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A theoretical study of Zn^{++} interacting with models of ligands present at the thermolysin active site

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SUMMARY

The binding energy and the geometrical arrangements of the complexes formed by the zinc dication with OH^- , one, four, five or six water molecules, SH^- , H_2S , formic acid, the formate anion, imidazole, its anion and formamide are calculated using the MNDO method. The comparison of the results obtained with those of ab initio computations on the same complexes induced us to propose for Zn^{++} a set of parameters different from the one determined by Dewar for the neutral metal atom. Using the two MNDO parametrizations, similar calculations are carried out for Zn^{++} interacting with two molecules of 2-aminoethanethiol and with models of the four ligands which are present at the thermolysin active site, in order to evaluate the possibilities and limitations of this semiempirical method for theoretical studies concerning zinc metalloenzymes. In the last case, the results obtained suggest that, in the crystal state, the water molecule could be deprotonated. This finding is discussed in relation with the mechanism of action of the enzyme which has been proposed.

INTRODUCTION

The presence of Zn^{++} at the active site of a large number of enzymes suggests that the action of this cation plays a determining role in the catalytic activity of the macromolecular systems concerned and/or in their binding with inhibitors [1,2]. Theoretical studies which could contribute to the understanding of the mechanism of these phenomena are difficult to carry out on such systems with nonempirical methods because of the number of electrons concerned, even if a pseudopotential treatment is retained for the metal [3-7]. On the other hand, only a very limited number of cal-

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culations using semiempirical methods, which can be used for large systems such as biomolecules, have been carried out on systems containing zinc atoms [8-13] and, to our knowledge, those concerning zinc dications have been limited to zinc porphins and zinc phthalocyanines [14,15], systems which are extremely different from zinc proteins and deal with only one type of Zn^{++} -ligand interactions.

Therefore, before undertaking even on a moderately large scale computations on zinc dications interacting with several ligands concerned in enzymatic reactions or inhibition, it appears necessary to carry out, with the semiempirical method chosen, a series of computations on Zn^{++} -ligand complexes for which the results obtained can be compared either to those of corresponding *ab initio* calculations and/or with experimental data.

The calculated values of the zinc-ligand(s) interaction energies will be checked by nonempirical calculations since the corresponding experimental data are not available, while the theoretical geometrical arrangements of the complexes studied will be compared as often as possible with X-ray diffraction data. However, for the complexes which have not been studied experimentally the comparison will again be done with results of nonempirical computations.

The semiempirical method used throughout this study is MNDO, the reason for this choice is twofold. It has been shown to give results which are, in a large number of cases, in reasonable agreement with *ab initio* ones and with experiment for chemical reaction studies [16, 17], this feature appears to be particularly satisfactory for a method which is to be used to tentatively contribute to the understanding of the mechanism of enzymatic reactions. Moreover, this method has given reasonable results for cations [18] and complexes containing ionic entities [19, 20] as is the case for the systems considered in this study.

The ligands for which the test calculations are reported are models of those coordinated to the zinc dication at enzyme's active site [1, 2]. The computations on Zn^{++} interacting with 4-6 molecules of water were undertaken in order to examine the ability of theory to give the evolution of the geometrical arrangements of the ligands of the dication first coordination shell as their number increases. Finally, the largest aggregates considered will be Zn^{++} with models of the 4 ligands which are present at the thermolysin active site and Zn^{++} interacting with 2 molecules of 2-aminoethanethiol, since the results of X-ray studies for complexes containing these entities, which can be considered representative of small models of active sites of enzymatic systems, are available in the literature [21, 22].

COMPUTATION DETAILS

The *ab initio* calculations have been carried out using for the ligands basis sets (contracted to minimal) [23] which have been shown to give good results for ligand-cations complexes [24]. We had to use a very limited number of Gaussian functions and of atomic orbitals because of the size of the systems for which we had to carry out geometrical optimization of the intermolecular arrangements, namely $\text{Zn}^{++}-(\text{H}_2\text{O})_5$ and $\text{Zn}^{++}-(\text{H}_2\text{O})_6$. For the zinc dication we have used the 3-21G basis set recently proposed by Dobbs and Hehre [25] without the more diffuse 4p orbitals. In the case of Zn^{++} it appeared that the reoptimization of the STO 3G scaling factors [24a] proposed for Mg^{++} and Ca^{++} leads to a basis set which in the case of the $\text{Zn}^{++}-\text{H}_2\text{O}$ complex gives a ligand \rightarrow dication charge transfer which is underestimated, and which does not have the proper variation with the distance when compared to corresponding results obtained with larger basis sets [5, 26].

Previous studies [5, 27] have shown that the anions are not properly described by basis sets appropriate for the corresponding neutral entities. Therefore, for the deprotonated molecules that we consider in this study, we have added to the minimal basis used for the neutral molecule the set of diffuse p functions determined by Dunning and Hay [28]. This set of diffuse p functions was also added to the basis of the neutral molecules but only for the calculations dealing with a single ligand, since Demoulin and Pullman [5] have shown that they do not modify significantly the cation-ligand binding energies nor the equilibrium distances.

In order to avoid the convergence difficulties which can be encountered to achieve self-consistency when the empty s orbital of the dication has an energy lower than the ligand's highest occupied orbital, we have used a modified version of MonsterGauss in which the eigenvectors used as initial guess are those of the orbitals actually occupied in the dication on one hand, and in the ligand(s) on the other.

With the MNDO method, two different sets of calculations have been run. One with the parameters proposed by Dewar and Merz [12] and the other with modified values of U_{ss} , U_{pp} , β_s and β_p ; the two sets of values are reported in Table 1. We were led to modify Dewar's original values because, as we shall see, they gave for Zn^{++} interacting with a single ligand results which differ not only quantitatively but also in some respect qualitatively with those of the corresponding ab initio computations. In order to overcome this discrepancy, we have varied the four parameters so as to obtain for the complex $Zn^{++} - H_2O$ values of the binding energy and of the equilibrium distance close to those given by nonempirical computations. Then we checked that this set of parameters gave results of comparable quality for $Zn^{++} - NH_3$ and $Zn^{++} - HCOOH$. Since the values calculated for these systems were also considerably improved we did not modify any additional parameter such as the orbital exponents, α or H_{sp} .

The molecular geometries used as input were taken from experimental data for H_2O , NH_3 , H_2S , $HCOOH$ [29], $HCOO^-$ [30], imidazole [31], formamide [32], Bis (2 amino ethanethiolato) zinc [22] and the models of the four ligands of the thermolysine active site [21]. For OH^- and SH^- the bond lengths used are those optimized by Demoulin and Pullman [5] and Kothekar et al. [33]. The same geometry was used for imidazole and deprotonated imidazole.

RESULTS AND DISCUSSION

Zn^{++} interacting with a single ligand

In Table 2 are reported the theoretical values of the equilibrium distances and binding energies between Zn^{++} and the different small ligands considered in this study, given by the two MNDO parametrizations and by the ab initio computations. Previous studies [33–38] having shown that

TABLE I
ORIGINAL (O) AND MODIFIED (M) VALUES OF THE MNDO PARAMETERS

Parametrization	U_{ss}	U_{pp}	β_s	β_p
O	−20.8397	−19.6252	−1.0	−2.0
M	−18.8	−17.6	−0.5	−0.1

TABLE 2

CALCULATED VALUES OF THE BINDING ENERGY (ΔE IN KCAL/MOL), EQUILIBRIUM DISTANCE (Re IN Å) AND ANGULAR DEPENDENCE (θ IN $d^{\circ a}$) IN COMPLEXES FORMED BY Zn^{++} AND SMALL LIGANDS

Ligand	MNDO						Ab initio		
	O			M					
	ΔE	Re	θ	ΔE	Re	θ	ΔE	Re	θ
H ₂ O	-141	1.84	0	-103	1.92	0	-105	1.9	0
NH ₃	-166	1.98	0	-116	2.04	0	-113	2.0	0
H ₂ S	-159	2.33	70	-97	2.42	65	-76	2.35	30
HCOOH	-167	1.76	10	-125	1.81	0	-115	1.8	10
ImH	-205	1.86	5	-152	1.92	5	-147	1.85	5
F	-206	1.72	10	-160	1.77	10	-148	1.75	0
OH ⁻	-525	1.61	0	-465	1.64	0	-401	1.7	0
SH ⁻	-84	2.08	80	-404	2.17	70	-355	2.2	70
HCOO ⁻	-451	2.01	0	-384	2.06	0	-381	2.0	0
Im ⁻	-422	1.79	5	-358	1.84	5	-344	1.8	5

^aFor H₂O, H₂S, OH⁻, SH⁻ and HCOO⁻, θ is the angle between the molecular symmetry axis and the X...Zn⁺⁺ direction; for ImH and Im⁻, it is the angle between the bisector of the CNC angle and the N...Zn⁺⁺ direction; for HCOOH and F, it is the angle between the C=O bond and (=)O...Zn⁺⁺ direction.

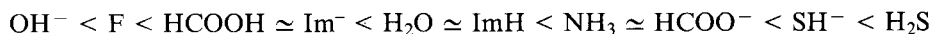
the binding of these molecules to the zinc dication modifies strongly the lability of their ionizable proton, the same results (except in the case of formamide) are reported for their deprotonated anion since they can also be present at the enzyme active sites.

The comparison of the semiempirical results with the corresponding ab initio values shows that the values of the binding energy are numerically closer with the modified parametrization than with the original values. The same improvement is obtained for the equilibrium distance with the exception of H₂S, SH⁻ and, to a smaller extent, the formate anion. The modification of the MNDO parametrization produces also a modification of the ordering of the interaction energies since, with the original values, ΔE is larger for H₂S than for water while the reverse order, given by the nonempirical calculations [33], is found with the modified parameters.

If we consider the results concerning the deprotonated molecules, we see from Table 2 that the agreement between the semiempirical and the ab initio results are not as good as in the case of the neutral molecules. In particular, MNDO overestimates by some 50 kcal/mol the interaction energy of Zn⁺⁺ with OH⁻ and SH⁻. The largest part of this difference is due to the use by MNDO of the same set of parameters for the anions and neutral molecules [18, 39]. The modification of the parametrization would correspond to the addition of the diffuse functions in ab initio computations [6, 27, 28, 40]. This is illustrated by the large ΔE value that we obtain for the Zn⁺⁺-OH⁻ system when we use the modified parameters which give an excellent value for the Zn⁺⁺-H₂O complex (Table 2). Therefore, the semiempirical method is not able to give in all cases the exact relative order of the binding energy of the zinc dication to the different deprotonated ligands considered.

The MNDO results do not compare favorably with the ab initio ones on another point, the energy required for deprotonation, especially when the molecule interacts with the zinc dication. The values of Table 3 show that the calculated values of the anions' proton affinities calculated by MNDO are overestimated by $\approx 20\%$ if we consider the free ligands but up to 100% in the case of their complexes with Zn^{++} since, in the case of water, the original parametrization gives a larger stability to $\text{Zn}^{++} - \text{OH}^- + \text{H}^+$ than to $\text{Zn}^{++} - \text{H}_2\text{O}$. This result shows that MNDO overestimates the decrease, produced by the presence of Zn^{++} , of the deprotonation energies. However, the values of Table 3 show also that the modified parametrization decreases the difference between the ab initio and the semiempirical values. Moreover, these two sets of calculations agree clearly to show that imidazole, when interacting with the zinc dication, has a much larger deprotonation energy than the three other molecules.

If, instead of the interaction energies, we consider the equilibrium distances (R_e) between Zn^{++} and closest heteroatom of the ligand, the comparison between the ab initio and the semiempirical values shows good agreement between the two sets of results for the deprotonated as well as for the neutral molecules since in every case we have for the values of R_e



Moreover, the two types of method give nearly identical values for the angles θ (see Table 2 for the definition of this angle in the different complexes).

The difference of agreement between the binding energies and the equilibrium distances is not surprising, since the results of Demoulin and Pullman [5], Kothekar et al. [33] and Pullman and Demoulin [34] have shown that the second quantity is much less sensitive than the first to the quality of the basis set used for the calculation and, therefore, to the degree of elaboration of the computational procedure.

The MNDO results concerning Zn^{++} interacting with one ligand tend to show that their geometrical arrangement is well given by the semiempirical method, and that the binding energies obtained with the 'modified parametrization' for the zinc dication are in fair agreement with the

TABLE 3
PROTONATION ENERGIES (IN KCAL/MOL) OF THE FREE AND COMPLEXED ANIONS

Anion	Experimental	Calculated		
		Ab initio	MNDO	
			O	M
OH^-	-390[41]	-387	-383	-383
SH^-	-352[42]	-336	-330	-330
HCOO^-	-345[42]	-351	-314	-314
Im^-	-353[51]	-355	-301	-301
$\text{Zn}^{++}\text{OH}^-$		- 83	+ 1	- 21
$\text{Zn}^{++}\text{SH}^-$		- 56	+ 4	- 23
$\text{Zn}^{++}\text{HCOO}^-$		- 89	- 31	- 52
$\text{Zn}^{++}\text{Im}^-$		-161	- 85	- 97

corresponding nonempirical values. In the case of deprotonated ligands, for which the differences between the values given by the two types of calculations are somewhat larger, our results lead us to think that MNDO energies which are supported by a satisfactory geometrical arrangement can be considered as being correct, at least at a semiquantitative level. This statement is further supported by the similarity of our nonempirical results, which are taken as reference with those obtained by other authors on some of the complexes that we have studied [5, 33, 34, 42].

In relation to the mechanism of action of thermolysin, the results of Table 2 show a feature worth underlining. Formamide, which can be considered as a model of the enzyme's substrate, has a binding energy larger than water on one hand and, on the other, formic acid and imidazole (which can be considered as models of Glu and His residues), the molecules which are coordinated to the metal at the active site. On the contrary, formamide has an interaction energy smaller by several hundreds kcal/mol than the deprotonated form of these molecules. This result shows clearly that a peptide group could definitely not displace the hydroxyl anion which has been postulated to be present during the reaction [43] while a neutral water molecule appears much more labile.

Zn⁺⁺ interacting with 4, 5 or 6 molecules of water

X-ray studies [44] as well as Monte Carlo calculations [45a,b] have shown that, in water, the zinc dication is coordinated to 6 molecules of solvent. But X-ray crystal studies have also shown that, aside from those in which Zn⁺⁺ is surrounded by 6 ligands [46], there exists a large number of complexes in which the dication interacts with four [1, 21, 22, 47] or five [43, 48] ligands. Furthermore, when five ligands are in the first coordination shell it appears that they adopt two types of geometrical arrangements depicted in Figs. 1A and 1B. The first one, called hereafter 5 (2-3), corresponds to a distorted trigonal bipyramid [48d, 49]. In the second, called 5 (1-4), the ligands form a pyramid with a square basis, the zinc being located somewhat out of the plane containing four of the ligands. The observed average value of the angle La-Zn⁺⁺-Le (La standing for the axial ligand and Le for the equatorial ones) is of 102° [49]. The optimization of the structure of Zn⁺⁺-(H₂O)_n complexes, with n running from 4 to 6, can give indications of the possibilities of the computational procedures used to reproduce the experimental data and of the differences be-

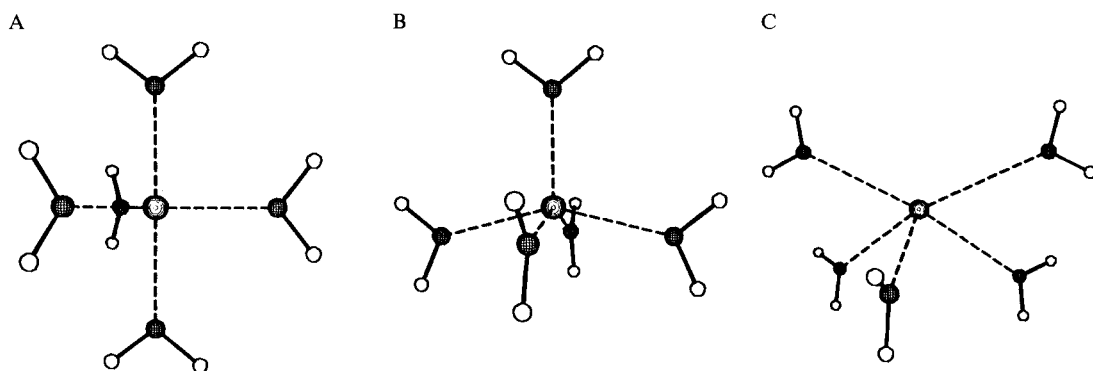


Fig. 1. Geometrical arrangements of the Zn⁺⁺-(H₂O)₅ complexes studied. A: trigonal bipyramid (5(2-3)); B: pyramid with a square basis (5(1-4)); C: configuration leading after optimization to the 5(1-4) arrangement.

tween the results of two types of computational methods or the two parametrizations, although the observed structures taken as references concern, except in the case of $\text{Zn}^{++}-(\text{H}_2\text{O})_6$, zinc dications interacting with different ligands.

In order to examine whether the two different types of structure observed for $\text{Zn}^{++}-(\text{H}_2\text{O})_5$ did correspond to theoretical energy minima we carried out, using MNDO, two optimizations using two very different initial geometrical arrangements. One was a 5(1-4) arrangement with a value of the $\text{La-Zn}^{++}\text{-Le}$ angle of 110° and a dication-ligand distance of 2.5\AA for the five of them. With this starting point, the minimization procedure gave us the 5(2-3) arrangement of the complex. The second starting point was the one shown on Fig. 1C; in this case the minimization gave the 5(1-4) arrangement. These results were obtained with both MNDO parametrizations, therefore the corresponding ab initio calculations have been limited to the optimization of the geometrical parameters of the two structures given by the semiempirical computations.

The results reported in Table 4 show that the three sets of calculations do give correctly the

TABLE 4
CALCULATED VALUES OF THE INTERACTION ENERGIES AND OF THE GEOMETRICAL PARAMETERS OF $\text{Zn}^{++}-(\text{H}_2\text{O})_n$ COMPLEXES FOR n RANGING FROM 4 TO 6*

No. of ligands		4	5		6
			5(1-4)	5(2-3)	
Ab initio	ΔE	-346	-395	-398	-446
	Re	1.96	2.00(a) 2.06(e) ($\theta = 102^\circ$)	2.06(a) 2.02(e)	2.09
MNDO O	ΔE	-350	-376	-378	-396
	Re	1.98	2.01(a) 2.06(e) ($\theta = 104^\circ$)	2.08(a) 2.04(e)	2.11
MNDO M	ΔE	-266	-285	-286	-301
	Re	2.04	2.07(a) 2.12(e) ($\theta = 104^\circ$)	2.07(a) 2.03(e)	2.17
Exp.	Re	2.01	2.07(a)	2.13(a)	2.08 (sol. [45])
		2	2.07(e) ($\theta = 102^\circ$)	2.02(e)	2.14 (cryst. [50])

* The values reported as experimental are average values obtained from a statistical study [50] except for the solution value [45]; (a) stands for axial and (e) for equatorial.

qualitative features of the geometrical arrangements of the complexes considered, that is, the increase of 0.13 Å of the Zn^{++} -ligand distance when going from 4 to 6 ligands [49] a smaller value of R_e for the equatorial ligands than for the axial ones in the 5(2-3) complex, and a value of 102–104° for the La- Zn^{++} -Le angle in the 5(1-4) one. However, in the 5(1-4) complex, theory gives a smaller R_e distance for the axial ligand than for the equatorial ones while, experimentally, these two distances are found to be statistically equal [49]. Concerning the two Zn^{++} -(H_2O)₅ complexes, we remark that the overall energy is in favor of the 5(2-3) arrangement by 1 (MNDO) or 5 (ab initio) kcal/mol. The larger difference obtained from the ab initio computations is due to the smaller average Zn^{++} -ligand distance given by this type of calculation. However, a closer examination of the tabulated values shows that the equilibrium distances obtained from the nonempirical calculations are smaller than the averages measured in crystals [46–49], but for Zn^{++} -(H_2O)₆ in excellent agreement with the value measured in liquid water [44]. The MNDO values are somewhat larger, especially with the modified parameters. The examination of the variation of the R_e values with the number of ligands shows that the ab initio calculations give a smaller increase of this quantity when going from 1 to 6 ligands than the MNDO ones, 0.19 Å (1.90–2.09) versus 0.27 Å (1.84–2.11) and 0.25 Å (1.92–2.17). We see also that the ab initio Zn^{++} -ligands interaction energy increases more rapidly with the number of ligands than the MNDO ones. These two differences are evidence that the MNDO method with the two parametrizations used in this work tends to underestimate the dication-ligand attraction with respect to the ligand-ligand repulsion, but is however able, as nonempirical computations are, to give the trends of the variations observed when the number of ligands increases.

Zn^{++} interacting with two 2-aminoethanethiol

We chose this complex as the next test for the MNDO method because, in this system, the metal cation is, as in the thermolysin active site, coordinated to four ligands which are not identical, as in the case of Zn^{++} -(H_2O)₄. Moreover, the crystal structure of a very closely related complex Bis (2 amino 1,1 dimethylethane thiolato) zinc is available in the literature [22]. Using the two MNDO parametrizations, keeping the bond lengths and angles of the two 2-aminoethanethiol molecules equal to the measured ones, we have optimized all the other geometrical parameters of the complex. The experimental and theoretical overall structures of the complex are shown in Fig. 2 and

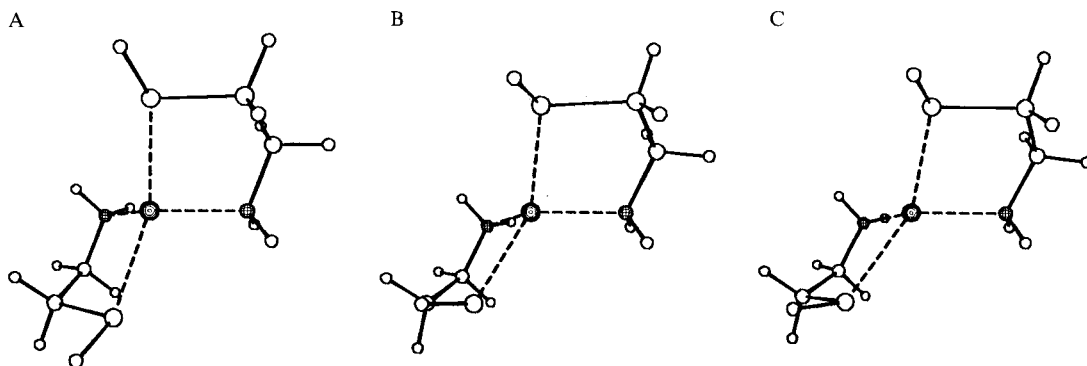


Fig. 2. Observed (A) and theoretical (B(O) and C(M)) conformations of the Bis (2 amino-ethanethiol)-zinc complex.

TABLE 5
EXPERIMENTAL^a AND THEORETICAL VALUES OF THE GEOMETRICAL PARAMETERS OF Zn^{++} -
(AMINO 2 ETHANETHIOL)₂

	Experimental ^b	Calculated	
		MNDO O	MNDO M
d Zn^{++} -N	2.06(0.01)	2.11	2.18
d Zn^{++} -S	2.30(0.004)	2.36	2.51
θ Zn^{++}NC	109(0.8)	114	116
θ Zn^{++}SC	93(0.4)	99	101
θ N(A) Zn^{++} N(B) ^c	123(0.6)	123	127
θ S(A) Zn^{++} S(B)	137(0.2)	128	126
θ N(A) Zn^{++} S(A)	91(0.3)	85	81
θ N(A) Zn^{++} S(B)	109(0.3)	119	124
φ NCCS	-58(1)	-50	-51
φ CN Zn^{++} S	-10(1)	-15	-19
φ NZn ⁺⁺ SC	-16(0.5)	-11	-8

^a The experimental data concern the Bis (2 amino 1,1 dimethylthiolato) Zinc [22].

^b Estimated standard deviations are given in parentheses.

^c The two ligands are labeled A and B.

the values of the main geometrical parameters which have been studied are reported in Table 5. The comparison of the three sets of values shows that the two computations give very similar results, which are in good agreement with the crystallographic data. The values of the Zn^{++} -ligands distances are somewhat overestimated by theory, as in the case of the complexes with 4 water molecules, but the conformation of the complex is given by the two computations with excellent precision. The only slight disagreement between theory and experiment concerns the relative value of the torsion angles about the NZn^{++} and SZn^{++} and 'bonds'. The larger value is calculated for the former angle while it corresponds to the smaller measured one. But the observed order of magnitude of these angles is well reproduced by theory. These results, if we keep in mind that the experimental data [22] do not concern 2-aminoethanethiol but its derivative, which has on carbon 1 two methyl groups which introduce repulsions which are not taken into account in our calculations, show that MNDO is able to give reliable results concerning a zinc dication interacting with moderately large ligands.

Zn^{++} interacting with models of the four ligands of native thermolysin active site

Using the two sets of MNDO parameters, the optimization of the intermolecular geometrical parameters was carried out for a zinc dication interacting with two imidazoles taken as models of His 142 and 146, a formate anion taken as model of Glu 166, and the molecule of water which is present in the native enzyme.

The two theoretical geometrical arrangements obtained are very similar and close to the experi-

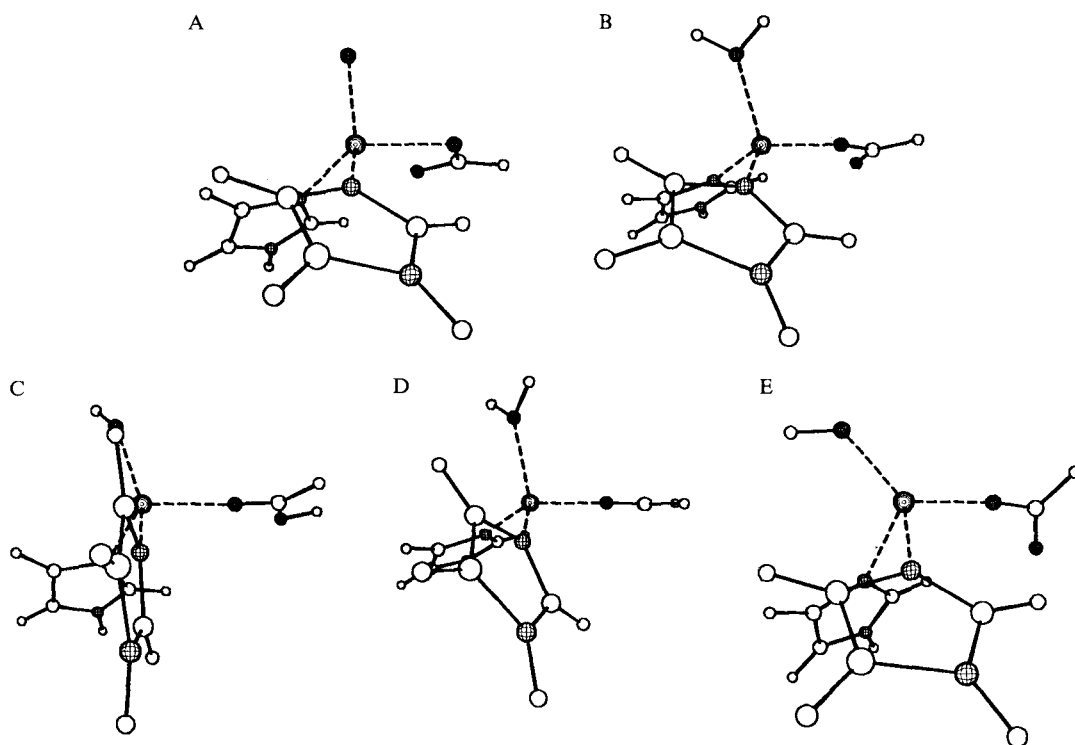


Fig. 3. Observed and theoretical geometrical arrangements of the four ligands coordinated to Zn^{++} at the thermolysin active site. A: experimental crystal geometry; B, C, D, and E calculated (with the modified parametrization) geometry for complexes in which the water molecule and the formic acid (Glu 166) are as $\text{H}_2\text{O}-\text{COO}^-$, $\text{OH}^- - \text{COOH}$, $\text{H}_2\text{O}-\text{COOH}$ and $\text{OH}^- - \text{COO}^-$ respectively. In the case of the experimental geometry the OH protons are not shown, since they are not available from the crystal data [21]. The imidazole ring at the foreground corresponds to His 146.

mental data as can be seen from Fig. 3A and B. The values reported in Table 6 show that the differences between the measured and calculated Zn^{++} -ligand distances are 0.02–0.21 Å with the original parametrization and 0.04–0.22 Å with the modified one. However, these results show some qualitative disagreements with the observed structure. The calculations lead to a carbonyl oxygen of the formate anion closer to the metal dication than the oxygen of the water molecule, whereas the reverse order is observed. In addition, the two histidines are calculated to be at the same distance from the metal when experiment shows that His 142 is somewhat further than His 146. On the other hand, the comparison of the different Zn^{++} -ligand distances which are observed in the native enzyme with the corresponding calculated values [5] (Table 2) suggests that the water molecule of the active site could be deprotonated since its distance to the zinc dication is smaller than the theoretical $\text{Zn}^{++}-\text{H}_2\text{O}$ Re value. These observations, added to the fact that the results reported in Table 3 show that if the deprotonation energy of formic acid is much lower than that of water, this difference no longer exists when these molecules are coordinated to Zn^{++} , induced us to rerun the optimization of the intermolecular arrangement of a complex in which the formic acid is neutral and the molecule of water deprotonated (replaced by a hydroxyl anion). The values reported in Table 6 show that the Zn^{++} -ligands distances given by these calculations are in excel-

TABLE 6

EXPERIMENTAL [21] AND THEORETICAL GEOMETRICAL ARRANGEMENTS OF THE COMPLEX FORMED BY A ZINC DICATION THE FOUR LIGANDS PRESENT AT THE ACTIVE OF NATIVE THERMOLYSIN

		Experimental	Theoretical							
			H ₂ O-COO ⁻		OH ⁻ -COOH		H ₂ O-COOH		OH ⁻ -COO ⁻	
			O	M	O	M	O	M	O	M
<i>Distances (Å)</i>										
Zn ⁺⁺ – OGlu166	2.08	1.87	1.90	2.08	2.17	1.99	2.05	1.91	1.94	
Zn ⁺⁺ – OWater	1.88	2.03	2.10	1.78	1.79	2.01	2.09	1.81	1.81	
Zn ⁺⁺ – NHis142	2.11	2.06	2.15	2.12	2.25	2.03	2.10	2.21	2.37	
Zn ⁺⁺ – NHis146	2.08	2.06	2.15	2.10	2.20	2.02	2.10	2.18	2.40	
<i>Angles (d°)</i>										
OGlu166 – Zn ⁺⁺ – OWater	94	104	105	105	108	101	100	124	130	
OGlu166 – Zn ⁺⁺ – NHis142	128	115	114	108	107	113	113	104	102	
OGlu166 – Zn ⁺⁺ – NHis146	95	109	111	99	100	110	110	101	101	
Zn ⁺⁺ – O – CGlu166	108	154	154	178	174	178	176	162	168	
Zn ⁺⁺ – N – C4His142	120	126	127	122	121	122	122	127	127	
Zn ⁺⁺ – N – C4His146	135	126	126	119	119	123	123	124	124	

The values calculated with the two MNDO parametrizations are reported for the ionization states of the water and formic acid molecules.

lent agreement with those observed in native thermolysin if we take into account that MNDO underestimates by ≈ 0.1 Å the Zn⁺⁺-OH⁻ Re, as can be seen from Table 2. We can remark that the agreement is particularly good for the distances given by MNDO original parametrization. This result could be expected since it is for Zn⁺⁺-(H₂O)₄, in other words a zinc dication interacting with four ligands as in the present case, that MNDO with Dewar's parameters and ab initio computations, which can be reasonably thought to give correct geometrical arrangements, give the closest results. But we see from Fig. 3C that the theoretical orientation of two ligands is in poor agreement with experiment, the discrepancy being particularly pronounced for His 146 imidazole. Moreover, the calculations indicate that this complex is less stable by ≈ 1.5 kcal/mol than the one in which the water molecule is neutral.

The differences between the results obtained from these two pairs of calculations induced us to rerun similar optimizations for the complexes in which both the water molecule and the formic acid are neutral or both deprotonated, in order to determine the origin of the differences obtained between the two preceding sets of computations. The corresponding values reported in Table 6 show that if, again, the calculated Zn⁺⁺-ligand distances are not very different from the measured ones, they are not as close as those obtained for the complex in which we have OH⁻ and HCOOH. However these last results show also that it is for the complex containing the two deprotonated molecules (OH⁻ and HCOO⁻) that the observed relative order of the Zn⁺⁺...O and Zn⁺⁺...N distances are correctly given by theory. Moreover, Fig. 3E shows that in this case there is no major difference between the calculated and observed orientations of the ligands.

These sets of results induced us to introduce into our calculations a model of the carboxylic group of the Glu 143 residue. The geometry optimization was run for two complexes, one in which both formic acids are anions and the water molecule neutral and one in which we have a hydroxyl anion and a neutral formic acid for Glu 143. The results of these calculations show that the second complex is more stable than the first by more than 15 kcal/mol, even when the modified parametrization, which gives a smaller $\text{Zn}^{++}\text{-OH}^-$ interaction energy than the original one, is used. The calculated values of the Zn^{++} -ligand distances are equal, and therefore not reported, to those obtained previously for the complexes containing only four ligands. The greater stability of the complex with an OH^- and a neutral Glu 143 could be expected from the values reported in Table 3. The protonation of a formate anion produces an energy gain of more than 300 kcal/mol [50] while the deprotonation of a water molecule coordinated to a zinc dication produces a decrease of energy of less than 100 kcal/mol [5, 35]. The difference in energy between the two complexes is much smaller than 200 kcal/mol, because Glu 143 has a nonnegligible interaction energy with the metal cation which is, in addition, coordinated to several ligands, not only to the hydroxyl anion.

Since in the theoretical arrangements reported in Figs. 4B and 4C, the absolute value of the Zn^{++} -ligand distances are somewhat different from the measured ones (the Zn^{++} -Glu 143 in particular is too large by $\approx 1 \text{ \AA}$), and could modify the relative energies of the two complexes considered, the computations were rerun, keeping the experimental geometry, the optimization being limited to the orientation of the OH bonds which are given by the X-ray data. In this case, the complex containing the hydroxyl anion is more stable by 10 kcal/mol than the one containing the water molecule. Therefore, our results strongly suggest that, in the crystal state, the water molecule which is at the active site of native thermolysin has transferred one of its protons, probably to Glu 143 since it is the closest formate group, and is in fact a hydroxyl anion. At this point we can mention that this result is in agreement with those of Nakagawa et al. [36]; their *ab initio* computations have shown that the most stable structure of a model of carboxypeptidase-A active site is the one in which the water molecule coordinated to the zinc dication has transferred one of its protons to the carboxylic anion of Glu 270.

If, in agreement with our theoretical results, it is a hydroxyl anion which is present at the ther-

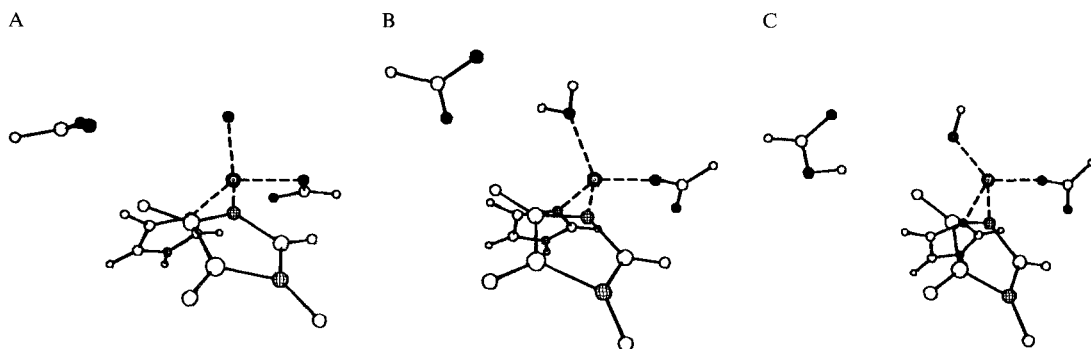


Fig. 4. Observed and theoretical geometrical arrangements of the complex formed by models of His 142, Glu 143, His 146 and Glu 166, H_2O and Zn^{++} . A: experimental crystal geometry [21]; B, and C calculated (with the modified parametrization) geometry for complexes in which the water molecule and Glu 143 are as $\text{H}_2\text{O-COO}^-$ and OH^--COOH , respectively.

molysin active site, the calculated relative values of the $\text{Zn}^{++}\text{-OH}^-$ and $\text{Zn}^{++}\text{-F}$ binding energies show that the carbonyl oxygen of the substrate peptide bond and the OH^- anion will be coordinated simultaneously to the metal, as proposed by Holmes and Matthews [43]. The hypothesis of the presence of an hydroxyl anion at the active site of thermolysin is to some extent supported by the results obtained on complexes between this enzyme and a number of inhibitors. They appear to be coordinated to the metal cation through anionic sulfur [47a] or oxygen [43, 48c] atoms which are the only ones to have an interaction energy of the same order of magnitude as OH^- . In relation to this interpretation we can add that the calculated values (MNDO with the modified parametrization) of the binding energies of the water molecule and of the hydroxyl anion at the thermolysin active site are, respectively, 12 and 326 kcal/mol, a result showing that the presence of the other ligands does not modify appreciably the differences found between these two entities when they are the only ligand coordinated to the zinc dication.

CONCLUDING REMARKS

The set of results obtained from the part of this study devoted to small model systems shows that MNDO is a semiempirical method which is able to give values of Zn^{++} -ligands binding energies and geometrical arrangements of the complexes formed which are in reasonable agreement with those obtained from *ab initio* calculations. After some minor modifications of some of the zinc parameters, most probably made necessary by the fact that we are dealing not with the neutral metal but its dication, it was possible to limit the differences between the two types of results. If MNDO tends to somewhat overestimate the Zn^{++} -ligand binding energy, it appears to give satisfactory geometrical arrangements for the complexes studied.

For larger systems, including several atoms coordinated to the metal dication, MNDO results do give all the features of the experimental data and results of the *ab initio* calculations. However, they tend to underestimate the increase of binding energy with the number of ligands; this shows that the variation, with the metal-ligand distance, of the binding energy given by the semiempirical method, with the two parametrizations used in this study, is not identical to the nonempirical one, and that some improvements might be desirable along this line.

The results concerning the model of a zinc metalloenzyme active site that we have studied show that the method does give a geometrical arrangement close to that observed in the crystal of the native protein. They show also that the increase of lability of the water protons which is produced by the coordination of the molecule to the zinc dication is most probably an important, if not determining, factor in the mechanism of action of thermolysin and for the structure of its (potential) inhibitors.

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