Inorg. Chem. **2003**, *42*, 4245–4247

Protonation State of Asp120 in the Binuclear Active Site of the Metallo-β-Lactamase from *Bacteroides fragilis*

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Received September 26, 2002

The determination of the protonation state of enzyme active sites may be crucial for the investigation of their mechanism of action. In the bizinc β-lactamase family of enzymes, no consensus has been reached on the protonation state of a fully conserved amino acid present in the active site, Asp120. To address this issue, we carry out here density functional theory (DFT) calculations on large models (based on *Bacteroides fragilis* X-ray structure) which include the metal coordination polyhedron and groups interacting with it. Our calculations suggest that Asp120 is ionized. The relevance of this finding for site-directed mutagenesis experiments on the 120 position and on the mechanism of action is discussed.

β-Lactam antibiotics counteract a great variety of bacterial infections.[1] Their beneficial therapeutic effect is based on their selective inhibition of enzymes involved in the biosynthesis of the bacterial cell wall.[2] Unfortunately, their action is severely limited by the bacterial expression of β-lactamases, which are able to hydrolyze the β-lactam ring moieties of these drugs.

Among the several classes of β-lactamases[1,3,4] so far characterized, Zn(II)-based metallo-β-lactamases have been spread on a wide number of pathogenic bacteria.[5−7] These enzymes share a common folding motif, and they are classified into B1, B2, and B3 subclasses, according to sequence homology.[8] They are active either as mononuclear or binuclear species. In the mononuclear species whose 3D structures have been determined so far,[9] the Zn(II) ion binds to three histidines and to a hydroxide group, which possibly acts as nucleophilic agent against the β-lactam ring.[10−12] The binuclear species features the same coordination geometry for one Zn(II) ion and a trigonal bipyramidal geometry for the other. The latter involves three protein ligands, a water molecule, and a hydroxide/water bridging the first Zn(II) ion.[13−15]

The currently available enzymatic reaction mechanisms for both species are based on an a priori assumption of the protonation state of a key residue, Asp120.[8] This residue is fully conserved,[8] and its replacement by noncharged groups impairs the catalytic efficiency.[16−18] Asp120 H-bonds either to the Zn1-bound OH (in both species) or to Zn2 (binuclear form, Figure 1).

Recent ab initio calculations suggest that, in the mononuclear species, Asp120 is ionized.[10] In the binuclear form, in contrast, the protonation state has not been unequivocally established.[19,20] Here we address this issue by theoretically investigating the energetics and structural properties of the

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10.1021/ic026059j CCC: $25.00
Published on Web 06/12/2003

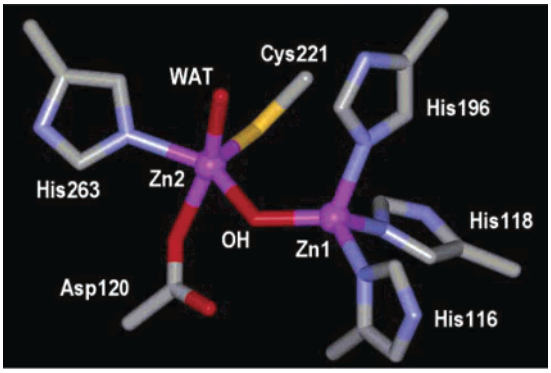


Figure 1. Active site of Zn(II)-β-lactamase from *B. fragilis* (1znb).[13] Zn1 (purple sphere) is tetrahedrally coordinated by His116, His118, His196, and a water/hydroxide molecule (OH). Zn2 is pentacoordinated in a trigonal bipyramidal conformation: His263, Cys221, and OH lie in a pyramidal plane; Asp120 and a water molecule (WAT) are the axial ligands. Asp120 H-bonds the nuclephilic agent OH.

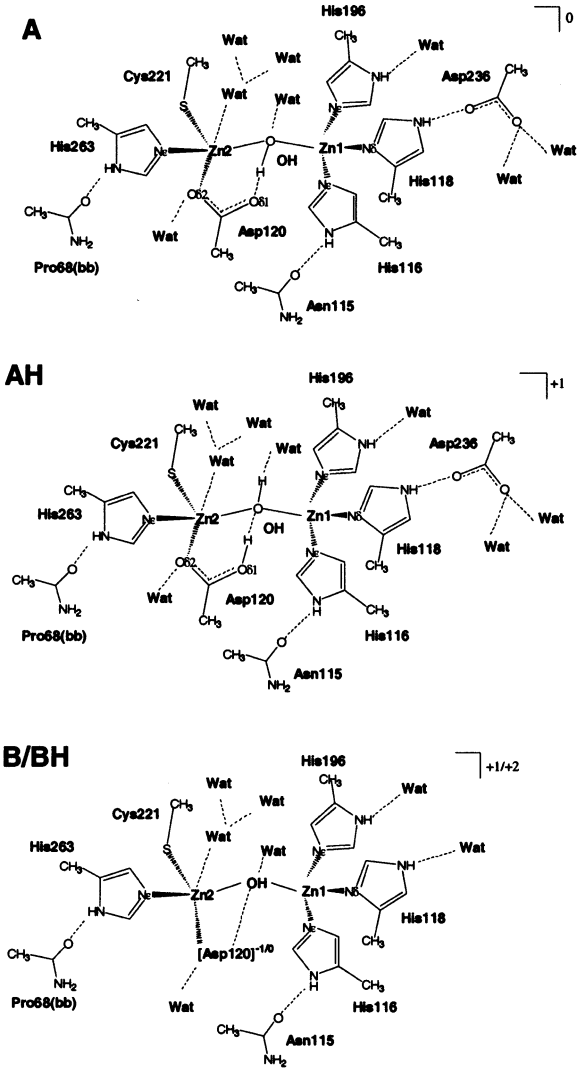
binuclear active site in the enzyme CcrA from *Bacteroides fragilis*, which represents the prototype of binuclear subclass B1 enzymes.

Density functional theory (DFT) based calculations are performed on the structure of the enzyme CcrA from *B. fragilis*, solved at 1.85 Å resolution at pH 7.0 (pdb entry 1znb).[13] This is the structure of a metallo-β-lactamase solved at the highest resolution so far. It belongs to the subclass B1. Two distinct chains are present in the unit cell (**A** and **B** hereafter), and they exhibit small structural differences: the rmsd of the metal coordination polyhedron is ≈0.5 Å. Our starting models include the Zn(II) coordination polyhedron (see Figure 1). Furthermore, the metal ligands H-bond to protein groups and/or to the solvent (Chart 1): His116 interacts with Asn115, His263 with the backbone of Pro68, His118 either with Asp236 (complexes **A** and **AH**, H stands for Asp120 protonated form) or to the solvent (**B** and **BH**). His196, Asp120 and Cys221 are also exposed to the solvent.

The Zn(II)-bound His residues have been all considered neutral; Cys221 was considered deprotonated, consistently with previous proposals[22] and with the observation that a significant decrease in the pK_a of methyl-thiolates occurs when they are bound to Zn(II) ions.[23]

Our calculations were performed in the framework of the density functional theory. The CPMD program V3.4 was used for the calculation.[24] The BLYP[25,26] generalized gradient approximation exchange-correlation functional was used. The valence shell electrons were described by a plane wave basis set up to an energy cutoff of 70 Ry. The interaction between

Chart 1. Quantum Chemical Models Used in This Work[21]



valence shell and core electrons was described by norm-conserving pseudopotential of the Troullier−Martins type.[27] The energetics of different protomers were corrected with an energy term which takes into account the proton hydration enthalpy, as in Dal Peraro et al.[10] The models were inserted in an orthorhombic box of variable size, and no constraints were imposed. The method of direct inversion in the iterative subspace was used for the optimizations of the ionic positions.[28] They were treated as isolated systems using the method of Martyna and Tuckerman.[29] This setup has been shown to accurately describe the structural determinants of the Zn(II) site in the mononuclear BLII enzyme[10] and in horse liver alcohol dehydrogenase.[30]

Calculations carried out with our computational setup on models representing only the metal coordination polyhedron (Chart 1SI, Supporting Information) turned out to provide different results from those obtained with the extended

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Table 1. Zn(II) Coordination Shell Distances

	A	AH	B	BH	X-ray (A/B)
Zn1−Nϵ@His116	2.08	2.05	2.07	2.03	2.14/2.09
Zn1−Nδ@His118	2.06	2.00	2.11	2.09	2.07/2.08
Zn1−Nϵ@His196	2.12	2.10	2.10	2.09	2.08/2.04
Zn1−O@OH	1.97	2.08	1.95	2.03	1.88/1.91
Zn1−Zn2	3.61	3.61	3.59	3.65	3.47/3.48
Zn2−O@OH	2.05	2.10	2.06	2.14	2.06/2.03
Zn2−Oδ2@Asp120	2.15	2.47	2.21	2.60	2.25/2.12
O@OH−Oδ1@Asp120	2.69	2.60	2.63	2.62	2.69/2.57
Zn2−Nδ@His263	2.11	2.09	2.12	2.08	2.10/2.13
Zn2−S@Cys221	2.34	2.31	2.36	2.31	2.30/2.37
Zn2−O@WAT	2.66	2.34	2.72	2.30	2.27/2.34

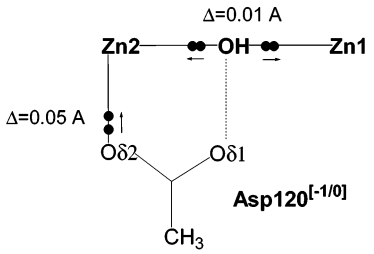
models **A/AH** and **B/BH** in Chart 1 (vide infra). Thus, our preliminary calculations provide further evidence that inclusion of second-shell ligands is required to correctly describe structure and energetics of Zn(II)-based enzymes.[10] We further notice that these calculations were in good structural agreement with previous DFT calculations on similar models with a localized basis set and a different recipe for the exchange-correlation functional.[19,20]

Two molecules (**A** and **B**) are present in the asymmetric unit in the crystal structure of the enzyme CcrA from B. fragilis[13] (Chart 1). They differ by the group H-bonding to His118, which is Asp236 and two water molecules in chains **A** and **B**, respectively. The inclusion of Asp236 in our models significantly affects the energetics: whereas the difference in stability between **A** and **AH** is very small (3.8 kcal/mol), **B** is more stable than **BH** by as much as 42.9 kcal/mol. Thus, it is impossible to conclude from these data alone whether Asp120 is deprotonated or not.

The structural features are more revealing. Indeed, in all circumstances, protonation of Asp120 disrupts the bonding features with the Zn2 (Table 1). In the deprotonated forms (**A** and **B**), instead, the bond is well reproduced. However, the bonding features of Zn2 with a terminal water molecule present in the active site channel and exposed to the bulk solvent are better reproduced in the protonated form (**AH** and **BH**). Test calculations on models in which water molecules not interacting with the metal ions are absent (see Chart 1SI and Table 1SI, Supporting Information) show that the displacement of the Zn2-bound water (WAT) is even larger. Thus, Zn2−WAT relatively large bond lengths (for models **A** and **B**) might result from the absence in our complexes of the overall bulk solvent effects present in the active site cavity. The rest of the structural features are fairly well maintained for all models investigated here (Tables 1 and 1SI[31]). Hence, our calculations indicate that a regular Zn2−Asp120 bond in the two chains in the 1znb crystal structure can only be reproduced by assuming that Asp120 is deprotonated.

This finding offers a rationale for the decrease of catalytic efficiency upon replacement of Asp120 with Asn.[16] Indeed, one may speculate that the change from one H-bond acceptor to an H-bond donor may highly affect the nucleophilic OH (Chart 1), which in turn may affect the interaction with the substrate. Furthermore, the fact that Asp is deprotonated may help explain why the Asp120Cys and Asp120Ser mutants[17] are even less active than the Asp120Asn mutation. Indeed, the removal of H-bond interactions between the Zn-bound OH and these residues further affects its nucleophilic properties.

Chart 2. Shifts of Relevant Boys' Orbital Maxima (●) upon Asp120 Deprotonation



The analysis of the electronic structure in terms of maxima of Boys' orbitals provides a vivid picture of chemical concepts such as electron lone pairs and chemical bonds.[32−34] In our models, deprotonation of Asp120 clearly decreases the polarity of the Zn2−Asp120 bond, as the Boys orbital maximum along this bond is significantly shifted toward the metal ion (Chart 2).

The deprotonation has also an effect, albeit smaller, on the electronic structure of the hydroxide group bound to the two Zn(II) ions, that is the nucleophilic agent.[35] Indeed, the replacement of an H-bond donor (protonated Asp120) with an acceptor (ionized Asp120) decreases the polarity of the Zn−O bonds.

In conclusion, our calculations support the mechanism of Wang et al., that rely on assuming a deprotonated Asp120.[35] Furthermore, they indicate that deprotonation of Asp120 is accompanied by a dramatic change of Zn2−Asp120 bonding and a small, yet significant, change of the chemical properties of the nucleophilic agent (OH), which in turn could be important for the enzymatic mechanism.

Acknowledgment. This work has been supported by grants from Istituto Nazionale di Fisica della Materia (INFM) and from Agencia Nacional de Promoción Científica y Tecnológica (99-01-6616), CONICET (PIP 0582/99), and HHMI to A.J.V. A.J.V. is a staff member from CONICET, a Carrillo-Oñativia fellow (Ministry of Health), and an International Research Scholar of the Howard Hughes Medical Institute. CINECA supercomputer center is also acknowledged for support.

Supporting Information Available: A further chart of minimal test models and an exhaustive table of structural data. This material is available free of charge via the Internet at http://pubs.acs.org.

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