Structure of the angiogenesis inhibitor ovalicin bound to its noncognate target, human Type 1 methionine aminopeptidase

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Abstract

Methionine aminopeptidases (MetAPs) remove the initiator methionine during protein biosynthesis. They exist in two isoforms, MetAP1 and MetAP2. The anti-angiogenic compound fumagillin binds tightly to the Type 2 MetAPs but only weakly to Type 1. High-affinity complexes of fumagillin and its relative ovalicin with Type 2 human MetAP have been reported. Here we describe the crystallographic structure of the low-affinity complex between ovalicin and Type 1 human MetAP at 1.1 Å resolution. This provides the first opportunity to compare the structures of ovalicin or fumagillin bound to a Type 1 and a Type 2 MetAP. For both Type 1 and Type 2 human MetAPs the inhibitor makes a covalent adduct with a corresponding histidine. At the same time there are significant differences in the alignment of the inhibitors within the respective active sites. It has been argued that the lower affinity of ovalicin and fumagillin for the Type 1 MetAPs is due to the smaller size of their active sites relative to the Type 2 enzymes. Comparison with the uncomplexed structure of human Type 1 MetAP indicates that there is some truth to this. Several active site residues have to move “outward” by 0.5 Å or so to accommodate the inhibitor. Other residues move “inward.” There are, however, other factors that come into play. In particular, the side chain of His310 rotates by 134° into a different position where (together with Glu128 and Tyr195) it coordinates a metal ion not seen at this site in the native enzyme.

Keywords: methionine aminopeptidase; fumagillin; cancer; angiogenesis

Angiogenesis, the growth of new capillary blood vessels, is important not only in physiological processes but also for tumor progression and metastasis. In 1971, Folkman (Folkman 1971) proposed that the inhibition of angiogenesis is potentially a promising approach for the treatment of cancer. The natural products fumagillin and ovalicin, and their synthetic analog TNP-470 (Fig. 1) were shown to have potent anti-angiogenic activity (Ingber et al. 1990; Kusaka et al. 1994; Yamamoto et al. 1994). TNP-

470 was effective against tumor growth and metastasis in a wide range of in vivo tumor models (Yamaoka et al. 1993a, b; Bergers et al. 1999). Human Type 2 methionine aminopeptidase (*HsMetAP2*) was identified as the molecular target of these fungal secreted (Griffith et al. 1997; Sin et al. 1997), and biochemical and structural studies showed that these compounds alkylate an active site histidine (Liu et al. 1998; Lowther et al. 1998).

There are >60 anti-angiogenesis inhibitors in clinical trials, of which TNP-470 (Fig. 1) is most promising (Ingber et al. 1990). TNP-470 has cleared up to 100% of the human tumors in mice. In humans, however, several patients experienced neurotoxicity at doses where anti-tumor activity was seen (Bhargava et al. 1999; Stadler et al. 1999; Logothetis et al. 2001; Herbst et al. 2002).

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Figure 1. Structures of the natural products fumagillin and ovalicin and the synthetic derivative TNP-470.

This led to the belief that the spiroepoxide, which is sensitive to hydrolysis, could be a target of epoxide hydrolases. Fumagalone, a synthetic analog of ovalicin in which the active epoxide has been replaced by an aldehyde, has been shown to be a promising alternative (Zhou et al. 2003).

Several studies have indicated that it is the Type 2 and not the Type 1 methionine aminopeptidase (MetAP1) that is the target of ovalicin, fumagillin, and TNP-470 (Griffith et al. 1997; Sin et al. 1997). Based on the structures of the complexes with human MetAP2 and the structure of uncomplexed *Escherichia coli* Type 1 MetAP, it was proposed that the difference in specificity was due to a more sterically restricted active site in Type 1 MetAP (Liu et al. 1998). This conclusion was also generally supported by the recent determination of the structure of Type 1 human MetAP (*HsMetAP1*) (Addlagatta et al. 2005). It has, however, been shown that ovalicin at high concentrations (>2 mM) can inhibit *E. coli* MetAP (*EcMetAP*), a Type 1 enzyme in vitro. This showed that the active site is accessible to these inhibitors but with lower affinity (Lowther et al. 1998). Biochemical and spectroscopic studies (Lowther et al. 1998; Cosper et al. 2001; D'Souza et al. 2005) have shed some light on the mode of binding of ovalicin to *E. coli* MetAP. At the same time the lack of a structure of the complex with Type 1 enzyme has limited the understanding of the difference in affinity between the Type 1 and the Type 2 enzymes.

In this report we show that ovalicin will bind to Type 1 ($\Delta 1-89$) human MetAP and describe the crystal structure of the complex at 1.1 Å resolution. This provides the first direct visualization of the mode of binding of one of these angiogenic compounds to a Type 1 MetAP and how it differs from the Type 2 complex.

Results

Ovalicin binding

The structure of human Type 1 MetAP (truncated at residue 89) has been described (Addlagatta et al. 2005). We therefore focus on aspects related to the binding of ovalicin. For simplicity, we refer, respectively, to the free

and the bound forms of the enzyme as *tHsMetAP1* and *tHsMetAP1-ov*. Liu et al. (1998) described the complex of Type 2 human MetAP with fumagillin and also deposited the coordinates of the ovalicin complex in the Protein Data Bank (PDB; code 1B59). We refer to the latter complex as *HsMetAP2-ov*.

His212 in *tHsMetAP1-ov* is covalently modified by the spiroepoxy group of ovalicin (Fig. 2A). His212 is homologous with His231 of *HsMetAP2* and with His79 of *EcMetAP*, which undergo similar reaction. The liberated hydroxyl group from the epoxide forms a short hydrogen bond with the bridging water/ μ -hydroxo anion (2.66 Å) (Fig. 2B). The hydroxyl group on the inhibitor does not interact directly with either of the active site metal ions (closest distance 3.5 Å), consistent with EPR and EXAFS studies of Mn^{+2} -loaded *EcMetAP* (D'Souza et al. 2005). The inhibitor is surrounded by protein side chains and makes only a single backbone contact (a hydrogen bond to amide N-H of Cys301) (Fig. 2A). The anisotropic thermal factor analysis of the ovalicin (Table 1; Fig. 2C) suggests that the isoprenyl group has more freedom than the rest of the inhibitor. The bound ovalicin displaces two water molecules. Three oxygen atoms of the ovalicin, the C2 hydroxyl, the C3 methoxy, and the intact epoxy group are solvent-exposed. Two partially ordered waters have been modeled into diffuse density that is within hydrogen-bonding distance to these oxygen atoms.

New metal center

Comparison of the free and bound structures of *tHsMetAP1* (Fig. 2D) shows that the conformational changes are generally modest (in contrast to the larger changes that were anticipated) (Liu et al. 1998; Addlagatta et al. 2005). His310, however, undergoes a major shift, moving away from its position in the native enzyme to accommodate the incoming ovalicin. In the native structure, $N^{\epsilon 2}$ of His310 is hydrogen-bonded to Tyr195 (3.0 Å), which in turn forms a short hydrogen bond with Glu128 (2.6 Å). In its new position, $N^{\epsilon 2}$ interacts with a new metal ion (2.2 Å), not seen in the native structure. This ion also interacts with Tyr195 (2.0 Å) and Glu128 (2.4 Å). The

Figure 2. (A) A stereo view of an “omit” electron density map within the active site of *tHsMetAP1-ov*. Coefficients are $(F_o - F_c)$, where F_o are observed amplitudes. The calculated amplitudes F_c and phases were obtained from the refined model with ovalicin removed. The map is calculated at 1.1 Å resolution and contoured at 4.2 σ . The spiroepoxy group of ovalicin covalently modifies His212. The isoprenyl group is buried deep in the active site and surrounded by several hydrophobic residues. In the ribbon diagram, the catalytic domain of the protein is depicted in gray; the N-terminal region, in red. (B) Ball-and-stick representation of ovalicin (yellow) covalently linked to His212 of *tHsMetAP1*. The newly released hydroxyl group forms a hydrogen bond with the bridging water/hydroxo anion (red) between the active site cobalt ions (purple). (C) ORTEP diagram of ovalicin (yellow) and His212 (green and blue). The size of the ellipsoids represents the thermal motion. The isoprenyl side chain is less ordered than the ring moiety. (D) Stereo view of the superimposed active site residues of *tHsMetAP1* in the native (gray) and ovalicin-bound forms (red). Ovalicin is represented with thicker bonds. His310 rotates away from the active site providing space for the inhibitor. Similarly, several other residues surrounding the ovalicin in *tHsMetAP1-ov* undergo outward movement. Cys301, Phe309, and Met338 move inward.

other side of the metal ion is solvent-exposed with diffuse electron density, which was refined as two partially occupied water molecules and a glycerol. The new metal site was confirmed in a Bijvoet difference Fourier map (not shown) and appeared to be a cobalt ion.

The hydrophobic sesquiterpenyl side chain of ovalicin is surrounded by several residues, many of which are hydrophobic (Pro192, Tyr195, Phe198, Cys203, His301, and Trp353) (Fig. 2D). The side chains of some of these residues experience slight “outward” movement on inhibitor binding, the largest being Tyr195 (0.65 Å) (Table 2). Trp353 moves 0.45 Å, this being transmitted to the surface of the protein, particularly in the neighborhood of Glu128 in the connector region that interacts with the new metal ion. There are main-chain shifts in the connector region as large as 2.0 Å.

In making the covalent adduct with ovalicin, His212 moves by 0.5 Å into the active site. Phe309 also tilts by

~0.5 Å toward the isoprenyl group. The side chain of Cys301 adopts two alternate conformations in an estimated 0.6:0.4 ratio with the major conformer closer to the ovalicin (Fig. 2D). In the native enzyme, the thiol group points away from the active site (Fig. 2A,D). Another residue that undergoes inward movement is Met338, whose C $^{\epsilon}$ rotates by 60°, increasing the hydrophobic interaction with C5 and C6 of the inhibitor.

Changes in the active site metal binding residues

Asp229, a bidentate ligand of Co2 in the native enzyme, exhibits a second “perpendicular” conformation with estimated occupancy of 25% (Fig. 3). In this conformation the carboxylate group contributes only a single oxygen to the coordination sphere of Co2. As a result, the geometry around Co2 has changed from distorted octahedral to nearly perfect trigonal bipyramidal in 25% of the molecules in the crystal.

Table 1. X-ray data collection and refinement statistics for the complex of ovalicin with *tHsMetAP1*

Space group	P2 ₁
Cell parameters	
<i>a</i> (Å)	47.45
<i>b</i> (Å)	77.56
<i>c</i> (Å)	48.58
β (°)	90.51
X-ray data collection	
Resolution range (Å)	20–1.10 (1.12–1.10)
Collected reflections	1,559,503
Unique reflections	136,234 (4639)
Completeness (%)	95.5 (65.2)
$\langle I/\sigma(I) \rangle$	17.3 (2.7)
R _{sym} (%)	4.9 (31.1)
Refinement statistics	
R (%)	11.7
R _{free} (%)	14.5
Δ_{bonds} (Å)	0.01
Δ_{angles} (°)	0.03
PDB code	2GZ5

Numbers given in parentheses correspond to the highest-resolution shell of data.

Based on theoretical calculations, a similar conformation was predicted for fumagillin bound to zinc-loaded *EcMetAP* (Klein et al. 2003). The other residue in the metal center that is affected upon binding of ovalicin is Glu336, which donates one of its carboxylate oxygens to Co1. In *tHsMetAP1-ov*, the noncoordinating oxygen moves away by 0.4 Å, to avoid a close contact (2.9 Å) with C5 of ovalicin.

Discussion

Based on homology modeling, all MetAPs were presumed to have similar active sites. In contrast, however, it was found that fumagillin and its relatives can inhibit the Type 2 MetAPs with more than a million-fold higher affinity than Type 1. Comparison of the *HsMetAP2-ov* complex with free *EcMetAP* suggested that this difference might be due to the smaller active site of the Type 1 enzymes (Liu et al. 1998). The crystal structure of the ovalicin-bound *tHsMetAP1* provides the first opportunity to test this hypothesis.

Comparison of ovalicin-bound Type 1 and Type 2 human MetAPs

Figure 4A compares the structures of ovalicin-bound human Type 1 and Type 2 MetAP. His212 in *tHsMetAP1-ov* is homologous with His231 in *HsMetAP2-ov*, both undergoing covalent modification. The residues in the respective active sites that surround the inhibitor are different. *HsMetAP1* has mostly aromatic residues (Pro192, Cys203, Tyr195, Phe198, and Phe309), whereas *HsMetAP2* has several nonaromatic residues (Phe219,

Pro220, Ile338, Met384, His382, and Ala414). Distances between the inhibitor and the surrounding residues are compared in Table 2. Ovalicin adopts a somewhat different orientation in the Type 1 complex compared with that in Type 2 (Fig. 4A). In the former, the ovalicin rotates by $\sim 20^\circ$ around the covalent linkage, which positions the isoprenyl group deeper into the active site. Despite this change in orientation, the lone hydrogen bond between the C4-carbonyl and the main-chain amide of the protein is preserved (Fig. 4A). The limited space in *HsMetAP1* between His212 and the opposite side of the active site (near Cys301) compared with the corresponding distance in *HsMetAP2* (Fig. 4B) dictates a change in orientation. In addition, His212 moves ~ 0.5 Å deeper into the active site, further contributing to the different alignment. In addition to the above-mentioned rotation, ovalicin also experiences a slight tilt ($\sim 14^\circ$) in the mean plane of the cyclohexanone ring toward C^{ε1} of His231. One of the reasons for this tilt is to avoid a steric clash with His310 in its new position. The overall change in alignment reduces the distance between the C2-hydroxyl oxygen of the ovalicin and C^{ε1} of His212 by ~ 0.2 Å in *tHsMetAP1-ov* from 3.0 Å in *HsMetAP2-ov*, demonstrating the tight fit in the former case.

Although fumagillin, ovalicin, and TNP-470 do not bind to Type 1 MetAPs as efficiently as they do to the Type 2 enzymes, the present study clearly demonstrates that the active site of human Type 1 MetAP can accommodate such compounds. There does not appear to be a single reason for the reduction in the affinity. The smaller size of the active site is clearly a factor. Consistent with this, several active site residues in *HsMetAP1* move “outward” in order to make room for the inhibitor. Also there is the reduced space across the active site shown in Figure 4B. Another contributing factor is the relative orientation of His310 in *tHsMetAP1-ov* compared with that of His339 in *HsMetAP2-ov*. A rotation of 134° along C^α–C^β bond in His310 of *tHsMetAP1-ov* is significantly less than that in the His339 of *HsMetAP2-ov* structure (248°). In the new position His310 of *tHsMetAP1-ov* is coordinated to a third cobalt ion (Fig. 2A). If the His310 of the *tHsMetAP1-ov* were to undergo a rotation of $\sim 250^\circ$ as in *HsMetAP2-ov*, it would result in severe steric clash with the methoxy group on C3 of ovalicin. The new metal ion presumably contributes to the binding of ovalicin to Type 1 MetAPs. This suggests that, apart from the size and shape of the active site, metal ions may also influence the affinity of ovalicin and related compounds toward the different MetAPs.

Thr334 in *HsMetAP1* vs. Ala364 in *HsMetAP2*

Recently, in a yeast-based screen, it was demonstrated that a single amino acid change (Ala362Thr) could render *HsMetAP2* resistant to ovalicin (Brdlik and Crews 2004). This position in human MetAP1 is Thr334, which upon

Table 2. Closest protein-inhibitor contacts for ovalicin bound to Type 1 and Type 2 human MetAP

Ovalicin	Contact distance (Å)					
	<i>tHsMetAP1-ov</i> ^a		Native <i>tHsMetAP1</i> ^b		<i>HsMetAP2-ov</i> ^c	
C1	His212 CE1	3.3	His212 CE1	3.5	His231 CE1	3.3
C2	His212 CE1	3.6			His231 CE1	3.6
C3			His310 CD2	3.4		
C5	Glu336 CD	3.6	Glu336 CD	3.4		
C6	His212 CE1	3.6			His231 CE1	3.6
C11	His212 CE1	2.6	His212 CE1	2.9	His231 CE1	2.5
	His212 CD2	2.5	His212 CD2	2.7	His231 CD2	2.5
	His212 NE2	1.5 ^d	His212 NE2	1.8	His231 NE2	1.5 ^d
C21			His310 CE1	2.8		
			His310 CD2	3.2		
			His310 CE1	3.5		
C23	Tyr195 CE2	3.3	Tyr195 CE2	2.8		
	Tyr195 CD2	3.4	Tyr195 CD2	3.0		
C24			His310 CE1	3.1		
			Tyr195 CD2	3.4	Ile338 CG2	3.6
			Tyr195 CE2	3.6		
			His310 CE1	3.5		
C2A			His310 CG	2.5		
			His310 NE2	1.0		
			His310 CE1	1.6		
			His310 CD2	1.8		
C2C	Phe198 CG	3.6	Phe198 CG	3.5	Tyr444 CG	3.4
			Phe198 CD1	3.6	Tyr444 CD2	3.6
C31			His310 CD2	3.4	His339 CD2	3.6
O11			His310 NE2	3.0		
O41	Cys301 N	2.9 ^e	Cys301 N	2.9	Asn329 N	3.1 ^e

The table lists all inhibitor-protein contact distances of 3.6 Å or less.

^aThis work.

^bDistances derived from model building. Ovalicin was placed in the active site of free *HsMetAP1* by superimposing the *tHsMetAP1-ov* complex on free *tHsMetAP1*.

^cPDB code 1B59 (Liu et al. 1998).

^dCovalent bond.

^eHydrogen bond.

mutation to alanine renders the enzyme sensitive to ovalicin. In *tHsMetAP1-ov* Thr334 and ovalicin are on opposite sides of the active site with closest atoms being 6.5 Å apart (Fig. 5). There are no apparent structural changes near this residue when ovalicin binds. However, inspection of the secondary structures of the Type 1 and Type 2 enzymes indicates some subtle differences around this residue, irrespective of the presence or the absence of ovalicin. Ala362 in *HsMetAP2-ov*, which is part of the long β -strand (358–369 residues), forms a hydrogen bond with the main-chain amide of His331 in the adjacent strand (Fig. 5). But in *tHsMetAP1-ov*, O ^{γ} 1 of Thr334 replaces the main-chain carbonyl in forming the hydrogen bond with the analogous amide (His303) (3.0 Å) (Fig. 5). This leaves the Thr334 main-chain carbonyl with no hydrogen-bonding partner. As a result of rearranged hydrogen bonding between the β -strands, loss of the β -sheet structure occurs in this region in *tHsMetAP1* (Fig. 5). His303 is adjacent to the Cys301 region that

protrudes ~ 1.0 Å into the entrance of the active site, restricting the access of ovalicin in *HsMetAP1*. The Thr334Ala mutation in *HsMetAP1* could result in changes in the residue 334 β -strand as well as in the neighboring strand including His303. This could expand the active site by pushing the region around Cys301 away from the entrance. Thus the mutant *HsMetAP1* could accommodate ovalicin more readily than the native enzyme. A similar argument can be made suggesting that the Ala392Thr mutation in the *HsMetAP2* results in contraction of the active site, reducing the affinity for ovalicin (Fig. 5).

Materials and methods

Cloning, expression, and purification

Truncated human MetAP1 ($\Delta 1$ –89) was cloned, expressed, and purified as described previously (Addlagatta et al. 2005).

Figure 3. Stereo diagram of the active site metal center is shown with an “omit” electron density map around Asp229. The map is contoured at 4.5 σ . The “native” conformation of Asp229 is shown in green. An alternative conformation shown in yellow is estimated to occur at 25% occupancy in the inhibitor complex.

Protein was dialyzed into storage buffer (25 mM HEPES at pH 8.0, 5 mM methionine, and 150 mM KCl), concentrated to 30 mg/mL, and stored at -80°C until further use. Diffraction quality crystals were obtained by mixing 5 μL apo protein with 5 μL reservoir solution (4%–6% PEG 10,000 or 12%–16% PEG monomethyl ether 2000, in 100 mM HEPES at pH 5.4–6.2) in a hanging drop that was incubated at 25°C . Crystals ($\sim 0.2 \times$

Figure 5. Superposition of the ovalicin complex with Type 2 human MetAP (gray) and the ovalicin complex with Type 1 human enzyme (green). The figure shows the vicinity of Thr334, and the numbering is for *HsMetAP1-ov*. In *HsMetAP2-ov* the hydrogen bond between the backbone of Ala362 and the backbone of His321 is shown in red. The analogous hydrogen bond in *tHsMetAP1-ov* between $\text{O}^{\gamma 1}$ of Thr334 and the backbone amide of His303 is shown in green.

0.2×0.6 mm) were harvested after 48 h. To the apo crystals, 2 mM of ovalicin (final concentration) dissolved in absolute ethanol and freshly prepared CoCl_2 (1 mM, final concentration) were added and incubated for 24 h. Crystals were then transferred into a cryoprotectant solution (6% PEG 10,000 or 16% PEG monomethyl ether 2000, 25% glycerol, and 100 mM HEPES at pH 6.0) for 5 min and then directly frozen in liquid nitrogen.

A

B

Figure 4. (A) Stereo view of the superimposed active sites of *tHsMetAP1-ov* (green) and *HsMetAP2-ov* (gray). The relative orientation of ovalicin differs somewhat in the two structures. Also, His339 in *HsMetAP2-ov* moves much farther than His310 of *tHsMetAP1-ov*. (B) The distance between $\text{N}^{\epsilon 2}$ of His212 and the entrance of the active site is shorter (8.1 Å) in *tHsMetAP1* than that in *HsMetAP2* (8.8 Å). This is the region occupied by the cyclohexanone ring of the inhibitor.

Data collection, processing, and structure refinement

X-ray diffraction data were collected on beamline 8.2.2 at the Advanced Light Source (ALS) using a radiation of wavelength 0.977 Å. Diffraction data were processed by HKL2000 and Scalepack (Otwinowski and Minor 1997). The structure was solved by molecular replacement with EPMR (Kissinger et al. 1999), using the coordinates of the isomorphous native enzyme (PDB code 2B3K) with all water molecules removed (Addlagatta et al. 2005). After initial rigid-body and simulated annealing refinement, coordinates and B-factors were refined and water molecules included using CNS (Brünger et al. 1998). Initial coordinates for ovalicin were adapted from the complex with the Type 2 enzyme *HsMetAP2* (PDB code 1B59) (Liu et al. 1998) and geometric restraints obtained from the hic-up server (Kleywegt and Jones 1998). Atomic resolution data made it possible to avoid any restraints on bond and torsion angles on the inhibitor during refinement. Final refinement (Table 1) with SHELX (Sheldrick and Schneider 1997) included anisotropic thermal factors. Figures for this manuscript were generated using PYMOL (DeLano Scientific) and PLATON (A.L. Spek, Utrecht University, Utrecht, The Netherlands).

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