Protonation States of Methionine Aminopeptidase and Their Relevance For Inhibitor Binding and Catalytic Activity

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Running title: Protonation states of methionine aminopeptidase
Summary

We have performed a computational study of different protomeric states of the methionine aminopeptidase active site using a combined quantum-mechanical/molecular mechanical simulation approach. The aim of this study was to clarify the native protonation state of the enzyme, which is needed for the development of novel, irreversible inhibitors – that can possibly be used as antiangiogenic and antibiotic drugs – by virtual screening and other drug design methods. The results of the simulations indicated that two protonation states are possible without disturbing the overall geometry of the active site. We then verified experimentally the presence of the two protonation states by studying the substrate hydrolysis and inhibitor binding reactions at different pH and come to the conclusion that one of the protomeric states is relevant for inhibitor binding, whereas the other is relevant for substrate hydrolysis. This result has implications for the development of other inhibitors of this class of enzymes and adds a new perspective to the pharmacological properties of the antiangiogenic drug fumagillin, which is an irreversible inhibitor of the human methionine aminopeptidase type II.

Introduction

Methionine aminopeptidases (MetAPs) play a central role for in vivo protein synthesis as they remove the starter methionine from newly synthetized proteins. The natural product fumagillin, an epoxide, covalently modifies one of the active-site histidines in the eukaryotic methionine aminopeptidase II (MetAP-II)(1-4) and other MetAPs (cf. Figure 1). Fumagillin inhibits the growth of vessels in tumors and a derivative of the compound has been evaluated in clinical trials as an anticancer drug. The antiangiogenic effect of fumagillin and other inhibitors of MetAP-II has been attributed to the inhibition of the Ets-1 transcription factor expression and the activation of the p53 pathway.(5,6) Besides from being anticancer drug targets, MetAPs have the
potential to become the target proteins of antibacterial substances, because the MetAP functionality is essential for cell growth and bacteria possess only one of the two known MetAP subtypes. (7)

MetAPs are metal-dependent enzymes. It is not clear which metal activates the MetAPs \textit{in vivo}. For \textit{in vitro} experiments, cobalt is commonly used because it activates all known MetAPs and the cobalt-substituted enzymes are usually the most active. (8) Zinc and iron(II) have also been shown to activate some MetAPs. (9,10) The metal-chelating residues in all known MetAPs are two glutamates, two aspartates and one histidine. The geometric arrangement of these residues is practically identical in all MetAP x-ray structures. (11,12) Several three-dimensional structures of MetAPs have been determined by x-ray diffraction methods, including the structure of human MetAP-II with a covalently bound fumagillin molecule. (13-15) Density functional theory (DFT) (16,17) has long been used to study the reactivity of organic molecules and recently began to find its way into biochemistry. (18) We present here an example in which the application of DFT to a biochemical problem led to a hypothesis which was subsequently verified by experiments. Our results do not only explain a couple of biochemical phenomena related to MetAPs, but also demonstrate the impact that modern quantum-chemical methods can have on the study of biological systems, where they can help fill the gap between mere conjectures and physical reality.

Our motivation for examining the MetAPs from a theoretical point of view stems from the highly selective, irreversible inhibition mechanism of fumagillin and related epoxides. Many enzyme inhibitors that form a covalent adduct with their target proteins (e.g., the beta-lactam antibiotics and acetyl salicylic acid) are important
drugs. We believe that the fumagillin/MetAP example is a good test case for theoretical methods that aim at rationalizing the development of covalent enzyme inhibitors, because a large amount of high-quality x-ray structural data, also with bound inhibitors, has been published over the last few years.

As a first step towards an understanding of the catalytic and inhibitor-binding mechanisms of MetAPs, we studied different protonation states of the active site water molecules by means of molecular dynamics simulations in a combined quantum-mechanical/molecular-mechanical (QM/MM) framework. In the 1.9 Å resolution x-ray structure of E. coli MetAP (pdb code: 2mat), one water molecule (or hydroxide ion) is bridging the two cobalt ions and another water molecule is bound to the cobalt ion that is not coordinated to the histidine. Three different protomeric states were examined (see Figure 2): one with two bound water molecules, one with a bridging hydroxide ion and a water molecule, and one with two hydroxide ions. For the quantum-mechanical part of the system, the ab-initio molecular dynamics method described by Car and Parrinello (CPMD) was used.(19) Because an accurate treatment of the open-shell cobalt(II) ion is very demanding in the framework of CPMD, we decided to use zinc(II) ions as the active-site metals in the CPMD simulations. The fundamental problem with cobalt is the uncertainty of its spin state and the multiplicity of the bi-cobalt system. A computational study of a di-cobalt system would therefore require the simulation of eight different electronic configurations, which is not feasible with CPMD and currently available supercomputer resources. Given the fact that at least some MetAPs have been shown to be active with zinc, and considering that a thorough theoretical analysis of the zinc- and cobalt-substituted truncated MetAP active sites did not reveal major geometrical differences between a di-zinc and a di-cobalt system,(20) we assume that the replacement of cobalt by zinc does not affect the validity of our results. Furthermore, preliminary CPMD simulations on a di-cobalt MetAP active site with one
water and one hydroxide ion did not show geometric or dynamic differences to a di-
zinc system (see supporting information).

INSERT FIGURE 2 HERE

Experimental Procedures

*Theoretical Methods:* The DFT-based Car-Parrinello (19) molecular dynamics program CPMD v. 3.5 was used for the QM/MM simulations of MetAP. (21-24) AMBER 94 force field parameters were used for the MM part. (25,26) All systems were fully hydrated with TIP3P water molecules in a periodic box and equilibrated by extensive classical-mechanical molecular dynamics (MD) simulations with AMBER prior to the ab-initio MD simulations. Visualization was done with VMD. (27) The quantum parts included the five metal-binding amino acids (truncated to acetate ions and imidazole, respectively; free valences were capped by adding hydrogen atoms), the two zinc ions and the two water/hydroxide molecules coordinating to them. The QM parts were minimized (annealed to a temperature below 0.1 K) before the start of the production MD phase. CPMD parameters were: Isolated system calculations; gradient-corrected exchange-correlation functionals due to Becke and Lee, Yang, and Parr (BLYP); plane waves basis set; kinetic energy cutoff: 70 Ry; soft normconserving Troullier-Martins pseudopotentials for the core electrons; timestep: 6 a.u.; Nosé-Hoover thermostat at 300 K, coupling frequency of 500 cm⁻¹; fictitious electron mass 800 a.u.. (28-32) The size of the orthorhombic QM box was 13.2 x 15.9 x 17.2 Å, corresponding to a minimum image distance of 6.4 Å. Simulations with cobalt were performed with a kinetic energy cutoff of 90 Ry and Becke-Perdew (BP) exchange-correlation functionals. (33,34)

*Experimental Methods:* E.coli MetAP was purified from an overproducer strain that was kindly provided by Drs. Lowther and Matthews. Assays were performed in 96-well microtiter plates using the method of Yang et al. with MGMM as substrate and
an enzyme concentration of 12 nM. The fumagillin concentration in the inhibition experiments was 66 μM, which is slightly below the IC\textsubscript{50} of fumagillin at pH 7 under otherwise identical conditions. Tris/maleate buffer was used for the incubation of MetAP with and without fumagillin at pH 5–8.5 (volume: 30 μL; 15 min.; 37 °C). For the ensuing MGMM hydrolysis and detection reactions, the pH was adjusted to pH 7.5 by adding 170 μL of four-fold concentrated tris/maleate buffer. The fluorescent reaction product (resorufin) was measured using a Wallac microtiter plate reader. All experiments were performed in triplicate. Detailed descriptions of the experiments are given in the supporting information.

Results

Simulations: In the CPMD simulations, the system with the two water molecules coordinated to the zinc ions showed a pronounced movement of the coordinating water molecules away from the x-ray structure of E. coli MetAP that has one water molecule located between the metals. The system evolved to a structure in which there was no more "bridge" between the metals and one water molecule started to form a strong hydrogen bond to a carboxylic acid. The simulation was stopped after about 5000 steps of simulation (0.85 ps), because the geometry remained essentially stable. In contrast, the structure with one bridging hydroxide was stable during the whole simulation time (about 10000 steps), showing only the expected thermal motions and a twist of one carboxylic acid group. Figure 3 gives a comparison of the active site geometries of the bi-water and the water-hydroxide system with the crystal structure. Hydrogens have been omitted for clarity. The final state of the bi-hydroxide system is not shown, because it deviates strongly from the crystal structure and a comparison with the other systems would not make any sense. After only about 1000 steps of simulation, large deviations from the crystal structure were observed in the bi-hydroxide system. After 3000 steps, the metal-coordinating residues were
considerably displaced from the crystal structure positions and the simulation was stopped.

(INsert Figure 3 HERE)

It becomes clear from Figure 3 that both the bi-water and the water-hydroxide system are intrinsically stable and quite similar to the 2mat x-ray structure. The rms deviations of the active site heavy atoms in comparison with the x-ray structure (hydrogens were not used because they are not present in the x-ray structure) are 0.81 Å for the bi-water and 0.85 for the water-hydroxide system. The main changes are: The bridging water leaves the position between the two metals in the bi-water simulation; In the water-hydroxide simulation, the bridging hydroxide remains in place and one of the metal-coordinating carboxylic acid groups twists to a position that is perpendicular to the x-ray structure. Nevertheless, this residue remains a binding partner for one of the metal ions.

(INsert Figure 4 HERE)

A picture of the final state of the simulation is shown in Figure 4. This Figure also shows the HOMO–1 orbital, which is localized at the hydroxide ion, the species that very likely acts as the nucleophilic agent in the substrate hydrolysis reaction. The HOMO orbital, which is about 0.5 eV higher in energy, is localized at one of the carboxylic acid groups.

*Experiments:* The pH profiles of the inhibitor binding and substrate hydrolysis reaction are given in Figure 5. It is evident that the pH optimum of the substrate hydrolysis reaction is at pH 7.5, whereas the binding of fumagillin becomes increasingly favored at lower pH.
Discussion

The results presented above allow the following conclusions: In the x-ray structure of E. coli MetAP (2mat), the bridging water molecule is most likely deprotonated. However, an active site with two (fully protonated) water molecules coordinated to the metal ions is also stable – albeit with a different coordination geometry. The x-ray structure of the E. coli MetAP (2mat) was determined at a pH larger than 7, whereas several other MetAP structures were determined at a more acidic pH. (Human MetAP-II: see ref. (15). The pH in the crystallization medium cannot be determined exactly, but the experimental (buffer) conditions described by Liu et al. indicate that the pH was below 7). Examining these, we found that no bridging water molecule is present between the two metals. Taken together, these x-ray structures and the results of the CPMD simulations indicate that two protonation states are possible without disrupting the active site geometry. At more basic pH one water molecule is deprotonated and bridging the two metal ions. From the electronic structure calculations this protonation state is expected to be relevant for the catalytic process. Two water molecules are present in the active site at more acidic pH, each one coordinating a different metal ion.

The covalent binding of fumagillin to MetAP requires protonation of the epoxide oxygen. Proton donation from an active site group, in particular from one of the active site water molecules, is more likely in the protonation state with two water molecules (i.e. at more acidic pH).

In other words: our calculations and the analysis of the crystal structures indicate that the active site of binuclear E. coli MetAP is characterized by the presence of a water molecule with a pKa of ~7. This is at the lower border of the pKa values reported for waters coordinating to zinc ions in organic complexes. (36) Binding of fumagillin is expected to be favored at more acidic pH, while a more basic pH would be required.
for catalysis. This finding opened the way to an experimental validation of the theory: we determined the pH-profile of the fumagillin inhibition reaction and the enzymatic activity of MetAP (see Figure 5). It is obvious that the binding of the inhibitor is favored under more acidic conditions as compared to those that are optimal for the substrate hydrolysis reaction. This observation provides strong substantiation to our interpretation of the QM/MM CPMD simulation results.

In summary, the mechanisms of the fumagillin binding and the initial step of the substrate hydrolysis reactions are shown in Figure 6. The substrate hydrolysis reaction starts with the attack of the metal-bound hydroxide ion to the carbonyl carbon of the scissile peptide bond. This mechanism differs slightly from the one that is given in the recent literature(12), where the deprotonation of the metal-bridging water occurs after the substrate has been bound in the active site.

INSERT FIGURE 6 HERE

Recent theoretical work on the reaction mechanism of imipenem binding to the dinuclear zinc-β-lactamase (37) and the substrate hydrolysis mechanism of the dinuclear bovine lens leucine aminopeptidase (38) indicate that the nucleophilic attack on the substrate originates from a “terminally” bound – and not a bridging – hydroxide ion. The nucleophilicity of a hydroxide ion that is complexed to one metal ion is higher than that of a bridging hydroxide. Considering these results, we suggest a mechanism (bottom of Figure 6) in which a bridging hydroxide (as in the crystal structure) is found in the resting state of the enzyme, and the binding of the substrate causes a rearrangement of the complexation pattern. Following this, a terminally bound hydroxide with increased nucleophilicity is the species that attacks the amide carbonyl carbon.

It may be argued that the acidic pH leads to the protonation of an active site residue other than the metal-coordinating water and that fumagillin binding is therefore
facilitated by a modification of the non-covalent complex that precedes the binding reaction. However, the only groups that could readily be protonated within the studied pH range would be His79 and His178. His79 is the binding partner of fumagillin: A protonated and therefore positively charged His79 would certainly be much less prone to act as a nucleophile on fumagillin and it can thus be assumed that His79 remains neutral. If one therefore assumes that His79 is not being protonated, then it is also very unlikely that His178 is protonated, because the chemical surrounding (which could influence the pKa of the histidine imidazole) is quite similar for both histidines. Furthermore, literature values for the histidine side chain pKa range from 6 to 6.4, which is below the value of about 7 for the protonation event in the MetAP active site.

The results of the CPMD simulations presented here do not provide quantitative free energy data of the different protomeric states. It would certainly be very useful to obtain such data, which could be used to calculate the pKa of metal-bound waters and other ionizable groups in the active site. However, one must be aware of the fact that the prediction of pKa values would require very exact free energy data of different protomeric states and a very complete sampling of conformational space. Even in the classical-mechanical simulation framework (where the computational cost of simulations is smaller by several orders of magnitude and a much more complete exploration of conformational space is possible), free energies are notoriously difficult to predict. Thus, we think that a meaningful prediction of free energy differences and pKa values by ab-initio simulation methods is currently not feasible. The situation may be somewhat different when less complex systems, such as pure water or small solvated ions are considered. In such cases, present computer capabilities can be sufficient to adequately determine pKa values by ab initio MD simulations.

Recent experimental work (40) has shown that E. coli MetAP may – under certain circumstances – function as a mononuclear enzyme, i.e. with only one metal ion
coordinating to the active site residues. The metal content and the identity of the metal in the MetAP enzymes in vivo is not known. With the sole exception of the publication mentioned above, all experimental data of E. coli, yeast and human MetAPs, including all published x-ray structures, have been obtained with binuclear enzymes, because enzymes with lower metal content had only marginal or no catalytic activity under the respective experimental conditions. We are therefore confident that it is reasonable to assume the presence two metal ions in the active site as we did in our simulation system.

Fumagillin and its congeners inhibit the growth of vessels in tumors. Considering the fact that the extracellular pH in tumors is more acidic than in normal tissues,(41) and assuming that the endothelial cells of vessels in tumors are under the influence of the acidic extracellular tumor pH, one may reason that the pH profile of the fumagillin–MetAP binding reaction leads to selectivity (or targeting) of the fumagillin effect to tumor vessels.

The results presented here have several implications for the rational design of MetAP inhibitors: First, virtual screening (docking/scoring) procedures should preferably be made on MetAP active sites with a net charge of -1, if inhibition at neutral pH is desired (e.g., for MetAP inhibitors with antibacterial activity). The active site net charge has a pronounced influence on the binding energetics of ligands in virtual screening and in physical reality, because electrostatic interactions are the strongest driving force for intermolecular attraction and repulsion. Second, irreversible inhibitors different from fumagillin should preferably be compounds that are activated by a nucleophilic attack (coming from the hydroxide ion) or by deprotonation. Such inhibitors would be much more active at physiological pH than fumagillin. For instance, the deprotonation of β-hydroxynitrile esters (cf. Figure 7) causes cleavage of the ester bond and subsequent formation of acrylonitrile, which can then bind (as a "Michaelis-like" electrophile) to one of the cysteine residues near the MetAP active
site. The sequence of reactions would be based on a "basic activation" – not acidic as with fumagillin.

In conclusion, we have clarified the protonation behavior of the MetAP active site and made an experimental proof of a hypothesis that was generated on the basis of quantum-mechanical calculations.

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Figure Legends

Figure 1. Binding of fumagillin in the MetAP active site. Me = metal ion. The numbering is for E. coli MetAP.

Figure 2. The three different protomeric states for which CPMD simulations were performed.

Figure 3. Final structures of the CPMD simulations of the bi-water and the water-hydroxide systems in comparison with the x-ray structure. Hydrogens and the surrounding residues of the enzyme are not shown for clarity. The x-ray-structure is shown in CPK colors, the bi-water system in yellow and the water-hydroxide system in green. Me = metal ion, Wat = water.

Figure 4. Final structure of the active site with one hydroxide ion and one water molecule coordinated to the metals (Me1, Me2, purple spheres). The HOMO–1 orbital, located at the bridging hydroxide ion, is also shown (red and blue clouds). The cutoff for the visualization of the HOMO–1 electron density was 0.1 e/au^3.

Figure 5. pH-profile for the substrate hydrolysis and fumagillin binding reactions of E. coli MetAP (mean values with standard deviation error bars).

Figure 6. Mechanisms of the fumagillin binding and substrate hydrolysis reactions in MetAP. Only the initial steps of the substrate hydrolysis are shown (see the literature references in the text for more details about the MetAP substrate hydrolysis mechanism).

Figure 7. De-protonation of a β-hydroxynitrile ester, subsequent ester hydrolysis and formation of acrylonitrile. Acrylonitrile then binds to nucleophiles in the active site, e.g. histidine or cysteine residues.
