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Published in:
Journal of Biological Inorganic Chemistry

DOI:
10.1007/s007750050171

1997

Document Version:
Peer reviewed version (aka post-print)

March 25, 2017

The active-site metal coordination geometry of cadmium-substituted alcohol dehydrogenase

A theoretical interpretation of perturbed angular correlation of γ-rays measurements

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Abbreviations: ADH, alcohol dehydrogenase; AOM, angular overlap model; DMSO, dimethylsulfoxide; ECP, effective core potentials; EFG, electric field gradient; NQI, nuclear quadrupole interaction; PAC, perturbed angular correlation of γ-rays.

Abstract

The structure of eleven complexes of cadmium-substituted alcohol dehydrogenase with or without coenzyme and with different non-protein cadmium ligands has been estimated by combined quantum chemical and molecular mechanical geometry optimisations. The geometry of the optimised complexes is similar to the crystal structure of cadmium substituted alcohol dehydrogenase, indicating that the method behaves well. The optimised structures do not differ significantly (except for the metal bond lengths) from the corresponding zinc complexes, which shows that cadmium is a good probe of zinc coordination geometries.

The electric field gradients at the cadmium nucleus have been calculated quantum chemically at the MP2 level with a large cadmium basis set, and they have been used to interpret experimental data obtained by perturbed angular correlation of γ-rays. The experimental and calculated field gradients (all three eigenvalues) differ by less than 0.35 a.u. ($3.4 \cdot 10^{21}$ Vm^{-2}), the average error is 0.11 a.u., and the average relative error in the two largest eigenvalues of the field gradients is 9 %. Calculated field gradients of four-coordinate structures agree better with the experimental results than do those of any five-coordinate model. Thus, the results indicate that the catalytic metal ion remains four-coordinate in all examined complexes. Two measurements are best explained by a four-coordinate cadmium ion with Glu-68 as the fourth ligand, indicating that Glu-68 probably coordinates intermittently to the catalytic metal ion in horse liver alcohol dehydrogenase under physiological conditions.

Key words: combined quantum chemical and molecular mechanical geometry optimisation, electric field gradient, five-coordination, nuclear quadrupole interaction, protein strain

Introduction

Alcohol dehydrogenase (ADH, EC 1.1.1.1) catalyses the reversible oxidation of primary and secondary alcohols using NAD^+ as coenzyme [1-3]. The active site of the enzyme contains a zinc ion that is essential for catalysis. Crystallographic studies [3-5] have shown that this zinc ion is bound to the enzyme through one histidine and two cysteine residues. In the free enzyme, the catalytic zinc ion appears to be tetrahedrally coordinated with a water molecule (or hydroxide ion, depending on pH) as the fourth first-sphere ligand.

In experimental studies, zinc is a notoriously problematic ion because it is invisible to all spectroscopic (except X-ray) techniques. Therefore, methods have been developed to replace the catalytic zinc ion in alcohol dehydrogenase by other metal ions, e.g. Co^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, Mn^{2+}, and Cd^{2+} [6-10]. Such metal-substituted enzymes have been used to study the ligand binding and coordination geometry by spectroscopic methods. The cobalt derivative has attracted special attention, since it has almost the same catalytic activity as the native enzyme. Unfortunately, it has recently been shown by X-ray crystallographic studies on carbonic anhydrase that zinc and cobalt (and most other metal ions) exhibit significantly different geometric preferences [11,12]. Thus, the biological relevance of results obtained on metal-substituted enzymes is unclear.

With this in mind, cadmium substitution becomes interesting. Being in the same group in the periodic table, cadmium and zinc would be expected to have similar chemical properties. This is confirmed by three low-resolution (0.24-0.29 nm) crystal structures of Cd-substituted alcohol dehydrogenase [13], which do not reveal any notable differences in the structure around the catalytic metal ion compared to the native enzyme (except for the 0.02 nm longer bond distances to cadmium). Furthermore, Cd-substituted alcohol dehydrogenase has a significant catalytic activity, 9-30 % of the native activity [9, 14, 15].

Cd-substituted alcohol dehydrogenase has been studied by NMR [16] and perturbed angular correlation of γ–rays (PAC) [17-20]. The latter method measures the nuclear quadrupole interaction (NQI) at the cadmium nucleus, i.e. the interaction between the nuclear quadrupole moment of the cadmium nucleus and the electric field gradient (EFG) from the surrounding charge distribution. The EFG is a very sensitive probe of the geometry of the ligands around the

cadmium ion (especially the angles around the ion) and can therefore provide detailed information about the coordination geometry.

Traditionally, ^{111}Cd-PAC experiments on biological systems have been interpreted using an approximate semiempirical method, the angular overlap model (AOM) [21]. With this method the NQI of a tentative coordination geometry, for example a crystal structure, can be estimated. Thus, changes in the experimental NQI may be correlated to changes in the geometry by estimating the NQIs of different structures using the AOM. However, the low resolution of the crystal structures of Cd-substituted alcohol dehydrogenase (rms. error of the angles of about 7° [13]) makes them less suitable for the interpretation of PAC spectra. Instead, structures of the native zinc enzyme have been used [17-20], which make the interpretations less certain. Furthermore, several geometries may give similar NQIs, which might lead to erroneous interpretations.

Most of the problems with the interpretation of PAC experiments can be overcome with modern quantum chemical methods. Recently, we have developed a procedure for the calculation of EFGs at cadmium ions in large complexes [22]. Moreover, methods have been developed to integrate quantum chemical and molecular mechanical geometry optimisations [23-25]. By such methods, metal ion complexes and other systems that are hard to handle in standard classical or semiempirical calculations may be more accurately treated. Recent examples of the reliability of such methods are the calculation of the geometry of the catalytic and structural zinc ions in alcohol dehydrogenase [25, 26].

In this paper, we combine these two techniques to determine the structure of cadmium-substituted alcohol dehydrogenase with different ligands and to calculate the EFGs of these complexes. The calculated EFGs are then used to interpret the PAC experiments. This is, to our knowledge, the first time the EFG at a metal site in a protein has been calculated by first principle methods, and consequently, the first time such an approach has been applied to the interpretation of experimentally determined nuclear quadrupole interactions at a protein active site in terms of the structure.

Methods

Quantum chemical geometry optimisations in vacuum

$Cd(SH)_2YX$ where *Y* is NH_3 or imidazole, and *X* is H_2O, $(H_2O)_2$, OH^-, dimethylsulfoxide (DMSO), $HCOO^-$, or $(HCOO^-)(H_2O)$, were chosen as models of the coordination sphere of the catalytic metal ion in Cd-substituted alcohol dehydrogenase. SH^- was used as a model of the cysteine ligands since earlier optimisations on the corresponding zinc complexes have shown that the SH^- and CH_3S^- give closely similar geometries [27]. The full geometry of the models was optimised until the change in energy and the internal coordinates were below 2.6 J/mole and 0.053 pm or 0.057°, using analytical gradient methods at the Hartree-Fock self-consistent field level. No symmetry restrictions were imposed and several starting structures were tested to reduce the risk of being trapped in local minima.

If not otherwise stated, the geometry optimisations were performed with the 36-electron effective core potential (ECP) and double-ζ basis set (21/21/31) of Hay-Wadt [28] for cadmium, the 6-31G basis for hydrogen [29], and the ECPs (10-electron for S and 2-electron for C, N, and O) and double-ζ basis sets (31/31) of Stevens et al. [30] enhanced with a polarising *d*-orbital (exponents: C, N, O: 0.8, S: 0.503) for the other atoms (this combination is called HW/Sp/31 below). For calibrations, the Cd basis of Gropen [31] (19s12p8d) was used, uncontracted and enhanced with a *p* and an *f* function with exponents 0.1172635 and 0.23383218, combined with the dζp basis sets of Dunning [32] for S, the sv basis set of Dunning and Hay [33] for H, and the svp basis sets of Dunning and Hay [33] for the other atoms (called Gpf/svp below). Only the pure five *d* and seven *f* orbitals were used. The calculations were performed using the quantum chemical program package TURBOMOLE 1.0 β [34].

Combined quantum chemical and classical geometry optimisations

Integrated quantum chemical and molecular mechanical geometry optimisations were performed using the program COMQUM [25]. In this program, the enzyme and solvent are divided into four subsystems. The central system 1 is optimised using the sum of the quantum chemical gradients within the system and molecular mechanical gradients from system 2. All

electrostatic interactions are included in the quantum chemical calculations; systems 2 and 3 are represented by partial charges, one for each atom, and system 4 by integer charges, i.e. one charge for each charged amino acid, located at the position of the N^{ζ}, C^{ζ}, C^{γ}, C^{δ}, S^{γ}, $C^{\varepsilon 1}$, N^{1N}, and both P atoms of Lys, Arg, Asp, Glu, Cys^{-}, His^{+}, NAD^{+}, and NADH, respectively. The integer charges are damped by a dielectric constant ε=4.0, while in systems 1-3 ε=1.0. In each step of the optimisation, system 2 is relaxed by molecular mechanics (keeping the other systems fixed), representing systems 1-3 with all atoms (using charges obtained from a quantum chemical Mulliken analysis for system 1 and partial charges for systems 2 and 3), and system 4 by damped integer point charges. Special care is taken at the junction between the classical and quantum chemical systems [25].

The full geometry of systems 1 and 2 was optimised until the changes in energy and the coordinates were below 26 J/mole and 0.53 pm, respectively. Then, system 2 was kept fixed and the optimisation was continued until the changes were below 2.6 J/mole and 0.053 pm. The quantum chemical computations were performed at the Hartree-Fock level with the HW/Sp/31 ECPs and basis sets. The geometries were corrected to the Gpf/svp basis set using the method of offset forces [35].

The program COMQUM is a combination of the quantum chemical software TURBOMOLE 1.0 β [34] and the molecular mechanics simulation package MUMOD [27, 36]. The potential function of the latter program contains a standard harmonic potential for bond stretches and angle bending, a truncated trigonometric series (n=1-3) for the dihedral angles, a Coulombic term for the electrostatic interactions and a 6-12 Lennard-Jones potential for the van der Waals interactions. The force field does not contain any specific terms for hydrogen bonds or improper dihedral angles. The interactions between the cadmium ion and its ligands were treated quantum mechanically; in the molecular mechanical gradients the terms from cadmium cancel out and in the classical optimisation of system 2 the cadmium ion interacts only by a non-bonded potential.

The enzyme

The coordinates of horse liver alcohol dehydrogenase in complex with NADH and dimethylsulfoxide at 0.18 nm resolution (R-factor=0.172; PDB file: 2OHX) [5] were used for the

NADH and NAD^+ complexes. This is at present the most accurate structure of alcohol dehydrogenase and it represents the catalytically active closed conformation of the enzyme. Charge assignment and equilibration were performed as described by Ryde [27, 37]. For the structure of free alcohol dehydrogenase without coenzyme, an unpublished crystal structure was used (Cedergren-Zeppezauer, E. S., personal communication), and it was equilibrated in the same way as the other two structures [27, 37].

In the geometry optimisations with COMQUM, system 1 consisted of $Cd(SH)_2$(imidazole)*X*, as a model of Cys-46, His-67, Cys-174, and the fourth (and possibly fifth) cadmium ligand (from subunit A of the enzyme; in some cases, $HCOO^-$ as a model of Glu-68 was also included in system 1). In system 2, all amino acids within 0.6 nm from any atom in system 1 were included, typically amino acids number 43-50, 52, 65-69, 91, 93, 140, 141, 143-146, 170-178, 203, 294, 319, 362, 369, 371, the nicotinamide, N-ribose, and pyrophosphate moieties of the coenzyme (if present), five crystal water molecules and three solvation water molecules (totally around 600 atoms). System 3 was composed of all atoms of residues within 0.3 nm of any atom in system 2, typically about 80 amino acids and 25 water molecules, or 1250 atoms. Finally, system 4 comprised 173 integer charges.

Electric field gradient calculations

The calculation of the EFGs follows the procedure developed by us [22]. The quantum chemical system 1 was enhanced by CH_3 groups on the cysteine and glutamate models (i.e. CH_3S^- and CH_3COO^-; the coordinates were taken from system 2 in the geometry optimisation) in order to include the full second coordination sphere of the cadmium ion. The EFGs of these models were calculated at the MP2 level using the generally contracted polarised basis set of Kellö and Sadlej [38] [19*s*15*p*9*d*4*f* / 11*s*9*p*5*d*2*f*] for Cd and 6-31G* for the other atoms [29]. The core orbitals on all atoms (1*s* through 3*d* on cadmium) were kept frozen. The surrounding enzyme was modelled by an array of point charges; all atoms on all amino acids within 0.9 nm from the cadmium ion and its ligands (systems 2 and 3) were modelled with charges from the Mumod library [36] (the same as in the geometry optimisations) and charged residues outside this radius (system 4) were represented by integer charges that were damped by a dielectric

constant ε=4.0. The calculations were performed with the quantum chemical program package Gaussian 94 (Rev. C.3) [39]. All calculations were run on IBM RISC RS6000 workstations.

The calculated EFGs are presented as the eigenvalues of the EFG tensor, ordered by the absolute value ( ). Since the tensor is traceless, only two of the elements are independent. In order to compare the results with experimentally measured nuclear quadrupole interactions, the quadrupole moment of the cadmium nucleus in spin 5/2 state has to be known. Values ranging from 0.74 to 0.83 barn have been published [40] and we used the average 0.78 barn. This gives the conversion factor 1 a.u. = 173 Mrad/s for ^{111m}Cd. The experimental measurements do not give the sign of the EFGs, therefore only absolute values are compared. All EFGs are reported in atomic units; 1 a.u. = $9.72 \cdot 10^{21}$ V/m^2.

Results

Calibration of the method

In order to test the influence of the basis sets on the geometry, $Cd(SH)_2(NH_3)(H_2O)$ was optimised with several different basis sets. The results in Table 1 show that polarising functions on S, N and C are necessary, and that a large basis set on Cd is mandatory. The addition of a *p* function to the Gropen [31] cadmium basis set is important, while additional functions have less influence. The Kellö-Sadlej [38] Cd basis set gives the best results, but it can be seen that the Gropen basis set enhanced with a *p* and an *f* function (Gpf) gives closely similar results. Therefore, the Gpf/svp basis was used for calibration of the geometry optimisations.

Cadmium contains so many core electrons that it was interesting to see if some sort of effective core potential (ECP) could be used. It can be seen from Table 1 that the HW/Sp/31 ECPs and basis sets give amazingly similar results to the largest all-electron basis set (differences in cadmium bond distances and bond angles are less than 4 pm and 3°). The result is not significantly improved when no ECPs were used on S, C, N, and O. On the other hand, Stevens' 28-electron ECP and (4211/4211/311) basis set for cadmium [30] gave much worse results. Therefore, we decided to use the HW/Sp/31 basis set for the optimisations with the protein.

To improve the results with the HW/Sp/31 basis set even more, the method of offset forces was used [35]. Thus, each complex was optimised in vacuum with the Gpf/svp basis set. Then the forces at this geometry were calculated with the HW/Sp/31 basis set, and these forces were subtracted from the forces in calculations with the HW/Sp/31 basis.

Table 2 shows the geometry of $Cd(SH)_2YX$, where Y=NH_3 or imidazole, and X=H_2O, $(H_2O)_2$, OH^-, DMSO, $HCOO^-$, or $(HCOO^-)(H_2O)$, calculated with the Gpf/svp and the HW/Sp/31 basis set, with or without offset forces. The results are very encouraging; the cadmium bond lengths with the Gpf/svp basis set and with the offset-corrected HW/Sp/31 basis sets differ by less than 1 pm, except for X=$(H_2O)_2$ where the largest difference is 3 pm.

The geometry of cadmium-substituted alcohol dehydrogenase

In order to interpret PAC measurements on Cd-substituted alcohol dehydrogenase [19], the geometries of eleven complexes of Cd-ADH were optimised by the combined quantum chemical and molecular mechanical program COMQUM [25] using the HW/Sp/31 basis set and offset forces. The complexes were selected to include several realistic alternative interpretations of the experiments. The cadmium bond lengths and angles of the optimised complexes are shown and compared with the corresponding vacuum and zinc complexes in Table 3. Energy data are collected in Table 4.

The geometry of the optimised complexes is reasonably similar to the crystal structure of Cd-substituted ADH [13]. For example, for the free enzyme with a water molecule bound to the cadmium ion, the average difference in the angles around the cadmium ion between the optimised and experimental structure is about 10° [13]. Considering the low resolution of the crystal structure, 0.24 nm, the differences may be due to uncertainties in the crystal structure as well as in the optimised structure.

The optimised structures also closely resemble the native zinc enzyme. Figure 1 shows the optimised structure of the binary complex of Cd-ADH and NAD^+ with a water molecule bound to the cadmium ion, compared to the experimental structure of the corresponding native zinc-enzyme complex with *p*-bromobenzyl alcohol bound to the zinc ion [41]. It is evident that except for the longer bond lengths to cadmium, the two structures are almost indistinguishable. This is most satisfying since the optimisation was started from another crystal structure with NADH as coenzyme. Thus, the choice of starting structure does not bias the result.

Likewise, the optimised structure of the binary Cd-ADH-NADH complex with a water molecule bound to the catalytic cadmium ion is almost identical to the optimised structure of the corresponding zinc enzyme [25]. As is shown in Figure 2, the only notable differences are in the hydrogen-bond pattern of the metal-bound water molecule and Ser-48. In the cadmium enzyme, H^{γ} of Ser-48 binds to the cadmium-bound water molecule, which in turn makes a hydrogen bond to a solvent water molecule. In the zinc structure, the latter water molecule is not present, and therefore the zinc-water molecule makes a hydrogen bond to O^{γ} of Ser-48 instead.

If the cadmium-bound water molecule is replaced by a hydroxide ion (Figure 3), the Cd-O distance decreases by about 20 pm, while the Cd-N and Cd-S distances increase by 4-10 pm and 1-3 pm, respectively. These changes are interesting because they indicate what happens when a

neutral metal ligand is exchanged with a charged ligand, which is assumed to occur during catalysis [1].

Electric field gradients of cadmium-substituted alcohol dehydrogenase

Table 5 shows the EFGs for all examined complexes, calculated according to the procedure developed by us [22]. Test calculations on $Cd(SH)_2(NH_3)(H_2O)$ indicate that geometries optimised with the HW/Sp/31 basis set give the same EFGs (within 0.01 a.u.) as those optimised with the KS+f/6-31+G** basis set.

The EFG is a very sensitive function of the geometry of the cadmium ion, especially of the angles between the ligands around the ion. This is clearly illustrated by the differences of the EFGs of the same complex with different coenzymes (or without coenzyme). The values of the two largest eigenvalues of the EFGs change by up to 0.7 a.u. (average 0.3 a.u.) when the coenzyme is changed or removed, both in experiments and in calculations. This is because the angles around the cadmium ion change substantially (up to 34°, average 11°) when the coenzyme is changed. The changes in cadmium bond lengths are much smaller (4 pm on average).

Discussion

Cadmium as a model of zinc

The results in Table 3 show that the active-site metal coordination geometry of Cd-ADH optimised by COMQUM is very similar to the geometry obtained with the native zinc enzyme, except for the longer Cd-ligand distances. The cadmium bond lengths increase by 16-19 pm for S, 24-29 pm for N, and 24-39 pm for O. These changes are consistent with the 23 pm larger ionic radius [42] and the larger softness of the cadmium ion. They are also in accord with the about 0.02 nm increased cadmium distances observed in the crystal structure of Cd-substituted alcohol dehydrogenase [13].

The calculated angles around the metal ion are the same for the two ions within 18° (the average difference is 6°). It should be noted that the present calculations have been performed with larger basis sets than the calculations with zinc (with polarisation functions on all atoms).

Therefore, the actual differences are probably smaller, especially for complexes with negatively charged oxygen ligands.

Furthermore, the general fold of the protein around the active site does not change significantly on metal substitution, as can be seen from the comparison of the optimised cadmium complexes with the native crystal structure (Figure 1) as well as optimised structures of the native zinc enzyme (Figure 2). Consequently, cadmium seems to be a good probe for the metal site coordination geometry in alcohol dehydrogenase, giving geometries that are relevant also for the native enzyme.

Interpretation of the PAC experiments

Table 6 shows the EFGs measured experimentally by the PAC method at different pH on Cd-substituted alcohol dehydrogenase with or without coenzyme (NAD^+ or NADH) and with different extraneous ligands [19]. The goal of the present investigation was to give a structural interpretation of these results.

Our calibration of the quantum chemical method showed that the calculated EFGs reproduce the three eigenvalue of the experimental EFGs within about 0.3 a.u. when the geometry is known [22]. Since we estimate also the geometry in this investigation, slightly larger differences can be expected. By comparing the three eigenvalues of the experimental EFGs with different calculated EFGs, a consistent interpretation of all the seven measurements could be reached. According to this interpretation, the cadmium ion is four-coordinate in all measurements. The fourth ligand is water in the free enzyme at low pH and in the ADH-NADH complex with DMSO as an extraneous ligand, it is a hydroxide ion in the two complexes at high pH, and it is one of the O^{ε} atoms of Glu-68 in the ADH-NAD^+ complex at low pH. The two slightly different NQIs observed with the ADH-NADH complex without any extraneous ligands can be reproduced by either water or Glu-68 as the fourth ligand, but the first (with V_{zz} = 1.88 a.u.) is best reproduced by Glu-68 and the second with water. The discrimination between different models is more thoroughly discussed below.

This interpretation is summarised in Table 6 together with the difference between the calculated and experimental EFGs. It can be seen that the average differences in the three

eigenvalues of the EFGs are 0.13, 0.12, and 0.08 a.u., respectively. The corresponding maximum errors are 0.35, 0.18, and 0.16 a.u., and the relative errors are 9, 10, and 103 %, respectively. The small absolute differences and large relative differences of the third eigenvalue are of course due to its small magnitude. It is very encouraging to note that the relative error in the calculated EFGs is within the experimental uncertainty, around 15 % . The latter originates almost entirely from the uncertainty in the quadrupole moment of the cadmium nucleus.

Comparison with the interpretations based on the AOM

Our interpretations of the experimental measurements differ in three cases from those of Hemmingsen et al. [19], which are based on the AOM. First, we assign the NQI from the binary ADH-NAD^+ complex at low pH to a four-coordinate cadmium ion with Glu-68 as the fourth ligand. Hemmingsen et al. give the more natural interpretation that it originates from a cadmium ion with a water molecule as the fourth ligand, but they then have to assume that the S-Cd-S angle has decreased at least 10° compared to the crystal structure and to the other water complexes [19]. Our calculations of the EFGs are incompatible with such an interpretation, however. The EFG of the ADH-NAD^+ complex with a cadmium-bound water molecule differ by 0.63 a.u. (average) from the measured EFG, which is almost three times larger than the average difference of any other complex.

Naturally, our interpretation leads to the question why no NQI is recorded from a cadmium ion with a water molecule as the fourth ligand for the ADH-NAD^+ complex. We suggest that there in fact may be such a signal. The experiments with NAD^+ complexes are complicated by that an unknown extraneous substrate converts NAD^+ to NADH [19]. Since NADH binds much stronger to Cd-ADH than NAD^+, NQIs from the ADH-NADH complex is present in all the measurements (up to 75 % of the enzyme may be in complex with NADH). This NQI has been subtracted from all the spectra with NAD^+. According to our calculations, the EFG of the NAD^+-complex with a cadmium-bound water molecule should be quite similar to the EFG of the corresponding complex with NADH. It is therefore possible that the NQI from a cadmium-bound water molecule in the NAD^+-complex has been erroneously taken for a NQI from the

corresponding NADH complex and has been subtracted away. After this subtraction, only the NQI from a cadmium ion with Glu-68 as the fourth ligand remains.

Second, Hemmingsen et al. assume that DMSO binds to the catalytic cadmium ion in the experiment with the ADH-NADH complex in 0.1 M DMSO solution [19]. The experimental EFG of this complex is closely similar to the EFG of the binary complex with NADH (and no extraneous ligands): the three eigenvalues differ by less than 0.07 a.u., i.e. much less than the average change in EFG when the coenzyme or ligands are changed. Moreover, the calculated EFG of the ternary ADH-NADH-DMSO complex fits the experimental EFG much poorer than does the NADH complex with water molecule as the fourth ligand; the average absolute differences are 0.37 and 0.07 a.u., respectively. We therefore suggest that the NADH-DMSO complex has a water molecule, and not DMSO, bound to the cadmium ion. The small, but significant change in the experimental NQI compared to the binary NADH complex may be attributed to small structural changes in the protein environment (DMSO may, for example, bind in the second coordination sphere of the cadmium ion) or dielectric changes in the solvent due to the rather high concentration of DMSO (0.1 M). This may seem contradictory to the fact that DMSO binds to the zinc ion in the crystal structure of native ternary NADH-DMSO complexes [5], but the concentration of DMSO in these crystals is higher than in the PAC experiments. Furthermore, it is likely that DMSO binds more weakly to the softer cadmium ion than to zinc.

Third, according Hemmingsen et al., the change in the NQIs when pH is raised for the free enzyme and the binary NAD^+ complex is caused by that the cadmium-bound water molecule is deprotonated to a hydroxide ion [19]. Intuitively, this would increase the ligand-ligand repulsion and therefore increase all the O-Cd-*X* angles and decrease the other angles, especially the S-Cd-S angle. In the interpretation of the experimental spectra using the AOM, a good fit was obtained if the S-Cd-S angle was decreased by 20° while the other angles were kept around the values encountered in the crystal [19].

Of course, this is a simplified view. A much more detailed picture of the changes due to the ionisation of the water molecule is obtained by the COMQUM optimisations. These show that the changes in the angles around the cadmium ion are quite erratic. Only in the binary ADH-NAD^+ complex are the changes as expected. In the free enzyme, the S-Cd-S angle even increases and the two S-Cd-O angles decrease. Nevertheless, the calculated EFGs of both these

two complexes are very close to the experimental ones. Thus, the interpretation by Hemmingsen et al. [19] seems to be correct, although their explanation in terms of changes in the S-Cd-S angle is not supported by our results.

Five-coordinate cadmium complexes

According to the most widely accepted reaction mechanism of alcohol dehydrogenase, the active-site zinc ion remains four-coordinate in all significant catalytic steps [1]. This mechanism is supported by crystallographic [2-5], spectroscopic [43-46], kinetic [1, 47], and theoretical [25, 27, 48] evidence. Alternative proposals have been put forward, however, according to which five-coordinate intermediates play an essential role during catalysis [49-53] and they build on spectroscopic studies of metal-substituted enzyme [51, 54-58] as well as kinetic experiments [47, 49, 52]. Early interpretations of PAC measurements indicated that several binary and ternary complexes of Cd-substituted alcohol dehydrogenase were five-coordinate [17, 18], but they have recently been reinterpreted [19, 20]. It was therefore of interest to see if our method can discriminate between four and five-coordinate cadmium complexes.

In accord with results obtained for the native zinc enzyme [25], five-coordinate structures with two water molecules as the non-protein metal ligands could be obtained also with Cd-ADH. As can be seen in Figure 4, one water molecule occupies the normal substrate site at the bottom of the substrate cleft, while the other occupies a cavity on the other side of the metal ion, inside the protein and near Glu-68 and Asp-49. This cavity is rather narrow, and therefore, the five-coordinate structures are more strained (with respect to the vacuum structure) than the corresponding four-coordinate structures, 83-110 kJ/mole compared to 44-63 kJ/mole (ΔE_{QC1} in Table 4).

Quite unexpectedly, it was also possible to obtain a five-coordinate structure with Glu-68 and a water molecule coordinating to the cadmium ion. Such a structure could not be obtained in vacuum, nor with the native zinc enzyme [38]. The structure is presumably legitimate, however, since during the optimisation, the geometry started out as five-coordinate state and went through a four-coordinate state before it finally became five-coordinate again. The structure is stabilised by a hydrogen bond from Ser-48 to the cadmium-bound water molecule. It is probably the size

and the softness of the cadmium ion that makes such a complex stable in the cadmium enzyme. Yet, the five-coordinate structure is appreciably more strained than the corresponding four-coordinate structure (173 kJ/mole compared to 101 kJ/mole), and it is therefore most likely of minor physiological relevance.

The calculated EFGs of the five-coordinate complexes differ significantly from those of the four-coordinate complexes. The EFG of the free enzyme with two water molecules bound to the cadmium ion fit the experimental data of the free enzyme at low and high pH appreciably worse than do the four-coordinate structures with a cadmium-bound water molecule or hydroxide ion; the average absolute differences are 0.52 a.u. and 0.30 a.u. compared to 0.23 and 0.11 a.u., respectively.

Similarly, the EFG of the ADH-NAD^+ complex with two cadmium-bound water molecules fit the experimental data at high and low pH much worse than do the four-coordinate structures with a cadmium-bound hydroxide ion and with Glu-68 bound to the cadmium ion; the average absolute differences are 0.61 and 0.39 a.u. compared to 0.02 and 0.11 a.u., respectively. Finally, the ADH-NADH complex with Glu-68 and a water molecule coordinated to cadmium gives an EFG that is less similar to the experimental results than are the four-coordinate complexes with a water molecule or Glu-68 coordinated to the cadmium ion; the average absolute differences are 0.17 and 0.25 a.u. to the two experimentally observed EFGs of the ADH-NADH complex compared to 0.14 and 0.11 a.u. for the water complex and 0.09 and 0.17 a.u. for the Glu-68 complex.

Thus, our results give no support to the suggestion that the active-site metal ion in alcohol dehydrogenase is five-coordinate in any kinetically significant steps. Instead, they are in accord with the recent suggestion that the low NQIs observed at elevated pH are due to cadmium-bound negatively charged ligands instead of five-coordinate cadmium complexes [19, 20].

Glutamate-68

Glu-68 is a residue in the second coordination sphere of the catalytic metal ion in alcohol dehydrogenase, located 0.47 nm from the ion, opposite to the substrate site. It is one of the most conserved amino acids among alcohol dehydrogenases from different sources, as often

conserved as the metal ligands [59]. Moreover, a mutation of Glu-68 to Gln yields an enzyme with only 1 % of the native catalytic efficiency [60]. It has therefore been suggested that Glu-68 plays an important role in the catalytic mechanism of the enzyme, e.g. by stabilising the geometry of the metal ligands [5] or by moderating the electrostatic potential at the active site [60]. The idea has also been advanced that Glu-68 may coordinate to the catalytic metal ion [18, 61] and recent quantum chemical calculations show that such a coordination is possible and not too energetically unfavourable [37]. Moreover, a very recent crystal structure of human χχ alcohol dehydrogenase in fact has Glu-68 bound to the zinc ion (0.20 and 0.29 nm Zn-O distance in the two subunits, respectively) [62]. It was therefore, interesting to see if the PAC experiments give any indications of such a coordination in the horse liver enzyme.

Structures with Glu-68 coordinating to the catalytic metal ion in alcohol dehydrogenase could be obtained also with the Cd-substituted enzyme. As can be seen in Figure 5, Glu-68 has rotated 70 ° degrees around the C^{δ}-C^{ε} axis and coordinates with one of the O^{ε} atoms to the ion. The cadmium ion and the C^{δ} atom of Glu-68 have moved 91 and 54 pm towards each other, while the other ligating atoms have moved much less (23-38 pm) and the rest of the enzyme has hardly moved at all. A water molecule in the substrate cleft either coordinates to the cadmium ion or resides in the second coordination sphere, 0.33-0.35 nm from the ion.

Quite surprisingly, it turned out that the calculated EFGs of the two four-coordinate structures with Glu-68 coordinating to the cadmium ion (with NAD^+ and NADH) gave the best fit to two experimental NQIs: the NQI of the ADH-NAD^+ complex at low pH and the first of the two NQIs of the ADH-NADH complex without any extraneous ligands. For the first NQI there is no alternative interpretation, while for the other, a four-coordinate cadmium ion with a water molecule as the fourth ligand gives a similar fit. Yet, the latter complex gives an even better fit to the *second* NQI of the binary ADH-NADH complex. Therefore, we suggest that the two former NQIs originate from complexes with Glu-68 coordinating to the cadmium ion.

If this proposal is correct, these PAC measurements show that such a coordination is possible also for horse liver alcohol dehydrogenase. Furthermore, it means that up to 40 % of the active sites have Glu-68 coordinated to the cadmium ion. This is probably higher than in the native enzyme, since the size of the cadmium ion allows Glu-68 to coordinate to the cadmium ion with smaller changes in the structure than does zinc.

Strain

It has been proposed that the metal site of alcohol dehydrogenase is in a strained (entatic) state [63]. Such a suggestion can be tested with the present method since the difference in energy of the quantum system between the vacuum geometry and the geometry calculated with COMQUM (ΔE_{QC1} in Table 4) is a measure of the strain induced by the enzyme onto the metal coordination sphere. The strain energy is low for the complexes with water bound to the cadmium ion 44-63 kJ/mole, which is comparable to strain energies observed for the native enzyme [25]. The strain energy is larger for the four-coordinate complexes with OH^- (70-129 kJ/mole) and Glu-68 (101 kJ/mole) and it is even larger for five-coordinate complexes (83-110 kJ/mole for the complexes with two water molecules and 173 kJ/mole for the complex with Glu-68 and a water molecule coordinated to the cadmium ion). Again, this is similar to the strain energies obtained with the native enzyme [25]. Thus, the moderate strain energies for the four-coordinate complexes show that the metal coordination sphere of alcohol dehydrogenase is not significantly strained.

Concluding remarks

We have presented a method to estimate the structure of metal substituted enzymes and calculate the electric field gradients at the metal ion. The method has been applied for the interpretation of PAC measurements on Cd-substituted alcohol dehydrogenase and the results are impressive; seven experimental EFG have been interpreted with an average error of only 0.11 a.u. in the three eigenvalues of the EFG (9 % relative error). Such an error is of the same magnitude as the uncertainty in the experimental values. It is notable that the two largest errors are encountered in the same complex, the free enzyme at low pH. The maximum error in the six other complexes is only 0.17 a.u. and the average error is 0.09 a.u. This may indicate that there are some problems with that complex, e.g. that the structure of the second-sphere water molecules is not the optimal ones.

It is notable that in all systems except one, the calculated EFGs are larger than or almost equal to the experimental ones; the average error with sign is +0.08 a.u. Thus, some sort of

systematic error seems to be present. It is also noteworthy that both the geometry optimisation and the point charge model decrease the absolute values of the EFGs. This is because the surrounding protein gives rise to a reaction field that screens the charges of the ligand atoms.

Unfortunately, our method is rather expensive in terms of the computer time needed (2-6 weeks of CPU time on IBM RS 6000 workstations per geometry optimisation). Yet, the result in Table 5 show that the optimisation is absolute mandatory for getting good geometries and therefore good EFGs; the average absolute difference between the experimental and calculated EFGs is four times lower if the COMQUM geometries are used than with vacuum geometries.

The EFG calculations are less time-consuming, but require much computer memory and disk space. Clearly, the point-charge model of the enzyme in the EFG calculations is important. It decreases the average absolute difference between the experimental and calculated EFGs from 0.19 a.u. to 0.10 a.u. Quite unexpectedly, the MP2 calculation and the addition of CH_3 groups to the cysteine and glutamate models have a small effect on the accuracy: the MP2 calculations decrease the average absolute error by 0.02 a.u., while the addition of the methyl groups in fact deteriorates the results slightly (0.10 a.u. average absolute error without the methyl groups), mainly due to the impaired results for the free enzyme at low pH. Thus, if time is critical or computer memory is limited, approximate EFGs could be calculated at the SCF level directly on system 1.

Acknowledgements

This investigation has been supported by grants from the Swedish Natural Science Research Council (NFR) and by the Danish Research Council for Natural Science.

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Legends to the figures

Figure 1. The optimised structure of the binary Cd-alcohol dehydrogenase–NAD^+ complex with a water molecule bound to the cadmium ion compared to the crystal structure of the ternary complex of the native zinc enzyme with NAD+ and *p*-bromobenzyl alcohol (no hydrogen atoms) [41].

Figure 2. The optimised structure of the binary Cd-alcohol dehydrogenase–NADH complex with a water molecule bound to the cadmium ion compared to the optimised structure of the same zinc complex [25].

Figure 3. The optimised structure of the binary Cd-alcohol dehydrogenase–NAD^+ complex with a hydroxide ion bound to the catalytic cadmium ion.

Figure 4. The optimised structure of free Cd-alcohol dehydrogenase with two water molecules bound to the catalytic cadmium ion in a five-coordinate fashion.

Figure 5. The optimised structure of the binary Cd-alcohol dehydrogenase–NADH complex with Glu-68 coordinating to the catalytic cadmium ion.

Table 1. The energy and geometry of $Cd(SH)_2(NH_3)(H_2O)$ optimised with different basis sets (C_s symmetry). The basis sets are: the 3-21G and 6-31G series of basis sets [29], the Dunning-Hay split valence + polarisation basis set (svp) [33], the Gropen [31] cadmium basis set, possibly enhanced with extra *s*, *p*, *d* and *f*-functions with exponents: 0.015360, 0.1172635, 0.130988, and 0.23283218, respectively, the cadmium basis set of Kellö & Sadlej [38] with (KS+f) or without *f*-functions, the Hay-Wadt [28] 36 electron ECP and double-ζ basis set for Cd together with svp (HW/svp) or the Stevens 10 (S) and 2-electron (C, O, N) ECP and double-ζ basis set enhanced with a polarising *d*-function for S, C, N, and O and 6-31G basis for H (HW/Sp/31), or the Stevens [30] 28 electron ECP and (4211/4211/311) basis set for Cd together with Sp/31 (S28/Sp/31).

Basis set	Energy	Distance to Cd (pm)			Angle subtended at Cd (°)			
Cd / other (/ H)	(Hartree)	N	S	O	N-S	N-O	S-S	S-O
3-21G / 3-21G	-6363.747583	235	257	229	103	98	148	97
KS / 3-21G	-6388.972347	236	255	231	104	95	148	97
Gropen / svp	-6393.525118	243	249	244	100	91	158	95
Gropen+p / svp	-6393.554021	244	251	250	104	87	149	96
Gropen+pf / svp	-6393.558512	245	249	251	103	86	152	96
Gropen+spdf / svp	-6393.567370	245	249	252	104	85	151	96
KS / svp	-6393.566079	245	251	251	104	86	149	97
KS / 6-31+G**	-6393.580760	245	251	251	103	86	151	97
KS+f / svp	-6393.573007	245	249	251	103	86	150	96
KS+f / 6-31+G**	-6393.587145	245	249	252	103	86	151	97
HW / svp / 31	-95.971451	243	245	254	104	83	151	97
HW / svp	-975.067607	243	246	252	104	84	150	97
S28 / Sp / 31	-231.766987	235	223	246	96	99	161	96

Table 2. The geometries of different complexes optimised with the Gpf / svp and HW / Sp / 31 basis sets (with and without offset forces). *A* and *B* denotes $Cd(SH)_2(NH_3)$ and $Cd(SH)_2$(imidazole), respectively.

Complex	Symmetry	Basis set	Distance to Cd (pm)			
			N	S_1	S_2	O
A(H_2O)	C_s	HW / Sp / 31	243	245	245	254
		Gpf / svp	245	249	249	251
B(H_2O)	C_1	HW / Sp / 31	237	246	247	251
		HW / Sp / 31 offset	238	250	252	248
		Gpf / svp	238	251	252	249
A(OH^-)	C_s	HW / Sp / 31	252	256	256	215
		Gpf / svp	254	259	259	215
B(OH^-)	C_1	HW / Sp / 31	251	257	257	211
		HW / Sp / 31 offset	248	261	262	214
		Gpf / svp	249	261	262	214
A(DMSO)	C_1	HW / Sp / 31	243	247	248	239
		Gpf / svp	245	251	253	237
B(DMSO)	C_1	HW / Sp / 31	237	249	249	235
		HW / Sp / 31 offset	238	253	254	233
		Gpf / svp	238	254	255	233
A($(H_2O)_2$	C_s	HW / Sp / 31	239	248	248	260,264
		Gpf / svp	242	253	253	255,258
B($(H_2O)_2$	C_1	HW / Sp / 31	236	249	251	261,265
		HW / Sp / 31 offset	237	254	256	254,257
		Gpf / svp	238	253	258	256,260
A($HCOO^-$)	C_1	HW / Sp / 31	243	253	253	223
		Gpf / svp	244	257	257	225
B($HCOO^-$)	C_1	HW / Sp / 31	245	253	254	222
		HW / Sp / 31 offset	244	257	258	223
		Gpf / svp	245	257	258	224
A($HCOO^-$)(H_2O)	C_1	HW / Sp / 31	241	253	256	222,422
		Gpf / svp	241	259	259	223,353

$B(HCOO^-)(H_2O)$	C_1	HW / Sp / 31	240	255	255	220,386
		HW / Sp / 31	240	260	260	223,379
		offset				
		Gpf / svp	241	260	260	222,379

Table 3. The geometry of Cd-substituted alcohol dehydrogenase with and without coenzyme and with different ligands of the catalytic metal ion. The structures are optimised with COMQUM and the HW/Sp/31 ECP and basis set using offset forces. For comparison, the corresponding vacuum geometries (with the Gpf/svp basis set) are also listed. Values in brackets show the change compared to the corresponding zinc complex (calculated with basis sets of double-ζ quality) [25, 37]. S_1 and S_2 represent the S^{γ} atom of Cys-46 and Cys-174, respectively. For the five-coordinate complexes, two numbers are given for the geometric parameters involving oxygen; the first corresponds to the water molecule in the substrate site and the second to the oxygen ligand in the alternative site.

Ligand	Coen-zyme	Distance to the metal (pm)				Angle subtended at the metal ion (°)						
		N	S_1	S_2	O	N-S_1	N-S_2	N-O	S_1-S_2	S_1-O	S_2-O	O_1-O_2
H_2O	Apo	223	260	249	241	105	130	101	119	97	95	
H_2O	NAD^+	222	258	250	240	115	115	101	124	97	97	
		(+24)	(+19)	(+18)	(+24)	(-2)	(-3)	(+2)	(+6)	(-10)	(+6)	
H_2O	NADH	228	255	249	249	110	112	95	134	106	88	
		(+29)	(+17)	(+16)	(+37)	(-5)	(-4)	(-1)	(+10)	(+7)	(-8)	
H_2O	vacuu	238	251	252	249	100	108	98	148	106	84	
	m	(+34)	(+16)	(+15)	(+38)	(-3)	(+1)	(-6)	(-3)	(+10)	(-10)	
OH^-	Apo	227	259	251	222	106	127	112	122	94	86	
OH^-	NAD^+	232	261	251	217	112	106	103	116	118	100	
OH^-	vacuu	249	262	261	214	93	114	85	113	129	115	
	m	(+39)	(+15)	(+15)	(+27)	(-3)	(-18)	(-10)	(+5)	(-3)	(+3)	
DMSO	NADH	228	258	251	233	110	116	89	124	113	99	
DMSO	vacuu	238	255	254	233	101	112	100	137	102	98	
	m	(+24)	(+22)	(+21)	(+23)	(-4)	(-2)	(+2)	(+6)	(-2)	(-5)	
$(H_2O)_2$	Apo	228	263	252	265,235	127	118	97,90	115	74,92	97,91	166
$(H_2O)_2$	NAD^+	233	257	254	250,237	102	122	79,88	136	108,93	80,91	157

$(H_2O)_2$	vacuum	238 (+32)	253 (+6)	258 (+11)	257,260 (+45,40)	102 (+6)	112 (+11)	91 (-10)	146 (-17)	96,103 (+8,14)	82,79 (-6,9)	160 (+2)
($HCOO^-$)	Apo	229	261	252	236	104	116	114	123	94	105	
($HCOO^-$)	NAD^+	233	256	255	229	111	101	116	130	94	105	
($HCOO^-$)	NADH	236 (+31)	264 (+21)	253 (+18)	237 (+35)	110 (+3)	99 (-8)	112 (-8)	140 (+16)	91 (-7)	103 (+2)	
($HCOO^-$)	vacuum	245 (+36)	257 (+16)	258 (+16)	224,363 (+29)	110 (+1)	97 (-5)	96 (+2)	120 (+6)	111 (+1)	118 (-3)	
($HCOO^-$)(H_2O)	NADH	235 (+25)	257 (+16)	253 (+17)	260,250 (+40[e])	109 (+2)	102 (-4)	82,100 (-21)	146 (+25)	92,89 (-10)	78,100 (-2)	161
($HCOO^-$)(H_2O)	vacuum	241 (+31)	260 (+19)	260 (+15)	379,222 (+29)	104 (-3)	104 (-1)	101 (+2)	113 (-12)	116 (+6)	116 (+4)	
H_2O[a]	Apo					106	106	96	133	113	96	
H_2O[b]	Apo	214	234	222	228	106	115	98	127	101	108	
alcohol[c]	NAD^+	220	220	225	200	103	108	92	125	114	98	
DMSO[d]	NADH	209	223	231	214	113	106	94	130	106	102	

[a] The crystal structure of Cd-substituted alcohol dehydrogenase without coenzyme at 0.24 nm resolution [13].

[b] The crystal structure of free native enzyme (Cedergren-Zeppezauer, E., personal communication).

[c] The crystal structure of the native enzyme in complex with NAD^+ and pentafluorbenzyl alcohol at 0.21 nm resolution [41].

[d] The crystal structure of the native enzyme in complex with NADH and DMSO at 0.18 nm resolution [5]. Average of the two subunits.

[e] The corresponding zinc structure is four-coordinate with the water molecule in the second coordination sphere.

Table 4. The energy of Cd-substituted alcohol dehydrogenase with of without coenzyme (NADH or NAD^+) and with different ligands to the catalytic metal ion. The structures are optimised by COMQUM with the HW/Sp/31 ECP and basis set using offset forces. Values in brackets represent the energies for the corresponding zinc complex [25, 27]. The MP2 energies (including point charges) are calculated with the KS+f/6-31G* basis set and CH_3 groups on the cysteine and glutamate models. E_c is the classical energy of system 2. E_{pol} is the energy due to the polarisation of system 2 by the wave function of system 1. ΔE_{QC1} is the difference in quantum chemical energy of the isolated system 1 between the geometry optimised in vacuum and with COMQUM.

Ligand	Coenzyme	MP2 Energy	E_c	E_{pol}	ΔE_{QC1}
		Hartree	kJ/mole	kJ/mole	kJ/mole
H_2O	Apo	-6672.977128	471.0	-34.5	63.4
	NAD^+	-6652.957867	602.6	-93.8	53.9
	NADH	-6652.980921	618.8	-79.6	44.2
			(635.1)	(-73.7)	(41.7)
OH^-	Apo	-6672.547222	591.5	-344.8	129.5
	NAD^+	-6652.489061	684.2	-240.9	69.5
DMSO	NADH	-7128.907433	571.9	-112.1	76.2
$(H_2O)_2$	Apo	-6749.153168	517.8	-105.8	110.5
	NAD^+	-6729.164453	693.0	-107.5	83.2
$(HCOO^-)$	Apo	-6822.144941	429.2	-235.9	111.0
	NAD^+	-6801.033613	581.5	-236.9	99.2
	NADH	-6801.525875	301.2	-226.1	101.5
			(338.5)	(-137.5)	(119.9)
$(HCOO^-)(H_2O)$	NADH	-6878.047244	614.4	-250.3	172.8
			(328.9)	(-127.8)	(113.2)

Table 5. EFGs of the optimised complexes in the protein and in vacuum. The EFGs are calculated as described in the Methods section. Vacuum structures are optimised with the Gpf/svp basis set, protein structures with the HW/Sp/31 basis set with offset forces. The EFG are given as the ordered three eigenvalues of the EFG tensor (V_{ii}, in a.u., 1 a.u. = $9.72 \cdot 10^{21}$ V/m^2). *A* and *B* denotes $Cd(SH)_2(NH_3)$ and $Cd(SH)_2$(imidazole), respectively.

Complex	Coenzyme	With point charges			Without point charges		
		V_{zz}	V_{yy}	V_{xx}	V_{zz}	V_{yy}	V_{xx}
A(H_2O)	Vacuum				2.37	-1.64	-0.73
B(H_2O)	Vacuum				2.16	-1.77	-0.39
	Apo	-1.91	1.69	0.21	-2.03	1.81	0.22
	NAD^+	-1.97	1.30	0.67	-2.07	1.70	0.38
	NADH	-2.09	1.90	0.19	-2.17	2.11	0.05
A(OH^-)	Vacuum				-1.76	1.34	0.42
B(OH^-)	Vacuum				2.22	-1.93	-0.28
	Apo	0.75	-0.51	-0.23	-0.74	0.64	0.10
	NAD^+	-0.67	0.64	0.03	0.99	-0.97	-0.02
A(DMSO)	Vacuum				1.94	-1.36	-0.58
B(DMSO)	Vacuum				1.67	-1.29	-0.38
	NADH	-1.62	1.28	0.34	-1.74	1.58	0.16
A($(H_2O)_2$)	Vacuum				1.81	-1.26	-0.55
B($(H_2O)_2$)	Vacuum				1.85	-1.49	-0.37
	Apo	-1.19	0.73	0.46	-1.47	1.18	0.30
	NAD^+	1.61	-1.48	-0.13	1.89	-1.65	-0.24
A($HCOO^-$)	Vacuum				-1.07	0.81	0.26
B($HCOO^-$)	Vacuum				-1.16	0.95	0.21
	Apo	-1.23	1.10	0.13	1.24	-1.03	-0.21
	NAD^+	1.18	-1.08	-0.09	1.36	-0.97	-0.39
	NADH	1.81	-1.55	-0.26	1.86	-1.37	-0.49

B($HCOO^-$)(H_2O)	NADH	1.81	-1.44	-0.37	1.91	-1.33	-0.58

Table 6. The experimentally determined EFGs [19] together with our interpretation and the differences between the experimental and the calculated EFGs. Since all interpretations involve a four-coordinate cadmium ion coordinated to Cys-46, His-67, Cys-174, only the fourth ligand is listed. The EFGs are given as the ordered three eigenvalues (V_{ii}) of the EFG tensor (in a.u.). Note that the experimental measurements do not give the sign of the EFGs, therefore only absolute values are compared. The uncertainty in the experimental values is about 15 %, originating mainly from the uncertainty in the nuclear quadrupole moment of the cadmium nucleus in the I=5/2 state. High and low pH means over or under the pK_a found experimentally [19].

Coenzyme	Extra-neous ligand	pH	Experimental EFGs (a.u.)			Suggested fourth ligand	Error in the calculated EFGs (a.u.)		
			V_{zz}	V_{yy}	V_{xx}		V_{zz}	V_{yy}	V_{xx}
No	No	low	1.56	1.51	0.05	H_2O	0.35	0.18	0.16
		high	0.74	0.67	0.07	OH^-	0.01	0.16	0.16
NAD^+	No	low	1.03	0.92	0.11	Glu-68	0.15	0.16	–0.02
		high	0.70	0.63	0.07	OH^-	–0.03	0.01	–0.02
NADH	No	7-11	1.88	1.69	0.19	Glu-68	–0.07	–0.14	0.07
			1.92	1.81	0.11	H_2O	0.17	0.09	0.08
NADH	DMSO	9.1	1.99	1.83	0.16	H_2O	0.10	0.07	0.03