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On the role of Glu-68 in alcohol dehydrogenase

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Abstract

Theoretical computations (molecular dynamics and combined quantum chemical and molecular mechanical geometry optimizations) have been performed on horse liver alcohol dehydrogenase. The results provide evidence that Glu-68, a highly conserved residue located 0.47 nm from the catalytic zinc ion, may intermittently coordinate to the zinc ion. Structures with Glu-68 coordinated to the zinc ion are almost as stable as structures with Glu-68 at the crystal position and the barrier between the two configurations of Glu-68 is so low that it can readily be bypassed at room temperature. There is a cavity behind the zinc ion that seems to be tailored to allow such coordination of Glu-68 to the zinc ion. It is suggested that Glu-68 may facilitate the exchange of ligands in the substrate site by coordinating to the zinc ion when the old ligand dissociates.

Keywords: combined ab initio and molecular mechanical geometry optimization; five-coordination; geometry imposed by enzyme; ligand exchange; molecular dynamics; reaction mechanism

Alcohol dehydrogenase (EC 1.1.1.1) catalyzes the reversible oxidation of primary and secondary alcohols using NAD^+ as the coenzyme (Eklund et al., 1981; Eklund & Brändén, 1983; Pettersson, 1987). The active site of the enzyme contains a zinc ion that is essential for catalysis. Crystallographic studies of the horse liver enzyme (Eklund et al., 1981; Eklund & Brändén, 1983; Al-Karadaghi et al., 1994) have shown that this zinc ion is tetrahedrally coordinated by two cysteine and one histidine residue, with a molecule from the solution as the fourth ligand (water, alcohol, aldehyde, etc.) (Kinemage 1).

The most widely accepted reaction mechanism for alcohol dehydrogenase (Pettersson, 1987) is indicated in Figure 1 and includes: (1) binding of NAD^+; (2) binding of the alcohol substrate by replacing a zinc-bound water molecule; (3) deprotonation of the alcohol; (4) hydride transfer from the alkoxide ion to NAD^+, yielding NADH and a zinc-bound aldehyde; (5) release of the aldehyde by a water molecule; (6) dissociation of NADH. In applicable steps (7–9), the zinc-bound water molecule equilibrates with a zinc-bound hydroxyl ion, which gives rise to dead-end complexes.

In the second coordination sphere of the zinc ion, there are two negatively charged residues, Asp-49 and Glu-68 (Kinemage 2). In the crystal structure, Glu-68 is located 0.47 nm from the catalytic zinc ion, opposite to the substrate site, with the carboxylate oxygens hydrogen bonded to HE and HH of Arg-369 and to H of Gly-175 (Al-Karadaghi et al., 1994). Glu-68 is one of the most conserved amino acids in alcohol dehydrogenases from different sources; it is equally conserved as the zinc ligands (Sun & Plapp, 1992). Furthermore, an enzyme with Glu-68 mutated to Gln had only 1% of the native catalytic efficiency (Ganzhorn & Plapp, 1988). This strongly indicates that Glu-68 plays an important role in the catalytic mechanism of the enzyme. It has been suggested that it may stabilize the folding of the enzyme at the catalytic zinc ion by the hydrogen bond to Gly-175 (Al-Karadaghi et al., 1994), or that it may moderate the electrostatic potential at the active site, increasing the pK_a of a zinc-bound water or substrate molecule (Ganzhorn & Plapp, 1988).

The idea has also been advanced that Glu-68 may coordinate to the catalytic metal ion (Bauer et al., 1991; Formicka et al., 1992). Model building has shown that such a coordination may be accomplished by a simple rotation around the CG–CD bond. It has been suggested that this may give rise to a five-coordinate active-site metal ion that has been implicated in spectroscopic experiments on metal-substituted enzyme. Yet, no convincing evidence has been presented that shows that Glu-68 actually coordinates to the zinc ion in vivo; neither has the kinetic significance of such a coordination been discussed.

This paper provides theoretical evidence to show that Glu-68 actually may coordinate to the zinc ion. The energetics of such a coordination are studied and it is shown that zinc complexes with Glu-68 cannot be five-coordinate. The physiological role of this coordination is discussed.

Results

The charge of the zinc ion

In molecular dynamics and molecular mechanics computations a charge of the zinc ion has to be specified. The charge of the

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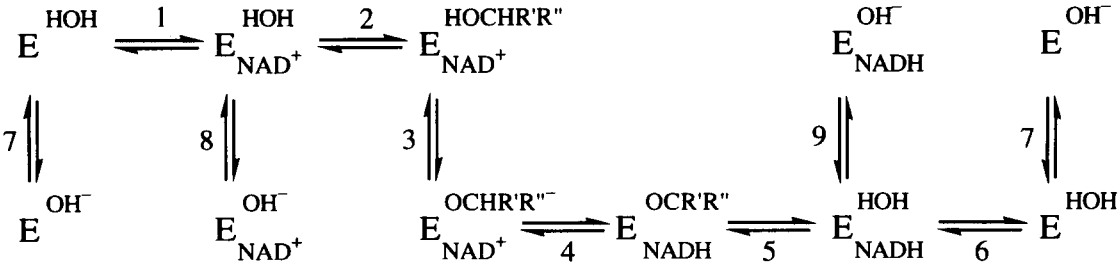

Fig. 1. Reaction mechanism proposed by Pettersson (1987). E denotes the enzyme.

zinc ion was estimated from a quantum chemical Mulliken analysis on the model system $Zn(HS)_2NH_3(H_2O)$ with different basis sets. From the typical values shown in Table 1, it can be seen that the charge distribution depends strongly on the basis sets. The charge of the N and O ligands does not differ much from the charge of the isolated ligand with the same basis set. For the sulfur ions, on the other hand, there is a considerable charge transfer to the zinc ion, which increases as the basis sets are enlarged. Therefore, the charge of the N and O ligands was taken from the standard Mumod library for His and water, whereas the charge of the zinc ion was taken directly from Table 1 (+1.584 to +0.488 in different runs) and the charges on the SG and CB atoms were uniformly changed (from −0.603 and −0.416, respectively, in the isolated amino acid) to ensure electroneutrality on the $ZnCys_2$ complex.

Molecular dynamics simulations of alcohol dehydrogenase

Molecular dynamics simulations and molecular mechanical minimizations were performed on alcohol dehydrogenase (in complex with NADH) with one or two water molecules coordinated to the catalytic zinc ion. With a four-coordinate zinc ion, the result of the simulations turned out to be strongly sensitive to the charge of the zinc ion. When the charge of the zinc ion was high (+1.6–1.0) the carboxylate group of Glu-68 tended to coordinate to the zinc ion, giving a five-coordinate zinc ion (Kinemage 2). As is shown in Figure 2, both the OE atoms may coordinate to the zinc ion, but never simultaneously; the ligating atoms change 12 times during a 40-ps simulation. The optimal distances of the two OE atoms to the zinc ion are 0.207 and 0.349 nm, respectively, and the average distances are 0.37 ± 0.09 and 0.35 ± 0.10 nm.

Clearly, these results are in variance with the crystal structure of the enzyme, but the same qualitative behavior was obtained with several different sets of partial charges and van der Waals parameters. Only if the charge of the zinc ion was decreased to +0.488 did the carboxylate group of Glu-68 remain near the position observed in the crystal structure of the enzyme, with 0.49 ± 0.08 and 0.49 ± 0.12 nm average zinc distances. Yet, as is shown in Figure 3, the carboxylate oxygens of Glu-68 still intermittently approach the zinc ion; five times during a 40-ps simulation are some of the carboxylate atoms within 0.3 nm from the zinc ion.

In simulations of alcohol dehydrogenase with two water molecules coordinated to the catalytic zinc ion, Glu-68 remains near the crystal position all the time because one of the water molecules sterically prohibits the carboxylate ion to approach zinc.

A similar behavior has frequently been encountered in other molecular dynamics simulations of transition metal atoms (Floris et al., 1992). In particular, Hoops et al. (1991) found in

Table 1. *Charge distribution around the zinc ion, calculated by a quantum chemical Mulliken analysis using different model complexes and basis sets*[a]

Complex	Basis set (Zn/other)	Charge on Zn	N	S	O
$A(H_2O)$	dζ/dζ	1.438	−0.853	−0.848	−0.758
	DZp/6-31+G**	1.020	−0.940	−0.629	−0.793
	DZpdf/6-31+G**	0.488	−0.878	−0.408	−0.772
$B(H_2O)$	dζ/dζ	1.480	−0.842	−0.851	−0.754
$A(H_2O)_2$	dζ/dζ	1.515	−0.843	−0.864	−0.740
	DZp/6-31+G**	1.070	−0.926	−0.664	−0.785
$B(H_2O)_2$	dζ/dζ	1.584	−0.825	−0.880	−0.745

[a] A and B denote $Zn(HS)_2(NH_3)$ and $Zn(HS)_2$(imidazole), respectively. Basis sets are described in the Methods.

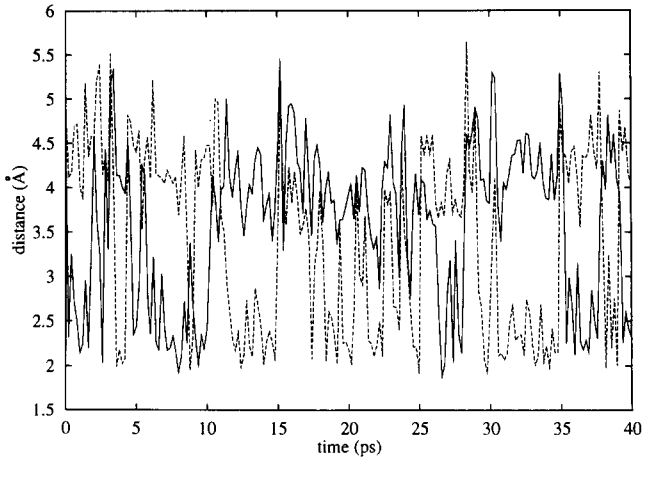

Fig. 2. Fluctuations in the distance between the two OE atoms (full and broken line) of Glu-68 and the catalytic zinc ion during 40 ps molecular dynamics simulations of alcohol dehydrogenase. The charge on the zinc ion was +1.48 *e*.

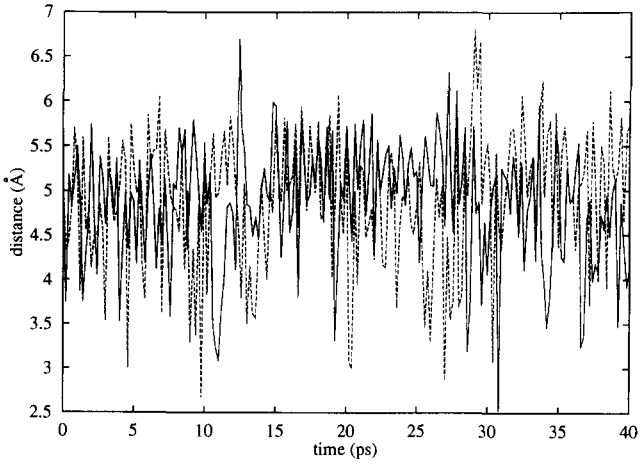

Fig. 3. Fluctuations in the distance between the two OE atoms (full and broken line) of Glu-68 and the catalytic zinc ion during 40 ps molecular dynamics simulations of alcohol dehydrogenase. The charge on the zinc ion was +0.488 *e*.

molecular dynamics simulations of carbonic anhydrase that the catalytic zinc ion becomes five-coordinate if the charge on the zinc ion is +1.02, but four-coordinate if the charge was +0.688–0.866. Clearly, more detailed studies are needed to establish the effective charge of the zinc ion in the active site of enzymes.

These results should *not* be taken to suggest that the crystal structure is incorrect, neither do they indicate that the zinc ion becomes five-coordinate when Glu-68 coordinates (the water ligand is *forced* to be a zinc ligand in these simulations; see below). Rather, the results reflect technical problems in the classical force field. Yet, the results undoubtedly and clearly show that there is no steric hindrance for the carboxylate group of Glu-68 to coordinate to the zinc ion, and that the barrier between the two conformations of Glu-68 is so low that it is easily bypassed at room temperature.

Combined geometry optimizations of the active site of alcohol dehydrogenase

With integrated quantum chemical and molecular mechanical geometry optimizations, several of the problems encountered in the molecular dynamics simulations can be overcome. No charge of the zinc or carboxylate ions has to be defined, and the nonbounded interaction between these atoms is described quantum chemically. Further, the coordination number of the zinc ion can change freely, and much more accurate geometric and energetic information is obtained.

Six different structures were studied with this method (Kinemage 2). First, the crystal structure was refined with a water molecule as the fourth ligand of the catalytic zinc ion (Fig. 4). This structure has been optimized before, but with Glu-68 in the classical region (Ryde, 1995b). There are only small differences between these two structures (the RMS difference [RMSD] is 3 pm). The zinc ligand distances and the positions of most atoms change by less than 1 pm. The atoms in the side chain of Glu-68 move about 30 pm, leading to slightly improved hydrogen bonds of the carboxylate group (0–20 pm shorter). Thus, the integrated optimization method behaves well; there is no significant difference between the potential of the quantum mechanical and the classical systems and the junctions do not introduce any discontinuities. Compared to the crystal structure (RMSD: 16 pm), the carboxylate group of Glu-68 has turned about 20° to improve the hydrogen bonds.

Secondly, the carboxylate group of Glu-68 was moved to the zinc ion and the structure was optimized again. The result in Figure 5 shows that Glu-68 may very well coordinate to the zinc ion. The carboxylate group of Glu-68 rotates 70° around the CD–CE axis, and the CD atom and the zinc ion move toward each other (60 and 130 pm, respectively), whereas the CG atom of Glu-68 hardly moves at all. The imidazole ring of His-67 bends to follow the zinc ion, with the CG atom almost fixed (NE2 moves 65 pm). The distance between the zinc ion and the two carboxylate atoms of Glu-68 is 202 and 421 pm, respectively, and the Zn–OE–CD angle is 157°. The large movement of the zinc ion ensures that the hydrogen bonds involving the carboxylate group of Glu-68 may remain. The hydrogen bond to the coordinating OE atom is slightly weakened (222 pm compared to 208 pm in the crystal structure), whereas the hydrogen bonds to the noncoordinating OE atom are unchanged or slightly strengthen (242 pm to H of Gly-175, 203 pm to HE, and 207 pm to HH2 of Arg-369 compared to 239, 201, and 229 pm in the crystal structure). As shown in Table 2, this structure is 1 kJ/mol (72 kJ/mol including E_{pol}) less stable than the one with Glu-68 in the crystal position.

This optimization was started as a five-coordinate structure with a water molecule coordinated to the zinc ion in the substrate site. Interestingly, this water molecule is displaced during the op-

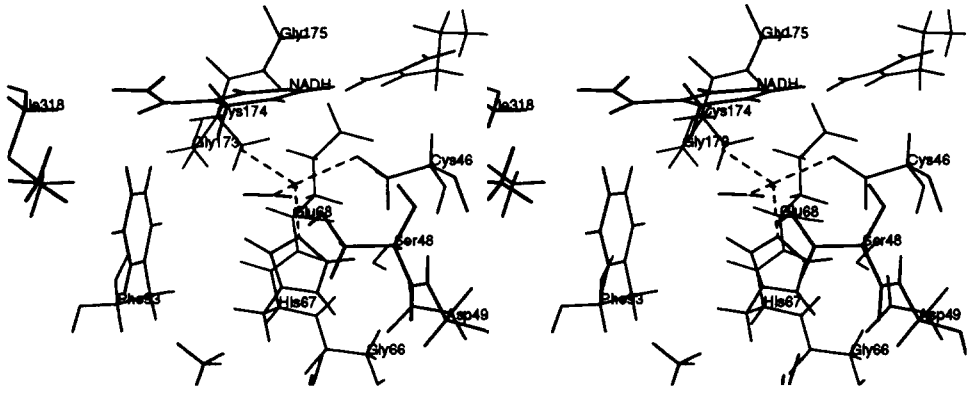

Fig. 4. Stereo view of the optimized structure of the active site of the alcohol dehydrogenase-NADH complex with a water molecule in the substrate site and Glu-68 at the crystal position. Amino acids in systems 1 and 2 are shown.

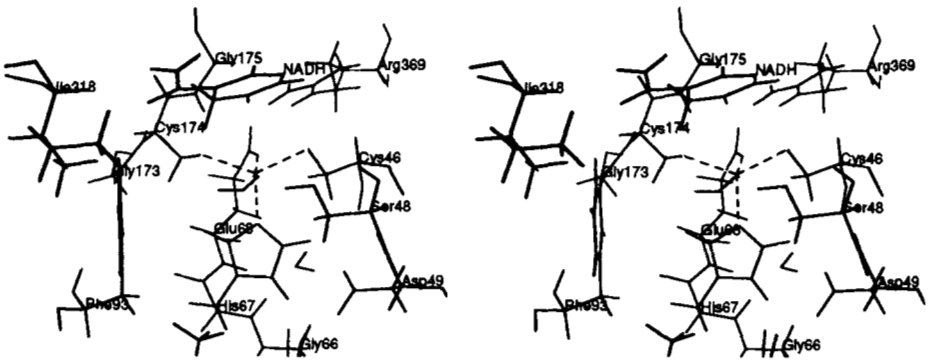

Fig. 5. Stereo view of the optimized structure of the active site of the alcohol dehydrogenase-NADH complex with one OE atom of Glu-68 coordinated to the zinc ion and a water molecule in the second coordination sphere. Amino acids in systems 1 and 2 are shown.

timization into the second coordination sphere of the zinc ion, so that the zinc ion becomes four-coordinate. This is in accord with the observation that in vacuum, a zinc ion with ligands similar to those found in alcohol dehydrogenase cannot be five-coordinate if the nonprotein ligand is negatively charged (Ryde, 1994). The water oxygen is 366 pm from the zinc ion and is weakly hydrogen bonded to HG of Ser-48 (230 pm). Both hydrogens form weak hydrogen bonds to the SG atoms of Cys-46 and Cys-174 (distances ≈300 pm) because no other hydrogen-bond acceptors are available in the hydrophobic substrate site.

If this second-sphere water molecule is removed and the structure is re-optimized, the geometry changes slightly (RMSD: 5 pm). The zinc ion and its ligands move 8–16 pm toward the substrate site and the HG atom of Ser-48 reorients to interact with SG of Cys-46 (instead of the water molecule).

For comparison, these structures were optimized also with NAD^+ bound to the enzyme (Figs. 6, 7; Kinemage 2). This substitution does not lead to any large alterations in the geometry (RMSD: 9 and 7 pm, respectively) and the changes are almost the same for both conformations of Glu-68. The positive charge on the nicotinamide ring of the coenzyme attracts the negatively charged SG atoms of Cys-46 and -174, and therefore most atoms in Cys-46, His-67, Glu-68, Cys-178, and Zn move 15–35 pm toward the nicotinamide ring. The nicotinamide ring itself tilts away from the zinc ion on the C5N side (30 pm), with N1N almost fixed. The interaction between the water molecule and Ser-48 is inverted, so that a hydrogen bond is formed between OG and a water hydrogen, whereas HG interacts weakly with SG of Cys-46 (360–380 pm). This is probably due to the repulsion between the water hydrogens and the nicotinamide ring. In the structure with Glu-68 coordinated to the zinc ion (Fig. 7), the second sphere water oxygen is strongly attracted by the nicotinamide ring. Because the carboxylate group of Glu-68 is nearer to the positively charged nicotinamide ring when coordinated to the zinc ion than in the crystal position (560 pm compared to 650 pm), the former structure is more favored by NAD^+, and it is in fact 38 kJ/mol more stable (26 kJ/mol *less* stable if E_{pol} is included) than the latter.

A water molecule may also coordinate to the zinc ion in a site opposite to the substrate cleft (termed the *alternative site*; Ryde 1995a, 1995b). In order to deduce the geometry of such coordination and to decide whether the cavity behind the zinc ion is designed for Glu-68 or for a water molecule, an optimization of a water molecule in the alternative site was also performed. It turned out that the alternative site is distinct from the Glu-68 site, as can be seen in Figure 8 (the sites are separated by about 290 pm). This is because Glu-68 sterically prohibits the water molecule to occupy the Glu-68 site. The water molecule in the alternative site is therefore forced down toward His-67 and pushes the imidazole ring 122 pm out into the substrate site. The zinc ion cannot follow this movement fully and is forced 45° out of the plane of the imidazole ring of His-67. Thus, a water molecule fits rather poorly into the alternative site and such a structure is 106 kJ/mol less stable than the structure with the water molecule in the substrate site (76 kJ/mol including E_{pol}). Similar conditions apply when both the substrate site and the alternative site are occupied by water molecules (Tables 2, 3).

In order to estimate the change in geometry and energy (i.e., the strain) imposed by the enzyme onto the zinc coordination sphere the systems were optimized also in vacuum (i.e., with only

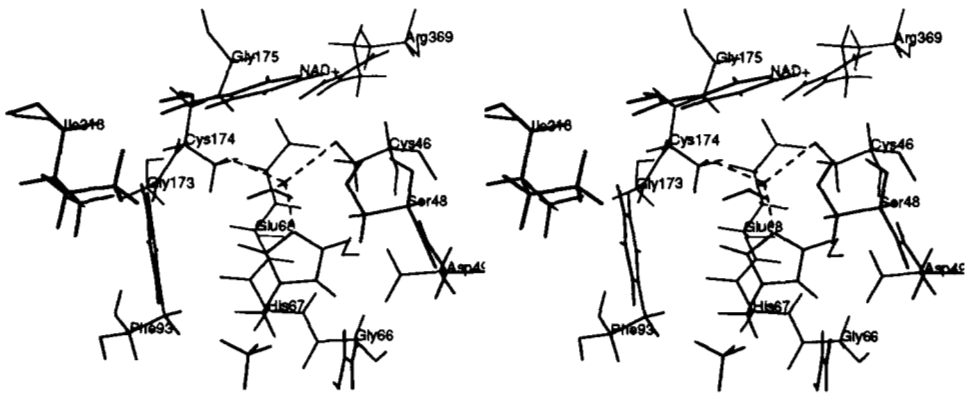

Fig. 6. Stereo view of the optimized structure of the active site of the alcohol dehydrogenase-NAD^+ complex with a water molecule in the substrate site and Glu-68 at the crystal position. Amino acids in systems 1 and 2 are shown.

Table 2. *Energies of structures optimized with Comqum*[a]

Quantum system	Coenzyme	E_{tot} (H)	E_{pol} (kJ/mol)	ΔE_{QC1} (kJ/mol)
$B(H_2O)$[d]	NADH	−2,877.879078	−73.7	41.7
$B(H_2O) + (HCOO)^-$	NADH	−3,065.147824	−198.4	[c]
	NAD^+	−3,066.124067	−221.7	[c]
$B(HCOO)^-$	NADH	−2,990.168497	−137.5	119.9
$B(HCOO)^- + (H_2O)$	NADH	−3,066.147477	−127.8	113.2
	NAD^+	−3,066.138372	−158.1	154.9
$B(H_2O)$[b]	NADH	−2,877.838778	−103.5	143.7
$B(H_2O)_2$[d]	NADH	−2,953.816308	−153.1	131.4

[a] B denotes $Zn(HS)_2$(imidazole). "+" indicates second sphere coordination. E_{tot} is the total (quantum chemical + classical) energy. E_{pol} is energy due to the polarization of system 2 by the wavefunction of system 1. ΔE_{QC1} is the difference in quantum chemical energy of the isolated system 1, at the geometry optimized in vacuum and with Comqum.

[b] The water molecule occupies the alternative zinc site

[c] Comparison with the vacuum structure makes no sense. because the formate ion is not primarily coordinated to the zinc complex but to molecular mechanic hydrogen bond partners.

[d] From Ryde (1995b).

system 1). In Tables 2 and 3, the energy and geometry of the systems optimized with and without the enzyme are compared. Quite naturally, second sphere zinc ligands are most strongly affected by the enzyme because there are many more binding sites for these molecules in the enzyme, and thus structures involving second-sphere ligands have a large strain energy: 113–155 kJ/mol. Similarly, the position of the noncoordinating OE atom of Glu-68 is also affected by additional binding sites and steric restrictions in the enzyme, leading to a rather high strain energy also for the third system in Tables 2 and 3 (120 kJ/mol). Furthermore, systems involving NAD^+ are significantly more strained than those with NADH, an effect of the strong electrostatic interactions with the positively charged nicotinamide ring.

Interestingly, systems with a water molecule in the alternative site are more strained than those with a water molecule in the substrate site or with Glu-68 coordinated to the zinc ion. This is especially pronounced in the angle of the zinc ion out of the His-67 imidazole plane. As shown in Figure 9 and Table 3, this angle is 35–45° in structures with a water molecule in the alternative site, whereas it is 1–4° in structures with a water molecule in the substrate site (6° in the crystal structure), and 4–8° when Glu-68 coordinates to the zinc ion. Altogether, this clearly shows that the alternative water site is much less favorable than the substrate site and the Glu-68 site. In other words, the cavity behind the zinc ion seems to be tailored for allowing Glu-68 (and no other molecule) to coordinate to the zinc ion.

Influence of the basis sets on the geometry

The basis sets used in the integrated geometry optimizations, although the largest afforded, are usually not sufficient to describe the zinc-ligand distances exactly (Ryde, 1995a). Therefore some vacuum geometry optimizations were performed with larger basis sets on $[Zn(HS)_2NH_3(HCOO)(H_2O)_{0-1}]^-$, where imidazole has been replaced by ammonia. The result in Table 4 shows that the size of the basis sets influences the geometry rather weakly, much less than in systems with first-sphere water ligands (Ryde, 1995a). The largest basis set gives 6–10-pm longer Zn–N and Zn–O distances and 3-pm shorter Zn–S distances. The carboxylate anion binds monodentately to the zinc ion also with large basis sets, and no five-coordinate complexes could be obtained; In fact, larger basis sets seem to disfavor five-coordinated complexes (Ryde, 1995a).

Discussion

The present investigation provides strong evidence that the carboxylate group of Glu-68 may intermittently coordinate to the catalytic zinc ion in alcohol dehydrogenase. The molecular dy-

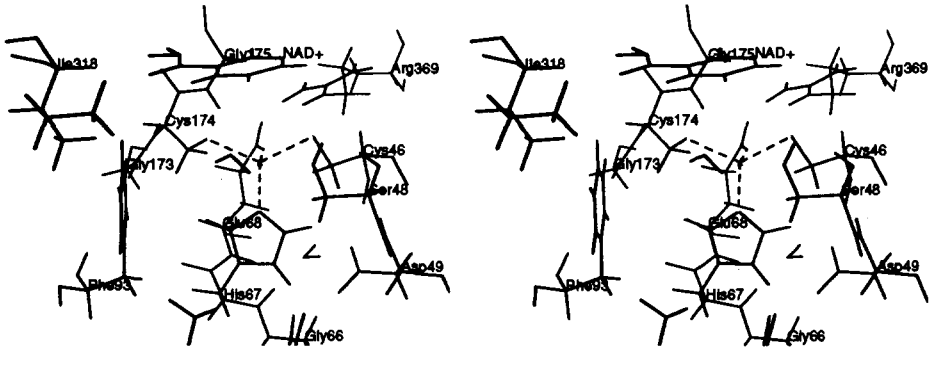

Fig. 7. Stereo view of the optimized structure of the active site of the alcohol dehydrogenase-NAD^+ complex with one OE atom of Glu-68 coordinated to the zinc ion and a water molecule in the second coordination sphere. Amino acids in systems 1 and 2 are shown.

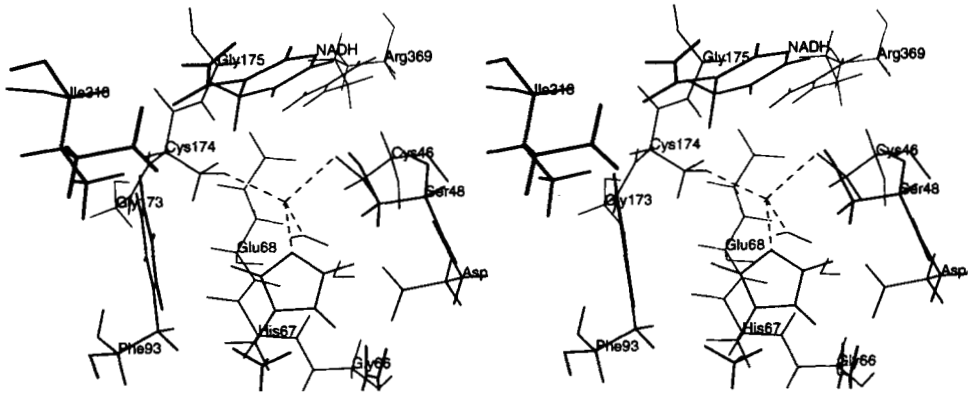

Fig. 8. Stereo view of the optimized structure of the active site of the alcohol dehydrogenase–NADH complex with a water molecule in the alternative site. Amino acids in systems 1 and 2 are shown.

namics simulations show that there is no steric hindrance for such a coordination and that the barrier between the two configurations of Glu-68 is so low that it is readily bypassed at room temperature.

The combined quantum chemical and molecular mechanical geometry optimizations show that such coordination actually is possible and can be accomplished with small changes in the geometry of the zinc coordination sphere. Furthermore, the complex with Glu-68 coordinated to the zinc ion is almost as stable as the crystal conformation. With NADH as coenzyme, the former structure is only 1 kJ/mol less stable than the latter, and with NAD^+, it is in fact 38 kJ/mol more stable. However, the polarization of system 2 by the quantum chemical system (E_{pol} in Table 2) seems to disfavor Glu-68 coordination by about 70 kJ/mol, so that the structures with Glu-68 coordinated to the zinc ion are 72 and 26 kJ/mol less stable than those with Glu-68 in the crystal position. Yet, it should be remembered that the forces due to the polarization of system 2 are not considered in the present version of Comqum, and thus system 2 is not optimized with respect to this polarization. Therefore, the difference in E_{pol} is probably too large and 26 kJ/mol can be considered as an upper limit of the energy difference between the two conformations of Glu-68 when NAD^+ is bound to the enzyme. If the difference in entropy between the two conformations is negligible, this indicates that more than one active site out of 34,000 has Glu-68 coordinated to the zinc ion, a small but significant ratio, especially because the interconversion of the two structures seems to be fast.

Furthermore, the active site of alcohol dehydrogenase appears to be tailored to make the coordination of Glu-68 to the zinc ion possible. There is a cavity behind the zinc ion opposite to the substrate site that has been conserved during evolution and that

Table 3. *Geometries of structures optimized with Comqum and in vacuum*[a]

			Distance to Zn					Angle subtended at Zn						
Quantum system	Protein	N	S1	S2	OE1	OE2	OH_2	S1–S2	S1–N	S2–N	S1–O	S2–O	N–O	Outp
Enzyme	A	214	224	235	468	610	219	130	113	104	106	102	94	5.7
	B	205	222	227	474	612	216	129	113	108	105	102	93	5.9
$B(H_2O)$[b]	No	204	237	235			211	145	103	107	96	94	104	0.6
	NADH	199	238	233			212	124	115	116	99	97	96	1.2
$B(H_2O) + (HCOO)^-$	NADH	199	239	233	501	541	209	125	114	116	98	97	98	0.4
	NAD^+	198	239	232	514	557	214	118	117	118	107	91	99	4.5
$B(HCOO)^-$	No	209	242	241	195	380		114	102	109	121	110	98	1.7
	NADH	205	243	235	202	421		124	107	107	98	101	120	5.7
$B(HCOO)^- + (H_2O)$	No	210	245	241	193	364	471	125	101	103	110	112	103	2.9
	NADH	206	241	236	201	420	366	121	107	106	99	102	121	3.9
	NAD^+	204	245	238	201	418	416	120	110	108	98	102	119	7.6
$B(H_2O)$[c]	NADH	206	241	234			215	113	120	117	98	124	81	44.7
$B(H_2O)_2$[b]	No	207	247	247			210	163	101	96	88	88	101	0.2
							211				89	88	101	
	NADH	204	246	238			223	110	127	123	83	89	90	34.8
							231				90	113	77	

[a] B denotes $Zn(HS)_2$(imidazole). "+" indicates second sphere coordination. In calculations with the enzyme, S1 and S2 represent the thiolates of Cys-46 and Cys-174, respectively. Outp is the angle (in degrees) of the zinc ion out of the plane of the His-67 imidazole ring. For the enzyme, A and B refer to the two subunits.

[b] From Ryde (1995b).

[c] The water molecule occupies the alternative zinc site.

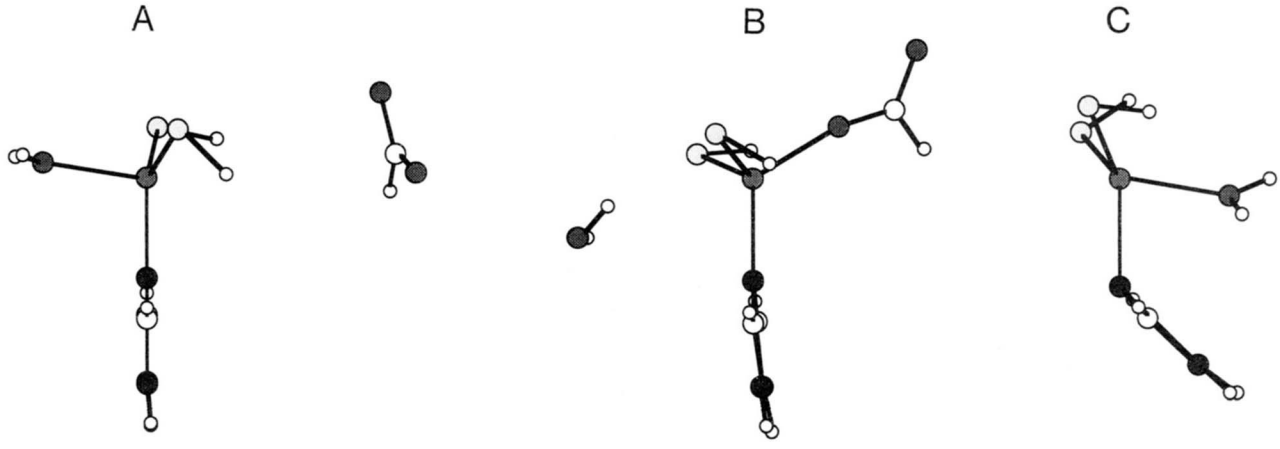

Fig. 9. Angle of catalytic zinc ion out of the plane of the His-67 imidazole ring in alcohol dehydrogenase in complex with NADH and **(A)** a water molecule in the substrate site and Glu-68 in the crystal position; **(B)** a water molecule in the second coordination sphere and Glu-68 coordinated to the zinc ion; **(C)** a water molecule in the alternative site. Only the quantum system is shown.

is perfectly sized to allow Glu-68 to flip between the zinc ion and the crystal position but is too small to allow any other molecule to occupy it. A water molecule can also coordinate at this side of the zinc ion, near the cavity, but such a coordination strongly distorts the geometry around the zinc ion (Fig. 8; Table 3) and is energetically disfavored by 76 kJ/mol.

Altogether this indicates that an intermittent coordination of Glu-68 to the zinc ion is kinetically desirable. The question then arises what the kinetic significance of such a coordination may be. Clearly, it cannot speed up the hydride transfer reaction or the deprotonation of the alcohol substrate (steps 3 and 4 in Fig. 1) because a zinc-bound substrate would be rejected by such a coordination and the pK_a of the substrate would be expected to increase as Glu-68 approaches. Rather, the explanation should be found among the ligand exchange steps (2 and 5 in Fig. 1).

Ligand exchange at metal ions can occur by three different mechanisms: the associative, dissociative, and interchange mechanisms, indicating that the new ligand binds before, after, or at the same time as the old one leaves (Cotton & Wilkinson, 1988). In vacuum, the five-coordinate intermediate involved in the associative mechanism and the three-coordinate intermediate in the dissociative mechanism are about 20 and 40 kJ/mol less stable than the original and final four-coordinate structures, respectively (Ryde, 1994). Thus, in vacuum the associative mechanism is energetically preferred.

In the enzyme, however, five-coordinate structures are disfavored by about 100 kJ/mol, whereas three-coordinate structures are hardly affected (Ryde, 1995a, 1995b). Furthermore, in the closed conformation of the enzyme (the catalytically active one), the substrate site is at the bottom of a 1-nm-deep cleft that is so narrow that the rather bulky substrate molecule (e.g., ethanol or benzyl alcohol) hardly can pass a water molecule. There is no room for more than four zinc ligands except behind the zinc ion, in the highly unfavorable alternative site. Therefore, the ligand in the substrate site probably has to dissociate and diffuse all the way through the substrate cleft before a substrate molecule may enter the cleft and bind to the zinc ion. Thus, if the Glu-68 was fixed in the crystal position, the conclusion must be that ligand replacement in the enzyme proceeds through the rather unfavorable dissociative mechanism.

Yet, Glu-68 is located opposite to the substrate site, in a perfect position to coordinate to the zinc ion when the ligand in the substrate site dissociates. This would give an interchange mechanism that might save up to 40 kJ/mol in the replacement re-

Table 4. *Quantum chemically optimized structures in vacuum*[a]

Complex	Basis (Zn/other)	Energy (H)	Distance to Zn					
			N	S1	S2	OE1	OE2	OH2
$A(HCOO)^-$	dζ/dζ	−2,818.163657	214	241	241	195	345	
	DZp/6-31+G**	−2,818.529341	220	238	238	202	344	
	DZpdf/6-31+G**	−2,818.538459	220	237	238	201	344	
$B(HCOO)^-$	dζ/dζ	−2,986.699586	209	241	242	195	380	
$A(HCOO)^- + (H_2O)$	dζ/dζ	−2,894.146809	214	238	243	197	345	341
	DZp/6-31+G**	−2,894.570576	224	235	239	203	320	362
$B(HCOO)^- + (H_2O)$	dζ/dζ	−3,062.677233	210	242	245	193	364	471

[a] A and B denote $Zn(HS)_2(NH_3)$ and $Zn(HS)_2$(imidazole), respectively. "+" indicates second sphere coordination. Basis sets are described in the Methods.

action. The present calculations show that Glu-68 is ideal for such a function; Glu-68 gives a rather relaxed geometry when it coordinates to the zinc ion (much less strained than when a water molecule coordinates to the zinc ion in the alternative site) and the energy difference between the two conformations of Glu-68 is small, as is the barrier between them. Thus, such a mechanism nicely explains the cavity behind the zinc ion, the behavior of Glu-68 in the simulations, and also gives a further reason why Glu-68 has been so conserved during evolution.

Several other roles of Glu-68 have been suggested, but none that explains why Glu-68 should be able to coordinate to the zinc ion. It is an inevitable effect of the negative charge of the carboxyl group that Glu-68 modulates the electrostatic potential at the substrate site (Ganzhorn & Plapp, 1988), and this is probably an important factor for the enzyme's capacity to discriminate between zinc-bound alkoxide and hydroxide ions by their pK_a values. Our mechanism is fully consistent with this proposal and also with the suggestion that the carboxylate group of Glu-68 may stabilize the folding of the enzyme around the zinc ion by the hydrogen bond to H of Gly-175 (Al-Karadaghi et al., 1994). In fact, our results show that this hydrogen bond remains even when Glu-68 coordinates to the zinc ion. However, the results give no support to the suggestion that the coordination of Glu-68 may lead to a five-coordinate active-site metal ion (Bauer et al., 1991; Formicka et al., 1992). On the contrary, the geometry optimizations show that when the carboxylate group of Glu-68 coordinates to the zinc ion, the coordination number of the zinc ion cannot exceed four, neither in vacuum nor in the enzyme (the zinc ion becomes five-coordinate in the molecular dynamics simulations only because the water molecule is *forced* to be a ligand by the potential). Thus, mechanisms assigning a significant role to a five-coordinate catalytic zinc complex still lack any theoretical support (Ryde, 1994, 1995a, 1995b).

Methods

Molecular dynamics and molecular mechanical calculations

Molecular dynamics simulations and molecular mechanical energy minimizations were performed by the program Mumod by Teleman and Jönsson (1986). The potential function of this 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 zinc ion and its ligands are treated as bonds and are described by an effective parameterization tailored for the active site of alcohol dehydrogenase (Ryde, 1995a). Mumod uses a double time-step Gear predictor-corrector algorithm of order 4 for the integration of the Newton equations of motion. The forces are divided into a slow (the nonbonded and dihedral terms) and a fast component. The slow forces were updated every five time steps. The time step was 0.05-0.2 fs. No cut-off in the nonbonded forces was applied. The neighbor list was updated every 40 time steps, and the temperature was scaled toward 300 K every 250 time steps.

In the molecular mechanics minimizations (Ryde, 1995a), the energy was minimized until the norm of the gradient was below 0.1 kJ/mol/nm. No cut-off was used for the nonbounded interactions. Several starting structures were tested but only the one with lowest potential energy is reported.

Combined quantum chemical and classical geometry optimizations

Integrated quantum chemical and molecular mechanical geometry optimizations were performed using the program Comqum (Ryde, 1995b). In this program, the enzyme is divided into four subsystems (see below). The central system 1 is geometry-optimized using 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 NZ, CZ, CG, CD, SG, CE1, N1N, 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, whereas in systems 1-3, ϵ = 1.0. In each step of the optimization, system 2 is relaxed molecular mechanically (keeping the other systems fixed), with systems 1-3 in all-atom representation (using charges obtained from a quantum chemical Mulliken analysis for system 1 and standard partial charges for system 2 and 3), and system 4 is represented by damped integer point charges. Special action is taken at the junctions between the classical and quantum chemical systems (Ryde, 1995b).

The full geometry of system 1 and 2 was optimized until the change in energy and coordinates was below 0.26 kJ/mol and 0.53 pm, respectively. Then, system 2 was fixed and optimization was continued until the changes were below 0.0026 kJ/mol and 0.053 pm. Several starting structures were tested in order to minimize the risk of being trapped in local minima. The quantum chemical computations were performed at the ab initio Hartree-Fock level with analytical gradients, using basis sets of double-ζ quality for all atoms (H: (31); C, N, O: (5111/31); S, P: (521111/4111); Zn: (62111111/51111/311)) (Huzinaga, 1965; Wachters, 1970).

For comparison, quantum chemical geometry optimizations were also performed in vacuum on $Zn(HS)_2X(HCOO)(H_2O)_{0-1}$, where X denotes NH_3 or imidazole (to the tighter thresholds). The basis sets were the same as above, or the 6-31+G* series of basis sets (Hehre et al., 1986) and the zinc basis (62111111/33111/311, called DZ) of Schäfer et al. (1992), enhanced with p, d, and f functions with exponents 0.162, 0.132, and 0.390, respectively. The calculations were performed using the semi-direct quantum chemical program package Turbomole (Ahlrichs et al., 1989) on an IBM RISC RS/6000 workstation.

The enzyme

Throughout, the coordinates of horse liver alcohol dehydrogenase in complex with NADH and dimethylsulfoxide at 0.18-nm resolution (R-factor = 0.172) (Al-Karadaghi et al., 1994) were used. 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

determination of the positions of hydrogen atoms and water molecules were performed as described by Ryde (1995a). Computations with NAD^+ bound to the enzyme were initiated by removing one H4N atom from NADH, changing the partial charges on the nicotinamide moiety (C1'N, 0.264; N1N, −0.528; C2N, 0.408; H2N, 0.262; C3N, −0.312; C7N, 0.693; O7N, −0.456; N7N, −0.719; H7N, 0.311; C4N, 0.092; H4N, 0.182; C5N, −0.182; H5N, 0.195; C6N, 0.307; H6N, 0.234; obtained from a Mulliken analysis on $[C_5NH_5CONH_2]^+$, adapted to the glycosylated form on N1N and C6N), and re-equilibrating the structure. After this project was initiated a high-resolution structure of the enzyme in complex with NAD^+ and substituted benzyl alcohols has been published (Ramaswamy et al., 1994). This structure confirms the assumption that the major structure of the complexes of alcohol dehydrogenase with NADH and NAD^+ is very similar and it also shows that Glu-68 is in the same position as in the other crystal structure.

In the integrated geometry optimizations, system 1 consisted of $[Zn(imidazole)(SH)_2(HCOO)(H_2O)_{0-1}]^-$, as a model of Zn, His-67, Cys-46, Cys-174, Glu-68 (from subunit A of the enzyme), and the zinc-bound water molecule. In system 2, all amino acids within 0.3 nm from any atom in system 1 were included, viz. Ser-48, Asp-49, Gly-66, Phe-93, Phe-140, Leu-141, Gly-173, Gly-175, Ile-318, Arg-369, H_2O-158, the nicotinamide moiety of NADH, and the rest of Cys-46, Cys-174, Glu-68, and His-67 (total of 206 atoms). System 3 was composed of all atoms of residues within 0.3 nm of any atom in system 2, viz. amino acids numbers 43–45, 47, 50–53, 57, 59, 63, 64, 69, 90, 92, 94, 95, 109, 110, 115, 116, 139, 142, 146, 170–172, 176, 178, 179, 202, 203, 292, 294, 317, 319–321, 345–348, 359, 368, 370, crystal waters numbers 5, 8, 21, 35, 55, 58, 59, 159–161, 167, 172, the N-ribose, and the pyrophosphate moiety of NADH and amino acids 309 and 310 from the other subunit of the protein, in total, 837 atoms. Finally, system 4 comprised 176 integer charges. The same systems were used in the molecular dynamics simulations; systems 1 and 2 were free to move and systems 3 and 4 were kept fixed.

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