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Horse Liver Alcohol Dehydrogenase: Zinc Coordination and Catalysis

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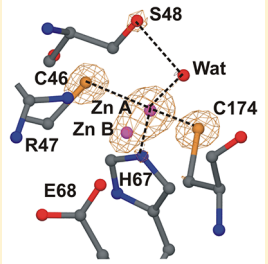
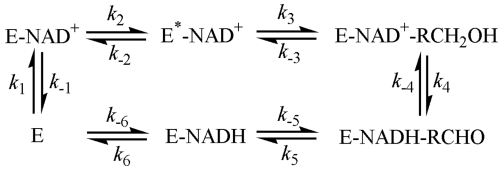
Supporting Information

ABSTRACT: During catalysis by liver alcohol dehydrogenase (ADH), a water bound to the catalytic zinc is replaced by the oxygen of the substrates. The mechanism might involve a pentacoordinated zinc or a double-displacement reaction with participation by a nearby glutamate residue, as suggested by studies of human ADH3, yeast ADH1, and some other tetrameric ADHs. Zinc coordination and participation of water in the enzyme mechanism were investigated by X-ray crystallography. The apoenzyme and its complex with adenosine 5′-diphosphoribose have an open protein conformation with the catalytic zinc in one position, tetracoordinated by Cys-46, His-67, Cys-174, and a water molecule. The bidentate chelators 2,2′-bipyridine and 1,10-phenanthroline displace the water and form a pentacoordinated zinc. The enzyme−NADH complex has a closed conformation similar to that of ternary complexes with coenzyme and substrate analogues; the coordination of the catalytic zinc is similar to that found in the apoenzyme, except that a minor, alternative position for the catalytic zinc is ∼1.3 Å from the major position and closer to Glu-68, which could form the alternative coordination to the catalytic zinc. Complexes with NADH and *N*-1-methylhexylformamide or *N*-benzylformamide (or with NAD^+ and fluoro alcohols) have the classical tetracoordinated zinc, and no water is bound to the zinc or the nicotinamide rings. The major forms of the enzyme in the mechanism have a tetracoordinated zinc, where the carboxylate group of Glu-68 could participate in the exchange of water and substrates on the zinc. Hydride transfer in the Michaelis complexes does not involve a nearby water.



Horse liver alcohol dehydrogenase (ADH) catalyzes the reversible oxidation of alcohols to aldehydes by an ordered mechanism (Scheme 1), with the coenzyme NAD^+

Scheme 1



binding first, isomerization to the E*−NAD^+ complex coupled to release of a proton and a conformational change that closes up the active site, binding of the alcohol, hydride transfer, release of aldehyde, and dissociation of NADH. Rate constants for each of these steps have been estimated for the EE isoenzyme (ADH1E), and transient kinetics and solvent isotope effects indicate that hydride is transferred from a zinc alkoxide.[1−3] The rate constants for the isomerization of the enzyme−NADH complex are too fast to determine with stopped-flow methods. The mechanism for the exchange of substrates for the water bound to the zinc also has not been established. Structures have been determined for the apoenzyme (E) and analogues of the ternary complexes, but structures for the binary enzyme−NAD^+ or enzyme−NADH complexes are needed to understand more details of the mechanism.

X-ray crystallography of horse ADH1E has determined the conformational states and the coordination of the catalytic zinc for the apoenzyme and for various complexes.[4−7] The catalytic zinc of the apoenzyme (no bound coenzyme) of liver ADH binds a water in a tetrahedral coordination with two cysteine sulfurs and one histidine imidazole.[4] In the holoenzyme complexes with NAD^+ and fluoro alcohols, the oxygen of the alcohol binds to the zinc and replaces the water.[8] The zinc acts as a Lewis acid and stabilizes the intermediate alkoxide, which forms a low-barrier hydrogen bond with the Ser-48 hydroxyl group and is connected by a proton relay system to His-51 that acts as a base.[1,9] In analogues of the ternary product complex with NADH and an aldehyde, the oxygen of a sulfoxide or a formamide also binds to the tetracoordinated zinc.[3,5,10−13]

The major issue addressed in this work is the coordination for the catalytic zinc in intermediate enzyme species and the mechanism for the exchange of the zinc-bound water (or hydroxide) with the substrate. A pentacoordinated zinc with adjacent oxygens from a water and the substrate was proposed for proton transfer in the enzyme−coenzyme−substrate complex to account for results available at that time.[14] The horse apoenzyme forms complexes with 2,2′-bipyridine and 1,10-phenanthroline, which appear to form a pentacoordinated zinc.[15−20] However, the proposed mechanism was abandoned when a structure of a ternary enzyme−NAD−substrate complex showed no such water and no space to accommodate

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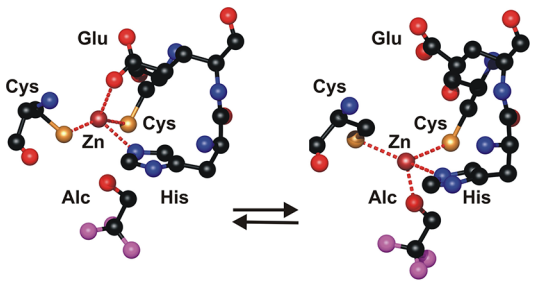
a water.[21] Nevertheless, later structural studies of enzyme−NADH complexes appear to show alternative positions for a water bound to zinc, with one water positioned close to C6N of NADH, and a mechanism in which a water bound to a pentacoordinated zinc activated the NADH was proposed.[22,23] As discussed below, nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) studies of the enzyme with Co(II) substituted for the catalytic zinc, NMR and perturbed angular correlation spectroscopy of the enzyme with ^{113}Cd(II) or ^{111}Cd(II), X-ray absorption spectroscopy, computations, and X-ray studies of other ADHs have led to various proposals about changes in metal coordination during the enzymatic reaction.[24−32]

X-ray crystallography has shown that the catalytic zinc has two different coordination environments in some ADHs. Complexes of human ADH3 with adenosine 5′-diphosphoribose or NAD(H) have an inverted tetrahedral configuration with coordination to the side chain carboxylate of Glu-67, two cysteines, and a histidine, whereas the apoenzyme and some ternary complexes have the classical coordination with the oxygen of a water or substrate analogue, two cysteines, and a histidine.[33−36] Some subunits of the apoenzyme of *Escherichia coli* ADH have the inverted coordination of the zinc with Glu-59, two cysteines, and a histidine, whereas complexes with NAD have a water displacing the glutamate.[37,38] The zinc in the yeast apoenzyme (in an open conformation) has an inverted tetrahedral configuration with coordination to the carboxylate of Glu-67, two cysteines, and a histidine, whereas the ternary complex with NAD^+ and 2,2,2-trifluoroethanol (in the closed conformation) has the oxygen of the alcohol displacing the glutamate.[39,40] The *Sulfolobus solfataricus* ADH also has these two different coordination geometries in apoenzyme and holoenzyme complexes.[41,42]

Thus, an alternative mechanism for the exchange of zinc ligands could involve a double displacement in which the side chain carboxylate of the nearby glutamate residue displaces the water, inverting the configuration of the tetrahedral zinc, and then the substrate oxygen displaces the carboxyl group.[34] Such a mechanism can be illustrated with two structures observed for yeast ADH [Scheme 2, from Protein Data Bank (PDB) entry 5ENV].[40] It is notable that the catalytic efficiency for alcohol oxidation is decreased when Glu-67 is substituted with neutral amino acids in yeast ADH1 and human ADH3.[36,43] Computational studies of horse ADH also suggest that the glutamate can intermittently move to coordinate to the zinc.[44]

Scheme 2



This study extends our knowledge of structures that are relevant to the mechanism. Partial data sets to 4.5 Å for binary complexes with NAD^+ or NADH were used previously to help define the alcohol binding site,[45] but higher-resolution structures are needed to describe the details. Some low-resolution structures for the horse ADH1E apoenzyme and complexes with adenosine 5′-diphosporibose and 1,10-phenanthroline have been determined; however, structure factors were not deposited in the PDB, and details of zinc coordination are lacking.[18,46] As noted above, some atomic-resolution structures of horse ADH complexed with NADH apparently show some partial adducts of water with the reduced nicotinamide ring,[22,23] but structures of ternary complexes of horse or human ADHs with NADH and formamides show no evidence of such a water.[11,47] Atomic-resolution studies of ternary complexes of horse ADH with NAD^+ and fluoro alcohols have no water near the zinc or the nicotinamide ring.[8] A new atomic-resolution structure of the enzyme−NADH complex shows that the catalytic zinc is mobile and has alternative positions, which suggest that Glu-68 participates in the exchange of water and substrates on the zinc. Although a pentacoordinated zinc may form transiently during the exchange of zinc ligands, the reactive ternary complexes are tetracoordinated. The conflicting results from the literature are analyzed in the discussion.

EXPERIMENTAL PROCEDURES

Crystallization. Wild-type (natural) crystalline horse liver alcohol dehydrogenase (EC 1.1.1.1, UniProt entry P00327, GenBank entry M64864) and $LiNAD^+$ were purchased from Roche Molecular Biochemicals. 1,10-Phenanthroline, 2,2′-bipyridine, and adenosine 5′-diphosphoribose were obtained from Sigma. 2-Methyl-2,4-pentanediol (MPD) was obtained from Kodak and treated with activated charcoal before being used.

The crystals of the apoenzyme were prepared by a modification of the published procedure in which 1 mL of 10 mg/mL enzyme was dialyzed against 10 mL of 50 mM Tris-HCl (pH 8.4) at 5 °C as the concentration of MPD was gradually increased to a final concentration of 25% over several days.[48] The complex with adenosine 5′-diphosphoribose (ADPR) was prepared by soaking some apoenzyme crystals in the final outer dialysate for 1 h at 5 °C with 2 mM ADPR, relative to a K_i of 41 μM.[49] Crystals were also soaked for 3 h at 5 °C with 30 mM 2,2′-bipyridine, relative to a K_d of 0.31 mM,[19] or for 2 h at 5 °C with 5 mM 1,10-phenanthroline, relative to a K_d of 9−31 μM.[15,19,50]

Crystals of the binary enzyme−NADH complex were prepared as described by the general procedure used previously.[8] The enzyme (10 mg/mL) was dialyzed against 50 mM ammonium *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonate buffer and 0.25 mM EDTA (pH 7.0) (measured at 5 °C, pH 6.7 at 25 °C) at 5 °C with 1 mM NAD^+ as the concentration of 2-methyl-2,4-pentanediol was increased over several days to 12% when crystals formed. The concentration of the diol was finally increased to 30%. As determined from the X-ray data, the NAD^+ was reduced to NADH in the final complex. Crystals of a ternary complex were prepared with 1 mM NADH and 10 mM racemic *N*-1-methylhexylformamide (MHF; K_i = 5.4 μM[51]) under the same conditions described above. Three months after the crystals were produced, an ultraviolet (UV) spectrum of the outer dialysate showed that almost all of the NADH had been converted to NAD^+, so the crystals were harvested from the dialysis bag and placed in a fresh solution of the crystallization medium with 25% MPD. Crystals were prepared similarly with 1 mM NADH and 10 mM *N*-benzylformamide (BNF; K_i = 0.74 μM[51]).

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Table 1. X-ray Data and Refinement Statistics for Horse Alcohol Dehydrogenases

enzyme complex, zinc ligand	apoenzyme, H_2O	ADPR, H_2O	2,2′-bipyridine	1,10-phenanthroline	NADH, H_2O	NADH, MHF	NADH, BNF
PDB entry	1YE3	5VKR	5VJG	5VJ5	4XD2	5VN1	5VL0
space group, no. of subunits per asymmetric unit[a]	$C222_1$, 1	$C222_1$, 1	$C222_1$, 1	$P2_12_12_1$, 2	P_1, 2	$P2_1$, 4	$P2_1$, 4
cell dimensions (Å)	55.8, 74.3, 181.3	55.4, 74.3, 181.0	55.5, 73.7, 181.1	55.3, 73.4, 180.8	44.2, 50.9, 92.7	50.2, 180.8, 86.8	50.2, 180.3, 86.9
cell angles (deg)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	92.0, 103.0, 109.6	90, 106.1, 90	90, 106.2, 90
mosaicity	0.92	0.74	1.2	1.1	1.1		0.94
resolution range (Å) (shell)	20.1−1.59 (1.63)	19.93−1.80 (1.86)	19.9−1.90 (1.97)	19.84−1.90 (1.97)	19.8−1.1 (1.13)	20.0−1.25 (1.30)	20.0−1.2 (1.24)
no. of reflections (total, unique)[b]	205694, 36732	245973, 30816	198230, 27148	300828, 55508	687793, 257968	611039, 325043	2260931, 361271
redundancy (shell)	5.61 (5.43)	7.64 (5.68)	6.99 (5.04)	5.30 (3.38)	2.8 (1.89)	1.79 (1.59)	6.19 (4.34)
completeness (%) (outer shell)	71.9 (54.0)	91.7 (85.6)	95.3 (87.4)	96.1 (93.3)	86.8 (71.1)	80.0 (72.7)	79.4 (57.4)
R_{pim} (%) (outer shell)[c]	3.8 (9.9)	4.6 (23.2)	4.8 (17.0)	3.8 (24.9)	2.9 (39.6)	8.9 (25.5)	3.8 (27.7)
mean $\langle I\rangle/\sigma\langle I\rangle$ (outer shell)	12.9 (4.9)	9.0 (2.1)	9.8 (3.5)	8.8 (2.9)	9.1 (2.3)	6.6 (2.2)	8.3 (1.7)
R_{value}, R_{free} (%) (test %, no.)[d]	20.1, 25.6 (3.1, 1143)	19.2, 24.8 (4.1, 1328)	20.9, 28.3 (4.1, 1174)	24.8, 29.4 (2.1, 1169)	17.7, 19.8 (0.5, 1292)	15.2, 18.7 (0.5, 1650)	15.9, 19.9 (1.0, 3775)
RMSD for bond distances (Å)[e]	0.014	0.019	0.016	0.016	0.014	0.017	0.019
RMSD for bond angles (deg)[e]	1.53	2.07	1.80	1..81	1.75	1.89	2.06
estimated error in coordinates (Å)	0.069	0.125	0.153	0.195	0.032	0.028	0.036
mean B value (Wilson, Refmac) (Å^2)	25.4, 35.4	43.6, 50.6	29.2, 41.4	22.5, 35.7	8.8, 19.1	19.0, 25.0	13.0, 20.3
total no. of non-H atoms (mean B value)	2995	2868	2895	5828	6719	12774	12940
protein	2748 (32.0)	2785 (51.5)	2799 (41.0)	5568 (36.8)	5764 (17.7)	11195 (24.8)	11343 (19.8)
heteroatoms, zinc, ligands	10 (54.0)	38 (59.5)	14 (46.5)	32 (27.7)	126 (21.2)	250 (26.0)	240 (19.6)
waters	200 (39.6)	45 (51.4)	96 (40.5)	228 (38.4)	829 (36.8)	1329 (37.0)	1357 (32.5)
Ramachandran (%) (favored, outlier)	97.6, 0	96.0, 0	95.2, 0.27	96.0, 0.54	97.2, 0	97.2, 0	97.1, 0
MolProbity (clash, score, rank %)	3.71 (97th), 1.33 (95th)	2.99 (99th), 1.72 (83rd)	1.77 (100th), 1.49 (96th)	1.94 (100th), 1.75 (85th)	1.5 (97th), 1.03 (98th)	1.34 (98th), 1.05 (99th)	1.36 (98th), 1.01 (99th)

[a]The biological molecule is a dimer of identical protein subunits. [b]Data cutoff, $\sigma F > 0$. [c]$R_{pim} = R_{merge}/(n-1)^{1/2}$, where n is data redundancy. [d]$R_{value} = (\sum|F_o - kF_c|)/\sum|F_o|$, where k is a scale factor. R_{free} was calculated with the indicated percentage of reflections not used in the refinement.[104] [e]Deviations from ideal geometry.

X-ray Crystallography. Crystals were mounted on fiber loops (Hampton Research) and flash-vitrified by being plunged into liquid N_2. The data for the apoenzyme at 100 K were collected on IMCA-CAT beamline 17-ID with the ADSC Quantum detector at the Advanced Photon Source (APS) with a wavelength of 1.000 Å. The data for the apoenzyme complexed with ADPR at 100 K were collected on SBC-CAT beamline 19-ID at APS with the ADSC CCD detector with a wavelength of 1.0093 Å and high- and low-resolution passes. The data for the enzyme complexed with 2,2′-bipyridine or 1,10-*o*-phenanthroline were collected at 100 K on the Rigaku R-Axis IV^{++} instrument with the image plate and rotating anode at The University of Iowa X-ray Crystallography Facility with a wavelength of 1.5418 Å. The data were processed with d*TREK.[52]

The data for the ADH−NADH complex were collected at 100 K on the SBC 19-ID beamline with the ADSC detector at APS with an X-ray wavelength of 0.9537 Å. High-resolution data were collected at a 120 mm crystal to detector distance, with 0.3° oscillations and 10 s exposures with 2θ at 20° over a total of 216°; medium-resolution data were collected at 120 mm, with 0.5° oscillations over 360° and 1 s exposures, and low-resolution data were collected at 300 mm, with 0.5° oscillations over a total of 360° and 0.5 s exposures. Data were processed with XDS.[53] The data for the ADH−NADH−*N*-1-methylhexylformamide complex at 100 K were collected at the ESRF synchrotron on beamline 14-ID with a wavelength of 0.936 Å and high- and low-resolution passes. Data were processed with XDS.[53] Data were collected on two crystals of the complex with NADH and *N*-benzylformamide at 100 K at IMCA beamline 17-ID at APS with a wavelength of 1.000 Å with high- and low-resolution passes. Data were processed with d*TREK. Some data sets were not as complete as desired, because of the geometry of the beamline station, crystal orientation, lack of beam time, and radiation decay; however, the structures are of high quality, and the electron density maps support the reported structural details.

The structures for the apoenzyme and the complexes with ADPR and 2,2′-bipyridine were determined by molecular replacement using as a model of the structure in PDB entry 8ADH, which has the same space group. The structure for the apoenzyme complexed with 1,10-phenanthroline was determined by molecular replacement using a model of the dimeric enzyme derived from PDB entry 1QLH, which has the same space group. Regions of the structures that lacked readily interpreted electron density were examined with omit maps. The structure of the enzyme−NADH complex was determined by molecular replacement with the coordinates for the wild-type ADH−NAD$^+$−2,3,4,5,6-pentafluorobenzyl alcohol complex (PDB entry 4DWV) after removal of the pentafluor-

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obenzyl alcohol.[8] The structures for the enzyme complexed with NADH and *N*-1-methylhexylformamide or NADH and *N*-benzylformamide were determined by molecular replacement with the structure in PDB entry 1P1R.[11]

The structures, with riding hydrogens, were refined by cycles of restrained refinement with REFMAC5.[54] The monomer dictionary used by REFMAC for NAD^+ was modified to remove the restraints on planarity and relax the restraints on bond distances (from ±0.02 to ±0.10 Å) for the nicotinamide ring so that puckered or reduced rings could be properly refined. We named this modified NAD as "NAJ" in several structures in the RCSB data bank (e.g., 4DWV, 4DXH, 1N92, and 1N8K) to distinguish it from the NAD (with tight restraints) used in other structures. After the bound coenzyme was identified as NADH in the structures presented here, the REFMAC dictionary for NADH (named "NAI" in the RCSB data bank), modified with relaxed restraints on bond distances and angles for the reduced nicotinamide ring, was used for the final stage of refinement. Occupancies for alternative positions were adjusted to make the temperature factors similar. Model building used the program O.[55] SHELXL-2013[56] was finally used for analysis of the structure of the enzyme–NADH complex, with no restraints on the coenzyme for 10 cycles of CGLS refinement and one full-matrix cycle of least-squares refinement (BLOC 1, MERG 4) to determine puckering angles and bond distances and their errors for the nicotinamide ring. The structures were checked with MolProbity.[57] EXCEL was used for statistical analyses. Figures were prepared with the Molray web server in Uppsala, Sweden.[58]

RESULTS

Tertiary Structures. Structures for three forms of horse liver alcohol dehydrogenase that have been deposited in the Protein Data Bank were re-determined at 100 K to extend the resolution of the data, secure the structure factors, and establish detailed structural features. Four structures of new complexes were also determined. Table 1 summarizes the X-ray data and refinements.

The apoenzyme structure determined here at 1.6 Å resolution (PDB entry 1YE3) is in the open conformation, with essentially the same structure (RMSD of 0.33 Å for α-carbon atoms) as in PDB entry 8ADH, which was determined at 278 K and 2.4 Å resolution.[4] Note the tetrahedral coordination of the zinc and the water molecule that is hydrogen bonded between Ser-48 and His-51 (Figure 1); this water was not identified in the first publication but was added when the structure was further refined and deposited in the PDB. This water is part of the proton relay system, which is replaced by the nicotinamide ribose O2D in ternary complexes.[21]

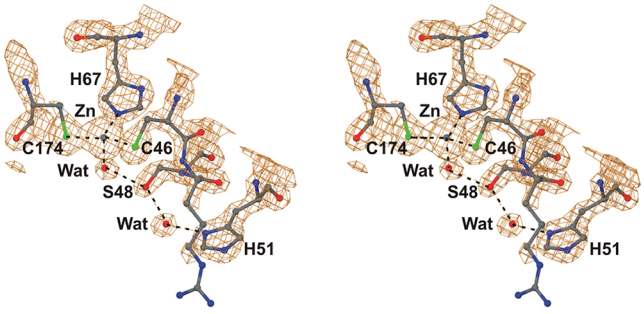

Figure 1. Zinc coordination in the apoenzyme. The stereoview is derived from PDB entry 1YE3. The $2|F_o| - |F_c|$ map is contoured at ~0.3 $e^-/Å^3$.

The structure of the enzyme–adenosine 5′-diphosphoribose complex is very similar to that of the apoenzyme (PDB entry 8ADH) and to that of the complex with ADPR determined at 2.9 Å at 278 K previously (PDB entry 5ADH[46]) except for the conformation of the terminal ribose, which is disordered and has relatively high *B* values (Figure 2). The position of the adenosine 5′-diphosphate moiety is similar to that found in PDB entry 5ADH, as well as in ternary complexes with NAD^+ or NADH, but because the protein conformation is open, there is no interaction of His-51 with the ribose and Arg-47 is not well-defined in density. The catalytic zinc is in the same location as in the apoenzyme, but the side chain of Glu-68 is not well-defined in density and has a *B* value higher than that of the protein backbone (~2-fold), as compared to other structures.

Structures for two complexes with the zinc chelators, 2,2′-bipyridine and 1,10-phenanthroline, were determined at 1.9 Å after the apoenzyme crystals had been soaked with the chelators (Figures 3 and 4). The structure with bipyridine was not determined before, whereas the structure with 1,10-phenanthroline was determined at 4.5 Å.[18] The tertiary structures of the subunits are very similar to those of the apoenzyme, except for the region between residues 294 and 299, which may be disordered as the electron density is not well-defined. The structure of the complex with bipyridine superimposes onto the apoenzyme structure (PDB entry 1YE3) with an RMSD of 0.23 Å for α-carbons, and each of the two subunits in the asymmetric unit of the complex with 1,10-phenanthroline superimposes with an RMSD of 0.46 Å. The binding of the chelators is well-defined and indicates that both nitrogens of the chelators are bound to the zinc, and the pentacoordination inferred from spectroscopic studies is confirmed.

The structure for the enzyme–NADH complex has the dimeric molecule as the asymmetric unit, with two subunits that differ only slightly in conformation, with a water ligated to the catalytic zinc (Figure 5). The protein structure is essentially identical to the atomic-resolution structure of the wild-type enzyme complexed with NAD^+ and 2,3,4,5,6-pentafluorobenzyl alcohol (PDB entry 4DWV[8]), as all α-carbons superimpose with an RMSD of 0.16 Å. The two subunits of the enzyme–NADH complex are very similar; the α-carbons of the coenzyme binding domain (residues 176–318) of subunit A superimpose onto subunit B with an RMSD of 0.08 Å, whereas α-carbons of all residues superimpose with an RMSD of 0.14 Å. Both subunits are in the closed conformation and should be able to bind substrates and be enzymatically active. As discussed below, there appear to be two, alternative positions for the catalytic zinc, but the zinc in the major position has the classical tetrahedral coordination.

The structure for the enzyme–NADH–(*R*,*S*)-*N*-1-methylhexylformamide complex was determined at a resolution (1.25 Å) higher than that for the structure described previously with (*R*)-*N*-1-methylhexylformamide (1.57 Å, PDB entry 1P1R[11]) to detect potential water molecules near the catalytic zinc, but there is no significant electron density for such a water. All four subunits of the two homodimers in the asymmetric unit have the closed conformation, typical of ternary complexes with NAD(H) and substrate analogues. For this structure, the racemic *N*-1-methylhexylformamide (an aldehyde analogue) was used for crystallography, and it was expected that the *R*

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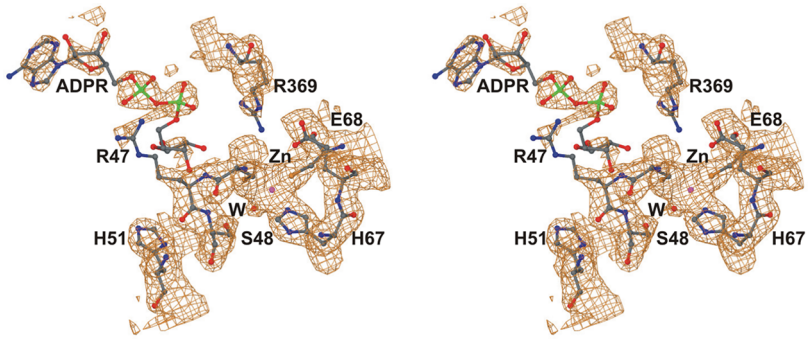

Figure 2. Complex of the apoenzyme with adenosine 5′-diphosphoribose (ADPR). The stereoview is derived from PDB entry 5VKR. The $2|F_o| - |F_c|$ map is contoured at ~0.25 $e^-/Å^3$.

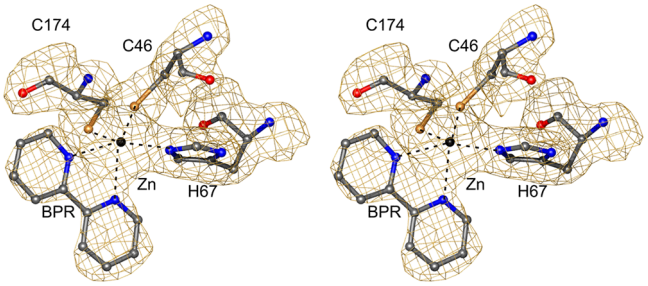

Figure 3. Complex of the apoenzyme with 2,2′-bipyridine (BPR). The stereoview is derived from PDB entry 5VJG. The $2|F_o| - |F_c|$ map is contoured at ~0.25 $e^-/Å^3$.

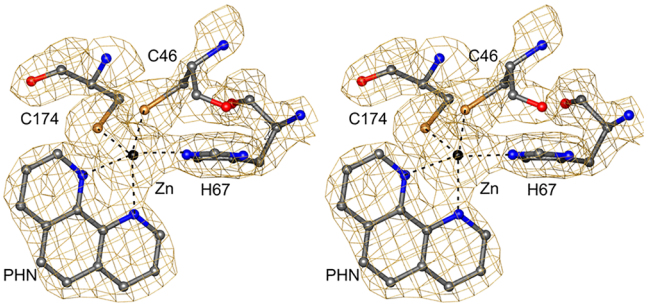

Figure 4. Complex of the apoenzyme with 1,10-phenanthroline (PHN). The stereoview is derived from PDB entry 5VJ5. The $2|F_o| - |F_c|$ map is contoured at ~0.25 $e^-/Å^3$.

isomer would be bound because it has an affinity 1.7-fold higher than that of the *S* isomer.[11] For the structure presented here, however, it appears that both isomers can bind because the electron density is fitted well by the *N*-isopropylformamide portion of the molecule, whereas the density for the C4−C7 tail is weak. Subunits B and D have predominantly the *S* isomer (named NWH in the PDB file). Figure 6 illustrates the binding of the *S* isomer in subunit B in the electron density map and shows the binding of the *R* isomer (named NMH in PDB entry 1P1R) in green, which is accommodated by alternative rotamers of the side chains of Leu-116 and Ile-318. The conformations of these residues are indicative of the binding of the two different isomers of *N*-1-methylhexylformamide. Such accommodations are also observed in binding different substrate analogues, such as trifluoroethanol or pentafluorobenzyl alcohol, and the isomers of 3-butylthiolane oxide.[8,12] It is interesting that the structure of human ADH1C2 complexed with NADH and *N*-1-methylhexylformamide (determined at 1.45 Å) has the *R* isomer, even though the crystallization medium contained both isomers.[47] For this human ADH, the *R* isomer binds 7-fold more tightly than the *S* isomer.[11] The catalytic zinc has one position, in the classical tetracoordination.

The structure of the complex with NADH and *N*-benzylformamide (an aldehyde analogue) has the closed conformation and is very similar to the atomic-resolution structures with NADH and *N*-1-methylhexylformamide (PDB entry 1P1R) or NAD^+ and 2,3,4,5,6-pentafluorobenzyl alcohol (PDB entry 4DWV) with an RMSD of 0.26 Å for the superposition of α-carbon atoms of one dimer (748 residues) on either of the two molecules in the asymmetric unit. The side chain of Leu-309 has alternative positions, reflecting differing energetics in the interaction with the benzylformamide, and Leu-116 has alternative conformations in subunits A and C (Figure 7). The structures determined at 2.5 Å for the complexes with NADH and *N*-cyclohexylformamide (PDB entry 1LDY) or *N*-formylpiperdine (PDB entry 1LDE) also differ in the conformation of Leu-116.[13] The structure of human ADH1B2 at 1.6 Å has the *N*-benzylformamide in a similar position in the active site, showing that the Thr/Ser-48 exchange can be accommodated in either the human or horse enzymes without a large change in affinity for the inhibitor.[47,51] The structure of horse ADH1E with NAD^+ and pentafluorobenzyl alcohol has alternative positions for Leu-57 and Leu-309, but not Leu-116.[8] The formamides have the *cis* configuration, with the *re* face of the carbonyl group oriented favorably for direct transfer of a hydride from C4N of the reduced nicotinamide ring at an average distance of 3.48 Å for benzylformamide and 3.55 Å for methylhexylformamide. These atomic-resolution structures provide good analogues of the structure expected for the active enzyme−NADH−aldehyde complex.

Identification of NADH in Structures. Figures 5−7 show views of the binary and ternary complexes with NADH, which were determined by refinements with relaxed or no restraints on the dictionaries for NAD^+ or NADH. A major feature is the puckered and reduced nicotinamide ring of the NAD; i.e., it is NADH. The enzyme−NADH complex was produced by crystallizing the enzyme with NAD^+, but apparently the enzyme reduced the NAD^+ with 2-methyl-2,4-pentanediol to produce NADH. The diol is a poor substrate; however,[45] the crystals were kept at 5 °C for 1 year, and formation of NADH would be favored as the solution contained excess diol and minimal concentrations of carbonyl compounds or inhibitors. Crystals of the F93A enzyme prepared in the presence of NAD^+, 2,3,4,5,6-pentafluorobenzyl alcohol, and methylpentanediol also contained NADH, apparently because the fluoro alcohol binds relatively weakly while NADH binds tightly (PDB

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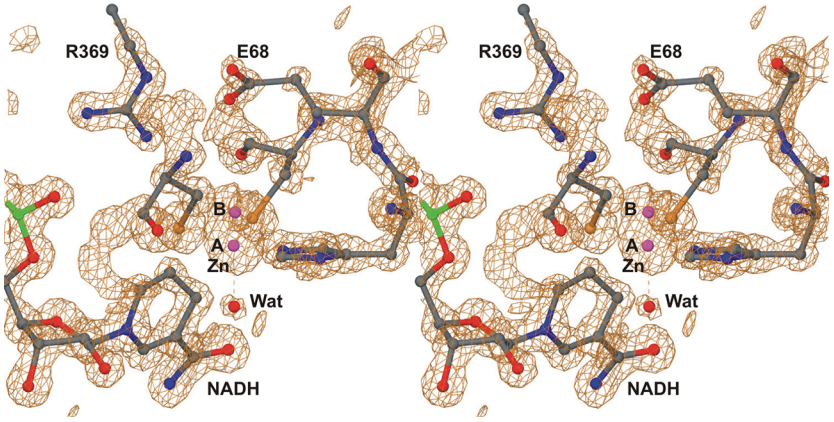

Figure 5. Complex of the holoenzyme with NADH. Stereoview of one active site, derived from PDB entry 4XD2. Atom coloring is used, gray for carbon and magenta for the zincs in alternative positions, A and B. The zinc in the major position is coordinated to Cys-46, Cys-174, His-67, and a water, "Wat", just as in Figure 1 for the apoenzyme. The $2|F_o| - |F_c|$ map is contoured at ~1.0 $e^-/Å^3$. The graphic in the abstract shows another view of the alternative positions for the zincs, with the $2|F_o| - |F_c|$ map contoured at ~2.0 $e^-/Å^3$.

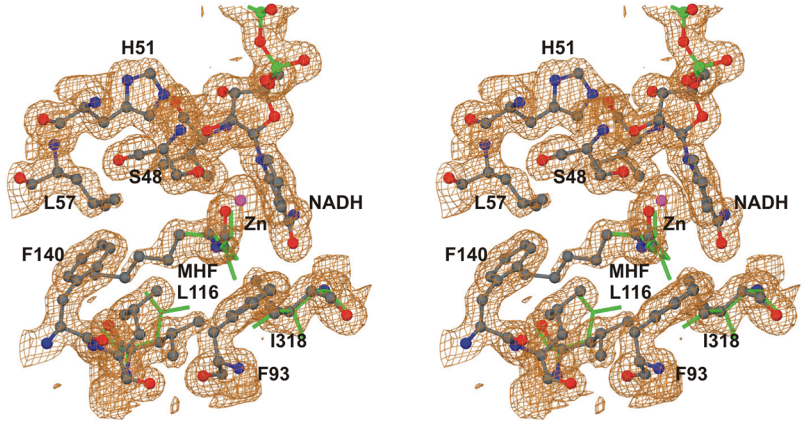

Figure 6. Complex of the holoenzyme with NADH and *N*-1-methylhexylformamide (MHF) based on PDB entry 5VN1. Subunit B with (*S*)-*N*-1-methylhexylformamide is shown in atom coloring with the $2|F_o| - |F_c|$ electron density map contoured at ~0.35 $e^-/Å^3$. Represented as green sticks are (*R*)-*N*-1-methylhexylformamide and the alternative conformations of Leu-116 and Ile-318 found in the structure crystallized with (*R*)-*N*-1-methylhexylformamide (PDB entry 1P1R).

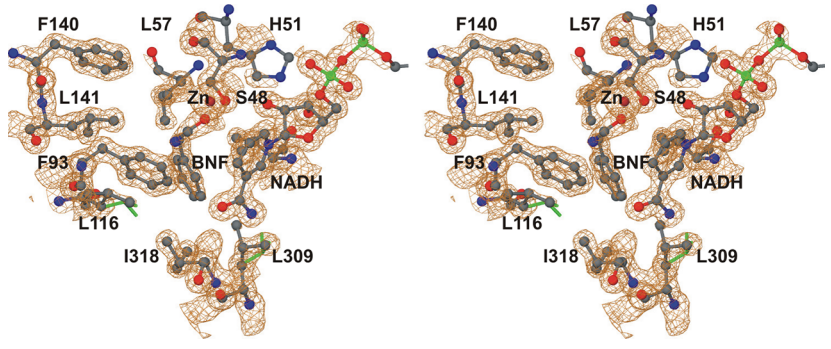

Figure 7. Complex of the holoenzyme with NADH and *N*-benzylformamide (BNF), based on PDB entry 5VL0. Subunit A is shown in atom coloring, except for the alternative conformations of Leu-116 and Leu-309, which are colored green. The $2|F_o| - |F_c|$ electron density map is contoured at ~0.7 $e^-/Å^3$.

entry 1MGO).[59] Spectroscopic studies show that the enzyme–NAD^+ complex reacts in solution with the diol to form NADH, as do crystals of the ternary enzyme–NAD^+–2,2,2-trifluoroethanol complex that are washed with buffer containing methylpentanediol, which replaces the inhibitory 2,2,2-trifluoroethanol.[60,61]

The puckering geometry of the nicotinamide rings for structures of ADH complexed with NADH or NADH and formamides, or NAD^+ and fluoro alcohols, is presented in Table 2.[8,62] The nicotinamide rings have essentially a boat conformation, with C4N directed toward the substrate binding site. Other atomic-resolution (1.0–1.2 Å) structures of the enzyme crystallized with NADH have very similar geometry,

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Table 2. Nicotinamide Ring Puckering in Complexes of Horse Liver ADH[a]

ligands	PDB entries	α-C4 (deg)	α-N1 (deg)	twist (Å)
NADH, H_2O[b]	4XD2	16.0 ± 1.6	12.6 ± 2.2	−0.26 ± 0.03
NADH, MHF[c]	1P1R, 5VN1	18.1 ± 4.2	11.2 ± 2.3	−0.04 ± 0.12
NADH, BNF[d]	5VL0	12.5 ± 3.9	13.1 ± 3.2	0.07 ± 0.07
NADH, various[e]	1HET, 2JHG	12.3 ± 2.5	11.0 ± 4.5	−0.31 ± 0.19
NAD^+, FALC[f]	4DXH, 4DWV	4.9 ± 2.3	5.8 ± 2.1	0.06 ± 0.03

[a]The structures were refined with relaxed or no restraints on the nicotinamide ring. Ring puckering geometry is defined as follows: α-C4, angle between the C3−C4−C5 and C2−C3−C6 planes; α-N1, angle between the C2−N1−C6 and C2−C3−C6 planes; twist, distortion of the boat conformation, defined as the distance between C5 and the C2−C3−C6 plane.[22] [b]Weighted average from two subunits as calculated with SHELXL-2013 with no restraints on the nicotinamide ring. [c]Average of eight subunits of the enzyme complexed with NADH and *N*-1-methylhexylformamide, an aldehyde analogue and potent inhibitor,[11] calculated with SHELXL-2013 after refinement with REFMAC. [d]Average of four subunits of the enzyme complexed with NADH and *N*-benzylformamide calculated with SHELXL-2013 after refinement with REFMAC. [e]Average of eight subunits from four structures of wild-type ADH with Zn(II) or Cd(II) at the active sites, complexed with NADH and other ligands, including water, methylpentanediol, dimethyl sulfoxide, or isobutyramide (PDB entries 1HEU, 2JHF, 1HET, and 2JHG).[22,23] [f]Weighted average of four subunits of the enzyme complexed with NAD^+ and 2,3,4,5,6-pentafluorobenzyl alcohol or 2,2,2-trifluoroethanol calculated with SHELXL-2013 with no restraints on the nicotinamide ring.[62]

but the structures with methylpentanediol, dimethyl sulfoxide, or isobutyramide apparently have partial occupancy of water molecules in close contact (1.7−2.0 Å) with C6N of the nicotinamide ring.[22,23] There is no clear evidence in the electron density maps of water molecules interacting with the nicotinamide ring in our new structures with NADH alone or with NADH and the aldehyde analogues, *N*-1-methylhexylformamide or *N*-benzylformamide. The nicotinamide ring can be even more puckered, as the enzyme−NAD−pyrazole complexes (PDB entries 1N92 and 1N8K, with a partial covalent bond of 1.7 Å between pyrazole N2 and nicotinamide C4N) have α-C4 = 21° and α-N1 = 14°.[63] In contrast, in the atomic-resolution (at 1.12 Å) structures with NAD^+ and fluoro alcohols, the nicotinamide rings are only slightly puckered and apparently strained.[8] The puckered nicotinamde rings in the enzyme complexes with NAD^+ and NADH and substrate analogues may represent ground states that could facilitate catalysis of hydride transfer, as nicotinamide C4N moves closer to the reactive carbon in the substrate.[8,64−67]

The bond distances in the nicotinamide ring (Table 3) are also consistent with the identification of the coenzyme as NADH in the enzyme−NADH and enzyme−NADH−formamide complexes. In particular, the C3N−C4N and C4N−C5N bonds are longer than the bonds in the complexes with NAD^+. The distances in these complexes also agree with those determined for other structures with NADH.[22,23] Comparison of bond distances for nicotinamide rings in enzyme complexes is problematic because of different dictionaries for crystallographic refinement procedures, uncertainty about the NADH/NAD^+ ratio in the complex, and the potential effects of binding of the coenzyme and ligands to the enzyme.[8] For the enzyme−NADH structure, the bond distances calculated with SHELXL-2013 with no restraints on the nicotinamide ring for a model of either NAD^+ or NADH in the complex agree within the stated errors, but the distances from refinement with REFMAC5 using the dictionary for NAD^+ with relaxed restraints are somewhat closer to the average of those found in five structures with NADPH or *N*-benzyl-1,4-dihydronicotinamide (see Table 4 of ref 8) We conclude that atomic-resolution crystallography can distinguish NAD^+ from NADH.

Catalytic Zinc Coordination. For all of the structures described here, except for the enzyme−NADH complex, the electron densities around the catalytic and structural zincs are essentially spherical. The catalytic zinc has tetrahedral coordination (e.g., see Figure 1), but the structures with the two chelators, 2,2′-bipyridine and 1,10-phenanthroline, are pentacoordinated with the two nitrogens binding on adjacent positions of the zinc. The ligation distances listed in Table 4 agree with those found in high-resolution X-ray structures,[68] except that the distances from Cys-46 SG and His-67 NE2 to the zinc in the chelators are somewhat longer than in the other structures and that the zinc−nitrogen distances are also relatively long, suggesting somewhat looser interactions in the pentacoordinated complexes. Three structures have a water ligated to the zinc, where a distance of 2.1−2.3 Å would be consistent with a neutral water. A p*K* value of 9.2, determined from the pH dependence for binding of NAD^+, 2,2′-bipyridine, or 1,10-phenanthroline to the apoenzyme has been attributed to the zinc-bound water.[20,69−71] The p*K* value is apparently

Table 3. Bond Distances (angstroms) in the Nicotinamide Rings of NADH or NAD^+ in ADH Complexes

complex	N1−C2	C2−C3	C3−C4	C4−C5	C5−C6	C6−N1
NADH, H_2O[a]	1.36 ± 0.02	1.35 ± 0.02	1.50 ± 0.02	1.43 ± 0.02	1.44 ± 0.02	1.35 ± 0.02
NADH, MHF[b]	1.40 ± 0.02	1.36 ± 0.03	1.49 ± 0.05	1.48 ± 0.02	1.33 ± 0.03	1.42 ± 0.06
NADH, BNF[c]	1.36 ± 0.02	1.35 ± 0.02	1.50 ± 0.03	1.42 ± 0.04	1.36 ± 0.05	1.42 ± 0.02
NADH, various[d]	1.40 ± 0.03	1.35 ± 0.02	1.48 ± 0.05	1.47 ± 0.03	1.41 ± 0.04	1.40 ± 0.03
NAD^+, FALC[e]	1.35 ± 0.01	1.36 ± 0.01	1.42 ± 0.01	1.38 ± 0.01	1.37 ± 0.01	1.39 ± 0.01

[a]Weighted average of the bond distances and errors for two subunits from refinement with SHELXL-2013 with no restraints on distances or planarity of the nicotinamide ring (PDB entry 4XD2). [b]Two structures (1.25 and 1.57 Å resolution) with *N*-1-methylhexylformamide, with an average of eight subunits and the standard deviation from refinement with REFMAC (PDB entries 1P1R and 5VN1). [c]Average of four subunits of the structure with NADH and *N*-benzylformamide (PDB entry 5VL0) after restrained refinement with REFMAC and calculation with SHELXL-2103. [d]Four structures (1.0−1.2 Å resolution) with an average of eight subunits and the standard deviation in complexes with NADH and various ligands (PDB entries 1HET, 1HEU, 2JHG, and 1JHF) refined with relaxed restraints on the nicotinamide ring.[22,23] [e]Weighted average for four subunits with SHELXL-2013 refinement with no restraints on the nicotinamide ring in complexes with NAD^+ and fluoro alcohols (PDB entries 4DWV and 4DXH).[62]

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Table 4. Coordination Distances (angstroms) from Ligands to Catalytic Zinc in ADH Complexes

enzyme complex	PDB entry	C46 SG	H67 NE2	C174 SG	ligand O	N1	N8 or N10
apoenzyme, H_2O	1YE3	2.42	2.05	2.21	2.11	–	–
ADPR, H_2O	5VKR	2.28	2.10	2.34	2.35	–	–
2,2′-bipyridine	5VJG	2.42	2.27	2.18	–	2.48	2.60
1,10-phenanthroline[a]	5VJ5	2.46	2.30	2.20	–	2.26	2.44
NADH, H_2O[b]							
"A"	4XD2	2.31	2.06	2.24	2.08	–	–
"B"		2.18	2.24	2.53	(3.35)	–	–
NADH, MHF[c]	1P1R, 5VN1	2.29	2.05	2.24	2.16	–	–
NADH, BNF[d]	5VL0	2.31	2.02	2.24	2.09	–	–
NAD^+, FALC[e]	4DWV, 4DXH	2.33	2.03	2.28	1.96	–	–

[a]Distances were averaged for two subunits; the average deviation is 0.025 Å. [b]Distances were averaged for two subunits. For the major A position, the average deviation is 0.045 Å and the distance to Glu-68 OE1 is 4.8 Å. For the minor B position, the average deviation is 0.11 Å and the distance to Glu-68 OE1 is 3.7 Å. The alternative zinc position is closer to Cys-46 SG by ∼0.1 Å than is the major position, whereas the alternative position is farther from His-67 NE2 or Cys-174 SG by 0.2−0.3 Å than is the major position. Some movement of the ligands could alter these distances, but refinements could not define alternative positions for the ligands. [c]Distances were averaged for eight subunits for structures complexed with NADH and *N*-1-methylhexylformamide; the average standard deviation is 0.021 Å. [d]Average for four subunits of the enzyme complexed with NADH and *N*-benzylformamide, with an average standard deviation of 0.015. [e]Distances were calculated with SHELXL-2013 refinement with data to 1.1 Å, and weighted averages for four subunits are reported, with an average standard deviation of 0.005 Å.

shifted to 7.6 in the enzyme−NAD^+ complex and to 11.2 in the enzyme−NADH complex.[72] The ternary complexes with NADH and *N*-1-methylhexylformamide or *N*-benzylformamide have a carbonyl oxygen ligated to the zinc (Zn−O distance of 2.1 Å), whereas the previously reported structures with NAD^+ and a fluoro alcohol probably have an alcoholate O bound to the zinc, with a Zn−O distance of 2.0 Å.[8] Solvent deuterium isotope effects suggest that the alcohol is deprotonated and participates in a low-barrier hydrogen bond (2.5 Å) to Ser-48 OG.[1,8] There is no evidence of an additional water near the zinc in these complexes.

Of particular interest is the fact that the electron density for the catalytic zinc in the enzyme−NADH binary complex is a prolate ellipsoid (or ovoid), rather than a sphere as for the catalytic and structural zincs in the other structures. Anomalous scattering difference maps show that the density is due to zincs, and not, for instance, a water (see the Supporting Information). We placed two zincs, separated by ∼1.3 Å, to illustrate the potential alternative coordination. The major position (80%) is located in the classical coordination with Cys-46 SG, His-67 NE2, Cys-174 SG, and a water molecule, as found in the apoenzyme (Figure 1, PDB entry 1YE3). The alternative zinc position is closer to Glu-68 OE2 (at 3.7 Å), as compared to the distance in the major position (4.8 Å), but the protein ligands were not shifted in the model because the electron density did not justify alternative positions (Table 4). Elongated densities were also observed for horse liver ADH with Cd(II) substituted for Zn(II) in a complex with NADH and 2-methyl-2,4-pentanediol (see Figure 5a of ref 23) and for human ADH3 in complex with NADH.[35,36] However, in those structures, Glu-68 (or Glu-67 in human ADH3) can also adopt an alternative conformation that brings OE2 into ligation with the alternative position of the metal. In the structure presented here, there is not sufficient electron density to place Glu-68 in an alternative position. Nevertheless, the structure suggests some flexibility in the zinc position, which can be relevant for the mechanism by which the water is replaced with alcohol or aldehyde in the enzyme−coenzyme complexes.

DISCUSSION

With the new results on the alternative positions of the catalytic zinc in the ADH−NADH complex, combined with higher-resolution structures of other enzyme complexes and extensive, previously published results, we can address controversial proposals about the role of water and zinc coordination in catalysis and suggest a mechanism that is most consistent with the results.

Does a Water Adducted to the Nicotinamide Ring Participate in Hydrogen Transfer? Meijers and co-workers[22] suggested that a water (as hydroxide) bound to the active site zinc and to C6N of the nicotinamide ring could activate NADH for the enzymatic reaction, but the proposed mechanism does not seem to be chemically necessary, reasonable, or supported by most structural studies. Although their computations support the conclusion that the NADH−hydroxide "adduct" in the X-ray structure is 6-hydroxy-1,4-dihydronicotinamide, it is not clear what this adduct is and how it was formed. The distance between the water oxygen and C6N is between 1.7 and 2.0 Å in six subunits of three different structures (PDB entries 1HET, 1HEU, and 2JHF) and thus is not typical of a C−O bond. They proposed that the water ligated to the catalytic zinc and directed by the hydroxyl group of Ser-48 could react with either the reduced or oxidized nicotinamide ring, but they did not explain why the redox state did not change or how the C6N hydrogen was displaced.

The origin of the electron density is not clear in the structures determined by Meijers and co-workers.[22,23] Hydration at C6N of the reduced nicotinamide ring occurs at low pH to form 6-hydroxy-1,4,5,6-tetrahydronicotinamide.[73,74] Glyceraldehyde-3-phosphate dehydrogenase can also catalyze formation of this adduct with NADH, apparently assisted by bound anions.[75] The oxidized nicotinamide ring of NAD^+ can be attacked under basic conditions by various nucleophiles at the C2N, C4N, and C6N atoms,[74] but the source of NAD^+ in the ADH structures is not clear. It may be difficult to assign waters in specific structures because of the presence of alternative positions and occupancies.

In the structure of the enzyme−NADH complex presented here, the water bound to the zinc is 3.40 Å from C6N (3.25 Å to C4N or C5N), and there is no significant electron density

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for additional atoms attached to the nicotinamide ring. It is not obvious why our studies differ from those of Meijers and co-workers.[22] We crystallized the enzyme with NAD^+ (which was apparently reduced to NADH by the precipitant, methylpentanediol) at pH 7.0, where addition of hydroxide to the nicotinamide ring would be minimized, whereas Meijers and co-workers crystallized the enzyme with NADH with polyethylene glycol 400 and methylpentanediol at pH 8.2, where acid-catalyzed addition of water would be slow.

We suggest that none of the structural evidence supports a catalytic mechanism for the dehydrogenase that involves a covalent NADH−hydroxide adduct. Structures for complexes of ADH with NADH and aldehyde analogues, e.g., with formamides (Figures 6 and 7) or sulfoxides, did not locate such a water.[10−13] Atomic-resolution structures of ternary complexes with NAD^+ and fluoro alcohols also show no evidence of a water near the zinc or the nicotinamide ring.[8] Crystallography could in principle locate a water, as one was found at 3.1 Å from C6N of the NAD^+ in the V203A ADH complex where a cavity is formed by the substitution of Val-203.[62] Computations apparently adequately describe the ADH reaction without invoking a water linked to the coenzyme.[65,76−78]

Does a Water Bound to Zinc Participate in Proton Transfer in the Central Complex? Several studies have proposed that a water bound to the zinc, either with the substrate in the second ligation sphere or with both the water and substrate oxygens bound to a pentacoordinated zinc, participates in acid/base catalysis in the ternary complex. Spectroscopic and kinetic studies in which the catalytic zinc was replaced with Co(II) and Cd(II) in horse liver ADH provide information about the metal coordination. An X-ray structure shows that Co(II) can replace the Zn(II) with minimal changes in the coordination of the metal.[79] NMR studies using the paramagnetic effect of Co(II) on the relaxation of the hydrogens of ligands suggested that a water (or hydroxide) binds to the metal whereas substrates and inhibitors bind at distances that are too long to allow direct coordination of the oxygen with the metal, and it was proposed that hydroxide on a tetracoordinated zinc acted as the base for alcohol oxidation.[80] However, a low-resolution X-ray crystallography study suggested that substrates and inhibitors bind directly to the zinc in enzyme−coenzyme complexes that resemble Michaelis complexes.[45] Spectra showed that *trans*-4-(*N*,*N*-dimethylamino)cinnamaldehyde binds to the Co(II) in the complex with 1,4,5,6-tetrahydronicotinamide adenine dinucleotide.[81,82] The NMR study was extended to ternary complexes with NADH and dimethyl sulfoxide or NAD^+ and 2,2,2-trifluoroethanol, and again it was concluded that the oxygens of the ligands were not directly ligated to the metal.[83] However, other studies concluded that the paramagnetic effects of Co(II) on the relaxation of water or alcohol protons were too small to give reliable determinations of the distance of the protons from the metal.[84,85] After higher-resolution X-ray studies clearly showed that the oxygens of the ligands are directly ligated to a tetracoordinated zinc and that there is no water near the zinc, we proposed that the oxygen of the substrate is bound to the zinc, and the proton is transferred to or from the substrate through a hydrogen-bonded system, including the hydroxyl group of Ser-48 and the imidazole group of His-51.[21]

EPR measurements of the Co(II) enzyme showed that the zero-field splitting energies were increased in enzyme−coenzyme complexes, including complexes with NAD^+, NADH, NAD^+−pyrazole, and NADH−trifluoroethanol, and suggested a pentacoordinated metal.[24,25] However, our study shows that the enzyme−NADH complex has a closed conformation with a tetracoordinated zinc, and it is likely that the enzyme−NAD^+ complex was reduced by contaminating alcohols to form the enzyme−NADH complex.[61] Furthermore, the enzyme−NAD^+−pyrazole complex has a closed conformation with a tetracoordinated zinc.[63] Subsequent EPR and magnetic circular dichroism studies suggested that changes in zero-field splitting reflect a highly distorted tetrahedral coordination related to the conformational change in the protein when coenzymes bind.[26] As shown in Table 4, however, the catalytic zincs in the apoenzyme and enzyme−coenzyme complexes have essentially the same coordination of the ligands, even if the environments differ because of the conformational change and the replacement of the water.

The Co(II) enzyme is about as active as the zinc enzyme; however, the p*K* values for the oxidation of benzyl alcohol (k_{cat} and k_{cat}/K_m) are shifted somewhat as compared to the zinc enzyme, and it was concluded that a neutral water ligated to a pentacoordinated metal acted as the base to deprotonate the bound alcohol.[25,86] However, these kinetic constants are controlled by dissociation of NADH and reaction of the enzyme−NAD^+ complex, respectively, in the ordered mechanism and do not report on the p*K* values in the central complex.[87] The transient oxidation of alcohols (ethanol, 1-propanol, and benzyl alcohol) shows that the hydride transfer rate is most rapid when a group with a p*K* value of ∼6.4 is deprotonated, which can be attributed to the ionization of the alcohol bound to the zinc mediated by the proton relay system.[1,9,71,82,88−90] The observed rate constant for the transient oxidation of ethanol is decreased to 66% with the substitution of the zinc with Co(II).[91]

The catalytic zinc can also be replaced with Cd(II) to form an enzyme with ∼14% of the maximal turnover activity of the zinc enzyme. NMR studies of the enzyme substituted with ^{113}Cd(II) showed that the resonances are shifted differently in binary complexes with NAD(H) or ternary complexes with NAD^+, but the chemical shift was the same in the complexes with NAD^+ and trifluoroethanol or pyrazole, leading to the conclusion that the substrate analogues did not bind directly to the metal because binding of oxygen or nitrogen atoms should cause different shifts.[92] However, the structures of the zinc enzyme with NAD^+ and either pyrazole or trifluoroethanol determined by high-resolution X-ray crystallography show that the ligands are bound to tetracoordinated zinc.[8,63] Perturbed angular correlation (PAC) spectroscopy of the enzyme substituted with ^{111}Cd(II) suggested that the apoenzyme and the complex with NAD(H) were tetrahedrally coordinated, but the complexes with NAD^+ and either pyrazole or trifluoroethanol were thought to be pentacoordinated with a water and the ligand bound to the metal.[27,85] However, X-ray crystallography shows that the apoenzyme and ternary complexes with NAD(H) have the same structures as the zinc enzyme, all tetracoordinated with a water or the oxygen of a ligand binding directly to the metal.[23,93] A more extensive PAC study of the Cd(II) enzyme with better instrumentation suggested that the geometry is tetracoordinated in the apoenzyme and in complexes with NAD^+, NADH, and NADH−dimethyl sulfoxide, but the apoenzyme and the enzyme−NAD^+ complex have two different pH-dependent forms that may result when a water bound to the metal ionizes and the Cys46 S−metal−Cys174 S bond angle changes. The enzyme−NADH complex has two pH-independent forms that

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have different coordination geometries.[28] A subsequent study suggested that the enzyme−NADH−imidazole complex also has two different forms even though X-ray crystallography shows that this complex crystallizes in an open conformation.[29] Some of these PAC results were reanalyzed by optimizing structures of the complexes with a combined quantum chemical and molecular mechanical program (starting with known X-ray structures) and calculating the electric field gradients.[30] It was concluded that all complexes had tetracoordinated Cd(II), but the two spectral forms could be due to different charges of the ligands on the zinc. The apoenzyme could have neutral water or hydroxide; the enzyme−NAD^+ complex could have hydroxide at high pH or one oxygen of the carboxyl group of Glu-68 at low pH, and the enzyme−NADH complex could have either water or the carboxyl group of Glu-68 ligated to the zinc. It was suggested that ∼40% of the Cd(II) was ligated to Glu-68, whereas the percentage should be lower for the Zn(II) enzyme because of the relative sizes of the metals. It is significant that an atomic-resolution structure of the Cd(II) enzyme complexed with NADH shows that the electron density for the Cd(II) is an ellipsoid extending toward Glu-68 and that there is an alternative position for Glu-68 that brings the shortest distance to the metal to 3.0 Å.[23] When dimethyl sulfoxide binds to the metal in this complex with NADH, Glu-68 reverts to the position found in the corresponding zinc enzyme. Additional calculations "quite unexpectedly" suggested that a strained pentacoordinated Cd(II) with water and Glu-68 on opposing sides of the metal could form, but such a species was not obtained with the zinc enzyme.[44] The proposed alternative coordination in the Cd(II)−enzyme−NADH complex is relevant because it appears in this study that the catalytic zinc can move closer to Glu-68. Such a complex could represent a transient in the exchange of water with the substrate, as shown in Scheme 2.

Although the spectroscopic studies suggest that the coordination of the catalytic zinc changes in various complexes, they do not provide strong support for a stable pentacoordinated zinc in the apoenzyme or ternary complexes. We think the X-ray structures that show the catalytic zincs in the apoenzyme and the ternary complexes are tetracoordinated are the definitive evidence of zinc coordination for these forms of the enzyme. Nevertheless, binary enzyme−coenzyme complexes and transient species may have pentacoordinated zinc or the alternative coordination with Glu-68.

Do the Catalytic Zincs in ADHs Become Pentacoordinated? The results in Figures 3 and 4 and Table 4 show that the catalytic zincs are pentacoordinated in the complexes with 2,2′-bipyridine and 1,10-phenanthroline, with some alteration of the bond distances and angles around the zincs as compared to those around the tetracoordinated zincs. The kinetics of binding of these zinc chelators are relevant. 2,2′-Bipyridine binds most rapidly to horse liver ADH at low pH and is slower at pH values above a p*K* value of 9.2, which can reflect ionization of the zinc-bound water.[20,94] Equilibrium binding of 2,2′-bipyridine and 1,10-phenanthroline is also tighter below the p*K* of 9.2, apparently because water binds more weakly than hydroxide in the competitive replacement by the chelators. (Our analysis of the binding data for 2,2′-bipyridine and 1,10-phenantroline suggests that at high pH the limiting rate constants for binding are ∼10-fold slower and the equilibrium binding constants are ∼10-fold weaker than at low pH, indicating that formation of zinc hydroxide does not prevent the binding.) In the presence of imidazole, which displaces the water from the zinc,[18,95] the pH dependence for equilibrium binding of bipyridine is abolished. Chelators bind with rapid association followed by a slower limiting isomerization of ∼200 s^{-1} at high concentrations of the chelator.[19,20,94,96] The limiting rate for bipyridine binding is pH-independent, as is the rate constant for dissociation of the complex, and imidazole abolishes the limiting rate.[20] Water is displaced from the zinc in the enzyme−bipyridine complex (Figure 3), but the mechanism of exchange is not clear; for hydrated metal ions, it involves rapid dissociation of water and rapid chelation.[97] For the zinc in ADH, the limiting kinetics could be due to slow dissociation of water (or hydroxide) and reaction of a tricoordinated zinc, reorganization from a tetracoordinated to a pentacoordinated zinc, or double displacement involving Glu-68 with inversion of a tetracoordinated zinc. In any case, a pentacoordinated zinc can form, and some step in the enzyme mechanism could involve both a water and a substrate oxygen bound adjacently to zinc.

ADHs can slowly oxidize aldehydes to the carboxylic acids,[61,98] and one or both oxygens of the hydrated aldehyde substrate or the carboxylate product might bind to the zinc. Both monodentate ligation and bidentate ligation are observed for zinc ions in protein structures.[68] However, a structure for *Aeropyrum pernix* ADH (PDB entry 1H2B) with NAD(H) has just one oxygen of octanoate bound to the zinc.[99] Human ADH3 complexed with NAD (PDB entry 1MAO) paradoxically binds the methyl group of dodecanoate close to the zinc that is coordinated by Cys-44, His-66, Glu-67, and Cys-173.[34] Perhaps both oxygens of a carboxyl group cannot easily bind to the zinc in ADHs.

The catalytic zinc in human sorbitol dehydrogenase forms a pentacoordinated complex with an inhibitor that mimics the vicinal 1,2-hydroxyl groups of the substrate and ligation to His-69 and Cys-44 from the protein and a water that is proposed to be the proximal proton donor and/or acceptor in the reaction at C2 of sorbitol (PDB entry 1PL6).[100] The inhibitor approximates an isosteric analogue of 2,2′-bipyridine by having the oxygen and a nitrogen, linked by two carbons, ligated to the zinc. The structure may resemble the reactive ternary complex for this ADH. It is notable that the binding of the inhibitor displaces the carboxyl group of Glu-67 that is ligated to the opposite side of the zinc in the enzyme−NAD^+ complex.

X-ray absorption spectroscopy (XAS) data on the tetrameric ADH from *Thermoanaerobacter brockii* led to the suggestion that binary complexes with $NADP^+$ or NADPH have zinc tetracoordinated with Cys-37, His-59, and oxygen atoms from Glu-60 and Asp-150, whereas the ternary complexes with coenzymes and dimethyl sulfoxide have the coordination expanded to five or six ligands with the addition of oxygen atoms.[32] Model building with an X-ray structure of an enzyme−coenzyme complex suggested that a trigonal bipyramidal coordination could represent a species poised for hydrogen transfer. The XAS studies were extended by studying the transient phase for oxidation of 2-propanol with $NADP^+$ where the coordination number apparently increased to five or six.[31] A mechanism was proposed in which the carboxylate of Glu-60 was replaced stepwise with a water and the substrate oxygen before hydride transfer, to explain two transient intermediates. The results with this enzyme are interesting, but they are not convincing because of the uncertainties in interpreting XAS data, the lack of data in the steady state phase where turnover of central complexes could be observed, the heterogeneity of zinc ligands in the structures of the enzyme−NAD(H) structures

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(PDB entries 1KEV and 1YKF), and the lack of an X-ray structure of a relevant ternary complex. A role for a mobile Glu-60 (homologous to Glu-68 in horse ADH) is intriguing, but the substitution of Glu-60 with an alanine or aspartic acid residue in the *T. brockii* enzyme decreases activity by only ~5-fold.[101] The mechanism with this enzyme may be different from that of the horse or yeast ADHs because the protein ligands to the zinc are different.

How Is the Water Bound to the Zinc Replaced by Substrate? The catalytic zincs in the horse ADH apoenzyme and holoenzyme complexes with coenzyme and substrate analogues have similar tetracoordinated geometry (e.g., Figures 1, 6, and 7). Transient kinetic studies suggest that the zinc-bound water in the enzyme–NAD^+ complex deprotonates to form zinc hydroxide, with a rate constant of 200–500 s^{-1}, which is coupled to the global protein conformational change and occurs before ligands bind to the zinc to form the ternary complexes.[2] An associative mechanism involving a pentacoordinated zinc with adjacent oxygens is theoretically possible. However, it seems more reasonable that Glu-68 OE2 participates in a double-displacement, interchange mechanism, via a trigonal bipyramidal geometry, to displace the water (or hydroxide) in the first step, and in the second step, the substrate oxygen displaces the Glu-68 and produces the structure observed in the ternary complexes (Scheme 2). The water would dissociate from the enzyme through the hydrophobic substrate binding barrel (illustrated in Figures 6 and 7), and then the substrate would enter the channel and bind to the zinc. Structural studies with yeast ADH, other tetrameric ADHs, and human ADH3, as well as computational studies with horse ADH, support such a mechanism.[2,3,33−36,39,40,44] In particular, the structure of the horse ADH–NADH complex (Figure 5) shows an alternative position for the catalytic zinc that is closer to ligating to Glu-68 OE2. This evidence of an inverted coordination in horse ADH is similar to that observed for the complex of human ADH3 complexed with NAD(H) or ADP-ribose where a loose trigonal bipyramidal coordination or alternative positions were modeled for the zinc.[35,36] Ligand coordination may change more readily in the binary enzyme–coenzyme complexes, as discussed previously for human ADH3 and yeast ADH.[36,39] Comparison of the structures of the apoenzyme (PDB entry 1YE3) and the enzyme–NADH complex (PDB entry 4XD2) supports the suggestion that binding of the coenzyme alters the energetics of the interaction of Glu-68 with Arg-47, because Arg-47 binds the pyrophosphate of the coenzyme (Figure 5), and Glu-68 could then more readily interact with the catalytic zinc.[35,36] In the structure of the enzyme–NADH complex presented here, the alternative position for the zinc has not moved sufficiently closer to Glu-68 to become coordinated in the inner sphere to OE2, and alternative conformations of the side chains of the other ligands (Cys-46, His-67, and Cys-174) could not be refined, but the elongated electron density for the catalytic zinc indicates the potential dynamics. It appears that a consequence of the conformational change accompanying coenzyme binding is that the zinc becomes more mobile, and it is reasonable that similar effects would occur upon binding of NAD^+. After the second substrate (alcohol or aldehyde) binds to the enzyme–coenzyme complex, the zinc position is restored to that found in various ternary complexes, which seems appropriate for catalysis of the hydride transfer.

The molecular dynamics and combined quantum chemical and molecular mechanical computational studies with horse ADH support the conclusions that only tetracoordinated Zn(II) species are stable, where Glu-68 and water are coordinated alternatively.[44,102] These computational studies suggest that the enzyme complexes with NAD^+ or NADH may differ somewhat in the propensity of Glu-68 to ligate the zinc and can be affected by the ionization of the zinc-bound water to form hydroxide, but further studies are required to characterize the dynamics of the exchanges in the apoenzyme and enzyme–coenzyme complexes.

Substitution of the homologous glutamate in yeast ADH (Glu-67) with a glutamine residue decreases the catalytic efficiency for both ethanol oxidation and acetaldehyde reduction by 100-fold.[43] Yeast ADH in the open, apoenzyme conformation (with or without the bound coenzyme) has the glutamate ligated to the zinc, while the ternary complex with NAD^+ and trifluoroethanol has the closed conformation and the classical zinc coordination.[39,40] Other ADHs also have the homologous glutamate residue ligated to the catalytic zinc in the apoenzyme and some complexes with coenzyme, whereas the ternary complexes with the coenzyme and a substrate analogue have the classical zinc coordination (see the Supporting Information, Table 6S, of ref 39) The differences in coordination in the various apoenzymes could be of crystallization, but we think that the different structures represent energetically accessible states that are relevant for the catalytic mechanism. There may be different mechanisms for ligand exchange in these ADHs, but a common mechanism for these related enzymes is more reasonable.

The exchange of an alcohol via an intermediate coordination with Glu-68 may involve several steps. Glu-68 might displace the zinc hydroxide in the enzyme–NAD^+ complex, or it might be kinetically more rapid to reprotonate the hydroxide through the proton relay system so that a neutral water dissociates.[21] Then, after an alcohol binds and displaces Glu-68, the hydroxyl group of the alcohol would be deprotonated again to facilitate the transfer of hydride from the alkoxide.[1] These steps may involve local conformational changes.[3] We do not think that a pentacoordinated intermediate, with both a water and the substrate bound, is likely because of the limited space to move a water from the zinc to the bulk solvent through the substrate binding site after alcohol is bound, and such complexes were not identified by the computations of Ryde.[44,103] Release of aldehyde product and replacement with water may also involve intermediate ligation with Glu-68. We note that binding of alcohols and aldehydes to the enzyme–coenzyme complexes (10^5–10^6 M^{-1} s^{-1}) is somewhat slower than diffusion and dissociation of these ligands from the ternary complexes is relatively slow (20–560 s^{-1}), so that various species may isomerize.[1] The mechanism still needs to be studied further to describe the structures of additional intermediate species and the rate constants for their interconversion; new techniques might demonstrate the transient ligation of Glu-67 to the catalytic zinc during the exchange of ligands.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b00446.

Electron density ($2|F_o| - |F_c|$) and anomalous scattering difference ($|F_o| - |F_c|$) maps for the catalytic and structural zincs in the enzyme–NADH complex (PDF)

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Accession Codes

The X-ray coordinates and structure factors have been deposited in the Protein Data Bank as entries 1YE3, 4XD2, 5VJ5, 5VJG, 5VKR, 5VL0, and 5VN1.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ADH, alcohol dehydrogenase; ADPR, adenosine 5′-diphosphoribose; MPD, 2-methyl-2,4-methylpentanediol; RMSD, root-mean-square deviation; XAS, X-ray absorption spectroscopy.

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