1 provide structural information about two intermediates in the preferred ordered reaction catalyzed by ADH1 [3] and support a proposal for the exchange of ligands to the catalytic zinc in the enzyme- NAD^+ -alcohol complexes. An open conformation of

apoenzyme probably binds the coenzyme, as the open subunits B and D of the previous structures (4W6Z.pdb) appear to allow coenzyme to dissociate when the crystal is soaked in the absence of coenzyme, whereas the closed form of subunits A and C retained coenzyme. In the present structure, the open form of subunit B has bound coenzyme because the concentration of NAD^+ was maintained at 2 mM. A structure of apoenzyme is not known, but it may have the open conformation for all four subunits with the catalytic zinc in the alternative coordination with Glu-67, as observed in subunits B and D of the previous structures (4W6Z.pdb) [1] and in related tetrameric apoenzyme structures from other species (1PED.pdb, 1JVB.pdb, 1E3J.pdb, 1PL7.pdb) [30–33]. However, it is also possible that the catalytic zinc of the yeast apoenzyme has the classical coordination as observed in the apoenzyme forms of dimeric horse liver ADH1E and human ADH3 (8ADH.pdb, 1YE3.pdb, 1M6H.pdb) [21,34], and after yeast ADH1 binds coenzyme, the zinc coordination changes to the alternative one observed in subunit B.

The open conformation of subunit B with bound coenzyme and alcohol, and the alternative coordination of the catalytic zinc, could represent an intermediate on the pathway toward forming the ternary complex that resembles the expected Michaelis complex, with a structure like the “closed” conformation (subunit A in the present structure, or subunits A and C of the previous structures). The movement of Arg-340 from the catalytic domain toward the coenzyme, as the conformational change closes up the cleft between domains, may be coupled to the change in coordination of the catalytic zinc when the alcohol binds to the zinc and displaces Glu-67, as suggested previously for yeast ADH1 and human ADH3 [1,15,23]. In the closed conformation of the ternary complex of subunit A, Arg-340 interacts with O2N of the pyrophosphate of the coenzyme and with OE2 of the carboxylate of Glu-67, but in the open conformation of subunit B, Arg-340 does not interact strongly with the coenzyme or Glu-67 as the electron density and *B* factors suggest that the side chain is more flexible. It is not known if the global conformation changes before the zinc coordination changes in the mechanism of yeast ADH1. However, transient kinetic studies suggest that deprotonation of the horse liver enzyme complexed with NAD^+ is coincident with the conformational change, and those results are consistent with a two step mechanism in which zinc-bound hydroxide is displaced by Glu-67 and then the alcohol binds to the zinc and displaces Glu-67 [15,35].

As noted above, other alcohol dehydrogenases complexed with NAD(H) have the alternative coordination of the catalytic zinc: human ADH3 (1TEH.pdb, 1MAO.pdb, 1MC5.pdb) [20–22]

and human sorbitol dehydrogenase (1PL8.pdb) [33]. It is significant that the catalytic zinc in the human ADH3 complexes appears to be “bipyramidal” as if the zinc positions are almost energetically equivalent [23]. For the present yeast ADH1 structure, there is no evidence for dual positions for the catalytic zinc as the electron density is essentially spherical and the occupancy is approximately full. The trifluoroethanol bound in subunit B does not appear to ligate the zinc, but small movements by the alcohol, the zinc, Cys-43, and Glu-67 could produce the classical coordination (Fig. 4).

Evidence for the participation of Glu-67 in the catalytic mechanism of yeast ADH was provided by an early site-directed mutagenesis study, where the E67Q substitution decreased catalytic efficiency (V/K_m) for ethanol oxidation and acetaldehyde reduction by ~100-fold and altered the pH dependence [36]. The (V/K_m) kinetic parameters report on the binding of the substrate, hydrogen transfer and release of the product, which could be affected if Glu-67 could not ligate the zinc. Similarly, the E67L substitution significantly affects the catalytic efficiency of human ADH3, and a structure of the E67L enzyme complexed with NAD⁺ (or NADH, oxidation state unknown) shows that the catalytic zinc has the classical coordination, but one subunit has the nicotinamide ring flipped out of the “productive” binding pocket [23].

Ternary complexes with coenzyme and relevant substrate analogs determined so far have the zinc coordinated in the “classical” manner as seen with subunit A of yeast ADH1, dimeric horse liver ADH1E complexed with NAD⁺ and trifluoroethanol (4DXH.pdb), abortive complexes of dimeric human ADH3 complexed with NADH and *S*-(hydroxymethyl)glutathione (1MC5.pdb), and tetrameric *Pseudomonas* and *Sulfolobus* ADHs complexed with NADH and ethylene glycol or ethoxyethanol (1LLU.pdb, 1R37.pdb) [22,26,29,37]. The structure in subunit A of yeast ADH1 appears to represent a ground state analogue of the Michaelis complex for the catalytic reaction, with the oxygen of the alcohol ligated to the zinc; a hydrogen bond system that includes the oxygen of the alcohol, the hydroxyl group of Thr-45, and the 2'-hydroxyl group of the nicotinamide ribose, which can relay a proton to the imidazole group of His-48; and the methylene carbon of the alcohol in position to transfer a hydride to C4N of the nicotinamide ring of NAD⁺ (Fig. 5).

The crystallographic and kinetic studies of the various ADHs suggest that the alternative coordination of the zinc may be relevant for the exchange of ligands (water, alcohol, aldehyde),

but further study is required. Rate constants for interconversions of the various species need to be determined. Structures of yeast ADH1 apoenzyme and other complexes, such as with NADH and substrate analogs, would be useful for characterizing other species in the mechanism, if good crystals could be obtained.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Supplementary material

Coordinate files used for figures and analysis are included: Fig. 1 (YADH-conf-change.pdb), Fig. 3 (YADH-AonB.pdb, Fig. 4 (zinc-ligation.pdb, analysis of hypothetical AA dimer (holodimer-model.pdb), and biological tetramer (YADH-tetramer.pdb). These files are based on the ones deposited with the RCSB (4W6Z and 5ENV).

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Table 1

X-ray Data Collection and Refinement Statistics

PDB entry	5ENV
space group	<i>P</i> 321
subunits in asymmetric unit	2
cell dimensions, Å	146.3, 146.3, 66.0
cell angles, deg	90, 90, 120
resolution range, Å (outer shell)	20.0–3.0 (3.00–3.05)
measured reflections: total, unique	74630, 15427
completeness, % (outer shell)	98.1 (75.1)
twin fraction, H, K, L; –H, –K, L	0.714, 0.286
R_{symm} , (outer shell) ^a	0.064 (0.326)
mean $\langle I \rangle / \sigma \langle I \rangle$, (outer shell)	18.0 (2.7)
R_{value} , R_{free} , test % ^b	0.14, 0.20, 4.5
rmsd for bond dist. (Å), angles (deg) ^c	0.013, 1.76
mean B value, (Å ²), Wilson, REFMAC	60, 57 ^d
non-H atoms, protein, mean B	5164, 58
4 Zn, 2 NAD, 2 TFE heteroatoms, B	104, 58
waters, B	20, 30
estimated coordinate error, Å	0.28

^a $R_{\text{symm}} = \Sigma(|I - \langle I \rangle|) / \Sigma I$. ^b $R_{\text{value}} = (\Sigma|F_o - kF_c|) / \Sigma|F_o|$, where k is a scale factor. R_{free} was calculated with the indicated percentage of reflections not used in the refinement [38]. ^c Root-mean square deviations from ideal geometry. ^d Refinement with 4 TLS groups gave a residual mean B value of 18 for all non-H atoms.

Figure Legends

Fig. 1. Stereoview (“walleye”) of the conformational differences between subunits A and B in the asymmetric dimer. A and B subunits with bound NAD and subunit B from the previous structure that does not have bound NAD were superpositioned by using selected residues, 165–200 and 206–291, from the coenzyme binding domain, which excludes some regions that differed among the three structures. The conformational change closes the cleft between the coenzyme binding and catalytic domains, approximately around the axis perpendicular to the plane shown by the “X”. This axis is close to residue 107 near the structural zinc, extending up toward residues 296–297, and farther up to a position between residues 143, 148, 308 and 309. Subunit B without coenzyme (4W6Z.pdb) is in red, subunit B with coenzyme (5ENV.pdb) is in green, and subunit A with NAD is in black. NAD, trifluoroethanol and the two zincs are shown as ball and stick models. The figures were made with the Molray web interface server in Uppsala, Sweden [39].

Fig. 2. Stereoviews of electron density maps for NAD, trifluoroethanol (TFE), catalytic zinc and associated amino acid residues in A (“A”) and B (“B”) subunits. The views were chosen to illustrate the differing interactions of amino acid residues from the catalytic domain with the NAD. The electron density map for subunit A is contoured at about $0.3 \text{ e}^-/\text{\AA}^3$, and for subunit B at about $0.16 \text{ e}^-/\text{\AA}^3$. The average *B* factor for the NAD in subunit A is $45 \text{ \AA}^2$, and in subunit B it is $75 \text{ \AA}^2$. Residues that interact with the NAD that are not labeled in “A” include His-44, His-48, Thr-45 and Asp-201. In “B”, His-48, Thr-45 and the catalytic zinc are not labeled.

Fig. 3. Stereoviews of the interactions of the nicotinamide nucleotide with the amino acid residues from the catalytic domain. Subunit A is shown in ball and stick, with atom coloring and red, dashed, lines for the hydrogen bonds. Subunit B is shown in green, but there are no hydrogen bonds to be shown, except for the ones involving the carbonyl oxygens of Val-268 and Ser-293, which are actually part of the coenzyme binding domain.

Fig. 4. Stereoviews of the coordination of the catalytic zinc in the active sites, showing the nicotinamide ribose of NAD, trifluoroethanol (TFE) and associated amino acid residues. The coenzyme binding domains of subunit A and subunit B were superpositioned. The classical coordination of the zinc in subunit A (ball and stick, atomic coloring) is shown by red, dashed lines, and the “alternative” coordination in subunit B (green) is implied. Separate electron

density maps for the differing coordination states of the zincs were shown previously [1]. Note that subunit A has TFE coordinated to the zinc, whereas in subunit B, the zinc has moved and interacts with Glu-67 in the inverted coordination, and the TFE is displaced from the zinc.

Fig. 5. Active site in subunit A of yeast ADH1. The structure appears to resemble the expected Michaelis complex with the methylene carbon of the alcohol (TFE) close to C4N of the nicotinamide ring of the NAD with good geometry for hydride transfer, and hydrogen-bonded system shown in dashed red lines for relaying a proton from the oxygen of the alcohol to solvent.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.