Binding and Hydrolysis of Ampicillin in the Active Site of a Zinc Lactamase

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Binding and hydrolysis of ampicillin are described in a model active site derived from dinuclear *B. fragilis* zinc lactamase. The protein binding site consists of the two zinc cations bound with a bridging hydroxide and ligands from the first-shell residues, conserved residues near the zinc site, and the moveable loop of residues from numbers 43−53. The model active site consists of the first-shell residues, the conserved residues, and glu45 and glu47 from the moveable loop. Ampicillin is primarily located in the active site by the binding of the thiazolidine ring's extra-cyclic carboxylate to the ammonium of conserved lysine 184 when water bound to Zn2 in the active site is retained. A comparable strong salt bridge is formed between the ammonium of the ampicillin zwitterion and glu45 on the flexible loop that moves mostly as a unit at least 10 Å to complete the binding site. The zwitterion character of this antibiotic influences the final docking arrangement and ultimate reaction path. Classical molecular dynamics, in the presence of Zn2 bound water, Wat1 and Wat2, and a limited number of waters placed around the ionic groups in the active site, determined a number of reactive docking conformations. One of the low-energy structures with strong interactions to glu45 and glu47 was chosen by the reactive proximity of the nucleophilic water, Wat2, to calculate the reaction path for binding reactant, intermediates, and product for the initial hydrolysis reaction. Water is added to solvate the classical reactant structure, and the reaction path was calculated quantum mechanically within a model chosen from the molecular mechanics structure. Two waters were found in a productive conformation for hydrolysis, the water bound to Zn2 (path 1) and water bound to the ampicillin carboxylate (path 2). In path 1, the hydrolysis product is only bound to the enzyme through hydrogen bonds and can be released by solvating these bonds. Additional proton-transfer steps from the initial product can occur, however, to create intermediates from this product stabilized by interaction with the Zn1 cation. The product formed in path 2 is bound directly to Zn2 suggesting that neither zinc is specially chosen for a catalytic role. Within this model the entire active site is utilized for both binding and catalysis in the case of ampicillin. Strong polar hydrogen bonds are found to the substrate, the waters in the active site, and the residue ligands present in the active site. Autocatalysis or assistance in water activation by the carboxylate of the antibiotic is found and likely to be general. The proton abstracted from the water can park on a number of anionic or polar atom sites in the active site leading to a range of intermediates. The lactam ring C−N bond does not break with prior protonation of the nitrogen or with the initial attack by the hydroxide abstracted from the nucleophilic water but requires attack of the hydroxide at the carbonyl carbon either prior to proton binding or concurrently. This study provides insight into a wider variety of antibiotic docking and shows that more than one reaction path is possible within the highly ionic active site of a bimetallic lactamase.

1. Introduction

Antibiotic resistance of microbial pathogens has developed in part due to the evolution of metallolactamases with wide substrate specificity. Study of the structure and reactivity of this class of enzymes has yielded considerable detail on the structure of the active site of this enzyme.[1,2] One zinc atom, denoted as Zn1 following the notation of Concha et al.,[1] is coordinated to four ligands, three His residues, and a bridging hydroxide denoted as Wat1. The second zinc atom denoted as Zn2, is coordinated to five ligands, one additional His residue, one Asp and one deprotonated Cys residue, the bridging hydroxide, Wat1, and a water molecule denoted as Wat2 (see Scheme 1a). The initial crystal structure of the bimetallic active site suggested two possible nucleophiles, the bridging hydroxide, Wat1, or the water bound to Zn2, Wat2.[1] Docking of antibiotic molecules[1] suggested binding to the conserved residues, lys184 and asn193. Stabilization of the developing charge on the acyl-intermediate in the first step of the hydrolysis was suggested by interaction with Zn1. The final protonation step was proposed from the water bound to Zn2. Kinetic analysis has supported a range of speculation on the hydrolysis mechanism[3,4] suggesting an anionic intermediate and rate-limiting proton transfer to the lactam N required to break the lactam C−N bond. However, none of these experimental or any theoretical studies has determined, as yet, the structure of any point along the reaction path of the hydrolysis of any antibiotic. The spectra of reactive intermediates of nitrocefin in a metallolactamase [5] and in an

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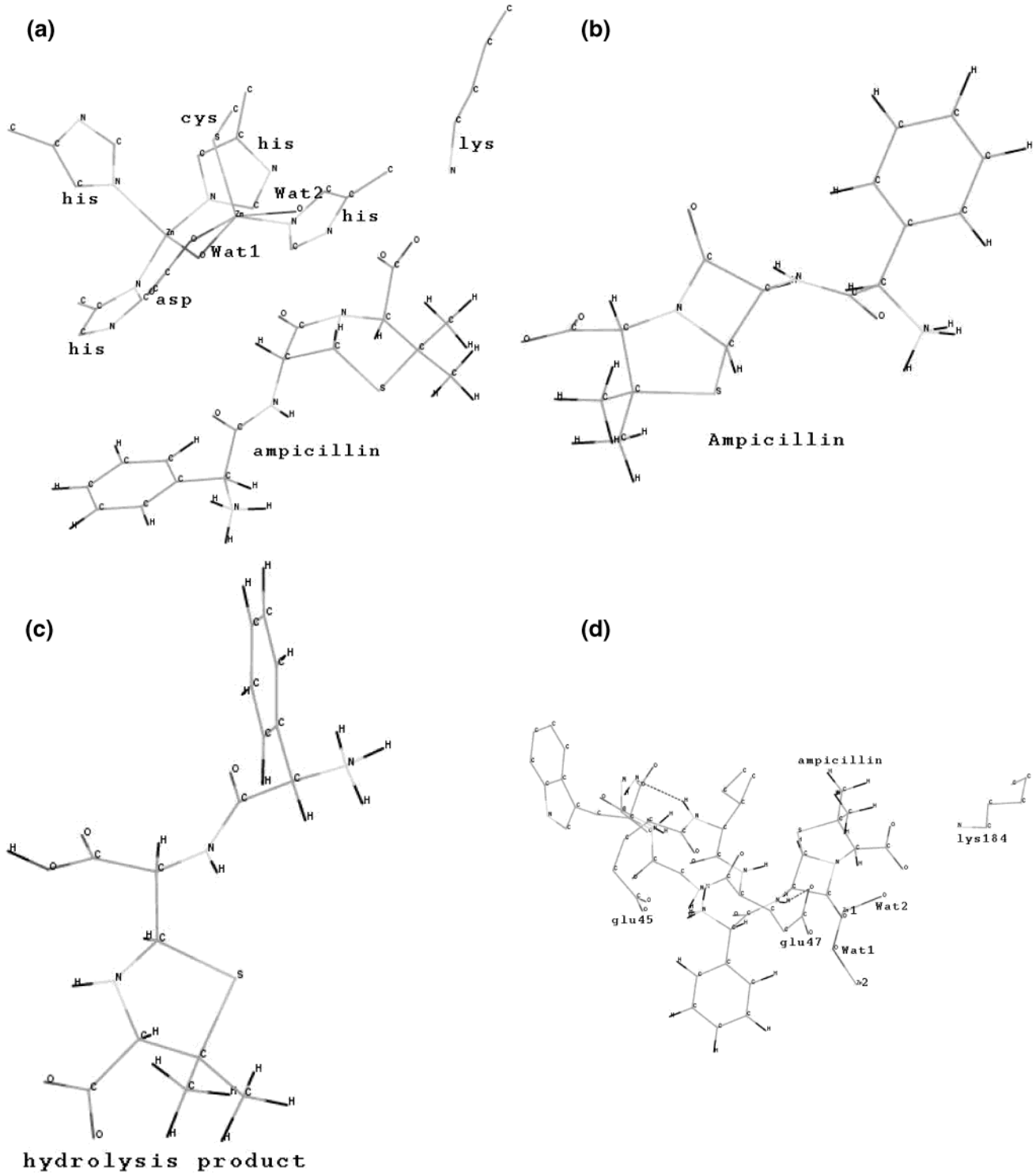
SCHEME 1 [a]



[a] (a) Representation of the metal active site of zinc lactamase relative to the bound substrate, ampicillin. (All figures were obtained with MOLDEN, G. Schaftenaar and J. H. Noordik, "Molden: a pre- and post-processing program for molecular and electronic structures", *J. Comput.-Aided Mol. Design* **2000**, *14*, 123−34.) (b) Zwitterion representation of ampicillin with carboxylate on thiazolidine ring and ammonium on the amide linkage. (c) Hydroxide attack at the lactam carbonyl carbon followed by protonation of the lactam nitrogen opens the lactam ring. (d) The ampicillin is docked between the binding of the lys184 to the carboxylate of the thiazolidine ring and the ammonium of ampicillin to the carboxylate of glu45.

enzyme mimic[6] provide insight but not a definitive picture of the structure along a reaction path. There is also the possibility that nitrocefin hydrolysis is atypical in metallolactamases.[7]

Concha et al.[1] also observed that residues 45−51 have large crystallographic temperature factors suggesting this loop could adjust upon substrate binding and form part of the binding pocket. Structural[8] and spectroscopic information[9,10] on the binding of inhibitors has been obtained which provides insight into the flexibility of loops in the enzyme that are required for binding. However, the electronic characteristics of the inhibitors differ from the lactam antibiotics and may not provide a clear picture of the binding of such a substrate. The binding of 4-morpholineethanesulfonic acid (MES)[8] and the NMR-derived chemical shifts[9] provide important insight into the movement

of a flexible flap that helps form the active site on the solvent side. However, the ionic character of ampicillin is sufficiently different from the inhibitors used in these studies that the binding is necessarily different. This is particularly true when an inhibitor like thiomandelic acid is used since the NMR data suggest that the inhibitor thiol binds to both zinc cations. The binding can compete with the bridging hydroxide for the zinc cations reminiscent of theoretical structures describing a lactamase first shell with two bridging ligands[11] or displaces the hydroxide as suggested in a modeling study[12] or for cysteinyl inhibitors.[13] Binding of a thiolate inhibitor directly to the metal is observed for the IMP-1 metallolactamase[14] and also suggested in a modeling study of the binding of captopril in zinc lactamase.[15]

A variety of theoretical calculations have leveraged the available experimental structural information to provide more information on the metal binding and the docking of an inhibitor. Binding of the substrate or an inhibitor is apparently dependent on the behavior of the flexible flap that covers the active site[8−10] otherwise open to solution. An intriguing result of a modeling study of inhibitor binding[12] is the movement of the flap toward the active site when water is removed from the simulation. The reaction path for a lactam substrate has been calculated only for a mono-zinc model of *B. cereus*[16,17] and the dynamics of the active site without the flap were explored for the dizinc enzyme[18] A molecular modeling study of the dinuclear enzyme[19] has examined the binding along the reaction pathway for benzylpenicillin and suggested that Zn2 can accept a direct bond from the lactam nitrogen. However, this note also suggests that the waters in the active site are to be identified more with Zn1 in a structural role. The water, W2, that was found bound to Zn2 in the fragilis structure, has often been identified with the nucleophile in the zinc lactamase. It is important to note that this water is maintained in the binding of the MES inhibitor and is also hydrogen bonded to the inhibitor.[8] In a recent analysis, we have suggested that additional waters in the active site may also play direct role in the hydrolysis. Theoretical analysis does suggest that a number of docking modes are possible for this dinuclear enzyme. The very ionic active site of the dinuclear enzyme, however, can support a number of water ligands that are not evident in a crystal structure, and it has been suggested that one of these waters may supply the nucleophile.[11] The binding of a number of waters in the active site was also found to stabilize the Zn1−OH−Zn2 bridge by preventing the activation of a single water allowing the transfer of a proton to the bridging hydroxide. W2 is calculated to be strongly hydrogen bonded to the bridging hydoxide which maintains the presence of this water in the binuclear active site. Protonation of either the bridging hydroxide or the aspartate hydrogen bonded to the bridging hydroxide leads to a substantial increase in the Zn−Zn distance.[11,18] The active site maintains its rigidity if it is not protonated and fully solvated. Activation of the water can also result from a number of protein residues either directly bound to the zinc cations or from ionic residues located on the loop region that can interact with the substrate. The aspartate and bridging hydroxide are mostly protected from protonation by the strong hydrogen bond found between these moieties.

This paper is intended to provide insight into the variety of docking modes and concomitant reaction paths that are possible. The *B. fragilis* crystal structure is used to initiate the calculation since the reactivity of this enzyme is optimal in the dinuclear structure. In the report of this structure, Concha et al. found that all three model lactams—ampicillin, ceftazidime, and imipenem—bind the omnipresent lactam carboxylate through the water bound to the Zn2 cation.[1] This water is in a position that it can function as the nucleophile with the proton removed by the substrate carboxylate, an example of substrate-assisted catalysis. Maintaining this water determines the docking behavior of the antibiotic. The bridging hydroxide is retained when the MES inhibitor is bound. The apical water is displaced in cases where the bridging hydroxide is displaced by a thiol ligand.[12−15]

This study will examine a model of the binding of ampicillin in the active site of *B. fragilis*. Ampicillin (Scheme 1b) was chosen because it is a zwitterion in solution and presents at least two sites for a salt bridge to the protein. The ionic binding also presents a strong interaction between the substrate and protein that suggests initial docking positions and facilitates strong interactions capable of being modeled with classical potentials. The hydrolysis of ampicillin is accomplished by hydroxide attack at the carbonyl carbon of the lactam ring and the subsequent protonation of lactam nitrogen which opens the ring leading to the product (Scheme 1c).

The calculational procedure is initiated by a molecular dynamics docking search and then uses the classical structure as the basis of quantum calculations similar to those utilized earlier in a study of the water binding in the active site of zinc lactamase.[11] In this case we are searching for reactive conformations of the docked substrate. The ligands, in this case ampicillin and additional waters, are docked with the carboxylate of the substrate bound through water to Zn2 as well as to the conserved lysine, lys184. A constrained optimization of the protein with ampicillin finds that the loop from residues 41−53 has moved about 10 Å. In addition to W2, only six waters, solvating ampicillin and neighboring ionic sites of the active site were retained. Removal of the water solvation shell of the protein should facilitate the movement of the flap as observed in a simulation of the apo-protein.[12] The flap motion would seem to be driven mostly by the removal of the water. There are several reactive binding modes to the ampicillin driven by the final ionic interactions between the flap and zwitterionic substrate. The reactive modes are found by examining the low-energy structures for the proximity of the Wat2 water to the carbonyl carbon of the lactam ring. The docked structures optimized with classical potentials cannot accurately describe the ionic interactions and, particularly, the hydrogen bonds between the ligands, water, and active site. The classical model structure (Scheme 1d) provides the initial starting point for ab initio Hartree−Fock determination of the reaction path of the hydrolysis reaction. Ab initio calculations are necessary for the reaction path and to ensure that the hydrogen bond arrangements are more accurately determined especially where proton transfers can occur. The two reaction paths presented here are representative of a number that can be calculated, but the docked reactant structure is a calculated low-energy structure and the two waters chosen as nucleophiles are closest to the lactam ring to be hydrolyzed. The reaction paths are exploratory and the structures obtained for the reaction path are new in detail. One path will be seen to be in the spirit of suggestions made from the experimental spectroscopic and structural data but the other is substantially different from experimental deductions.

2. Method

Classical MD simulations were performed using the CFF91 force field and Discover software.[20] The starting structure is chain A of structure 1znb[1] in the protein data bank.[21] The missing residues 48 and 49 were built into the loop region from 41 to 53. The end residues of the loop were fixed and the loop

alone was energy minimized at the end of a 500 ps molecular dynamics (MD) simulation. The lowest energy structure of the loop is then replaced into the protein. Water (Wat1 and Wat2) bound to Zn2 and a restricted number of active site waters are kept in the model. The pH was assumed to be 7 so that glutamate and aspartate are −1; lysine and arginine are +1. All other amino acids are neutral except for cys181 which is −1 because it is ligated to Zn2. Ampicillin (Scheme 1b) is docked with the carboxylate on the thiazolidine ring bound to the lys184. Without additional waters bound to the flap, a minimization protocol is run on only the loop contribution to the binding of the substrate. A series of 500 ps MD simulations are done at 300 K with an energy minimization of ampicillin, amino acids 42−51, lys184, asn193, and the hydrogens of the bridging hydroxide and Wat2 from each 5 ps snapshot. Explicit solvation of the protein was avoided by using a value of 4 for the dielectric constant. Docking structures are examined along the entire trajectory. Depending on the initial docking position, a number of docking conformations are obtained. The lactam and thiazolidine rings are in similar positions along the trajectory because of the ion-pair interaction with lys184 that is maintained throughout the trajectory. A low-energy structure from the simulation in which two salt-bridge bonds are formed was chosen as the starting docked structure for a quantum calculation of the reaction path because two waters are in position for nucleophilic attack on the lactam ring. With the flap already closed over the substrate, the energy minimization is driven by the strong interaction between the ammonium and carboxylate moieties on the ends of ampicillin acting with their charge counterparts in lys184 and glu45 in the loop. The other conserved residue allowed to optimize, asn193, is found to bind weakly to glu47 in this structure but is over 4 Å from the ampicillin amide. It is neglected in the construction of the quantum model based on the conformation where glu45 and glu47 are the strongest binding partners to ampicillin. In other docking conformations asn193 does bind directly to the ampicillin but these higher energy cases will not be considered here. With the loop or "flap" conformation already covering the active site, the zwitterion character of ampicillin drives the final binding of glu45 and glu47 relative to ampicillin. Van der Waals interactions are less significant in the binding of ampicillin.

The docking conformation obtained classically is used to initiate the quantum calculations of the reaction path. The quantum model includes the dizinc hydroxide-bridged binding site with all first-shell ligands including the three histidines bound to Zn1 and the aspartate, histidine, and cysteine bound to Zn2. All side chains are cut at the Cβ atom or the imidazole Cγ carbon that is frozen in the subsequent optimization calculations. Three additional residues bound to ampicillin, the conserved lys184, and glu45 and glu47 from the loop, are included in the quantum optimization with the Cβ atom again frozen for these side chains. A total of eight waters are included in the quantum region including the water (W2) bound to Zn2. The presence of W2 was maintained during the classical minimization calculation but is freely variable in the quantum optimization. As noted above, it was found in the crystal structure[1] and even in the presence of an ionic inhibitor.[8] Theoretical calculations show a strong hydrogen bond between W2 and the bridging hydroxide when other waters are also present in the bimetallic active site.[11] The additional waters were distributed to solvate three carboxylate groups and the ampicillin carbonyl present in the active site. Except for freezing the Cβ atoms and the imidazole Cγ carbon atom, the optimization at the Hartree−Fock level is unconstrained. Effective core potentials were used with the concomitant CEP 4-31G basis set[22,23] which is equivalent to a double-ζ basis. Earlier calculations on ligand binding to a transition metal cation have shown that larger basis sets, including two 3d polarization functions, do not qualitatively change energy-optimized structures[11,24] nor even alter relative energetics substantially.[25,26] Even with this relatively small basis, a total of 820 Gaussian basis functions were required. All quantum chemistry calculations were done on the Biowulf/LoBoSIII parallel processing system[27] using the GAMESS code.[28]

Two nucleophilic waters are identified from the energy-optimized reactant structure. Wat2, the water bound to Zn2, is one and has long been identified as a candidate[1] (path 1) and the other is Wat4, bound to the glu47 (path 2). These waters are identified in Figure 1 describing the binding of ampicillin in the active site. The figure is in two parts with the first including all atoms in the model for the energy-optimized structure. The second part describes just those atoms involved in hydrogen binding to the ampicillin emphasizing the nucleophiles for path 1 or path 2, respectively. Activation of the Wat2 is, in part, a case of autocatalysis where the anionic group on the substrate assists in the catalysis after it is partially desolvated when bound. W2 is bound to the ampicillin carboxylate as well as Zn2. Activation of the transphosphorylation reaction in ribonuclease is calculated to involve the substrate phosphate,[29] and substrate catalysis is now widely believed for guanine binding proteins.[30] Autocatalysis has also been shown to play a role in the class-C β-lactamases.[31,32] The structures of the reactant, intermediates, and product of the two reaction paths have been calculated. Once the reactant structure has been optimized, the hydrolysis reactions are initiated by the positioning of partially dissociated water in a reacting conformation toward the carbonyl carbon of the lactam. The proton of the water is directed toward the nearest-proton-accepting site that may be an atom in an anionic group or an atom that grows a negative charge during the hydroxide attack. Energy optimization either returns the dissociating water to its original conformation or leads to the first step in the hydrolysis. Although the energy data will map out a region for the transition state, the calculation of the Hessian is too expensive for the large number of atoms and basis functions. The calculation of the transition state is not considered necessary for this exploratory study since this procedure can map out the hydrolysis step and in a similar fashion the subsequent proton-transfer steps that lead to the intermediates and products. In all cases the proton is moved toward the nearest acceptor site on the flap residues or the ampicillin itself. The structures of the reactant, intermediates, and products of the two reaction paths have been calculated.

In path 1 the product achieved immediately upon the hydrolysis is bound to the enzyme active site or flap by only hydrogen bonds. Solvating the active site would release the hydrolysis product but this could take sufficient time to allow for proton transfers from the product that lead to binding to the zinc in the active site. A dianionic product is possible by moving the proton from the attacking hydroxide from the hydrolyzed ampicillin to the enzyme. After a number of proton transfers the product binds to Zn1. A figure describing the entire model will be provided to illustrate the complex of hydrogen bonds found for all steps in the reaction. However, since the figures are complicated another figure will be provided that focuses on the important reaction or hydrogen bonding for that step in the reaction. In path 2 the product is immediately bound to Zn2. In a remarkable demonstration of the strong ionic bonds driving

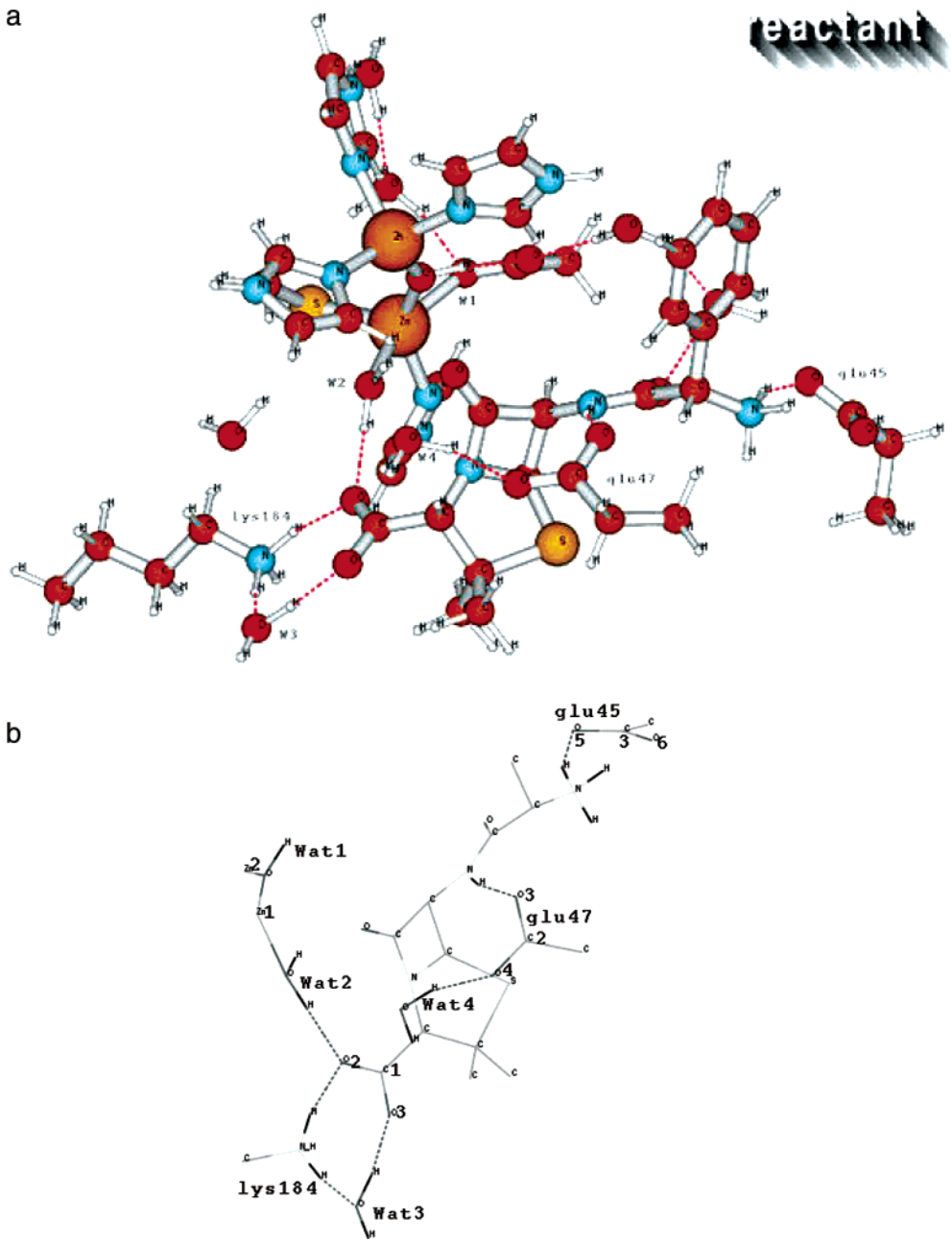


Figure 1. (a) Reactant conformation of active site binding ampicillin to dizinc residues, lys184, and "flap" residues, glu45 and glu47. (b) The relevant hydrogen bonds to ampicillin from the protein are from lys184 and glu45 which anchor the substrate and from the carboxylates of the thiazolidine ring and glu47 which bond to the nucleophiles, Wat2 and Wat4, respectively.

TABLE 1: Energies of Quantum Model for Complete Active Site with Ampicillin (−*E* au with Relative Energies in kcal/mol in Parentheses)[a]

reactant (1)	1154.26677 (0.0)
path 1	path 2
product (2)1154.26729 (−0.3)	(4) 1154.34139 (−46.8)
intermediate	
(3) 1154.17981 (54.6)	
product/intermediate	
(5) 1154.28483 (−11.3)	
(6) 1154.30640 (−24.9)	
(7) 1154.28089 (−8.9)	
(8) 1154.28795 (−13.3)	

[a] The figure describing the structure is also provided in parentheses before the energy.

the hydrolysis, the proton on the attacking hydroxide is abstracted by the carboxylate of glu47 resulting in a dianionic product that binds strongly to Zn2.

The energies along the path are given in Table 1. Since the local environment of the protein around the active site is not used in this calculation and a limited number of waters are included, the energetics are only suggestive of the possibilities. The complexity of the present model and its computational cost was one reason to perform this exploratory calculation to ascertain the structure of the intermediates and the local hydrogen-bonding pattern to expect. Optimization of the total system in a QM/MM calculation must be preceded by an exploratory analysis.

3. Results and Discussion

Binding of Ampicillin. Binding of the carboxylate of ampicillin to lys184 is expected from the conserved nature of this residue and was chosen as the initial binding site for ampicillin in the classical minimization. The salt bridge found between the lys184 ammonium and the carboxylate (C1O1O2)

in the classical structure is maintained in the quantum-optimized structure and shown in Figure 1 to be mediated by the water bound to Zn2, Wat2, and an additional water molecule, Wat3. The entire structure is found in Figure 1a to show the whole network of hydrogen bonds but a close-up view of the binding around the two nucleophiles, Wat2 and Wat4, is shown in Figure 1b. There is only one short NH−O1 bond (1.67 Å) in the salt bridge formed by lys184 and the carboxylate of the thiazolidine ring (see Figure 1b). The carboxylate (C1O1O2) is also directly hydrogen-bonded to Wat2 (O8H−O1, 1.71 Å). Lys184 and Wat2 provide an effective anchor for the ampicillin carboxylate. The carboxylate side chain of glu47 (C2O3O4) is bound to Wat4 (O9H−O3, 1.75 Å) and to the amide NH bond of ampicillin (NH−O4, 1.68 Å). The ammonium end of the ampicillin is approximately 10 Å away with another salt-bridge formed between the ampicillin ammonium and the carboxylate (C3O5O6) side chain of glu45 with one short NH−O5 bond (1.64 Å) with the other carboxylate oxygen, O6, of glu45 within 2.2 Å of another HN ammonium bond.

This orients ampicillin so that the nucleophillic hydroxide, O8H(Wat2), is 3.18 Å from the carbonyl carbon, C4O7, of the lactam ring. The bridging hydroxide oxygen is 3.80 Å from C4 in this conformation. However, the bridging hydroxide is rigidly locked into the dizinc active site structure[11,18] and cannot attack the lactam without a substantial active site reorganization or change in the protonation of either the bridging hydroxide or asp103. The lactam carbonyl oxygen, O7, is 3.42 Å from Zn1 that suggests a role for Zn1 in stabilizing any intermediate that is formed. Asp103 remains hydrogen-bonded to the bridging hydroxide. Anionic asp103 remains deprotonated and hydrogen bonded to the bridging hydroxide throughout the hydrolysis reaction. Two additional waters are hydrogen bonded at one end of the carboxylate side chain of asp103 and ultimately to both the ampicillin amide carbonyl and glu45 carboxylate that is bound to the ampicillin ammonium moiety. This is an indication of the extended hydrogen-bond chains found in all the calculated structures even with a restricted number of water molecules. With all the anionic and polar bonds available on both the antibiotic and the first shell ligands, the available waters link these polar and ionic bonds in hydrogen-bonded chains. The dizinc active site is maintained and acts as the stable base of the active site with the loop moving approximately 10 Å to provide the top of the active site. Since ampicillin is a zwitterion, the anionic ligands in the loop, glu45 and glu47, are attracted to the ammonium end of ampicillin and bind the molecule tightly. Without the ammonium, an antibiotic like benzylpenicillin will bind to other residues such as asn193 as has been suggested.[1] In this model asn193 is bound weakly to glu47 and does not compete with the stronger ionic bonds. The Zn−Zn distance is 3.71 Å. This is a small increase from the experimental and molecular dynamics value of 3.54 Å and the optimized distance of 3.61 Å for the model structure with one water in the active site.[11]

Glu47 is hydrogen-bonded to the ampicillin amide NH with one branch of the carboxylate, O3(glu47), and the other carboxylate oxygen, O4(glu47), is bound to a water, Wat4, whose oxygen is 4.12 Å from C4(C4O7) (see Figure 1b). This water, Wat4, is chosen as the other nucleophile. The reaction paths that result from nucleophilic attack of Wat2 and Wat4 are obtained from a low-energy binding structure. In both cases the hydrolysis results from the concurrent attack of the hydroxide and proton from either Wat2 or Wat4. The dizinc environment provides a "target" rich environment for the binding and activation of water with many proton binding sites available after the water is cleaved. This active site is rigidly maintained primarily by the bridging hydroxide between the zinc cations. If the nucleophile would be the hydroxide, Wat1, there is also a requirement to replace the bridging hydroxide by having the proton removed from the closest water, Wat2, by the ampicillin carboxylate. Since activation of Wat2 already presents a nucleophile even closer and more energetically accessible to the C4(C4O7), utilization of Wat1 is not necessary or energetically efficient.

Reaction Path 1. The nucleophile, Wat2, is activated by both the Zn2 cation and the carboxylate of ampicillin. Binding of Wat2 to Zn2 lowers the proton affinity of the hydroxide extracted from Wat2 while the partially solvated thiazolidine carboxylate accepts the proton from Wat2. The entire structure obtained by energy minimization from the concurrent movement of the proton toward the lactam nitrogen as the hydroxide attacks the carbonyl carbon is shown in Figure 2a with the relevant hydrogen-bonding interactions to the hydrolysis product shown in Figure 2b. The direct formation of the product is much lower in energy, as seen in Table 1a, than the stepwise formation starting with the transfer of the proton from Wat2 to the thiazolidine carboxylate, C1O2O3 (see Figure 3). The energy lowering results primarily from the transfer of the proton bound to the carboxylate to the lactam nitrogen. Binding the proton to the lactam nitrogen neutralizes the lactam ring breaking the C−N bond. The product is still attached to the lys184 ammonium by the same water mediated salt bridge. It should be noted that the all the hydrogen bonds in the reactant complex with the active site are retained with one additional hydrogen bond resulting from the hydrolysis. The hydrolyzed product is attached to the enzyme by the additional hydrogen bond from the hydroxide that attacked the lactam ring carbonyl. This hydroxide forms a hydrogen bond (C4O8H−OH(Wat1), 1.68 Å) to the bridging hydroxide, Wat1. The hydrolyzed lactam ring and the hydrogen bond to the bridging hydroxide of the active site are provided in more detail in Figure 2b.

When the Wat2 proton is moved first toward the thiazolidine carboxylate, a high-energy intermediate is energy optimized with the hydroxyl binding at the lactam carbonyl carbon and its proton hydrogen bonded to the bridging hydroxide, Wat1 (C4O8H−OH Wat1, 1.90 Å) (Figure 3). The proton abstracted from Wat2 is bound to the ampicillin carboxylate but its presence raises the energy of this intermediate since it reduces the binding energy of the salt-bridge to lys184. In the course of the energy optimization, the proton that had translocated to the extracyclic carboxylate group of ampicillin moves from an initial hydrogen bond to the attacking hydroxide from which it was abstracted to a strong hydrogen bond to the lactam nitrogen (1.64 Å). The H−O distance is also elongated to 1.03 Å suggesting the movement to the lactam nitrogen is possible in a subsequent step or by a concurrent attack. In this high-energy structure, the lactam and thiazolidine rings are not disrupted although the C−N bond distance has increased from 1.38 Å in the reactant complex to 1.55 Å due to the delocalization of the anionic charge resulting from the hydroxyl attack at the carbonyl carbon. The C−O bond distance of the COOH group on the lactam is 1.31 Å indicative of a charged bond with a comparable lengthening of the C−O(H) bond distance from 1.35 Å to 1.47 Å. Attack by the hydroxide at the lactam carbonyl carbon is insufficient by itself to break the lactam ring. As we showed in Figure 2, the concurrent attack of Wat2 results in the hydrolysis product and is energetically preferred.

Reaction Path 2. The carboxylate of glu47 activates Wat4 as shown in Figure 4b. As in the case of Wat2, the nucleophilic

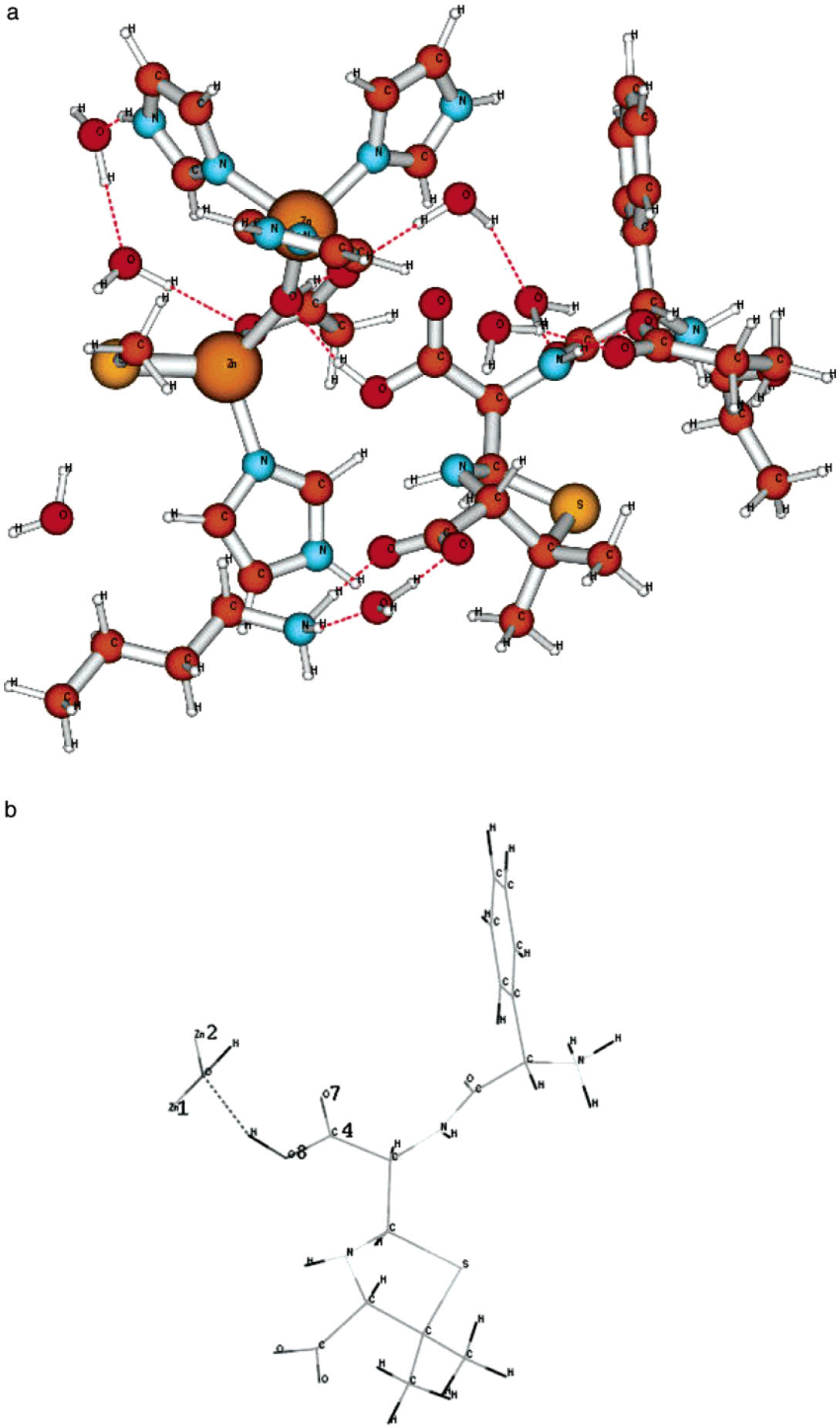


Figure 2. (a) Hydrolysis product on path 1 after concerted attack of water on ampicillin. (b) Hydrolysis product from the concurrent attack of Wat2 on ampicillin.

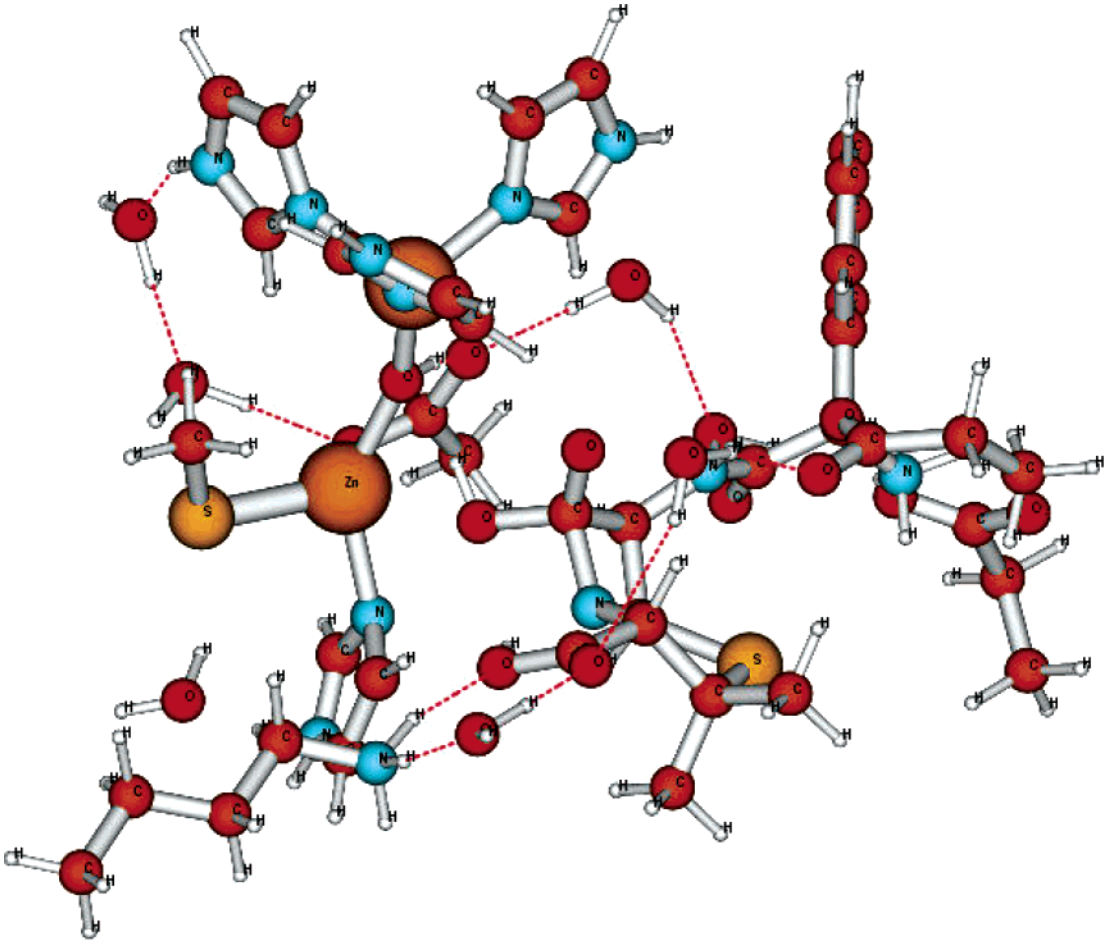


Figure 3. First intermediate on path 1 with proton abstracted by ampicillin carboxylate.

attack of Wat4 is primarily by concurrent binding of the Wat4 hydroxide at the lactam carbonyl (C4−O9H) and the proton binding to the lactam N which opens the ring. The concurrent attack geometry was deduced from the structure obtained from the stepwise attack of Wat4 that moved the proton to the carboxylate of glu47 (C2O3HO4). After nucleophilic attack, the ring carbonyl oxygen is then very negative with the energy-optimized formation of the tetrahedral intermediate leading to the binding to Zn1. The entire bonding network holding the hydrolysis product is seen in Figure 4a and the relevant hydrogen bonds from Wat2 and glu47 are shown in Figure 4b. During the energy optimization, the proton of the nucleophilic hydroxide moved toward the carboxylate of glu47 while maintaining a strong hydrogen bond to the oxygen of the hydroxide (O3H−O9, 1.62 Å). There are now two carboxylates found on the hydrolysis product. One is the thiazolidine carboxylate whose hydrogen bonding to lys184, Wat2, and Wat3 has not changed qualitatively from the reactant. The other carboxylate originates in the nucleophilic attack (C4O7O9) to the former carbonyl carbon with the subsequent transfer of the hydroxide proton to the carboxylate of glu47 (HO3). Wat2 also hydrogen bonds to the arm of this carboxylate that binds to Zn1 (Zn1−O7, 2.42 Å). The binding of O7 to Zn1 drives asp103 away from Zn1 and it binds with a bifurcated hydrogen bond to the bridging hydroxide, Wat1. The oxygen in the bridging hydroxide is now more negative and the Zn−Zn distance shortens slightly to 3.58 Å. As seen from Figure 4b, there is an extended set of hydrogen bonds that bind the product to the active site in addition to the Zn1−O7 bond.

Deprotonated Nucleophile Intermediates/Product. Moving the proton from the hydroxyl nucleophile now attached to the path 1 product in Figure 2 creates a range of other intermediates or products with comparable energies. It also permits the direct binding of the hydroxylated ampicillin product in path 1 to Zn2. Although the path 1 product (Figure 2) is not covalently bound to the active site and could be released by solvating the hydrogen bonds holding it in the active site, such a process may require sufficient time to allow the proton transfers to be described below. The first intermediate/product (Figure 5a) is obtained by transferring the proton from the hydroxyl nucleophile (O8H) to the carbonyl oxygen (O7H) and then energy optimizing the modeled structure. All subsequent structures are obtained by partial movement of the transferring proton toward the acceptor site followed by energy minimization. The proton movement is determined to be sufficient that the proton does not return to its initial position. The hydrogen bond from the hydroxyl, O7H, to the bridging hydroxyl, Wat1, is maintained in the energy-optimized structure (Figure 5a) but now the resulting carbonyl oxygen, O8, is attracted toward the Zn2 cation at a distance of 3.16 Å. The symmetry of the interaction of the O8C4O7H moiety to the bridging hydroxide and zinc cations is suggested by the fact that the hydrogen-bond distance to the bridging hydroxide remains 1.68 Å. The transfer of the proton stabilizes this intermediate substantially relative to its symmetric partner. The central position of the bridging hydroxide in stabilizing the hydroxylated ampicillin supports the maintenance of the dizinc active site with the aspartate carboxylate still bonded to H(O) of the bridging hydroxide (Wat1) which orients the hydroxide to accept the proton from the hydroxylated ampicillin. This region of the structure is shown in more detail in Figure 5b. The remainder of the network of hydrogen bonds from the active site to the ampicillin is not changed with the exception

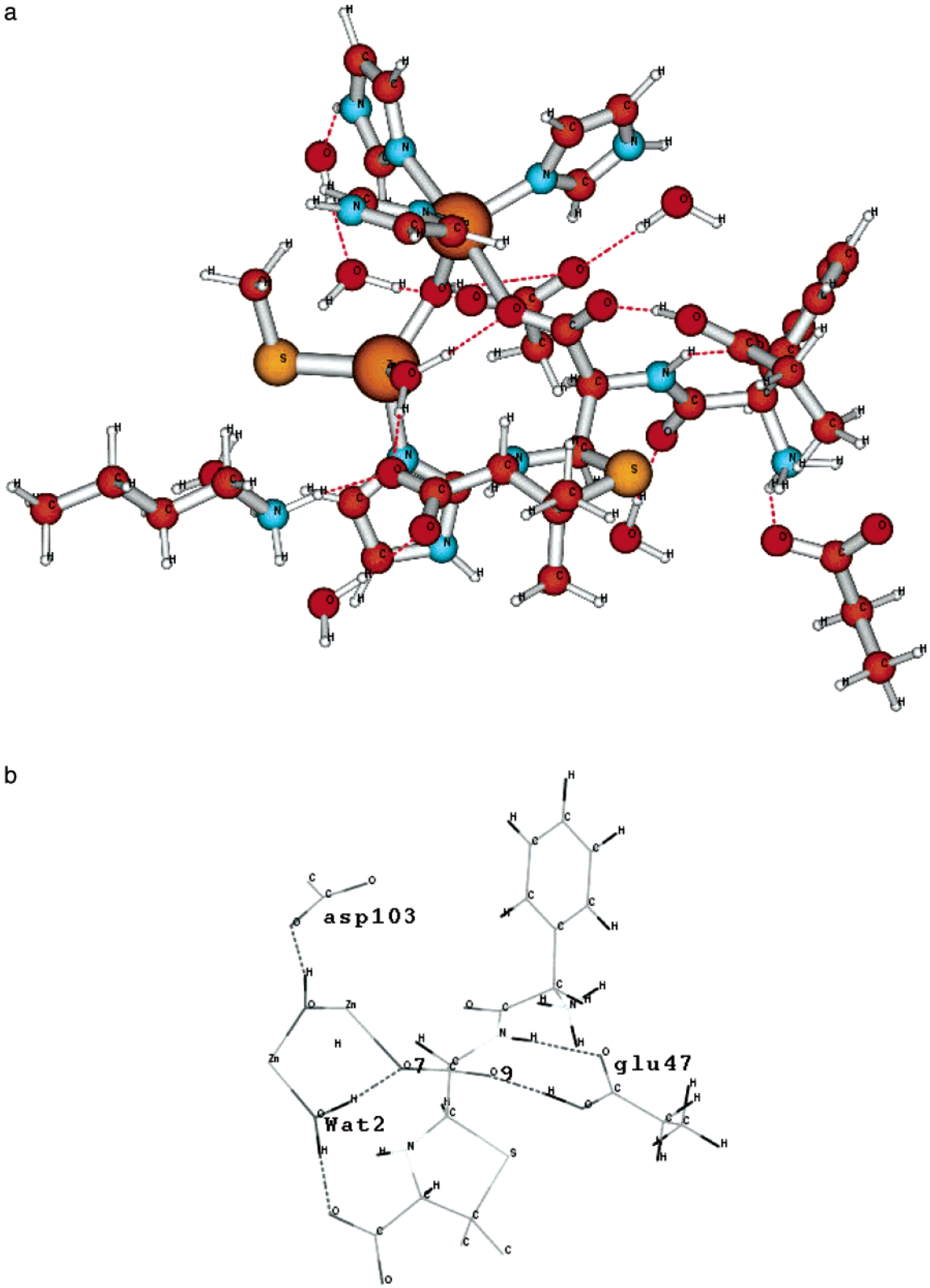


Figure 4. (a) Path 2 product with concurrent attack of proton to thiazolidine nitrogen and hydroxide on carbonyl carbon of lactam run that results in dissociation of the lactam ring. (b) Path 2 hydrolysis product has covalent bond from O7 to Zn2 and hydrogen bonds from O7 through Wat2 to the thiazolidine carboxylate.

of a proton transfer between the salt-bridge components from the ampicillin ammonium and the carboxylate of glu45. Proton transfers between salt-bridge components have small barriers with little local energy stabilization resulting from the transfer.

The proton can also be rotated away from the hydrogen bond to the bridging hydroxide (Figure 6a). If we rotate the proton in Figure 5a 180°, this allows the carbonyl oxygen to come a little closer to the Zn2 cation. All subsequent structures are obtained by partial movement of the transferring proton toward the acceptor site followed by energy minimization. Since the carbonyl, C4O8, is not ionic and the effective charge on the zinc is small, the loss of the hydrogen bond to the bridging hydroxide leads initially to a substantially higher energy structure. However, the hydrogen bond between Asp103 and

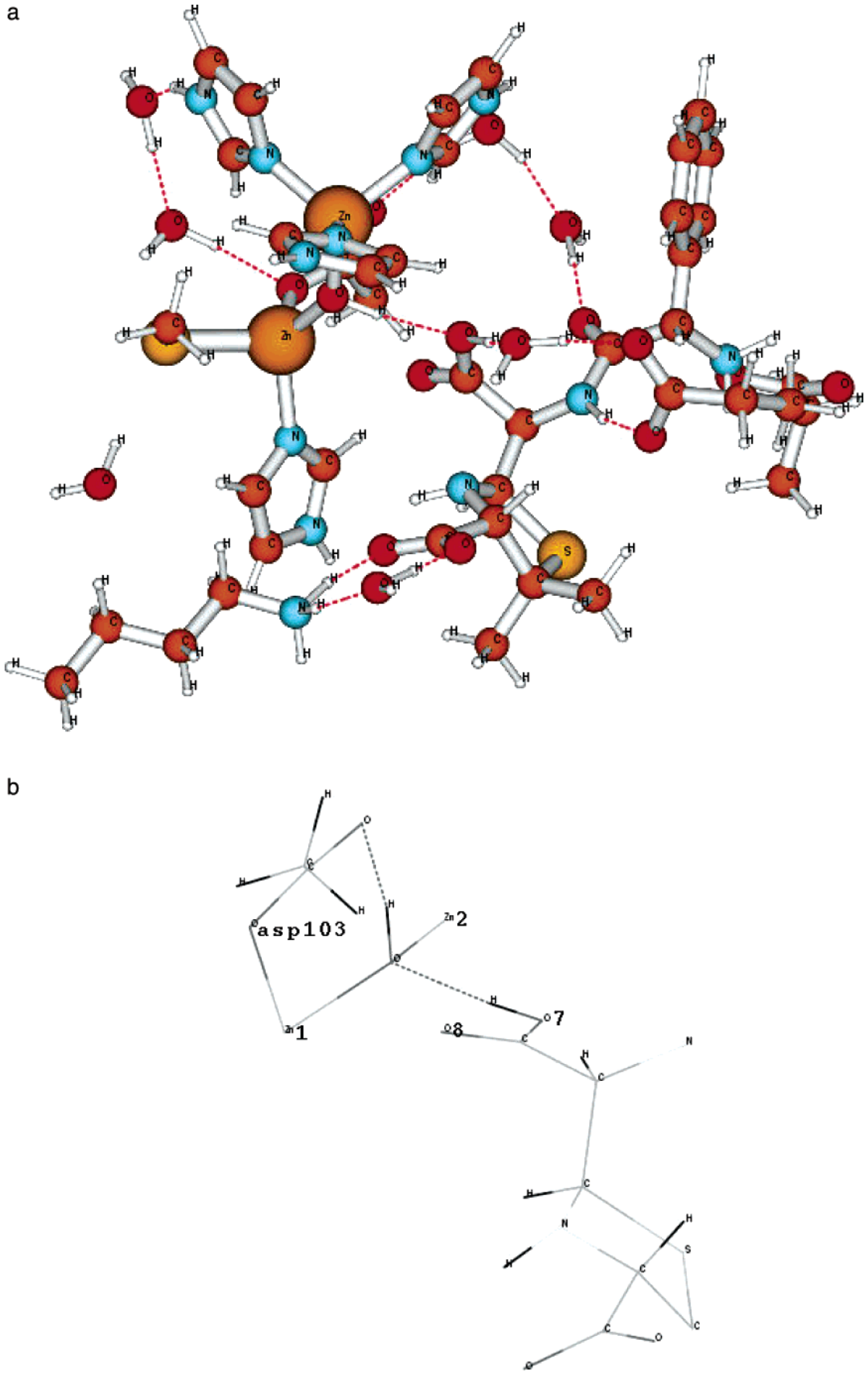


Figure 5. (a) Intermediate with hydrogen bond to bridging hydroxide. (b) Proton transfer from O8 to O7 with the hydrogen bond to Wat1 maintained.

the bridging hydroxide is broken as the structure optimizes leading to a bond between the bridging hydroxide, Wat1, and the nucleophilic oxygen, O8(C4), on the ampicillin. This has little effect on the Zn−Zn distance, 3.74 Å, or on the approach

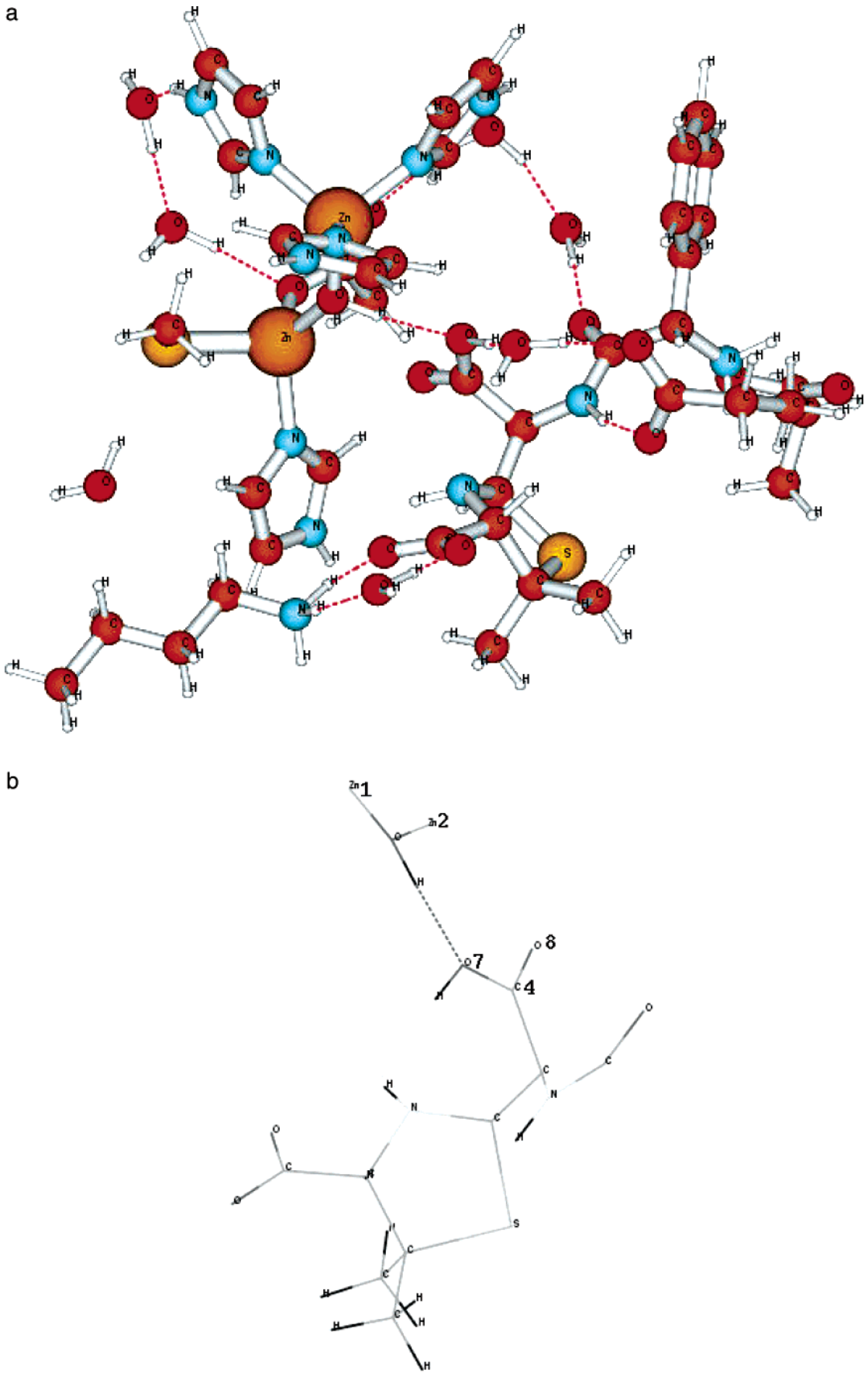


Figure 6. (a) Product/intermediate with no hydrogen bond to bridging hydroxide; O7H directed toward the thiazolidine nitrogen and the ampicillin amide. (b) Proton on O7H directed toward ampicillin with thiazolidine nitrogen and ampicillin amide the closest proton acceptor sites.

of the thiazolidine oxygen carbonyl to the Zn2 at 3.05 Å. Maintenance of the relatively short Zn−Zn distance relies significantly on a strong ionic hydrogen bond to the bridging hydroxide but the asp103 interaction can be replaced by proton

donation hydrogen bond from HO7C4. The water bound to glu47, Wat4, does hydrogen bond to O8C4 with a short proton-donated bond of 1.55 Å. The other hydrogen bonds remain the same except the ampicillin ammonium transfers one proton to the carboxylate of glu45. These shifts in the hydrogen-bond pattern, however, do lower the energy substantially as seen in Table 1 illustrating the importance of the hydrogen bonding to the energy. Subtle shifts in the hydrogen-bond distances lead to energy changes that would be difficult to predict. A more complete model with extended shells of protein around the active site and solvent outside the flap will also affect total energies but probably not the relative energetics. This structure has the lowest energy calculated for this path but still should be considered an intermediate product since there is no direct bonding to Zn2 although the energy-optimized distance, Zn2-O8, is now 2.91 Å.

Moving the nucleophilic proton toward the nitrogen in the thiazolidine ring and energy minimization ultimately leads to a pattern of hydrogen bonding from the two protons now attached to the lactam nitrogen to the glu47 carboxylate that also involves the amide NH of ampicillin (Figure 7a). The initial structure formed by the proton movement retains the asp103 bond to the bridging hydroxide but this is broken in the course of the optimization. The final optimized structure has a hydrogen bond from the deprotonated nucleophile to the bridging hydroxide. The hydrogen bonds from the thiazolidine ring to the bridging hydroxide and glu47 are shown in focus in Figure 7b. Once again we find that the bridging hydroxide plays a role in stabilizing the intermediate or a product structure.

Proton transfer from the hydroxide in Figure 6 is also possible to the amide nitrogen since the distance is 2.6 Å. The proton is moved toward the amide nitrogen and the structure energy minimized. The deprotonated nucleophilic hydroxide forms a rotatable carboxylate that now binds to Zn2 (2.31 Å) in the energy optimized structure creating the lowest energy structure bound directly to the active site zinc (see Figure 8a). The other arm of the carboxylate binds to the bridging hydroxide again replacing the hydrogen bond to asp103 with another anionic hydrogen bond. There is also a hydrogen bond from asp103 to a water that is bonded to the amide carbonyl of ampicillin. In the course of the energy optimization the amide proton transfers in turn to the carboxylate of glu47. A proton involved in the ampicillin ammonium bound to glu45 also transfers in the energy-optimized structure. In this structure ampicillin is directly bound to Zn2 and is now the counterpart of the product obtained directly in path 2. The binding arrangement to Zn2 is focused in Figure 8b with the hydrogen bonding toward Wat2 and glu47.

4. Conclusion

Binding of ampicillin requires the movement of the flexible loop to complete the construction of the active site. This was done by a classical constrained dynamics simulation and energy minimization of the loop and the conserved lys184 and asn193 residues. The Zn−Zn distance and much of the first-shell active site must also be constrained since the metal effective potentials are not accurate enough to model the dizinc system. The charge state of the substrate determines the final flap conformation and the binding to the conserved residues. Ampicillin is a zwitterion that can form two salt bridges in the active site. The classical energy minimization is initiated and maintained with the thiazolidine carboxylate interacting with the ammonium of lys184. The two glutamate residues, glu45 and glu47, on the flap interact with the ammonium and amide moiety of ampicillin, respectively. The lowest energy structure is found with a salt bridge between glu47 and the ammonium which was selected as the basis of the quantum model. This model is a docking arrangement that has a nucleophilic water close to the hydrolysis attack site, the carbonyl carbon, on the lactam ring. The quantum model with additional water solvating ionic and polar sites provides an additional nucleophile to consider for this docking arrangement. Binding to lys184 is essential in this model and has been found experimentally for a penicillin.[32] This is a first step in the understanding of the construction of the complete active site and needs to be repeated with a more complete QM/MM optimization. The present calculation is still a substantial undertaking considering the large size of the quantum region and the present classical construction was used to gain insight into the reaction path expected for a substrate like ampicillin. Ampicillin can form the salt bridge because there is no steric constraint from any hydrophobic moiety near the thiazolidine carboxylate. This is not true in other antibiotics and cephalosporins where such substituents are attached disrupting the direct lys184-carboxylate and water-mediated salt bridge.

Two reaction paths are described for the two activated waters. The quantum model is optimized for the reactant, a number of intermediates, and products depending on the final position of the proton abstracted from the nucleophile. The water bound to Zn2, Wat2, was suggested as a possible nucleophile once it was identified in the crystal structure of *B. fragilis*.[1] However, the other water, Wat4, only becomes a candidate when the flap binding is described. Wat2 is hydrogen-bonded to the thiazolidine carboxylate and activation and proton abstraction is assisted by the partially desolvated carboxylate of the substrate. Wat4 is primarily activated by glu47 but is also near the thiazolidine carboxylate. Both Zn1 and Zn2 effectively participate in the stabilization of the hydroxylated product. The path initiated by attack of the water, Wat2, bound to Zn2 has the activated carbonyl on the thiazolidine ring binding to the Zn2 after appropriate proton transfers while the second path has the hydroxylated ampicillin bound to Zn1.

In reaction path 1 the lactam ring is hydrolyzed by a concurrent attack of the hydroxide and proton of Wat2 on the lactam ring. This product is bound to the enzyme by hydrogen bonds alone and could be released by solvating the flap and the active site. The detailed reaction pathway described by the quantum-energy-optimized structures has similarities and differences from the mechanism deduced from the observed kinetics for another antibiotic, nitrocefin.[4] This study shows that activated waters, rather than the bridging hydroxide, provide the nucleophile. It is also important to note that the ampicillin carboxylate is deprotonated in order to form the salt bridge to lys184. The first product formed by the concurrent attack of hydroxyl and proton of Wat2 does not form a zinc bound intermediate. However, there are a number of hydrogen bonds retaining this product in the active site and solvation release may take sufficient time for deprotonation of the nucleophile hydroxide yielding a zinc bound long-time intermediate/product.

In path 2 hydrolysis of ampicillin by Wat4 also occurs by concurrent attack of the hydroxide and proton on the lactam ring. In this case a much lower energy product is obtained than that found for path 1, in part because the product is covalently bound to Zn2. There is also a network of hydrogen bonds that connect Wat2, the thiazolidine carboxylate, and the carboxylate formed by proton transfer from the nucleophilic hydroxide, O9H. The low-energy of the product assists in the concurrent nucleophilic attack of Wat4 that suggests that this path is competitive with the one initiated from Wat2.

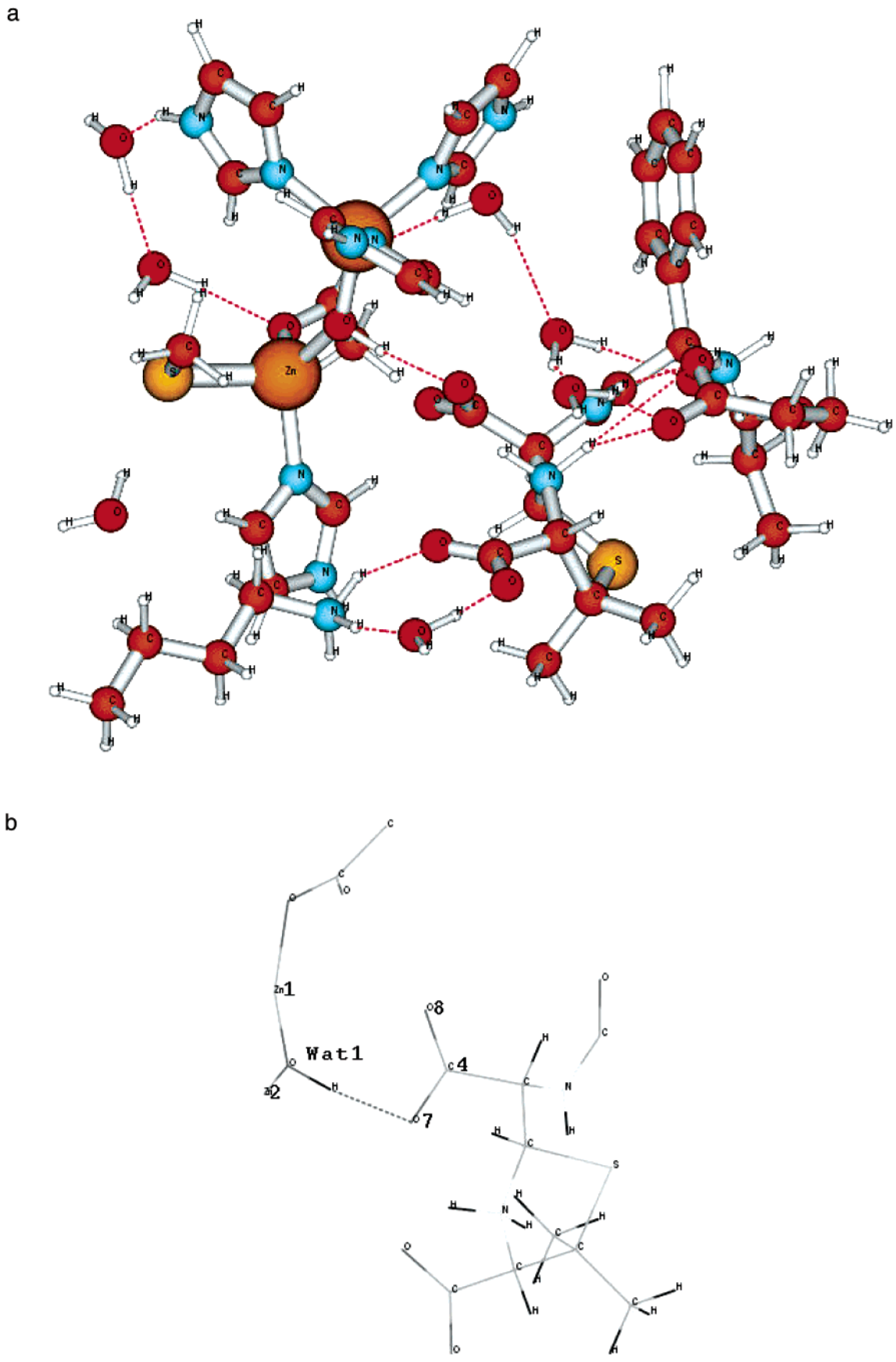


Figure 7. (a) Product/intermediate with protonated ammonium on thiazolidine ring finally hydrogen bonded to carboxylate of glu47. (b) Starting conformation after transfer of proton from C4O7H to thiazolidine nitrogen.

The dinuclear kinetics finds a wide range of pH independence. The coupled hydrogen bonding in the active site will maintain anionic aspartate and glutamate side chains so there will be no deprotonation at high pH nor protonation at low pH. As the pH goes below 5, the dizinc active site will be protonated and disrupted.[11] Zinc binding to the anionic thiazolidine nitrogen[4,19]

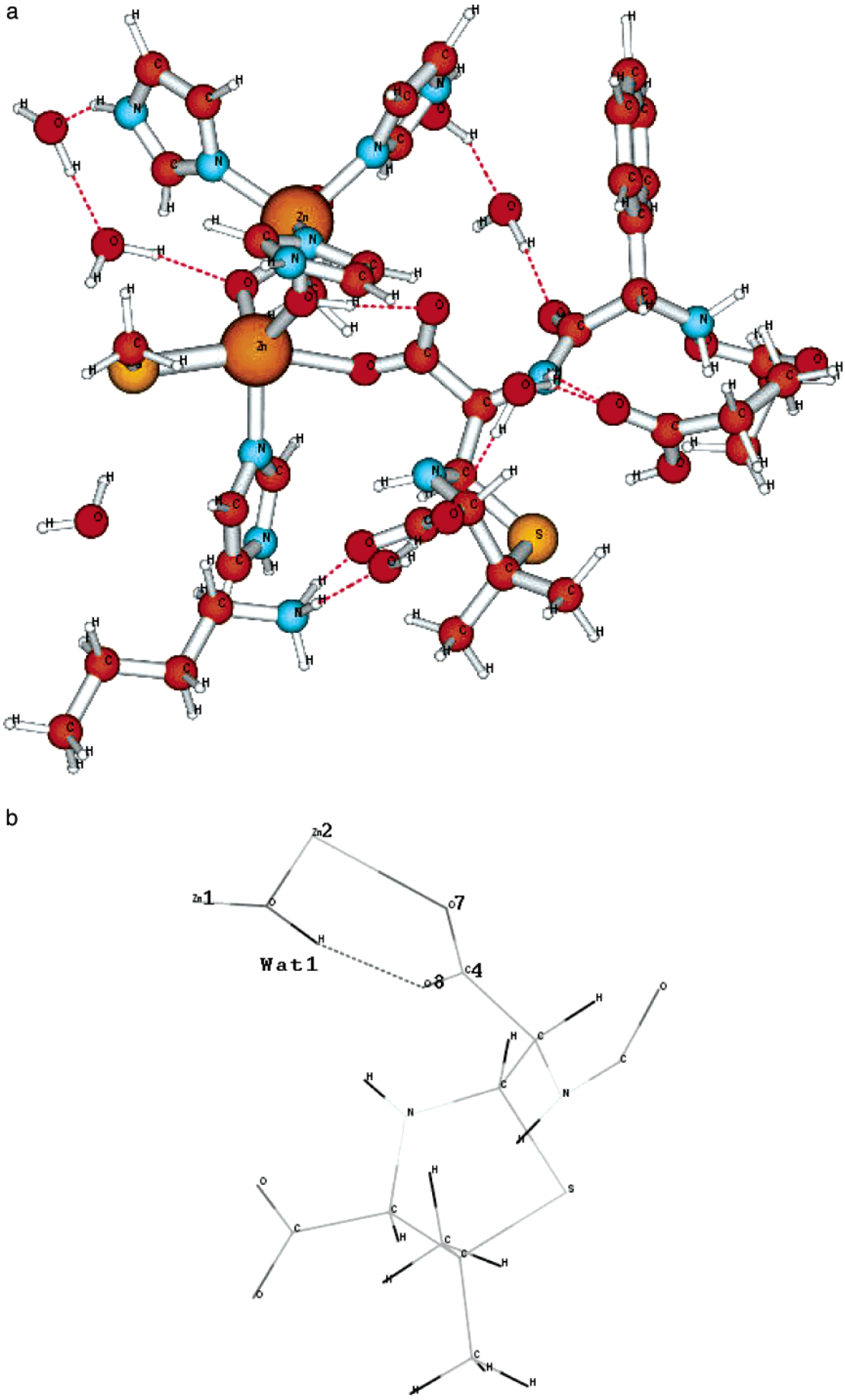


Figure 8. (a) Product for path 1 with hydroxylated ampicillin bound to Zn2. (b) Path 1 product with covalent bond from C4O7 to Zn2 and ionic hydrogen bond from bridging hydroxide to O8C4.

does not result from our model since the zinc does not get close enough in the solvated active site and the proton from the nucleophilic water will quickly attack the nitrogen to produce a low-energy product. Asp103 is not protonated since that would disrupt the dizinc active site. It can be displaced by another ionic ligand that also orients and enhances the binding of the bridging hydroxide between the two zinc cations.

The bridging hydroxide is necessary for this dizinc mechanism and an anionic asp103 binding to this moiety initially stabilizes and orients the dizinc metal complex. For ampicillin, lys184 is critical for binding but asn193 is not. This is also suggested by the kinetics for lys184 mutants.[32] Because of the ammonium group on ampicillin, asn193 does not play an important role in binding but is displaced by either glu45 or glu47. The side chain of asn193 is found weakly bound to the glutamates instead of ampicillin. Removing the ammonium finds the asn193 does play a binding role for benzylpenicillin that supports in part mutant data on the binding.[32] The experimental data also find that asp103 is very important for the rate constant. However, it does not explicitly help move the protons within the hydrogen-bonded network. The maintenance of an anionic asp103 is supported by the importance of asp103 and the bridging hydroxide to maintain the dizinc structure which is critical to the development of the overall coupled network that is found in the reactant initial structure for both path 1 and 2. The charge state of the antibiotic is significant for the type of active site created with the flexible loop. In this case the active site, bound ampicillin, and waters form a network of interacting hydrogen bonds allowing for protons to move readily between binding sites. Two water nucleophiles initiated two different reaction paths and the energetics of the intermediates are similar. Moving protons between charged or polar atoms does not alter the energies much within the coupled network.

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