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The coordination chemistry of the structural zinc ion in alcohol dehydrogenase studied by ab initio quantum chemical calculations

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Abstract

The coordination chemistry of the structural zinc ion in horse liver alcohol dehydrogenase has been examined by quantum chemical geometry optimisations. It is shown that all the four cysteine ligands are deprotonated in the enzyme, not only two of them as has been suggested. The Zn-S bond lengths are very sensitive to the theoretical treatment; in vacuum they are predicted to be 15 pm longer than in the crystal structure. Half of this discrepancy is due to electronic correlation, the rest can be attributed to screening of the negative sulphide charges by the enzyme, in particular by N-H···S hydrogen bonds. The potential surface is rather flat, so the large difference in geometry between the crystal and the vacuum structure corresponds to an energy change of less than 35 kJ/mole. The crystal bond lengths can be reproduced only with methods that accounts explicitly for the enzyme. A dielectric continuum model gives too long bond lengths, indicating that the enzyme solvates the coordination sphere better than water. Thus, the structural zinc ion can be used as a sensitive test of methods trying to model the surrounding medium in quantum chemical computations.

Introduction

Alcohol dehydrogenase (EC 1.1.1.1) catalyses the reversible oxidation of primary and secondary alcohols using NAD^+ as coenzyme (Pettersson, 1987). Each subunit of the dimeric enzyme contains two zinc ions, one that is essential in catalysis, and one that has been suggested to play a structural role (Cedergren-Zeppezauer et al., 1985). Crystallographic studies of the horse liver enzyme (Eklund et al., 1981, Eklund & Brändén, 1983, Al-Karadaghi et al., 1994, 1995) have shown that the latter zinc ion is bound to the enzyme by four cysteine residues. As can be seen in **Table 1**, the coordination geometry around this zinc ion is almost tetrahedral, with small but reproducible differences between the ligands. A characteristic feature of the structure is the extensive hydrogen bonding to the cysteine sulphur atoms, illustrated in **Fig. 1** and **Table 2**. Each sulphur atom interacts with at least three polar hydrogens in the surrounding enzyme, mostly NH-amide groups of the backbone. Similar hydrogen bonds have been observed in several other proteins containing a metal bound to sulphur atoms, e.g. ferredoxins (Adman et al., 1975).

It is usually assumed (Eklund & Brändén, 1983, Pettersson, 1987) that all the four cysteine zinc ligands are deprotonated, i.e. that the total charge of the coordination sphere is $-2\ e_0$. Yet, Garmer and Krauss (1993) recently suggested that only two of the cysteine residues are deprotonated on the basis of the interpretation of the electronic spectra of the Co-substituted enzyme. From a theoretical point of view, such a proposal may seem legitimate. The pK_a of a free cysteine ligand is about 8.3. It is easy to conceive that for the first two cysteines coordinating to the zinc ion, this pK_a decreases to well below 7 through the electrostatic interaction with the positively charged zinc ion. However, for the third and forth ligands, the total charge of the zinc-complex is 0 and $-1\ e_0$, respectively, and it is then less clear why the pK_a should be significantly lowered. Furthermore, the lengths of the Zn-S bonds are significantly different (221-246 pm), indicating that the bonds are not equivalent, and they are also substantially longer than the average zinc-cysteine bonds in protein crystal structures (212 pm according to Chakrabarti, 1989).

The structural zinc ion in alcohol dehydrogenase is well suited for theoretical calculations. The enzyme has been thoroughly studied and the crystal structure is known

for several complexes (Eklund et al., 1981, Eklund & Brändén, 1983, Al-Karadaghi et al., 1994, 1995). Furthermore, if all the cysteines are deprotonated, there are eight equivalent Zn-S bonds in the enzyme, which gives a very accurate estimate of the average Zn-S bond length. We therefore decided to estimate the number of deprotonated cysteine residues by quantum chemical geometry optimisations. During the course of the calculations, it turned out that the system is very sensitive to the surrounding medium and that it therefore can be used as test of different methods to model the surrounding enzyme.

Methods

Quantum chemical vacuum computations

$[Zn(HS)_{4-n}(HS_2)_n,]^{-2+n}$ where n=0-2, was chosen as a model of the structural zinc ion and its ligands in alcohol dehydrogenase. 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°, respectively, using analytical gradient methods at the Hartree-Fock self-consistent field (SCF) 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 results refer to optimisations with the double-ζ zinc basis (62111111/33111/311) of Schäfer et al. (1992), enhanced with a p function with exponent 0.162 (called DZP), and the 6-31G* basis sets on all other atoms (Hehre et al., 1986). The contaminant d and f functions were always removed. Electronic correlation effects were included through second order perturbation theory (MP2), keeping the core orbitals frozen. The calculations were performed using the quantum chemical program packages TURBOMOLE 2.0β (Ahlrichs et al., 1989) and Mulliken 2.18d (MP2 geometry optimisations, Rice, et al., 1995) on IBM RISC RS/6000 workstations.

Geometry optimisations in a dielectric cavity

Reaction field calculations (Tapia & Goscinski, 1975, Mikkelsen et al., 1988) were performed by placing $[Zn(HS)_4]^{2-}$ in a spherical cavity (radius r_0), surrounded by a dielectric medium with a dielectric constant, ε. The charge distribution of the ligand sphere introduces an electric field acting on the dielectric medium. This reaction field interacts with the charge distribution of the ligand sphere and perturbs the one-electron Hamiltonian. This perturbation is calculated by a multipole expansion of the electron distribution, truncated after the fifth term. The Pauli repulsion due to the medium is treated by use of spherical well integrals added to the one-electron Hamiltonian (a sum of four spherical gaussian shell functions with exponents 5.0, 3.5, 2.0 and 1.4 at the radius r_0 + 106, 159,

265, and 370 pm (r_0 + 2, 3, 5, and 7 atomic units), respectively (Andersson, et al., 1994). The origin of the cavity was at the centre of mass of the ligand sphere, which almost coincide with the zinc ion. The radius of the cavity was optimised by minimising the total energy of the system at a fixed geometry and ε=80.0 and was found to be 133 pm plus the average Zn-H distance. The optimal Zn-S bond lengths were then determined by varying the mean Zn-S distance (and r_0) stepwise at six different dielectric constants between 1.0 and 80.0, keeping the rest of the geometry fixed. The calculations were performed both at the SCF and MP2 level using the program package MOLCAS-3 (Andersson et al, 1994). At the MP2 level, the reaction field from SCF calculations at the same geometry was used. This treatment is valid as long as the electron structure does not change much from SCF to MP2, an approximation that is acceptable in these rather crude computations.

Combined quantum chemical and classical geometry optimisations

Integrated quantum chemical and molecular mechanical geometry optimisations were performed using the program COMQUM (Ryde, 1996). In this program, the enzyme is 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; system 2 is represented by partial charges, one for each atom, and system 3 and 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}, $C^{\varepsilon 1}$, and both P atoms of Lys, Arg, Asp, Glu, Cys^{-}, His^{+}, 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 system 1-3 with all atoms (using charges obtained from a quantum chemical Mulliken analysis for system 1 and partial charges for system 2 and 3), and system 4 by damped integer point charges. Special action is taken at the junction between the classical and quantum chemical systems (Ryde, 1996).

The full geometry of system 1 and 2 was optimised until the change in energy and the coordinates were below 0.26 kJ/mole and 0.53 pm, respectively. Then, system 2 was fixed and the optimisation was continued until the changes were below 2.6 J/mole and 0.053 pm. In some cases, system 2 was kept fixed all the time and the tighter thresholds were used. The quantum chemical computations were performed at the Hartree-Fock level with the DZP/6-31G* basis sets. The effect of correlation was simulated by the method of offset forces (Fogarasi et al., 1992). A factor of +0.010 atomic units was added to each Zn-S bond (SCF–) gradient before the geometry relaxation. This factor is the average negative gradient of the Zn-S bond with the same basis set at the MP2 optimised geometry in vacuum.

The calculations were performed using the quantum chemical program TURBOMOLE (Ahlrichs et al., 1989) combined with the molecular dynamics simulation package MUMOD (Teleman & Jönsson, 1986, Ryde, 1995a). The potential function of the latter program contains a 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 zinc ion and its ligands were treated purely quantum mechanically; in the molecular mechanical gradients the zinc terms cancel out and in the classical optimisation of system 2 the zinc ion interacts only by a non-bonded potential.

The enzyme

Throughout, the coordinates of horse liver alcohol dehydrogenase in complex with NADH and dimethylsulfoxide at 0.18 nm resolution were used (R-factor=0.172; Al-Karadaghi et al., 1994). This is at present the most accurate structure of alcohol dehydrogenase and it represents the catalytically active closed conformation of the enzyme. Charge assignment was performed as described by Ryde (1995a). To determine the positions of the hydrogen atoms and the solvent water structure around the structural

zinc ion, a series of classical simulations was performed on all amino acids within 0.3 nm from any atom in system 3 (see below) plus a spherical cap of water molecules within 1.0 pm from the zinc ion: three 6 ps molecular dynamics simulations at 300, 100, and 0 K, followed by a molecular mechanics optimisation until the change in energy and the norm of the gradient were below $4.2 \cdot 10^{-4}$ kJ/mole and $4.2 \cdot 10^{-6}$ kJ/mole/pm, respectively. All heavy atoms were kept fixed except the oxygen atoms of the solvent water molecules. The non-bonded cut-off radius was 1.0 nm and the program package AMBER 4.0 (Pearlman et al., 1991) was used.

In the integrated geometry optimisations, system 1 consisted of $[Zn(SH)_4]^{2-}$, as a model of Zn, Cys-97, Cys-100, Cys-103, and Cys-111 (from subunit A of the enzyme). In system 2, all amino acids within 0.6 nm from any atom in system 1 were included, viz. Thr-94, Pro-95, Gln-96, Gly-98, Lys-99, Arg-101, Val-102, Lys-104, His-105, Asn-109, Phe-110, Leu-112, Lys-113, Asn-114, Ile-155, Lys-323, 8 crystal water molecules and the rest of four cysteine zinc ligands (totally 339 atoms). System 3 was composed of all atoms of residues within 0.3 nm of any atom in system 2, viz. amino acids number 92-93, 106-108, 115-117, 124, 153-154, 156-157, 318-319, 321-322, 324-327, amino acids number 258-259, 261, 283-286, 310 from the other subunit of the enzyme, and 28 crystal water molecules, in total, 500 atoms. Finally, system 4 comprised 171 integer charges.

Results and Discussion

The protonation status of the structural zinc ion

In order to determine the number of deprotonated cysteine ligands of the structural zinc ion in alcohol dehydrogenase, $[Zn(HS)_{4-n}(HS_2)_n]^{-2+n}$, with n=0-2, was optimised quantum chemically. As shown by the results in **Table 3** and in **Figure 2**, $[Zn(HS)_4]^{2-}$ is four-coordinate with 248.3 pm Zn-S distances. All four hydrogen atoms form weak hydrogen bonds to a sulphide ion (373 pm), which make the S-Zn-S bond angles different: either 106° (the four SH···S pairs) or 117° (the two non-hydrogen-bonded pairs).

$Zn(HS)_2(H_2S)_2$ in **Figure 3** is also four-coordinate but with very dissimilar Zn-S bond lengths; 226 pm for HS^-, and 293 pm for H_2S. This reflects that the interaction between the zinc ion and HS^- is much stronger than with H_2S, due to the charge-charge attraction. Analogous results were obtained with OH^- and H_2O at the catalytic zinc ion of alcohol dehydrogenase (211 and 187 pm bonding distance, respectively, according to Ryde, 1994). The structure is a distorted tetrahedron with a large (148°) HS^--Zn-SH^- angle.

$[Zn(HS)_3(H_2S)]^-$, on the other hand, is three-coordinate with the three HS^- ligands at 233 pm Zn-S distance and the H_2S ligand in the second coordination sphere of the zinc ion (625 pm from Zn), hydrogen bonded to a sulphur ion. This is due to the negative charges on the HS^- ligands, which make the total charge of the complex negative, thereby destabilising the Zn-SH_2 bond so much that the HSH···S hydrogen bond becomes energetically more favourable. Similar lowering of the coordination number of Zn^{2+}-complexes when the total charge becomes negative has been observed in both experiments (Irish et al., 1963, Cotton & Wilkinson, 1988), and theoretical calculations (Tossell, 1991, Ryde, 1994). It was impossible to find any four-coordinate structure of $[Zn(HS)_3(H_2S)]^-$; no such local minimum exists in vacuum.

These results clearly show that all the cysteine ligands of the structural zinc ion in alcohol dehydrogenase must be deprotonated. If only two of the cysteine ligands were deprotonated as suggested by Garmer & Krauss (1993) the bond lengths would have been

very different (226-290 pm) and the angles would have been more distorted (92-148°). If only one ligand was protonated, the differences would have been even larger.

Influence of the ligand choice, basis set, and correlation on the Zn-S bond lengths

Even if it is clear that all the cysteine ligands are deprotonated, there still remains one problem: the calculated average Zn-S bond length in $[Zn(HS)_4]^{2-}$ is 15 pm longer than in the crystal. Therefore, more detailed calculations were performed on this complex. First, the effect of improving the cysteine models was tested by replacing HS^- with the more realistic, but also appreciably more expensive CH_3S^- ion. This ligand gave 1.4 pm shorter Zn-S bond lengths (246.9 pm), probably due to a decreased charge on the CH_3S^- groups (–0.752 e_0 compared to –0.761 e_0, see **Table 4**). In this structure there are twelve C-H···S hydrogen bonds (243-245 pm), which are weaker than the HS···S bonds and therefore give a difference in the S-Zn-S bond angles of only 1.8° (four 108.9° angles and two 110.7°). Thus, the ligand choice has a small but significant influence on the Zn-S bonds, larger than the one observed for the catalytic zinc ion in alcohol dehydrogenase (0.8 pm change according to Ryde, 1994). This can probably be ascribed to the larger net charge of the coordination sphere at the structural zinc ion.

Secondly, the basis sets were improved. It turned out that the basis sets do not affect the geometry very much; the bond lengths obtained with basis sets enhanced with optimised s, p, and d functions on sulphur and s, p, d, and f functions on zinc, differed by less than 0.1 pm from the calculations with the normal basis set (Table 3).

Finally, the effect of electronic correlation was tested. A correct treatment of the correlation has been shown to be crucial for the geometry of first-row open-shell transition metal complexes (Langhoff & Bauschlicher, 1988, Lüthi et al, 1985), and it turned out to be equally important also for zinc complexes. When the Zn-S bond lengths were optimised at the MP2 level, the Zn-S bond lengths decreased by 6.8 pm to 241.5 pm. Thus, correlation accounts for approximately half of the discrepancy between the calculated structure and the crystal.

These calculations indicate that in vacuum, the Zn-S bond lengths of the structural zinc coordination sphere should be about 240 pm, still 6 pm longer than in the enzyme. Thus, the surrounding enzyme seems to shorten the Zn-S bonds by about 6 pm. A prominent characteristic of the enzyme around the structural zinc ion in alcohol dehydrogenase is the large number of hydrogen bonds to the cysteine sulphur ions in the enzyme. We therefore tested the effect of adding a hydrogen bond donator, NH_3, to the second coordination sphere of the zinc complex. In the optimised structure (Table 3), the NH_3 molecule forms three hydrogen bonds to three sulphur ions (309-327 pm) and the average Zn-S bond length decreases by 0.4 pm. This decrease is probably due to that the electrons on the SH^- groups are slightly shifted against the NH_3 molecule, which leads to a lowered effective charge on the SH^- groups. Therefore, the effect is largest on the sulphur ion that does not receive any NH-hydrogen bond. As can be seen in Table 4, the average SH^- charge decreases by 0.007 e_0, which is compensated by a 0.010 e_0 lower charge on the zinc ion and -0.019 e_0 net charge on the ammonia.

Geometry optimisations in a dielectric cavity

Most probably, the major factor decreasing the Zn-S bond lengths in the enzyme is screening of the negative charges on the sulphur ions. In vacuum, these charges strongly repel each other and push the sulphur ions away from the zinc ion. If the dielectric constant was larger than 1.0, this repulsion would be smaller, and the Zn-S bond lengths would decrease. A popular method to account in an average way for an increased dielectric constant is the cavity reaction field approach (Tapia & Goscinski, 1975, Mikkelsen et al., 1988). This technique is well suited for the present model system, since $[Zn(HS)_4]^{2-}$ fits well into a spherical cavity with little uncertainty in the location of the origin.

Reaction field calculations were performed on $Zn(HS)_4]^{2-}$ by first determining the optimal radius of the cavity (by energy minimisation) and then optimising the Zn-S bond lengths at different dielectric constants. The method behaves well; the monopole term accounts for 99.4 % of the total reaction field energy, while the dipole and octopole terms

account for 0.2 and 0.3 % respectively, indicating that the multipole expansion is well converged. As expected, the Zn-S bond lengths decrease appreciably, about 5 pm, showing that screening of the sulphide charges is important in the enzyme. Thus, the structural zinc site is very polar, with an effective dielectric constant much larger than 3.0-4.0 as is usually assumed for the enzyme interior (Sharp & Honig, 1990).

In fact, it turned out that even with an infinite dielectric constant of the surrounding medium, the average Zn-S bond length could not become smaller than 236.0 pm, see **Figure 4** (243.3 pm at the SCF-level), which is still 2 pm longer than in the crystal. Thus, the enzyme solvates the coordination sphere of the structural zinc ion better than water. This is most probably due to detailed directed atomic interactions in the enzyme, and that atoms can come in between the sulphur ions and screen the charges (i.e. that the model system is not perfectly spherical).

Combined quantum chemical and molecular mechanic geometry optimisations

Apparently, a continuum model can in an average way model the screening of charges around the structural zinc ion and alcohol dehydrogenase, yet it does not manage to account for the effect fully, and obviously it can not reproduce the differences between the ligands either. Therefore an approach that explicitly accounts for all the detailed interactions in the enzyme was tested. In the program COMQUM by Ryde (1996), the course of a quantum chemical geometry optimisation is influenced by classical forces exerted by the enzymic environment. In this way, it is possible to quantify changes in geometry induced by an enzyme onto a bounded subsystem. The effect of electronic correlation was simulated by the offset force method, which has been successfully applied to many small and medium sized molecules (Fogarasi et al., 1992). From Table 3, it can be seen that it is a very effective technique to obtain a quasi MP2-quality geometry at the SCF level; the average Zn-S bond length in the calculation using offset forces differs from the optimal MP2 bond lengths by only 0.4 pm and the energy difference is 1 kJ/mole.

The average Zn-S bond length of the structural zinc ion optimised with COMQUM is 235 pm irrespective if the enzyme is allowed to relax or not (**Table 5**), i.e. very near the bond length in the crystal. Again, this shortening of the Zn-S bond length correlate with a lowered average charge on the SH^- groups (–0.719 e_0 compared to –0.761 e_0, see Table 4), and the SH^- group with the shortest zinc distance also has the lowest charge. The S-Zn-S angles are also well reproduced: 105, 121, 102, 103, 119, 104° compared to 106, 115, 102, 107, 119, 108° in the crystal (same order as in Table 1) and the calculated structure (**Figure 5**) is quite similar to the one found in the crystal. The S-Zn bond length of Cys-97 is correctly predicted to be the longest one, and the calculations show that this can be ascribed to the interaction of this group with the positively charged amine group of Lys113 (hydrogen bond distance 260 pm). This depicts the importance of hydrogen bonds from the enzyme backbone amide groups to the cysteine sulphur ions. Hydrogen bonds from dipoles, such as amide groups and water molecules shorten the Zn-S bonds, while hydrogen bonds from positively charged groups lengthen them.

The simulations, however, fail to reproduce the very short Zn-S bond length of Cys-103, and instead predict that Cys-111 should have the shortest bond length. The explanation of this problem, and also of why the average Zn-S bond length still is about 0.8 pm too long is most probably the neglect of the polarisation of the classical system by the quantum system. The energy of this polarisation can be approximately calculated after the geometry optimisations (E_{pol} in Table 5) and it turns out that it largely decreases with the Zn-S bond length; the polarisation energy of the crystal structure is about 10 kJ/mole lower than in any of the optimised systems. Thus, if this energy had been allowed to influence the optimisation of the geometry, the Zn-S bonds had most probably become shorter.

Since the effect of correlation and the surrounding enzyme was so large on the $[Zn(HS)_4]^{2-}$ system, similar calculations were performed on $Zn(HS)_2(H_2S)_2$ also. The optimal Zn-S bond lengths decreased at the MP2-level to 221 and 275 pm. In the COMQUM calculations, where Cys-97 and Cys-111 (which have the longest Zn-S bonds in the crystal) were assumed to be protonated, the Zn-S bond lengths decreased further to 217 and 249-266 pm (the offset forces were 0.0133 and 0.0056 au for the SH^- and the SH_2 groups,

respectively). The average bond length, 238 pm is rather similar to the one found in the crystal, but the span of the bond lengths is all too large and the structure is more strained than the structures with all cysteines deprotonated (88 kJ/mole compared to 35-55 kJ/mole). Consequently, correlation and the enzyme do not change the conclusion that all the four cysteine ligands to the structural zinc ion in alcohol dehydrogenase are deprotonated.

The strain induced by the enzyme onto the zinc coordination sphere

The importance of strain in enzyme structure and function has been much discussed in the literature (e.g. Fraústo da Silvia & Williams, 1991, Warshel, 1991). The comparison of a structure optimised quantum chemically in vacuum and with COMQUM provides an estimate of the change in geometry and energy when it is inserted into the enzyme. Thus, ? E_{QC1} in Table 5 is a measure of the strain forced by the enzyme onto the zinc coordination sphere in alcohol dehydrogenase. This strain amounts to 35-55 kJ/mole for the structural zinc ion. This is equal to the energy of one or two hydrogen bonds, and it is not more than what was observed for the catalytic zinc ion in alcohol dehydrogenase (Ryde 1995b, 1996), i.e. in energy terms the enzyme does not influence the structural zinc ion very much. Nevertheless, the change in the geometry is rather large. Thus, the potential surface is rather flat; shortening the Zn-S bond lengths the first 3 pm, for example, costs only 0.8 kJ/mole.

Warshel (1991) has strongly argued that strain plays a very modest role in enzyme catalysis. By strain he then understands only contributions of bonds, bond angles, and torsional deformations plus the repulsive van der Waals interaction (i.e. forces that vary fast with small molecular deformation), as opposed to the electrostatic interactions (that change slowly with distance). In order to quantify how much of the distortion of the zinc geometry is due purely to electrostatics, another simulation with COMQUM was run with the C^{α}, C^{β}, and H^{β} atoms of the four cysteine zinc ligands removed (thereby eliminating the covalent strain effects; the charges were the same in both calculations). As can be seen

in Table 5, this change increases the average Zn-S bond length by only 1.5 pm, keeping the trends between the different ligands. Yet, the strain energy $?E_{QC1}$ is only 9 kJ/mole. Thus, strain in the restricted meaning of Warshel, dominates $?E_{QC1}$, while the electrostatic interactions are responsible for most of the change in the geometry of the zinc coordination sphere. This is an effect of the flatness of the potential surface; the strong and steep covalent interactions are needed to force the Zn-S bond lengths the last 2 pm that costs about 30 kJ/mole.

Concluding remarks

The present calculations undoubtedly show that all the cysteine ligands of the structural zinc ion in alcohol dehydrogenase are negatively charged; $[Zn(HS)_4]^{2-}$, with an appropriate treatment of electronic correlation and the enzymic environment, reproduces the crystal geometry satisfactorily, while any other system with a total charge less than –2 e_0 assume a structure very different from that found in crystals. The calculations also show that the divergences among the Zn-S bond lengths in the crystal are due to differences in the immediate neighbourhood of the ligand, i.e. the number and character of hydrogen bonds to the ligand. The long average Zn-S bond length of the structural zinc ion, compared to the average zinc-cysteine bond lengths in other proteins (Chakrabarti, 1989), can be ascribed to the repulsion between the negative charges of the sulphur ions. In analogy, it was found for the catalytic zinc ion in alcohol dehydrogenase that if the zinc-bound water molecule was deprotonated, (giving a total charge of –1 e_0), the distance to zinc of the other ligands increased by 6-10 pm (Ryde, 1994). Consequently, it must be concluded that the improvements on the electronic spectra observed by Garmer and Krauss with the $Co(SH)_2(SH_2)_2$ model of the structural zinc site in alcohol dehydrogenase were only fortuitous.

Naturally, the fact that all the cysteine ligands of the structural zinc ion in alcohol dehydrogenase are deprotonated strongly influences the physical properties of the enzyme.

The isoelectric point of the protein is a function of the number and acid constants of ionisable residues in the protein (the total charge of the dimer at normal pH becomes about +8 e_0, instead of +12 e_0 if only two of the ligands were deprotonated). Similarly, the geometrical and spectral properties of native or substituted structural metal ion is directly affected by the charge of the metal ligands. Several other properties may be indirectly modulated through the electrostatic potential, e.g. the pK_a values of nearby residues and the stability and structure of the whole protein. Furthermore, a correct charge assignment and a proper description of the electrostatic potential are absolutely essential for an accurate description of the enzyme in theoretical simulations.

The results also have implications on other zinc proteins. Since the surrounding of the structural zinc ion in alcohol dehydrogenase is not very special, it seems safe to assume that all cysteine ligands are deprotonated also in other proteins with a four-coordinate zinc ion. Four-coordinate zinc ions with several cysteine ligands are present in many enzymes, e.g. aspartate carbamoyltransferase, methionine-tRNA synthetase, metallothionein (all $ZnCys_4$), and the zinc finger proteins ($ZnCys_2His_2$, $ZnCys_3His$, and $ZnCys_4$) (Fraústo da Silvia & Williams, 1991, Brändén & Tooze, 1991). Such zinc ions are usually assumed to play an important stabilising role as cross links in intracellular proteins (Fraústo da Silvia & Williams, 1991).

The calculations clearly illustrate that danger of studying an isolated subsystem of a larger structure in vacuum. In general, if the net charge of the system is high, the vacuum geometry may be very different from the actual structure. An even more obvious example of this is $[ZnS_4]^{-6}$ which has a Zn-S bond length of 235 pm in zincblende but dissociates in vacuum calculations.

Our investigations have also shown that the coordination geometry of the structural zinc ion is very sensitive to the theoretical method. A balanced treatment of correlation, electrostatics and polarisation is needed to obtain a correct structure. Clearly, dielectric continuum models are inappropriate to mimic the protein matrix. In fact, the probably most important factor for the protein function is the detailed placement of charged groups and dipoles in well-defined positions. Therefore, only methods that incorporate the details of

the enzyme structure during the geometry optimisation, such as QUEST (Singh & Kollman, 1986) and COMQUM, can be expected to give geometries that are relevant to the protein environment.

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Table 1. Experimental geometry around the structural zinc ion. Bond lengths and bond angels involving the structural zinc ion are given for the complex between horse liver alcohol dehydrogenase, NADH and dimethylsulfoxide at 0.18 nm resolution (Al-Karadaghi et al., 1994) and the same complex with the catalytic zinc ion (but not the structural zinc ion) replaced by Cu^{2+} at 0.21 nm resolution (Al-Karadaghi et al., 1995). Distances are given in pm, angles in degrees.

	LADH-NADH-DMSO			Cu-LADH-NADH-DMSO		
parameter	A	B	average	A	B	average
C97S$^{\gamma}$-Zn	237.8	240.7	239.2	245.9	246.3	246.1
C100S$^{\gamma}$-Zn	234.1	234.3	234.2	234.6	230.0	232.3
C103S$^{\gamma}$-Zn	224.2	230.1	227.1	221.5	224.3	222.9
C111S$^{\gamma}$-Zn	234.1	236.4	235.2	236.0	234.0	235.0
average	232.55	235.38	233.96	234.50	233.65	234.08
C97S$^{\gamma}$-Zn-C100S$^{\gamma}$	106.4	105.4	105.9	110.5	109.0	109.8
C97S$^{\gamma}$-Zn-C103S$^{\gamma}$	113.7	116.5	115.1	114.6	115.1	114.8
C97S$^{\gamma}$-Zn-C111S$^{\gamma}$	100.9	103.4	102.1	102.3	100.6	101.4
C100S$^{\gamma}$-Zn-C103S$^{\gamma}$	108.4	106.2	107.3	109.8	108.0	108.9
C100S$^{\gamma}$-Zn-C111S$^{\gamma}$	118.7	118.4	118.5	116.4	119.6	118.0
C103S$^{\gamma}$-Zn-C111S$^{\gamma}$	108.8	107.6	108.2	103.0	104.8	103.9
average	109.48	109.58	109.53	109.43	109.52	109.48

Table 2. Hydrogen bonds to the sulphur ligands of the structural zinc ion in alcohol dehydrogenase. All hydrogen bonds with a S–donor distance < 365 pm in the crystal structure of horse liver alcohol dehydrogenase in complex with NADH and dimethylsulfoxide (Al-Karadaghi et al., 1994) are included. D is the hydrogen bond donator, A and B are the two subunits of the enzyme. Hydrogen atoms were added with standard algorithms and relaxed by molecular dynamics and energy minimisation.

sulphur	hydrogen	dist (S-H) pm		dist (S-D) pm		angle(SHC)		angle(C_βSD)	
		A	B	A	B	A	B	A	B
C97S$^\gamma$	K99H	248	243	336	332	148	148	96	97
	C100H	246	239	328	323	139	142	151	154
	K113H$^{\zeta 3}$	294	607	348	616	113	91	67	95
	H_2O	470	268	537	304	127	101	42	108
C100S$^\gamma$	C100H	302	289	328	316	96	96	60	63
	V102H	291	297	358	367	125	127	93	91
	C103H	240	244	338	340	165	161	123	122
	L112H	257	268	355	365	167	164	116	115
C103S$^\gamma$	C97H	305	291	348	346	107	115	150	143
	G98H	234	241	323	335	148	158	96	91
	H_2O	241	246	341	332	171	143	96	95
C111S$^\gamma$	C97H	249	252	345	345	162	155	106	104
	K113H	242	249	339	343	164	156	105	105

Table 3. Optimised structures (at the SCF level, if not otherwise stated) of different models of the structural zinc ion in alcohol dehydrogenase.

Complex	Energy (H)		Distance to Zn				
	SCF	MP2	S1	S2	S3	S4	mean
$[Zn(HS)_4]^{2-}$	–3370.207843	–3370.965142	248.2	248.3	248.3	248.3	248.27
(MP2 optimum)	–3370.205069	–3370.967772	241.4	241.4	241.5	241.6	241.45
(offset forces)	–3370.205293	–3370.967318	241.1	241.1	241.1	241.1	241.07
(enhanced basis)[a]	–3370.257139		247.7	247.7	247.7	247.7	247.66
(optimised basis)[b]	–3370.266560		248.3	248.3	248.3	248.3	248.31
(cavity, ε=80, SCF)	-3370.437877		243.4	243.4	243.4	243.4	243.35
(cavity, ε=80, MP2)	-3370.435495	-3371.201482	236.1	236.1	236.1	236.1	236.08
$[Zn(CH_3S)_4]^{2-}$	–3526.238378		246.9	246.9	246.9	246.9	246.93
$[Zn(HS)_3(H_2S)]^{-}$	–3370.859343		232.2	232.7	233.2	624.9	330.75
$[Zn(HS)_2(H_2S)_2]$	–3371.338701	3372.106205	225.9	225.9	292.8	293.1	259.42
(MP2 optimum)	–3371.335299	3372.109190	221.5	221.5	275.2	275.8	248.51
$[Zn(HS)_4(NH_3)]^{2-}$	–3426.408344		247.0	248.0	248.2	249.4	248.15

[a] The zinc basis was enhanced with d and f functions with exponents 0.132 and 0.390, respectively, and the 6-31+G** basis sets (Hehre et al., 1986) were used on the other atoms.

[b] The same basis as in footnote [a], but the exponents of one s, p and d function on each sulphur and two s, p and d functions and one f function on zinc were optimised during the course of the geometry optimisation (always the most diffuse functions). Resulting optimal exponents: S: 0.206-0.217, 0.0413-0.0417 and 0.611-0.613; Zn: 0.173, 0.166, 0.905, 0.180, 0.590, 0.200 and 0.270.

Table 4. Average charges (in units of the electron charge, e_0) on the groups in different models of the structural zinc ion in alcohol dehydrogenase. The charges were estimated by a standard Mulliken analysis.

System	Average group charge (in units of e_0)		
	Zn	SH^- (or CH_3S^-)	NH_3
$[Zn(HS)_4]^{2-}$	1.044	–0.761	-
$[Zn(CH_3S)_4]^{2-}$	1.006	–0.752	-
$[Zn(HS)_4(NH_3)]^{2-}$	1.036	–0.754	-0.019
$[Zn(HS)_4]^{2-}$ + protein[a]	0.875	–0.719	-

[a] The protein is modelled by the program COMQUM, with the enzyme allowed to relax and correlation effects simulated offset forces.

Table 5. Geometry optimisations of different models of the structural zinc ion in alcohol dehydrogenase using the program COMQUM. The optimisations were performed at the SCF-level with and without offset forces, and with and without the cysteine C^{α}, C^{β} and H^{β} atoms (Cys; see the Result section). $?E_{QC1}$ is the (SCF+MP2-) energy difference of the quantum system optimised in the enzyme and in vacuum. E_{pol} is the energy due to the polarisation of the classical system by the quantum system calculated after the geometry optimisation from the wave function of system 1 and point polarisabilities of the atoms in system 2, (Ryde, 1996). For comparison, the experimental average Zn-S distances for both subunits of horse liver alcohol dehydrogenase in complex with NADH and dimethylsulfoxide at 0.18 nm resolution (Al-Karadaghi et al., 1994) and a single-point COMQUM calculation on the crystal structure of subunit A are also included.

Protein	Offset forces	Cys	Energy (H) SCF	Energy (H) SCF+MP2	E_{pol} (kJ/mole)	$?E_{QC1}$ (kJ/mole)	Distance to Zn S97	S100	S103	S111	mean
fix	no	yes	–3376.700448	–3377.464123	–409.5	34.6	249	238	239	232	239.6
fix	yes	yes	–3376.698764	–3377.466374	–410.6	41.8	243	233	234	229	234.8
free	yes	yes	–3376.927167	–3377.695830	–398.7	54.7	239	239	232	229	234.9
fix	yes	no	–3376.606602	–3377.466374	–376.6	8.8	246	234	235	230	236.3
fix[a]	yes	yes	–3377.025430	–3377.803069	–99.6	87.8	266	217	217	249	237.6
A, single-point		yes	–3376.670552	–3377.439627	–419.8	36.8	238	234	224	234	232.5
Crystal structure							239	234	227	235	234.0

[a] The quantum system was $[Zn(HS)_2(H_2S)_2]$ instead of $[Zn(HS)_4]^{2-}$.

Legends to the figures

Figure 1. Stereo view of horse liver alcohol dehydrogenase around the structural zinc ion, showing the extensive hydrogen bonding to the cysteine sulphur ions. Data from the crystal structure of the complex between horse liver alcohol dehydrogenase, NADH and dimethylsulfoxide at 0.18 nm resolution (Al-Karadaghi et al., 1994) with hydrogen atoms and water molecules inserted as described in the Methods.

Figure 2. Stereo view of the optimal structure of $[Zn(HS)_4]^{2-}$ at the MP2 level in vacuum.

Figure 3. Stereo view of the optimal structure of $Zn(H_2S)_2(HS)_2$ at the MP2 level in vacuum.

Figure 4. The optimal average Zn-S bond length as a function of the dielectric constant ε of the dielectric medium surrounding a cavity enclosing $[Zn(HS)_4]^{2-}$ in the reaction field calculations. The upper curve is calculated at the SCF level, the lower curve at the MP2 level. The fitted curves are (243.3 + 4.83/ε) pm and (236.0 + 4.35/ε) pm.

Figure 5. Stereo view of the structural zinc ion, optimised by COMQUM (free enzyme with offset forces). All amino acids in System 1-2 are shown and the structure is compared to the crystal structure of the complex between horse liver alcohol dehydrogenase, NADH and dimethylsulfoxide at 0.18 nm resolution (no hydrogen atoms; Al-Karadaghi et al., 1994).