On the enzymatic activation of NADH

Rob Meijers*, Richard J. Morris*, Hans W. Adolph†, Angelo Merli‡, Victor S. Lamzin* & Eila S. Cedergren-Zeppezauer§

*European Molecular Biology Laboratory, c/o DESY, Notkestrasse 85, 22603 Hamburg, Germany

†Fachrichtung 12.4, Biochemie, Postfach 151150, Universitaet des Saarlandes, 66041 Saarbruecken, Germany

‡Instituto di Scienze Biochimiche, Universita di Parma, viale delle Scienze, 43000 Parma, Italy

§Biokemi, Kemicentrum, Lunds Universitet, Box 124, 22100 Lund, Sweden

Corresponding author: Victor S. Lamzin, EMBL c/o DESY, Notkestrasse 85, 22603 Hamburg, Germany, email: victor@embl-hamburg.de, tel: +49-40-89902121, fax: +49-40-89902149.

Running title: On the enzymatic activation of NADH
Summary

Atomic (1 Å) resolution X-ray structures of horse liver alcohol dehydrogenase in complex with reduced nicotinamide adenine dinucleotide (NADH) revealed the formation of an adduct in the active site between a metal bound water and NADH. Furthermore, a pronounced distortion of the pyridine ring of NADH was observed. A series of quantum chemical calculations on the water-nicotinamide adduct showed that the puckering of the pyridine ring in the crystal structures can only be reproduced when the water is considered a hydroxide ion. These observations provide fundamental insight into the enzymatic activation of NADH for hydride transfer.
**Introduction**

Nicotinamide adenine dinucleotide NAD(H) is the most abundant electron carrier in cell metabolism. It exists in an oxidized (NAD⁺) and a reduced (NADH) form and both species are stable under physiological conditions. Its capacity as a redox agent is exploited by numerous enzymes that catalyze reactions in which NAD⁺ is reduced to NADH and *vice versa*. The interconversion of NAD⁺ and NADH is achieved by the transfer of a hydride ion (two electrons and a proton) between a substrate and NAD(H). A study on deuterated model compounds has shown that the hydride carrier is the C4 atom on the pyridine ring of the nicotinamide and that the transfer process is stereospecific (1), Figure 1. Theoretical calculations on the nicotinamide indicated that deformation of the pyridine ring into a boat conformation enhances hydride transfer (2). It has been observed by UV-Vis spectroscopy and NMR that the puckering of the pyridine ring changes upon enzyme binding (3). Until now it was unclear how an enzyme could steer the puckering of the ring to facilitate hydride transfer. Here, we present for the first time an example of enzymatic activation of NADH as it was found in a complex of NADH with horse liver alcohol dehydrogenase (LADH; EC.1.1.1.1).

LADH is an NAD(H) dependent enzyme that catalyses the oxidation of primary and secondary alcohols to aldehydes (4). It belongs to the Class I of the alcohol dehydrogenase family (5) which also contains yeast alcohol dehydrogenase and the human zinc dependent alcohol dehydrogenase. The yeast enzyme is the key mediator of alcohol production, whereas the human enzyme cleanses the blood from poisonous compounds. Other functions that have been assigned to the human enzyme are the involvement in the hormonal household by switching on and off steroids like testosterone. It has been associated with the production and neutralization of free radicals, thereby protecting the organism against DNA damage (6).
Finally, alcohol dehydrogenase is thought to play a role in the development of the organism through retinoid regulation (7). Retinoids are vitamin A derivatives which regulate the expression of various genes involved in embryonic growth. It has been observed that heavy drinking leads to retarded growth and night blindness. This can be explained by the fact that the continuous oxidation of ethanol interferes with the reduction of carotene to vitamin A.

LADH is a homodimer with 374 residues and two zinc sites in each monomer. One zinc is thought to play a structural role, the other forms the core of the active site. The latter is liganded to two cysteine residues (Cys 46 and Cys 174) and a histidine (His 67). The X-ray structure of the apo-enzyme indicated that the fourth metal ligand was a water molecule (4). The enzyme has been the focus of intensive studies involving kinetics, X-ray crystallography and spectroscopic methods (8). Much is known about the kinetics of the assembly of the enzyme-cofactor complex (9), but the actual mechanism for hydride transfer and proton release is still under debate (10).

Alternative reaction mechanisms have been proposed for LADH. In one of these (4), LADH is regarded an exception among zinc dependent enzymes in that no water molecule is involved in the reaction. This is the standard textbook description of the LADH mechanism (11). In the second mechanism (12), the water molecule is linked to the zinc ion during the reaction and goes through a cycle of (de)protonation. This mechanism demands a five coordination around the metal. Such coordination of the metal is thought to be highly improbable, because a fifth ligand would cause collisions with surrounding residues (4). EPR (13) and NMR (14) data on a cobalt substituted enzyme and perturbed angular correlation of \( \gamma \)-rays (PAC) measurements (15) on cadmium substituted enzyme though indicate the existence of a five coordinate intermediate.
Unbound NADH displays an absorption maximum at 340 nm in the UV-Vis range. When NADH is bound to LADH, the maximum shifts to 325 nm. Early on, it was proposed that this blue shift is caused by the formation of a bond between the catalytic zinc ion and the nicotinamide (16). This proposal was discarded after elucidation of the 3 Å resolution X-ray structure of the NADH-LADH complex since it was concluded that the distance between the zinc ion and the nicotinamide was too long.

To access the minute structural changes that an enzymatic environment might impose on NAD(H), it is necessary to collect structural data at a truly atomic level. Atomic resolution X-ray data provide an accuracy in atomic positions in the range of 0.03 Å (17,18) and detailed features of the active site of the protein structure become visible that are lost at lower resolution. Once the precise positions of all the atoms that are essential for the reaction to proceed are known, detailed theoretical studies can be launched to unravel the reaction mechanism.
Experimental Procedures

Sample preparation, X-ray data collection and refinement. EE-isozyme of LADH was prepared according to procedures described in (19). Data were collected on native enzyme in complex with 2-methyl 2,4-pentanediol (MPD) and NADH further referred to as the Zn-MPD-NADH-LADH complex. The zinc was replaced by cadmium following protocols described in (13,20) A cadmium substituted enzyme was complexed with MPD and NADH and is further referred to as the Cd-MPD-NADH-LADH complex. The Zn-MPD-NADH-LADH complex was crystallized with PEG 400 at a concentration of 20 % in 0.05 M Tris/HCl buffer at pH 8.2 with 0.1 % MPD present. The Cd-MPD-NADH-LADH complex was crystallized by dialysis against 50 mM Tris/HCl pH 8.2 and stepwise addition of MPD up to a concentration of 12 %. Subsequently, PEG 400 was added as a cryoprotectant to a final concentration of 20 %. Diffraction data were collected on the BW7B beamline at the EMBL Outstation Hamburg, DESY, from flash-cooled crystals, using an image plate from MAR X-RAY RESEARCH GmbH, Hamburg. An additional data set was collected on the Cd-MPD-NADH-LADH complex at 277 K. Data were processed, merged and scaled with the HKL suite (218). Statistics are summarized in Table 1.

$R_{\text{free}}$ was used to cross-validate the refinement protocol. The 'free' reflections were included in the final refinement round. The Zn-DMSO-NADH-LADH structure (PDB reference code 2OHX (22) determined to a resolution of 1.8 Å was used as an initial model for refinement. The 1 Å resolution structures were refined with SHELX-97 (23) and REFMAC (24) using anisotropic displacement parameters and H atoms at idealized positions. The resolution of the X-ray data allowed the geometry of the nicotinamide of NADH to be freed from restraints in order to get an accurate picture of the puckering of the pyridine ring. No restraints were applied either to metal-ligand distances. The Cd-MPD-LADH-NADH complex collected at
277 K was refined using the cryo structure as a starting model. Due to the low number of observations, it was not possible to release the restraints in the active site in this case.

X-ray structure factor amplitudes and the derived atoms coordinates have been deposited in the Protein Data Bank under accession numbers R1HEUSF, 1HEU, R1HF3SF, 1HF3, R1HETSF and 1HET.

**UV-Vis spectroscopy on the crystals.** Single crystal polarized absorption spectra were recorded at 283 K using a Zeiss Mpm800 microspectrophotometer on a crystal placed in a quartz flow cell with the incident beam perpendicular to the face of the crystal (25). The crystal was oriented with either of the two principle optical directions parallel to the electric vector of the polarized light.

**Theoretical calculations.** The nicotinamide moiety was truncated in such a way that the oxygen connecting the ribose to the pyrophosphate group was replaced by a hydrogen. Starting coordinates for this nicotinamide moiety were taken from the Zn-MPD-NADH-LADH crystal complex and the geometry was optimized at the Hartree-Fock level using the GAMESS (26) software package with a 6-31+G** basis set. The hydrogen bonds that influence the geometry of the nicotinamide moiety were initially mimicked by fixing the dihedral angles $X_N$ and $X_{AM}$, Figure 1(a). A water molecule was placed at 1.7 Å from C6 of the pyridine ring of the nicotinamide. The geometry of the nicotinamide moiety was optimized to fit the new conditions with constraints applied to the oxygen of the water molecule to keep the water in a fixed position relative to the pyridine ring as observed in the Zn-MPD-NADH-LADH X-ray structure.

For the reduced nicotinamide system these calculations were then extended to further approximate the electronic environment of the nicotinamide moiety by the introduction of pre-calculated potentials placed at all hydrogen bonding positions. Figure 1(b) shows the
enzyme hydrogen bonding environment in the crystal structure and the fragments used for the electronic potential calculations. Their potential, dipole and quadropole moments were incorporated at fixed positions. Finally, the zinc atom was included in a similar manner together with the two SH groups and an ammonia mimicking its cysteine and histidine ligands in the crystal structure.
Results

Active site interactions. X-ray data were collected to a resolution of 1 Å on native zinc LADH and the cadmium substituted enzyme in complex with NADH and MPD, Table 1. MPD was used as a precipitating agent during crystallization of the cadmium substituted protein but only as a minor addition in case of the zinc enzyme. MPD formed an abortive complex in both cases. Two complexes were measured under cryogenic conditions. A separate data set was collected on the Cd-MPD-NADH-LADH complex at 277 K to a resolution of 1.95 Å that has shown that there were no gross structural changes in the active site induced by flash cooling.

In both subunits of the Zn-MPD-NADH-LADH complex there is a residual density peak close to the C6 atom of the nicotinamide, W1 in Figure 2(a) and Table 2. This peak is within inner coordination sphere distance of the catalytic zinc ion. Another peak of similar height and distance from the metal, W2, is also seen in both monomers. The second location corresponds to the position otherwise taken by a zinc-interating atom of a substrate (4). The distance between the two peaks is 1.47 Å. In the Cd-MPD-NADH-LADH complex, the distances are essentially the same, Table 2. In both monomers and in both complexes the W2 site is remote from the C6 NADH atom. Similar peaks are observed in the active site of the Cd-MPD-NADH-LADH complex measured at 277 K. Accurate distances cannot be obtained from this structure as a result of the limited resolution.

The proton shuffling residue Ser 48 is equidistant to both peaks in all complex structures. In each case, the pyridine ring of NADH is puckered in a twisted boat conformation. We interpret the two peaks together to represent a metal bound water molecule occupying two alternative positions, partially forming an adduct with the pyridine ring of NADH.
**Substrate conversion.** UV-Vis spectra were recorded on crystals from the same batch as those used for X-ray data collection. Figure 3 shows an absorption maximum at 325 nm typical for enzyme bound NADH. Subsequent treatment with substrates shows that the crystalline enzyme is active. This allows us to interpret the crystal structures in terms of the enzymatic reaction mechanism.

**NADH adducts.** Water involvement in (de)protonation reactions in LADH has played a central role in explaining pH dependencies for enzymatic activity (27). In many other zinc enzymes (28), the metal bound water ought to be deprotonated to facilitate catalysis. Due to the fact that the water is disordered, it is not possible to assign its protonation state directly from the X-ray structure. In order to establish whether the adduct observed involves plain water or a hydroxide ion, quantum chemical calculations were performed on a nicotinamide model system, Figure 1(b).

Our calculations indicate that the pyridine ring structure is a highly sensitive system in terms of basis set and of its electronic environment. The system was initially modeled by fixing the two dihedral angles $X_N$ and $X_{AM}$, Figure 1(a), as described in the experimental section and presented in Tables 3 and 4. The unperturbed oxidized nicotinamide ($NIC^+$) and its adducts with $H_2O$ and $OH^-$ are essentially planar. The unperturbed reduced nicotinamide ($NIC^-$) shows some deviations from planarity. Pronounced puckering is seen in both NIC adducts. It can be seen in Figure 4 that the NIC-$OH^-$ mostly resembles the puckering observed in the pyridine rings of the crystal structures. Furthermore, an increase in bond length for C5-C6 is observed in the reduced NIC adducts. In the calculated structure of the unperturbed reduced nicotinamide, the bond length is 1.33 Å corresponding to a double bond, Figure 2 (b). In the calculated structure of the reduced OH$^-$ adduct, the bond length is 1.46 Å, Figure 2 (c). In the atomic resolution crystal structures, the C5-C6 bond length is 1.41 Å.
For the NADH-OH\textsuperscript{−} adduct fixation of the two dihedral angles proved to be a valid approximation. The geometry of the system did not change when the constraints on the dihedral angles $X_N$ and $X_{AM}$ were released and hydrogen bonding network was introduced in the form of fragments. For the H\textsubscript{2}O adduct, the removal of the dihedral angle constraints resulted in significant deviations from the crystal structure. This is a possible indication that an NADH-H\textsubscript{2}O adduct is unlikely to acquire the observed crystal structure geometry. The introduction of zinc to the NICH-OH\textsuperscript{−} system affected mainly the charge distribution and the electronegativity of the HR hydrogen became more profound.
Discussion

Since the crystal structures considered here all contain NADH, we restrict ourselves to describe the implications of adduct formation for NADH oxidation and aldehyde reduction. The forward reaction will be ruled by the same mechanism of adduct formation, but NAD\(^+\) might be oriented towards the water molecule in a different manner. No crystal structure of a binary LADH-NAD\(^+\) complex is available so far.

In the reduced OH\(-\)-ring adduct, the \(\text{Pro}R\) hydrogen at the C4 atom is in a pseudo-axial position. The bonding order of the C4-H\(R\) bond decreases indicating a tendency for the \(\text{Pro}R\) to leave according to the stereospecific requirements, Table 4. Based on the results of the calculations and comparison with the crystal structures, we suggest that OH\(-\) adduct formation is part of the activation process for hydride transfer from the reduced cofactor in the LADH-NADH complex. The hydrogen bonding between the nicotinamide moiety and the protein plays mainly a structural role of holding NADH in place.

In Figure 5 a revised reaction mechanism is proposed which is an extension of the "dissenting" mechanism that incorporates the metal bound water (12). It was expected that a five coordination of the metal leads to collisions with surrounding residues, or with the cofactor NAD(H) (4). As can be seen in the crystal structures presented here such a "collision" is indeed observed. The revision of the mechanism alludes to the functional displacement of the water and the formation of a nicotinamide derivative. The metal bound water alternates between two positions. The movement of the water/OH\(^-\) is directed at the C6 of the nicotinamide guided by Ser 48. The formation of a nicotinamide derivative leads to a redistribution of electric charge along the ring, inducing hydride transfer. The (de)protonation of the alcohol goes through the metal bound water. In this manner, the enzyme can influence
the course of the reaction by changing the coordination chemistry of the metal and the proton flow towards the active site.

The new reaction mechanism resolves controversy over spectroscopic data on the metal coordination during the reaction. PAC measurements on the binary LADH-NADH complex revealed a mixed geometry of equal occupation (29). This would imply that already in the binary complex, water displacement and adduct formation occurs. Large shifts are observed in the metal spectra of Co (30) and Cu (8) substituted enzymes upon NADH binding. These shifts can now be attributed to geometrical differences in the metal coordination sphere.

The presence of a water molecule in the vicinity of the nicotinamide has been found in several other protein structures. In a complex of dihydrofolate reductase, folate and NAD$^+$ at a resolution of 1.6 Å (31), a water is situated at 3.2 Å of C2 of the pyridine ring. In a complex of ferredoxin reductase with NADP$^+$ and FAD (32), a close interaction between a protein residue (Thr 166) and the C6 (3.2 Å) of the pyridine ring is found. The distances quoted here are from X-ray structures with a restrained geometry, which implies that all the atoms that are expected to be non-bonding are kept at a distance that is in agreement with a van der Waals radius of 3.3 Å. Given sufficiently high resolution of the X-ray data, the release of such restraints might reveal other enzyme-coenzyme complex structures with close interactions between the pyridine ring and neighboring oxygen atoms.

The work presented here highlights an essential aspect of the NAD(P) dependent dehydrogenation: the enzymatic activation of the cofactor. The combination of truly atomic resolution X-ray crystallography and quantum chemical calculations provided the opportunity to study enzymatic catalysis in unprecedented detail and enabled us to detect that NADH is indirectly linked to the catalytic metal ion. This observation is independent of the nature of the transition metal and the temperature of the environment. We propose that the link
established by the enzyme activates the NAD(H) molecule to become involved in hydride transfer. Further atomic resolution X-ray studies and extended theoretical calculations on other dehydrogenases will certainly lead to a more complete understanding of NAD(P) dependent hydride transfer.
References

1. Loewus, F. A., Westheimer, F. H. & Vennesland, B. (1953) *J. Am. Chem. Soc.* **75**, 5018-5023.

2. Almarsson, O. & Bruice, T. C. (1993) *J. Am. Chem. Soc.* **115**, 2125-2138.

3. Burke, J. R. & Frey, P. A. (1993) *Biochemistry* **32**, 13220-13230.

4. Eklund, H. & Bräündén, C. I. (1987) In *Biological macromolecules and assemblies* F. A. Jurnak, A. McPherson, eds. (John Wiley Sons, New York) pp. 73-143.

5. Jörnvall, H., Persson, B. & Jeffrey, J. (1987) *Eur. J. Biochem.* **167**, 195-201.

6. Mantle, D. & Preedy VR (1999) *Adverse Drug React Toxicol Rev* **18**, 235-52.

7. Foglio, M.H. & Duester G (1999) *Biochim Biophys Acta* **1432**, 239-250.

8. Bertini, I., Luchinat, C., Maret, W. & Zeppezauer, M., eds. (1986) *Zinc Enzymes* (Birkhäuser Stuttgart,) pp. 377-492.

9. Adolph, H. W., Kiefer, M. & Cedergren-Zeppezauer, E. S. (1997) *Biochemistry* **36**, 8743-8754.

10. Northrup, D.B. & Cho, Y.-K. (2000) *Biophys. J.* **79**, 1621-1628.

11. Fersht, A. (1999) *Structure and Mechanism in Protein Science* (W. H. Freeman and Company, New York) pp. 460-465.

12. Dworschack, R. T. & Plapp, B. V. (1977) *Biochemistry* **16**, 111-116.

13. Maret, W. (1989) *Biochemistry* **28**, 9944-9949.

14. Sloan, D. L., Young, J. M. & Mildvan, A. S. (1975) *Biochemistry* **14**, 1998-2008.

15. Hemmingsen., L., Bauer, R., Bjerrum, M. J., Zeppezauer, M., Adolph, H. W., Formicka, G. & Cedergren-Zeppezauer, E.S. (1995) *Biochemistry* **34**, 7145-7153.

16. Mahler, H. R. & Douglas, J. J. (1957) *J. Am. Chem. Soc.* **79**, 1159-1166.

17. Dauter, Z., Lamzin, V. S. & Wilson, K. S. (1997) *Curr. Op. Struc. Biol.* **7**, 681-688.

18. Kachalova, G. S., Popov, A. N. & Bartunik, H. D. (1999) *Science* **284**, 473-476.
19. Hubatsch, I., Maurer, P., Engel, D. & Adolph, H.W. (1995) *J. Chromatogr. A* **711**, 105-112.
20. Maret, W., Andersson, I., Dietrich, H., Schneider-Bernlöhr, H., Einarsson, R. & Zeppezauer, M., (1979) *Eur. J. Biochem.* **98**, 501-512.
21. Otwinowski, Z. & Minor, W. (1997) Macromolecular Crystallography, Part A, vol. 276 of *Methods in Enzymology*, C. W. Carter Jr., R. M. Sweet, eds. (Academic Press, San Diego CA) pp. 307-326.
22. Al-Karadaghi, S., Cedergren-Zeppezauer, E.S., Hovmöller, S., Petratos, K., Dauter, Z. and Wilson, K.S. (1994) *Acta Crystallogr.* **D 50**, 793-807.
23. Sheldrick, G. M. & Schneider, T. R. Macromolecular Crystallography, (1997) Part A, vol. 276 of *Methods in Enzymology*, C. W. Carter Jr., R. M. Sweet, eds. (Academic Press, San Diego CA) pp. 319-343.
24. Murshudov, G. N., Vagin, A. A., Lebedev, A., Wilson, K. S. & Dodson, E. J. (1999) *Acta Crystallogr.* **D55**, 247-255.
25. Merli, A., Brodersen, D. E., Morini, B., Chen, Z., Durley, R. C .E., Matthews, F. S., Davidson, V. L., and Rossi, G. L. (1996) *J. Biol. Chem.* **271**, 9177-9180.
26. Schmidt, M.W., Baldridge, K. K., Boatz, J. A., Elbert, S. T., Gordon, M. S., Jensen, J. H., Koseki, S., Matsumaga, N., Nguyen, K. A., Su, S. J., Windus, T. L., Dupuis, M. & Montgomery, J. A. (1993) *J. Comput. Chem.* **14**, 1347-1363.
27. Kvassman, J. & Pettersson, G. (1980) *Eur. J. Biochem.* **103**, 565-575.
28. J. E. Coleman, (1998) *Curr. Op. Chem. Biol.* **2**, 222-234.
29. Hemmingsen, L., Bauer, R., Bjerrum, M. J., Adolph, H. W., Zeppezauer, M. and Cedergren-Zeppezauer, E.S. (1996) *Eur. J. Biochem.* **241**, 546-551.
30. Dunn, M. F., Dietrich, H., MacGibbon, A.K.H., Koerber, S.C. & Zeppezauer, M. (1982) *Biochemistry* **21**, 354-363.
31. Sawaya, M. R. & Kraut, J. (1997) *Biochemistry* **36**, 586-603.

32. Deng, Z., Aliverti, A., Zanetti, G., Arakaki, A. K., Ottado, J., Orellano, E. G., Calcaterra, N. B., Ceccarelli, E. A., Carrillo, N., Karplus, P. A. (1999) *Nature Struct. Biol.* **6**, 847-853.

33. Cruickshank, D. W. J., (1999) *Acta Crystallogr. D* **55**, 583-601.

34. Bader, R. F. W. (1990) *A Quantum Theory* (Oxford University Press, Oxford).

35. Esnouf, R. M. (1997) *J. Mol. Graph Model* **15**, 132-134.

36. Schaftenaar, G. (1999) MOLDEN, Centre for Molecular and Biomolecular Informatics, The Netherlands; available at http://www.caos.kun.nl/schaft/molden/molden.html
Acknowledgements We thank Z. Dauter for help in data collection; ECZ thanks Rector Inge Jonsson and Dr. Jan Lundgren at Stockholm University for constant support; RJM is supported by the EC grant number BIO4-CT96-0189; ECZ received support from the EC TMR/LSF grant ERBFMGECT980134. We thank the Associated Editor and referees for their constructive critique.
Figure Legends

Figure 1. a) Definition of the puckering of the nicotinamide moiety. Hydride transfer takes place at the C4 atom of the pyridine ring and is stereospecific. In LADH, the H\textsubscript{R} hydrogen is exchanged. X\textsubscript{N} describes the dihedral between the ribose and the nicotinamide moiety. The dihedral X\textsubscript{AM} describes the orientation of the carboxamide in relation to the nicotinamide ring. The puckering of the ring is described by \( \alpha \text{N} \) accounting for the pyramidalization of N1. It is the angle between the planes of N1-C2-C6 and C2-C3-C6. The tendency for C4 to be out of plane is represented by the angle \( \alpha \text{C} \) between the planes of C3-C4-C5 and C2-C3-C6. Twist T displays the distortion in the boat form, calculated from the distance between C5 and the plane formed by C2-C3-C6. b) The hydrogen bonding network between the nicotinamide moiety and the enzyme as observed in the Zn-MPD-NADH-LADH crystal structure. The fragments used in the quantum chemical calculations to mimic this environment are given in parentheses.

Figure 2. a) The active site of the Zn-MPD-NADH-LADH complex. The active site metal is coordinated to the protein residues Cys 46, His 67 and Cys 174. A water molecule occupies two alternative sites, W1 and W2. W1 is at a distance of 1.76 Å from the C6 atom of the nicotinamide. W2 is in a position normally taken by the oxygen atom of a metal bound inhibitor. Both water sites are within hydrogen bond distance to Ser 48. For W1, the distance to the O\textsubscript{γ} of Ser 48 is 2.63 Å and for W2 2.68 Å. Superposition with the X-ray structure of an inhibitor complex containing di-methyl sulfoxide (DMSO) (22) reveals that the position of W2 coincides with the position of the oxygen of DMSO. Density maps are shown at contour levels of 0.8 and 1.3 eÅ\textsuperscript{-3} to point out the double bonds in the nicotinamide. It can be seen that the double bond between C5 and C6 is weakened upon adduct formation. The picture of the crystal structure was produced with BOBSCRIPT (35); b) The theoretical electron density for
a reduced nicotinamide; c) The effect of the formation of a hydroxide adduct. The theoretical density was calculated with the MOLDEN package (36).

Figure 3: Polarized spectra of a single crystal of Cd-substituted LADH in complex with NADH taken from the same batch as the ones used for X-ray data collection. Spectra of the Zn-NADH-LADH complex show similar features. Spectra are shown for the crystal in the original crystallization medium MPD (thin line), in the presence of 40 mM benzaldehyde (dashed line) where all NADH disappeared and after washing the crystal and treatment with 200 mM ethanol (thick line) which indicates recovery of reduced NADH.

Figure 4. Superposition of the nicotinamide of the crystal structure of the Zn-MPD-NADH-LADH complex, subunit A (yellow) and the structures calculated from a quantum mechanical energy optimization (cyan) of a) the NADH-H$_2$O adduct (RMSD 0.257 Å for the pyridine ring atoms) and b) the NADH-OH$^-$ adduct (RMSD 0.043 Å). In the NADH-OH$^-$ adduct, the pyridine ring assumes a twisted boat conformation and the ProR hydrogen is in a pseudo-axial position ready for hydride transfer, whereas in the NADH-H$_2$O the ProR hydrogen is pseudo-equatorial.

Figure 5. On the reaction mechanism. Protein residues are omitted for clarity. The mode of the proton and hydride transfer cannot be resolved with the present data. Aldehyde reduction: (A) The zinc bound water is displaced towards NADH to make place for the reactant to act as a fifth ligand of the zinc atom. Upon water displacement, an adduct with NADH-OH$^-$ is formed; (B) Hydride transfer between the activated nicotinamide and the substrate; (C) Protonation of the zinc bound hydroxide. (D) Proton transfer between the zinc bound water and the alcoholate. Alcohol oxidation: (D), (E), (F) and (A) correspond to the inverse reaction.
Table 1 Data collection and refinement statistics

|                                | Zn-MPD-NADH | Cd-MPD-NADH | Cd-MPD-NADH |
|--------------------------------|-------------|-------------|-------------|
| **LADH Complex**               |             |             |             |
| Temperature (K)                | 120         | 120         | 277         |
| Resolution (Å)                 | 20 to 1.10  | 20 to 1.15  | 15 to 1.95  |
| Measured reflections           | 684,025     | 637,693     | 94,521      |
| Unique reflections             | 267,243     | 254,817     | 45,721      |
| Space group                    | P1          | P1          | P21         |
| Unit cell a, b, c (Å)           | 50.6, 44.1, 93.6 | 51.2, 44.6, 94.1 | 43.9, 179.0, 50.5 |
| α, β, γ (°)                    | 104.2, 101.2, 70.9 | 104.3, 101.1, 70.7 | 90.0, 107.5, 90.0 |
| Overall completeness (%)        | 89.5        | 94.5        | 86.6        |
| Overall I/sigma                | 13.1        | 19.5        | 19.0        |
| I/sigma highest resolution shell| 2.0         | 1.9         | 4.1         |
| Rmerge (%)*                    | 5.4         | 4.2         | 3.7         |
| R factor (%)†                  | 12.5        | 11.9        | 16.7        |
| R free (%)‡                    | 15.9        | 14.5        | 21.4        |
| RMSD bonded distances (Å)      | 0.016       | 0.015       | 0.014       |
| (Target value: 0.020)          |             |             |             |
| RMSD angle bonded distances    | 0.035       | 0.034       | 0.040       |
| (Å) (Target value: 0.040)      |             |             |             |
| e.s.u. (Å) §                   | 0.03        | 0.03        | 0.20        |

\* \( R_{\text{merge}} = \frac{\sum_{hkl} \sum_{i} |I_i - \langle I \rangle|}{\sum_{hkl} \sum_{i} < I >} \) where \( I_i \) is an intensity for the \( i \)th measurement of a reflection with indices \( hkl \) and \( < I > \) is the weighted mean of the reflection intensity.
† $R_{\text{factor}} = \sum_{hkl} |F_o(hkl)| - |F_c(hkl)| / \sum_{hkl} |F_o(hkl)|$ where $F_o$ and $F_c$ are the observed and calculated structure factors respectively.

‡ $R_{\text{free}}$ is the crystallographic $R_{\text{factor}}$ calculated with 0.4 % of the data that were excluded from the structure refinement.

§ as estimated from Cruickshank’s diffraction precision indicator (33).
Table 2 Distances (Å) between peaks W1 and W2 (as shown in Figure 2a) and the key atoms in the active site of the Zn/Cd-MPD-NADH-LADH crystal structures.

| Atom          | W1 Subunit A | W1 Subunit B | W2 Subunit A | W2 Subunit B |
|---------------|--------------|--------------|--------------|--------------|
| C6 NADH       | 1.76         | 1.70         | 3.28         | 3.25         |
| Zn            | 2.23         | 2.25         | 2.12         | 2.11         |
| OG Ser 48     | 2.75         | 2.70         | 2.70         | 2.76         |
| C6 NADH       | 1.77         | 1.93         | 3.68         | 3.85         |
| Cd            | 2.44         | 2.41         | 2.29         | 2.36         |
| OG Ser 48     | 2.62         | 2.86         | 2.65         | 2.64         |
Table 3 Conformation of the nicotinamide in the X-ray structures and the *ab initio* calculated models.

| System                        | RMSD (Å)† | α<sub>C</sub> (°)‡ | α<sub>N</sub> (°)‡ | Twist (Å)‡ |
|-------------------------------|-----------|---------------------|--------------------|------------|
| Zn-MPD-NADH A (X-ray)*        | 0.16      | 9.2                 | 4.9                | -0.47      |
| Zn-MPD-NADH B (X-ray)         | 0.17      | 10.5                | 3.0                | -0.57      |
| Cd-MPD-NADH A (X-ray)         | 0.14      | 10.4                | 8.9                | -0.37      |
| Cd-MPD-NADH B (X-ray)         | 0.16      | 13.8                | 8.0                | -0.42      |
| NICH                          | 0.06      | 13.0                | 7.6                | 0.04       |
| NICH-HOH                      | 0.10      | 9.7                 | 6.6                | 0.25       |
| NICH-OH                      | 0.15      | 14.7                | 10.3               | -0.36      |
| NIC<sup>+</sup>              | 0.01      | 1.6                 | 1.9                | 0.01       |
| NIC<sup>+</sup>-HOH            | 0.01      | 2.6                 | 3.5                | -0.03      |
| NIC<sup>+</sup>-OH<sup>−</sup> | 0.01      | 3.4                 | 2.9                | 0.02       |

* A and B refer to the two subunits of the enzyme.

† RMSD is the root mean square deviation from the least squares plane drawn through the non-hydrogen atoms of the pyridine ring.

‡ The α<sub>C</sub>, α<sub>N</sub> and Twist are the puckering parameters defined in Figure 1(a).
Table 4 Electronic charges based on Bader’s topological definition of an atom (34) and the bond order for atoms involved in hydride transfer.

| System        | Charge (e) | Bond order |
|---------------|------------|------------|
|               | C6  | C4  | H<sub>R</sub> | H<sub>S</sub> | C4-H<sub>R</sub> | C4-H<sub>S</sub> |
| NICH          | 0.473 | 0.260 | -0.058 | -0.046 | 0.942 | 0.934 |
| NICH-HOH      | 0.747 | 0.262 | -0.107 | -0.050 | 0.940 | 0.944 |
| NICH-OH       | 0.764 | 0.275 | -0.123 | -0.118 | 0.929 | 0.957 |

The C6 atom is positively charged in a reduced pyridine ring, thereby attracting the metal bound hydroxide. The charge on the C4 atom remains relatively equal upon adduct formation, whereas the charges on the respective hydrogens H<sub>R</sub> and H<sub>S</sub> become increasingly negative. The bond order for the bond between C4 and the leaving hydrogen, H<sub>R</sub>, decreases when the NICH-OH<sup>-</sup> adduct is formed whereas the bond order increases for the C4-H<sub>S</sub> bond.
Figure 1
Figure 2
Figure 5
