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STRUCTURE OF A MICROSPORIDIAN METHIONINE AMINOPEPTIDASE TYPE 2 COMPLEXED WITH FUMAGILLIN AND TNP470

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Abstract

Microsporidia are protists that have been reported to cause infections in both vertebrates and invertebrates. They have emerged as human pathogens particularly in patients that are immunosuppressed and cases of gastrointestinal infection, encephalitis, keratitis, sinusitis, myositis and disseminated infection are well described in the literature. While benzimidazoles are active against many species of Microsporidia, these drugs do not have significant activity against *Enterocytozoon bienersi*. Fumagillin and its analogues have been demonstrated to have activity *in vitro* and in animal models of microsporidiosis and human infections due to *E. bienersi*. Fumagillin and its analogues inhibit methionine aminopeptidase type 2. *Encephalitozoon cuniculi* MetAP2 (EcMetAP2) was cloned and expressed as an active enzyme using a baculovirus system. The crystal structure of EcMetAP2 was determined with and without the bound inhibitors fumagillin and TNP470. This structure classifies EcMetAP2 as a member of the MetAP2c family. The EcMetAP2 structure was used to generate a homology model of the *E. bienersi* MetAP2. Comparison of microsporidian MetAP2 structures with human MetAP2 provide insights into the design of inhibitors that might exhibit specificity for microsporidian MetAP2.

Keywords

Microsporidia; X-ray crystal structure; Methionine Aminopeptidase; *Encephalitozoon cuniculi*; *Enterocytozoon bienersi*; therapeutics

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Atomic coordinates and structure factors for the *E. cuniculi* MetAP2c-apo, MetAP2c-fumagillin, and MetAP2c-TNP470 structures have been deposited in the RCSB protein data bank with the PDB entries 3FM3, 3FMQ, and 3FMR, respectively.

1. Introduction

Microsporidia are a group of obligate, intracellular, parasites containing over 1200 species in at least 150 genera [1,2]. These organisms are significant pathogens with impacts on medicine, agriculture and aquaculture. The Microsporidia are eukaryotes containing a nucleus with a nuclear envelope, an intracytoplasmic membrane system, chromosome separation on mitotic spindles, a Golgi apparatus [3] and cryptic mitochondria [4]. While the initial studies on small subunit rRNA genes led investigators to conclude that the Microsporidia were “primitive” protozoa; it is now appreciated that the Microsporidia are degenerate protists related to fungi [5,6].

These organisms have been identified as parasites in all classes of vertebrates (including most mammals) as well as in most invertebrates [2]. Infections have been described in the gastrointestinal, reproductive, respiratory, muscular, excretory and nervous systems of their hosts [2]. Serosurveys have demonstrated a high prevalence of antibodies to microsporidia suggesting asymptomatic infection may be common [7]. Infections in humans have been described with the genera *Nosema* and *Vittaforma* [8], *Pleistophora* [9], *Encephalitozoon* [10], *Enterocytozoon* [11], *Septata* (now *Encephalitozoon*) [12], *Trachipleistophora* [13] and *Anncaliia* (previously *Brachiola*) [14-16].

Enterocytozoon bienersi is the most common cause of microsporidiosis in humans causing malabsorption, diarrhea and cholangitis [11]. The average prevalence of *E. bienersi* infection in patients with AIDS and chronic diarrhea was 30% prior to the widespread use of highly active antiretroviral therapy [2] and similar prevalence rates have been reported in both children and patients with AIDS in the developing world [17]. *E. bienersi* infection also occurs as a complication of organ transplantation [18] and has been reported in the elderly, travelers and immunocompetent hosts [19,20]. *Nosema*, *Vittaforma* and *Microsporidium* have been reported in immunocompetent patients with stromal keratitis associated with trauma [2]. *Pleistophora*, *Anncaliia* and *Trachipleistophora* have been reported in cases of myositis [9,13,15]. The Encephalitozoonidae (*E. cuniculi*, *E. hellem*, and *E. intestinalis*) have been reported in cases of keratoconjunctivitis, sinusitis, respiratory disease, prostatitis, hepatitis, encephalitis, diarrhea, superficial keratoconjunctivitis and disseminated disease [2,10,12].

Two groups of drugs have been identified as effective in the treatment of the various species of Microsporidia which infect humans [21]. The first group is the tubulin binding benzimidazoles which are effective for microsporidiosis due to the Encephalitozoonidae [21]. These drugs, however, are not effective for the treatment of microsporidiosis due to *E. bienersi* [2]. The second group of compounds is fumagillin and its derivatives. Fumagillin and its derivatives have activity against several groups of parasites including the Microsporidia [22,23]. It is used for the treatment of nosematosis, a microsporidiosis of honeybees, and was utilized to treat *Entamoeba histolytica* prior to the development of more effective amebicides [24]. Fumagillin has *in vitro* and *in vivo* activity against both Encephalitozoonidae and *Vittaforma corneae* [2,21,22]. Fumagillin has been demonstrated to have efficacy for the treatment of diarrhea due to *E. bienersi* in AIDS patients [25]. TNP-470, a fumagillin derivative developed as an anti-angiogenesis drug, is active both *in vitro* and *in vivo* against several microsporidia including Enterocytozoonidae [22,23].

Fumagillin, TNP-470 and ovalicin bind to and irreversibly inhibit methionine aminopeptidase type 2 (MetAP2)¹ [26,27]. The crystal structures of free and inhibited human MetAP2 demonstrated that a covalent bond is formed between a reactive epoxide of fumagillin and His231 (human MetAP2 numbering) in the active site of MetAP2 [28]. In yeast and higher eukaryotes two isoforms (type 1 and type 2) of MetAP exist. Fumagillin and its analogues do

not bind or inhibit the activity of methionine aminopeptidase type 1 (MetAP1) or other aminopeptidases. *Saccharomyces cerevisiae* deficient in MetAP1 (i.e. *map1* yeast dependent on MetAP2) are killed by ovalicin, but yeast deficient in MetAP2 (*map2* yeast dependent on MetAP1) are not [26,27]. Deletion of both MetAP1 and MetAP2 is lethal. These results confirm that fumagillin selectively targets MetAP2 and not MetAP1. The microsporidium *Encephalitozoon cuniculi* lacks MetAP1 based on the published *E. cuniculi* genome [29]; therefore, inhibition of MetAP2 by fumagillin is the most likely cause of cell death, analogous to the situation in *map1* yeast. It is likely, that other Microsporidia also lack MetAP1.

Our data [30] as well as that of other groups [31] indicate that the cellular target for fumagillin and its analogs in the Microsporidia is a MetAP2 ortholog and that this is an essential enzyme for these organisms. The crystal structure of human MetAP2 demonstrated extensive hydrophobic and water-mediated interactions that provide a mechanism for the observed selectivity of fumagillin for MetAP2 over MetAP1 [28]. Studies have also demonstrated that fumagillin derivatives display tissue and species selectivity in their ability to inhibit MetAP2 [32], suggesting that it should be possible to design selective MetAP2 inhibitors. The experiments described in this manuscript were, therefore, undertaken to obtain the crystal structure of a microsporidian MetAP2 in order to provide data that could facilitate the development of more selective MetAP2 inhibitors for the treatment of microsporidiosis.

2. Materials and methods

2.1. Cloning, Expression, and Purification

Encephalitozoon cuniculi methionine aminopeptidase type 2 (*EcMetAP2*) was cloned as previously described [30]. The domain of MetAP2 encompassing residues 2-358 (numbered relative to reference sequence NP_586190) was amplified by PCR and TOPO cloned into a custom TOPO adapted pFastBac (KF) vector (Invitrogen Corporation). Expression in this vector generated MetAP2 residues 2-358 fused to a TEV protease-cleavable N-terminal 6X histidine tag. Following cleavage, the protein generated is GSL–MetAP2 (2-358), where the GSL residues originated from the vector sequence. Standard baculovirus expression using the Bac-to-Bac system (Invitrogen) was used to generate recombinant virus. A 6 liter 48hr fermentation in Sf9 cells was carried out and cells harvested by centrifugation and pellets were stored at –80°C for purification.

For purification, the Sf9 cell pellet from a six liter fermentation was lysed in 240 ml of lysis buffer (0.05 M Tris-HCl pH 8.0, 0.25 M NaCl). HIS-tagged aminopeptidase was purified by Ni-NTA affinity chromatography in batch mode. After cleavage of the N-terminal his-tag by TEV protease, the aminopeptidase was further purified by a second Ni-NTA step. This material was concentrated and applied to a Sephadex S200 gel filtration column equilibrated with 0.01 M Hepes pH 7.5, 0.15 M NaCl, 0.01 M L-methionine, 10% glycerol, and 5 mM DTT. Fractions containing the aminopeptidase were pooled and concentrated to 2 mg/ml. As previously published, purification of recombinant *EcMetAP2* yields an approximately 95% pure enzyme as judged by SDS-PAGE [30]. Protein was stored at –80°C for long term storage.

2.2. *Homo sapiens* MetAP2 (HsMetAP2)

Recombinant human MetAP2 was purchased from Mediomix (St Louis, MO).

¹Abbreviations: MetAP2, methionine aminopeptidase type 2; Tris-HCl, Tris(hydroxymethyl)aminomethane hydrochloride; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); DTT, DL-Dithiothreitol; AAO, L-amino acid oxidase; HRP, horseradish peroxidase; MOPS, 4-Morpholinepropanesulfonic acid; CAPS, 3-(Cyclohexylamino)-1-propanesulfonic acid; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; PEG, polyethylene glycol.

2.3. Enzyme Activity Assays

Methionine aminopeptidase activity was measured spectrophotometrically by monitoring the free L-methionine formation after peptidolysis, using coupled enzyme assay comprised of L-amino acid oxidase (AAO) and horseradish peroxidase (HRP) [33]. Oxidation of o-dianisidine was monitored as an increase in absorbance at 450nm. This assay was adapted to 96-well assay format with a reaction volume of 50 μ l. To determine the optimum temperature the assay was performed at different temperatures in HEPES buffer pH 7.5 and for optimum pH the assay was performed at different pH (using MOPS, HEPES, Tris and CAPS buffers). After determining the optimum temperature and pH of activity to be at 37°C and pH 8.5 (Tris buffer) for *EcMetAP2* and pH 7.5 (HEPES) for *HsMetAP2* all assays were done under these conditions. A typical assay contained 50mM of buffer (either Tris pH 8.5 or HEPES pH 7.5), 100mM NaCl, 100 μ M CoCl₂, 1mM o-dianisidine, 0.5U HRP, 0.02U L-AAO, 0.5 μ g MetAP2, BSA at 0.1mg/ml and glycerol at 2% final concentration. The assay was initiated by the addition of substrate (2mM Met-Ala-Ser). The reaction was carried out at 37°C for 45min and absorbance at 450nm was measured at 5min intervals. Standard concentrations of o-dianisidine (oxidized by 0.6% H₂O₂ and 1U HRP) were used to calibrate the assay and determine the stoichiometry of the MetAP2 reaction. The initial rate was corrected for the background rate determined in the absence of MetAP2 enzyme but with all the other components of the assay. Assays were done in triplicates. To determine the optimum substrate concentration, K_m and V_{max} , assays were carried out at optimal conditions ie. pH 8.5 for *EcMetAP2* and pH 7.5 for *HsMetAP2*. Assays were performed with the substrate (Met-Ala-Ser) concentration ranging from 0.1mM to 8 mM. Analysis of the kinetic data was performed by using KaleidaGraph, fitting the Michelis-Menton double reciprocal plot.

To examine the inhibition of MetAP2 enzyme activity by Fumagillin and TNP470, fresh solutions of Fumagillin and TNP470 in 100% DMSO were prepared for the assay. Human MetAP2 or *EcMetAP2* (0.5 μ g) were incubated on ice for 30min with or without fumagillin or TNP470 in enzyme dilution buffer (20mM HEPES pH7.5, 40mM NaCl, 100 μ g/ml BSA, 2% DMSO). Reaction mixtures (50 μ l) contained 50mM assay buffer, 100 μ M CoCl₂, 4mM Met-Ala-Ser substrate, 0.2U L-AAO, 0.5U HRP and 1mM o-dianisidine. The reaction was started by adding the enzyme to the assay components, increase in absorbance at 450nm was measured every 5min at 37°C. To determine the IC₅₀ values of the inhibitors, 0.5 μ g of *EcMetAP2* or *HsMetAP2* enzyme was incubated for 30 min on ice with different concentrations of inhibitors diluted in enzyme dilution buffer having 2% DMSO.

2.4. Metal Analysis

The metal content of purified *EcMetAP2* was determined by X-ray absorption spectroscopy at the X3B beamline at the National Synchrotron Light Source (NSLS) [34]. The X-ray energy was set to 10 keV and the available instrumentation allowed for the detection of Mn, Fe, Co, Cu, Ni, and Zn. The appropriate volume of aqueous protein solution containing 0.1-0.15 mg of a protein was loaded in a sample well and dried overnight. Each sample was screened for the selected metals with sixty one-second-long scans using a 13-element Germanium X-ray fluorescence detector. Metal-to-protein stoichiometry was inferred by the comparison of the detected counts for a specific metal to an appropriately chosen set of standards for each metal under the same experimental conditions.

2.5. Crystallization

Diffraction quality crystals were grown by sitting-drop vapor diffusion at 20°C by mixing *EcMetAP2* (2mg/mL) and 30%(w/v) polyethylene glycol 4,000, 0.2 M ammonium sulfate. Crystals formed in seven to ten days with approximate dimensions of 0.1mm x 0.05mm x 0.01mm. Prior to data collection, crystals were cryoprotected by brief transfer to mother liquor supplemented with 10% (v/v) 2-methyl-2,4-pentanediol) and flash cooled in liquid nitrogen.

Crystals of *EcMetAP2* bound with fumagillin and TNP-470 were grown by co-crystallization. Prior to co-crystallization, *EcMetAP2* protein (2mg/mL) was dialyzed against one liter of (10 mM Hepes, 150 mM NaCl, 10% glycerol, 5 mM DTT, pH 7.5) for three hours with a change to fresh buffer every hour in order to remove any L-methionine from the original protein buffer. For co-crystallization, 100 mM stock solutions of fumagillin or TNP-470 (in 100% ethanol) were diluted to a final concentration of 0.5 mM in (27% PEG 4,000, 0.2 M ammonium sulfate). Co-crystals of *EcMetAP2* with either fumagillin or TNP-470 were grown as described for the free enzyme.

2.6. Data Collection and Processing

X-ray diffraction data (Table 1) were collected to resolutions of 2.18, 2.50, and 2.89 Å for the apo, fumagillin and TNP-470 bound crystals, respectively. All data were collected at beamline X29A at the National Synchrotron Light Source, Brookhaven National Laboratory (BNL). Data integration and scaling was carried out using HKL2000 [35]. Diffraction from these crystals is consistent with the monoclinic $P2_1$ space group with two molecules in the asymmetric unit.

2.7. Structure Determination and Refinement

The apo structure of *EcMetAP2* was determined by molecular replacement with the program MOLREP [36], using the structure coordinates of the human methionine aminopeptidase type 2 (*HsMetAP2b*) (PDB I.D.: 1YW9) as the search model (45% sequence identity). Residues not conserved between *EcMetAP2* and *HsMetAP2b* were pruned to the last common atom using CHAINSAW [37] to generate the search model. Two monomers are present in the asymmetric unit with a V_m of 2.4. The molecular replacement solutions were refined using rigid-body followed by restrained refinement using the program REFMAC5 [38]. In order to reduce any model bias, automated model building and refinement was carried out using the initial refined model in the program ARP/warp [39]. After the last cycle of refinement and model building, water molecules were added using ARP/wARP. At this stage of refinement two large spherical features (7σ and 9σ) present in difference Fouriers calculated with $F_o - F_c$ coefficients were modeled as iron ions. A final round of restrained refinement using all atoms was carried out. Final refinement statistics are summarized in Table 1.

Structure determination of *EcMetAP2* with either fumagillin or TNP-470 in the active site was determined by difference Fourier methods using the coordinates of the native *EcMetAP2* structure [39]. After the last cycle of refinement and model building, water molecules were added using ARP/wARP and iron ions were modeled manually. Significant features in difference Fourier maps were readily modeled as fumagillin and TNP-470. Atoms for which no electron density was observed in fumagillin and TNP-470 include (C44, C45, C46, C47, C48, C49, C4A, O4B, O4C) and (O4B, C44, CL4), respectively. Refinement statistics are summarized in Table 1. For all structures of *EcMetAP2*, electron density for residues 3 to 358 was clearly visible.

Model building was carried using the program Coot [40]. The stereochemical quality of the final refined models was evaluated using Procheck [41] and MolProbity [42]. Figures of X-ray structures were produced using Pymol [43]. The program ESPript [44] was used to produce sequence alignment figure 3.

3. Results and discussion

3.1. Expression of active recombinant *E. cuniculi* MetAP2 (r*EcMetAP2*)

Purified r*EcMetAP2* had enzymatic activity similar to that exhibited by Human MetAP2 (*HsMetAP2*) (Table 2). The highest activity of r*EcMetAP2* was exhibited using CoCl_2

(11.7 μ moles/min/mg protein), decreasing with MnCl_2 (7.9 μ moles/min/mg protein) and the lowest with FeCl_2 (2.9 μ moles/min/mg protein). *rEcMetAP2* was inhibited by both Fumagillin and TNP470 (Figure 1). Using the assay described in materials and methods the IC_{50} values of fumagillin and TNP470 exhibited by *rEcMetAP2* was 11.1 nM and 10.6 nM, respectively; while recombinant *HsMetAP2* demonstrated significantly higher IC_{50} values for Fumagillin (188.5 nM) and TNP470 (95.0 nM).

3.2. Overall Structure

We have determined the X-ray crystal structure of *rEcMetAP2* in its apo, fumagillin, and TNP-470 bound states at resolutions of 2.2, 2.5, and 2.9 Å, respectively. *E. cuniculi* MetAP2 exhibits a classic 'pita-bread' fold present in other methionine aminopeptidases (MetAP) from *E. coli*, *S. aureus*, *M. tuberculosis*, *T. maritima*, *P. furiosus*, and *H. sapiens* [28,45-51]. The 'pita-bread' fold of *EcMetAP2* is composed of a central beta-sheet with anti-parallel beta-strands β_3 , β_5 , β_6 , β_9 , β_{10} , β_{15} and flanking alpha-helices α_1 , α_2 , α_3 , and α_4 (Figure 2). A dinuclear metal center and the active site are located on the concave face of the central beta-sheet. Unlike the type-1 MetAP's which possess a 'pita-bread' fold with pseudo-two fold symmetry, this symmetry is broken in *EcMetAP2* by the insertion of a sixty-one residue subdomain (β_{12} , α_5 , α_6 , α_7 , β_{13}) found in type-2 MetAP's and a two-stranded anti-parallel β -sheet (β_7 , β_8) which is present only in the eukaryotic MetAP2 family members (Figures 2 and 3). As predicted by sequence alignment and confirmed by this crystallographic analysis, the insertion of the helical subdomain into the 'pita-bread' fold categorizes *EcMetAP* as a type 2 methionine aminopeptidase (*EcMetAP2*). Superposition of the α -carbon positions of *EcMetAP2* with Human MetAP2 (*HsMetAP2b*) result in a root-mean-squared deviation of 1.35Å over 355 α -carbon atoms, and further supports the inclusion of *EcMetAP* in the methionine aminopeptidase type 2 family (Figure 4). The members of the MetAP family are further differentiated based on the presence of an N-terminal extension of the catalytic domain. MetAP's possessing only the catalytic domain are designated 1a (*E. coli*) or 2a (*P. furiosus*), those with an approximately 100 residue N-terminal extension composed of two zinc finger domains followed by a linker are 1b (yeast/human) or composed of polybasic/acid residues are 2b (human), and those having relatively short extensions of approximately 40 residues have been designated 1c (*M. tuberculosis*) [45,47,52-55]. To date, X-ray structures for three MetAP type 2 enzymes have been determined from *Pyrococcus furiosus*, *Homo sapiens*, and in this work from *Encephalitozoon cuniculi* with each enzyme differing at the N-terminus of the catalytic domain. Like *HsMetAP2b*, *EcMetAP2* possess an N-terminal extension of the catalytic domain, albeit shorter (approx. forty residues) and with no polybasic/acidic character, while *P. furiosus* has no extension (Figure 3). In addition, structural sequence alignment of a homology model of a second microsporidian MetAP2 from *Enterocytozoon bieneusi* [Genebank accession number EED44036], having 48% sequence identity with *EcMetAP2*, suggests that the *E. bieneusi* enzyme would also have an N-terminal extension that is shorter than *EcMetAP2* (Figure Sequence alignment). With the addition of the *E. cuniculi* MetAP2 structure into the MetAP2 family and the differences at the N-terminus of the catalytic domains, a further subdivision of type 2 MetAP's should follow suit with *E. cuniculi* MetAP2 being designated 2c as its type 1c counterpart. Therefore, *E. cuniculi* MetAP2 will be designated as *EcMetAP2c*.

3.3. Active Site

During the process of model building and refinement two large spherical features (7σ and 9σ) present in difference Fouriers, calculated with $F_o - F_c$ coefficients, were observed amongst the putative metal coordinating residues suggesting the presence of two bound metal ions. In addition, these assignments are consistent with anomalous difference Fouriers, which revealed two large peaks of electron density (6σ and 8σ) amongst the putative metal coordinating residues (Supplementary Figure 1). As described in the experimental procedures, *EcMetAP2c*

was overexpressed in Sf9 cells infected with recombinant baculovirus and purified from cell lysate. At no time during expression, purification and crystallization was exogenous divalent metal added. Therefore, the metal bound in the active site could have been acquired from contaminating metal in the purification buffers since trace metals were not chelated from the buffers or alternatively metal could have been bound in the cytoplasm of the Sf9 cells during expression and carried through during purification. This same phenomenon of acquiring divalent metals from Sf9 cells during baculovirus expression and coordination in the active site has been observed by two separate groups overexpressing human MetAP2b (*HsMetAP2b*) [33,56]. A consensus for the physiologically relevant divalent metal ion for MetAP's has yet to be reached and both Mn^{2+}/Co^{2+} have been suggested for human MetAP2b, Fe^{2+} for *Pyrococcus furiosus* MetAP2a, Zn^{2+} for yeast MetAP-Ib, and Fe^{2+} for *E. coli* MetAP-Ia [33, 56-60]. Metal analysis of purified recombinant *EcMetAP2c* based on the method of Shi et. al. [34] determined the metal-to-protein ratios for Fe, Zn and Co to be 0.6, 0.2 and 0.1, respectively. Since Fe is the most abundant metal present in purified recombinant *EcMetAP2c* and enzymatic activity is observed using this metal ion, although four fold less activity than with Co^{2+} , we have modeled Fe^{2+} into the $F_o - F_c$ difference Fourier maps. These data do not identify or attempt to identify the physiologically relevant divalent metal ion for *EcMetAP2c* as Fe^{2+} , but support the existence of two metal ions bound by the putative metal coordinating residues in the active site and suggests that Fe^{2+} could be modeled into the corresponding electron density. As with other members of the MetAP family, the active site of *EcMetAP2c* resides on the concave face of the central beta-sheet, in a deep pocket with two metal ions bound and a hydrophobic side pocket that presumably provides specificity for binding of the N-terminal methionine of natural substrates. All the residues coordinating the Fe^{2+} ions are absolutely conserved in all MetAP sequences and in *EcMetAP2c*, including D130, D141, H210, E243, and E339, which are located on β -strands $\beta 5$, $\beta 6$, $\beta 9$, $\beta 10$, $\beta 15$ (Figure 5). Metal coordination is similar to other MetAP's with D141 and E339 coordinating both metal ions in a bidentate fashion, D130 is bidentate with metal 2, and H210 and E243 are monodentate with metal 1, and a single water molecule bridges both metal ions.

3.4. Binding of L-Methionine

The *EcMetAP2c*-apo structure was determined without L-methionine bound. However, superposition of the *EcMetAP2c*-apo structure with the product bound human *HsMetAP2b*-L-methionine structure (PDB I.D. 1KQ9) suggests the location of the methionine-binding site in the active site of *EcMetAP2c*. The methionine side chain is bound in a hydrophobic pocket that lies above the dinuclear metal center (Figure 6). Loops located on the periphery of the concave face of the central beta-sheet and from the subdomain insert provide the residues making up the S1 subsite hydrophobic methionine-binding pocket. The residues making up the methionine-binding pocket include F97, P98, H109, I217, H218 from the peripheral loops of the central beta-sheet and H261, V263, P292, and Y324 from the subdomain insert. Only two residues (H109 and H218) are absolutely conserved amongst the entire MetAP family while the remaining residues are exclusive to type 2 family members (Figure 3). The methionine side chain is bound in the hydrophobic pocket with H109 and H218 positioned behind the side-chain. Residues H109 and H218 are likely involved in catalysis as mutation of the structural counterparts in *HsMetAP2b* (H231 and H339) and *E. coli* MetAP1a (H79 and H178) result in abrogation or significant reduction in activity of the recombinant enzymes [53,61-63]. The structures of *E. coli* MetAP1a with bound transition state analogs, product, and product analogs and comparison with other 'pita-bread' enzymes such as aminopeptidase P has led to the proposal that H178 (H218 in *EcMetAP2c* and H339 in *HsMetAP2b*) stabilizes a tetrahedral intermediate of the transition state by hydrogen bonding to the carbonyl oxygen of methionine, while H79 (H109 in *EcMetAP2c* and H231 in *HsMetAP2b*) hydrogen bonds to the amide proton of the scissile bond [64]. In all structures of MetAP's with L-methionine bound in the active site, H178 of *E. coli* MetAP1a and its structurally equivalent residue in other MetAP's

form a hydrogen bond with the carbonyl oxygen of L-met. In *EcMetAP2c* H218, H178 in *E. coli* MetAP1, is just outside of hydrogen bonding distance to the carbonyl oxygen of the superimposed L-met from *HsMetAP2b*. In addition, in *EcMetAP2c* metal coordinating interactions between the carboxylate and amino group of the superimposed L-met from *HsMetAP2b* are observed and include N-terminal coordination with iron 2 and a bridging coordination between a carboxyl oxygen of L-Met and both metal ions; these interactions are conserved in all L-Met bound MetAP structures. The structurally conserved positions of *EcMetAP2c* H109 and H218, deleterious effects on hydrolysis by mutation of corresponding residues in *E. coli* MetAP1a and *HsMetAP2b*, and predicted contacts to L-Met strongly suggest that H109 and H218 are involved in N-terminal methionine hydrolysis.

3.5. Fumagillin and TNP-470 inhibition

In an effort to understand how fumagillin and/or TNP-470 (Figure 7A) could be modified to generate specificity for microsporidian MetAP2 over human *HsMetAP2b* we have solved X-ray structures of *EcMetAP2c* with fumagillin and TNP-470 bound in the active site to a resolution of 2.5 Å and 2.9 Å, respectively (Table 1). Electron density for fumagillin and TNP-470 was clearly visible within the active site, with $2F_o - F_c$ density visible for the cyclohexane ring and epoxide bearing isoprenyl substituent (Figure 7B and 7C). However, electron density for both the unsaturated decanoic acid side chain on fumagillin and the O-(Chloroacetylcarbamoyl) group on TNP-470 was visible up to carbon-43. Methionine aminopeptidases, specifically type 2, have been shown to be irreversibly inhibited by fumagillin and its derivatives through covalent modification of an active site histidine residue with the carbon atom of the spirocyclic epoxide at position C3 on the cyclohexane ring of fumagillin [27,28,46,61,65]. In the X-ray structures of *EcMetAP2c*-fumagillin and *EcMetAP2c*-TNP-470 electron density is clearly visible for a covalent bond between the Nε2 imidazole nitrogen atom of, the absolutely conserved residue, H109 and carbon 2 of the cleaved spirocyclic epoxide. Cleavage of the spirocyclic epoxide also results in the liberation of the oxygen atom and coordination to iron 1 (3.25 Å). The epoxide bearing isoprenyl sidechain of fumagillin/TNP-470 is bound in the methionine-binding pocket and makes hydrophobic contacts with F97, P98, I217, H261, V263, P292, and Y324 (Figure 7B and 7C). No significant movement of the active-site residues are observed upon binding fumagillin or TNP-470 with the exception of H218 which adopts a side-chain rotamer that is positioned away from the inhibitor in order to avoid close contact (Figure 7D).

The ordered portion of the unsaturated decanoic acid side chain of fumagillin and the O-(Chloroacetylcarbamoyl) group of TNP-470 protrudes from the active site and make hydrogen bonding contacts with the backbone amide and side-chain Nδ1 atom of H208. The O-(Chloroacetylcarbamoyl) group of TNP-470 is completely ordered in the human *HsMetAP2b*-TNP-470 structure (PDB ID. 1B6A) making hydrogen bonding contacts to ordered water molecules. In our *EcMetAP2c*-TNP-470 structure no electron density is observed for the terminal three atoms of the O-(Chloroacetylcarbamoyl) group, which is consistent with the absence of any additional potential interactions that might stabilize a single conformation. The unsaturated decanoic acid side chain of fumagillin in the human *HsMetAP2b*-fumagillin structure (PDB ID. 1BOA) is positioned in a single conformation by hydrophobic contacts with conserved residues L328, V374, and L447 and a single hydrogen bond with the main-chain amide of D376. Superposition of the *HsMetAP2b*-fumagillin structure (PDB ID. 1BOA) onto the *EcMetAP2c*-fumagillin structure suggests that the terminal carboxylate group of the decanoic acid side chain of fumagillin would be repelled by the negative charge from the side chains of D254 and D255 in *EcMetAP2c* (Figure 8A) and prevent the stabilization of a single conformation; this is consistent with a lack of electron density for a majority of the fumagillin decanoic acid side chain in our structure.

Sequence and structural comparison of *EcMetAP2c* and *HsMetAP2b* show that the active sites are virtually identical with very few changes in the residues making up the S1 and S1' subsites (Figure 8A). Comparison of a homology model of a MetAP2 from the microsporidia *Enterocytozoon bienewisi* with the *HsMetAP2b* structure, 43% sequence identity, shows that the active site residues are even more similar, than in *E. cuniculi* MetAP2c, to those in the human enzyme (Figure 8B). The microsporidian MetAP2 active sites are not, however; without amino acid differences compared to the human enzyme. Within the S1 hydrophobic methionine-binding subsite, *E. cuniculi* and *E. bienewisi* possess V263 and A245, respectively, while a methionine (M384 and M207) is present in the same position in the human and *P. furiosus* MetAP2 enzymes (Figure 8A and 8B). These changes to smaller amino acid residues in the S1 subsite of the microsporidian enzymes might provide some opportunities for the generation of fumagillin analogues that are specific for microsporidia MetAP2. Fumagillin analogues, active against human MetAP2b, have been synthesized with ring substitutions in place of the epoxide bearing isoprenyl group and with the isoprenyl group lengthened by one or more carbon atoms [66]. These existing fumagillin analogues could take advantage of the differences within the S1 subsite in human and microsporidian MetAP2 by changing the register of the reactive spirocyclic epoxide on fumagillin to prevent covalent modification of the active site histidine residue on *HsMetAP2b* (H231) and retain inhibition of the microsporidian enzymes. The importance of the amino acid residue differences found within the S1 hydrophobic methionine-binding subsite between the microsporidian, *E. cuniculi* (V263) and *E. bienewisi* (A245), and human (M384) MetAP2 enzymes is supported by fumagillin and TNP-470 resistant single site mutants of human MetAP2b. It has been suggested that the fumagillin and TNP-470 specificity determinants lie in the S1 hydrophobic methionine-binding subsite as shown by single site mutations (H382Y and Y444C) in the S1 subsite of human MetAP2b that result in inhibitor resistance [67].

Within the S1' subsite, where the unsaturated decanoic acid side chain of fumagillin lies, only modest sequence variation is observed, with *E. cuniculi* MetAP2c possessing D206, H208, and D254, while in human *HsMetAP2b* the structurally equivalent residues are N327, N329, and H375, respectively (Figure 8A). Superposition of the *HsMetAP2b*-fumagillin structure onto the *EcMetAP2c*-fumagillin structure suggests that the decanoic acid side chain of fumagillin could adopt a bound conformation in *EcMetAP2c*, as observed in the human enzyme, if the terminal carboxylate was modified to have functional groups that could make interactions with residues D254 and D255 in *E. cuniculi* MetAP2c. Modification of the terminal carboxylate group on the decanoic acid side chain of fumagillin to have a basic group (e.g., amino or guanidinium group) in place of a carboxyl oxygen atom might allow interactions with D254 on *E. cuniculi* and lock the fumagillin side chain into a single conformation in order to generate specificity for *EcMetAP2c*. Superposition of the homology model of *E. bienewisi* MetAP2 onto the *HsMetAP2b*-fumagillin structure suggest that the only amino acid variation in the S1' subsite is a I235 and K236 in *E. bienewisi* and V374 and H375 in the structurally equivalent positions in the human enzyme (Figure 8B). For *E. bienewisi* MetAP2, specificity would be better sought by directing attention to changing or modifying the epoxide bearing isoprenyl group on fumagillin and taking advantage of the S1 subsite amino acid differences where *E. bienewisi* has a much smaller residue (A245) than human MetAP2b (M384).

4. Conclusions

The methionine aminopeptidase type 2 from *Encephalitozoon cuniculi* has been shown to cleave the N-terminal methionine from substrates with kinetic values that are comparable to human MetAP2b. As with human MetAP2b, *E. cuniculi* MetAP2c is inhibited by the MetAP2 specific inhibitors, fumagillin and TNP-470. The X-ray structures of *E. cuniculi* MetAP2c and metal analysis of the purified recombinant enzyme have confirmed the presence of a dinuclear metal center occupied with two iron ions in the active site. Comparison of the *E. cuniculi*

MetAP2c and human MetAP2b structures has revealed amino acid differences in the active sites that may be exploited to design fumagillin analogues that are specific for microsporidian MetAP2. A homology model of the microsporidian MetAP2 from *Enterocytozoon bienersi* has been constructed, using the *EcMetAP2c* structure, which has provided a picture of the active site for design of specific fumagillin analogues. With the X-ray structures of the *E. cuniculi* MetAP2c enzyme, structure based drug design can now be carried out in order to develop a fumagillin class of inhibitor that has increased specificity for microsporidian MetAP2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Inhibition of EcMAP2 and HsMAP2 by Fumagillin (A) and TNP470 (B)

MAP2 enzyme (0.5 μ g) was incubated with or without Fumagillin or TNP470 in enzyme dilution buffer (20mM HEPES pH7.5, 40mM NaCl, 50 μ M CoCl₂, 100 μ g/ml BSA, 2% DMSO) for 30min on ice before starting the assay. Assay was performed as described in materials and methods. 50 μ l of reaction mixture contained 50mM assay buffer, 100 μ M CoCl₂, 4mM Met-Ala-Ser substrate, 0.2U L-AAO, 0.5U HRP and 1mM o-dianisidine. Reaction was started by adding the enzyme to the assay components, increase in absorbance at 450nm was measured every 5min at 37°C.

Figure 2. Ribbon diagram of *E. cuniculi* MetAP2

E. cuniculi MetAP2 is shown as a ribbon diagram with the catalytic domain and subdomain insert shown in dark and light pink, respectively. Two metal ions coordinated within the active site of the 'pita-bread' fold are represented as silver spheres.

Figure 3. Structural sequence alignment of *E. cuniculi* MetAP2 with other MetAP family members
 Structure-based sequence alignment of *E. cuniculi* MetAP2 with selected MetAP2 and MetAP1 family members. Numbering on the top is based on the sequence of *E. cuniculi* MetAP2. Identical and similar residues are on a red background and colored red, respectively. Residues coordinating metal ions and contacting fumagillin/TNP470 are indicated with a blue star and purple triangle, respectively. Protein data bank (PDB) structure coordinate ID's are: Human MAP2 (1BOA), *Pyrococcus Furiosus* MAP2 (1XGS), Human MAP1 (2GZ5), *Escherichia coli* MAP1 (1MAT), *Mycobacterium tuberculosis* MAP1 (1Y1N). *Enterocytozoon bienusi* Genebank accession number EED44036 [68].

Figure 4. Superposition of *E. cuniculi* and Human MetAP2 structures

Ribbon diagram of the superposition of *E. cuniculi* and human MetAP2 X-ray structures supporting the classification of *E. cuniculi* MetAP as a type 2 methionine aminopeptidase. *E. cuniculi* and human MetAP2 are shown in pink and green, respectively and active-site metal ions are depicted as spheres. The human MetAP2 structure coordinates are derived from PDB ID 1BOA.

Figure 5. Metal coordination within the *E. cuniculi* MetAP2c active site

Residues coordinating two iron ions within the active site are shown as sticks with black dashes representing coordination bonds. Iron ions and water molecules are represented as silver and red spheres, respectively. Bonding distances are in units of angstroms (Å).

Figure 6. *E. cuniculi* MetAP2c-apo with superimposed L-methionine

The active site of *E. cuniculi* MetAP2c-apo is shown superimposed with the bound L-methionine from *Hs*MetAP2b (PDB ID 1KQ9). Residues likely involved in catalysis (H109 and H218) and those making up the hydrophobic methionine-binding pocket are shown as pink sticks. L-methionine is shown as a stick with predicted coordination bonds to the iron ions represented as black dashes.

Figure 7. *E. cuniculi* MetAP2c complexed with fumagillin and TNP-470

(A) Structures of the natural product fumagillin and semi-synthetic product TNP-470. (B) The active site of *E. cuniculi* MetAP2c with fumagillin covalently bound to H109. (C) TNP-470 covalently bound to H109 in the active site of *E. cuniculi* MetAP2c. In (B) and (C) residues making covalent, hydrophobic, and hydrogen bonding interactions to fumagillin/TNP-470 are shown as sticks with hydrogen bonds depicted as black dashes and iron ions as spheres. Electron density ($2F_o - F_c$; 1σ contour) for both inhibitors is shown as red mesh. (D) Superposition of the *E. cuniculi* MetAP2c-apo (pink) and MetAP2c-fumagillin (blue) structures. The structures have a root-mean-squared deviation of 0.16 Å over 356 α-carbon atoms.

Table 1
X-ray Data Collection and Structure Refinement Statistics.

	EcMetAP2c-apo	EcMetAP2c-fumagillin	EcMetAP2c-TNP470
		Data Collection ^a	
space group	P2 ₁	P2 ₁	P2 ₁
a, b, c, (Å)	62.2, 94.3, 66.5	62.0, 94.2, 66.3	61.9, 94.1, 66.4
β(°)	99.2	99.4	99.3
resolution (Å)	48.92 - 2.18 (2.26 - 2.18)	50.00 - 2.50 (2.59 - 2.50)	50.00 - 2.89 (3.00 - 2.89)
R _{merge}	0.101 (0.302)	0.126 (0.294)	0.173 (0.473)
average I/σI	7.0 (2.2)	5.2 (2.2)	3.0 (2.4)
completeness (%)	94.7 (78.3)	97.2 (80.1)	99.2 (98.8)
redundancy	3.2 (2.0)	4.7 (2.8)	5.3 (5.3)
		Model Refinement ^b	
no. of reflections	37,498	25,385	16,710
R _{work} /R _{free}	0.19/0.25	0.18/0.23	0.18/0.26
no. of non-H atoms			
protein	5,539	5,525	5,516
ions/inhibitor	19	67	72
solvent	324	227	17
ave. B factors (Å ²)			
protein	22.75	18.10	25.41
inhibitors	NA	25.20	28.43
metals	35.33	21.17	36.73
sulfate ions	57.09	49.72	59.80
solvent	25.18	16.59	11.20
rms deviations			
bond lengths (Å)	0.015	0.008	0.009
bond angles (°)	1.583	1.186	1.198
estimated coordinate error (Å)	0.167	0.202	0.321

^aValues in parenthesis are for highest resolution shell.

^bData cutoff for refinement of all structures was F > 0.

Table 2Kinetic Parameters of Recombinant *E. cuniculi* and Human MetAP2^a.

	<i>EcMetAP2</i>	<i>HsMetAP2</i>
Specific Activity (μ moles/min/mg protein)	11.7	8.3
K_M (mM)	1.95 $\pm$ 0.14	2.08 $\pm$ 0.61
V_{max} (nmoles/min)	7.3 $\pm$ 1.7	2.3 $\pm$ 0.002

^a MAP2 assay was carried out at 37°C, pH 7.5 for HsMAP2 and pH8.5 for EcMAP2.^b Typical 50 μ l assay components include assay buffer (50mM HEPES pH7.5/50mM Tris pH8.5, 100mM NaCl, 100 μ g/ml BSA, 2% glycerol), 0.5 μ g MAP2, 2mM Met-Ala-Ser substrate, 0.02U L-aminoacid oxidase, 0.5U horseradish peroxidase, 100 μ M CoCl₂, 1mM o-dianisidine.