

Kinetic and docking studies of inhibitors targeting the catalytic zinc in MA clan enzymes



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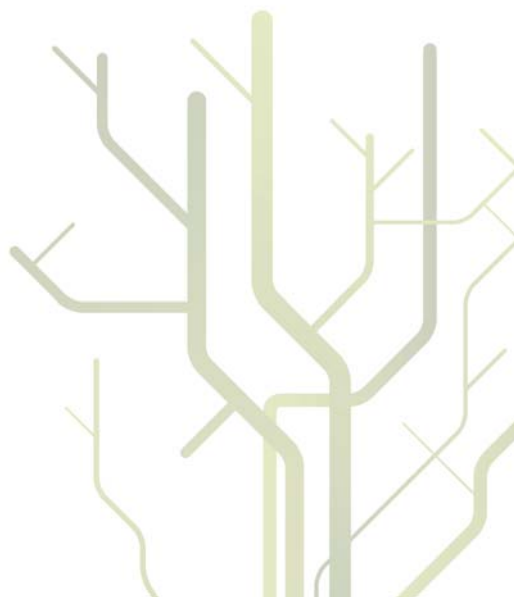


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List of Manuscripts

- I. Stian Sjøli, Øyvind Wilhelm Akselsen, Yang Jiang, Eli Berg, Trond Vidar Hansen, Ingebrigt Sylte and Jan-Olof Winberg. PAC-1 and Isatin 1,2,3 triazoles act as inhibitors in μM range of enclosed M10A, M13 and M4 enzymes. *Manuscript 1*.
- II. Stian Sjøli, Elisa Nuti, Francesca Casalini, Irina Bילו, Armando Rosello, Jan-Olof Winberg, Ingebrigt Sylte and Olayiwola A. Adekoya. Synthesis, experimental evaluation and molecular modelling of a series of hydroxamate derivatives as pseudolysin and thermolysin inhibitors. *Manuscript 2*.
- III. Olayiwola A. Adekoya *, Stian Sjøli * , Irina Bילו, Sérgio M. Marques, M. Amélia Santos, Elisa Nuti, Giovanni Cercignani, Armando Rosello, Jan-Olof Winberg and Ingebrigt Sylte. The inhibition of pseudolysin and thermolysin by hydroxamate based MMP inhibitors. *Manuscript 3*. * shared first authorship.

List of Abbreviations

ECE: Endothelin converting enzyme

ECM; Extracellular Matrix

IC₅₀; is the half maximal inhibitory concentration of a process.

k: rate constant

K_m or Michaelis-Menten constant; the equilibrium dissociation constant for an enzyme and substrate

K_i; the equilibrium dissociation constant for an enzyme and an Inhibitor

LIE: Linear interaction energy

MA(E): MA clan sub-group of families with a glutamic acid (E) as one of the zinc-tethering residues together with two histidines

MA(M): MA clan sub-group of families with three histidines as zinc-tethering residue. The MA (M) designation is based on a conserved methionine downstream of the catalytic triad that is incorporated in a structural fold - the «met-turn».

MePs; Metalloendopeptidase

MM: Michaelis-Menten

MM/QM: Molecular modelling/Quantum mechanical modelling

MMP; Matrix Metalloproteinases

MMPI; Inhibitor of a MMP

MT-MMP; Membrane tethered MMP

PDB: Protein database

proMMP; Inactive precursor of MMP

TIMP; Tissue inhibitor of MMPs

TLN; Thermolysin

ECM; Extracellular Matrix

V_{max}; Highest (initial) velocity, or turnover rate, of an enzyme, and is the enzyme saturation point.

ZBG: Zinc binding group

Preface

The subjects presented in the following could be combined in many ways depending on the reader's preferences and pre-existing knowledge. The chosen outline is a circle starting with description of the smallest unit – the enzyme, the enzymes form families or clans of families, then certain pathological environments that they are known to act in is presented. This creates a step-wise biological complexity, which somewhat decreases afterwards from drug-treatment in the body to inhibitor design and to theoretical modelling of individual enzymes. Theoretical modelling of the enzyme-inhibitor complex calculates approximations of free energy, which then builds on from the enzyme-section in the start. Catalysis (“Breaking peptide-bonds with water”) and substrate binding (“binding substrates”) might come prematurely for those that are not familiar with metalloproteases, and these readers might benefit from reading about the different enzyme families first.

Aim of Study

The aim of this thesis was to characterise inhibitors anticipated to target the catalytic zinc-ion found among other in enzymes from the M4, M10A and M13 families. This aim would be supported by the following smaller aims;

- I. Establish suitable FRET-assays and parameters for analysis of enzyme activity and inhibition
- II. Acquire numerical values of inhibition, IC_{50} and K_i
- III. Find and incorporate relevant enzyme-structures deposited in the Protein Database (PDB)
- IV. Dock the inhibitors in the different enzymes
- V. Correlate the numerical values of inhibition with the dockings, and investigating potential structure-function relationships of significance.

Introduction

1. Enzymes

Enzymes are predominantly proteins catalysing chemical reactions in living organisms, and have occupied the minds of 17th century researchers as well as present day scientists [1-5]. These biocatalysts are life essential, and were initially seen as vital forces contained within, as well as in need of, the cellular environment. Before the eighteenth century, the process of digestion was believed to be solely a mechanical process, similar to a meat grinder. The digestion of meat by stomach secretions and the conversion of starch to sugars by plant extracts were explored. In 1750 Rene-Antoine Reaumur fed his pet falcon pieces of meat enclosed in a metal tube with holes in it. He wanted to protect the meat from the mechanical effects of the bird's stomach friction [6]. When he removed the tube a few hours later, the meat had been digested, but the tube was still intact. It was evident that the digestion had resulted from chemical, not mechanical, action. Louis Pasteur coined the term «ferments» as the life force enabling yeast cells to ferment sugar to alcohol, and saw it as an act related with the life and organization of the yeast cell [7], while Wilhelm Kuhne first used the term «enzymes». This term was later used to refer to catalysis in non-living substances, while ferment was used to refer to catalysis by a living organism. Eduard Buchner described cell-free fermentation, which he attributed to enzyme «zymase» [8, 9]. Enzymes are, following his example, usually given the suffix -ase added to the name of the substrate they catalyse or the type of reaction when naming them. Enzymes can be crystallised, which allow structures to be solved by x-ray crystallography, and the structure of lysosyme was published in 1965 [10]. The biochemical studies on single enzymes began in the 19th century, but novel enzymes are still found and characterised on a daily basis today. Enzyme studies often investigate kinetic or thermodynamic properties of the reactions that the enzyme catalyses. Thermodynamical predictions do not require knowledge of the pathway between reactants and products, and give information on current properties. Thus, thermodynamics would focus on the initial state and final state, and potentially transition states that are long-living and stable enough to be identified (figure 1). Kinetics properties are dependent on the pathways from reactants to product. Kinetic properties can also reveal state-values as will be apparent in the following (figure 1).

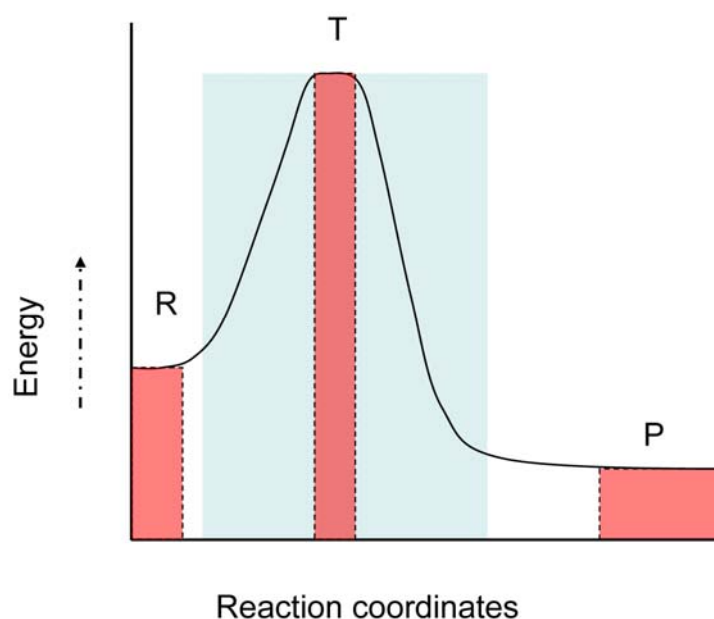


Figure 1: Diagram of a reaction, showing the energy niveau with reaction coordinates. The reactants R are transformed into product P. This is nomenclature for chemical reactions, and in the next figures reactants will be named substrates. The areas of thermodynamics, marked in red, are internal properties of the stable states such as initial state (R) and final state (P) and potentially long-lived transition states (T). Kinetics, marked in blue, is dependent on the reaction pathway between R and P.

1.1 Kinetics of Catalysis

Enzyme kinetics is the study of the chemical reactions that are catalysed by enzymes [1]. A catalyst increases the speed to achieve equilibrium of a reaction. Rate laws studies the net turnover rate of a given reaction, and thereby connects the reaction equation to a time factor [11]. The turnover rate must be determined experimentally, and normally either the reduction of substrate or generation of product is followed over a defined time period. The rate law will be proportional to both of these quantities.

1. $r = k[A]^x \cdot [B]^y$
2. $k = A \cdot e^{(E/(RT))}$

Equation 1,2: The rate. The equation consists of the rate (r), a rate constant (k), the concentration of two generic chemical species A and B (as an example), while x and y are coefficients that needs to be determined experimentally. In equation 2, A is the pre-exponential factor, E is the activation energy, and R is the gas constant, T is the temperature in kelvin.

The enzyme catalyses both directions, meaning that it is reversible (figure 2), but one wants to treat it as composed of two irreversible reactions [1]. This can for instance be done by starting far from equilibrium in the early phase with only substrate, and remove the reverse reaction catalysis.

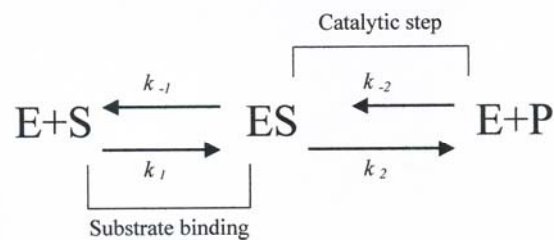


Figure 2: Illustration shows the two steps in an enzyme-catalysed reaction. Enzyme (E) binds to substrate (S) and form a complex (ES). The ES dissociates into E + S or E + P or associates from them. Total association is governed by $k_1 + k_{-2}$ and total dissociation by $k_{-1} + k_2$.

The assumption of quasi-steady state, in which substrate-bound enzyme ([ES]) changes much more slowly than substrate ([S]) and product ([P]), allows the amount of formed complex to be regarded as fixed. The total amount of enzyme, $[E]_{\text{tot}}$, is always the sum of free, $[E]_{\text{free}}$ and bound forms, [ES] and [EP] when the dissociation of product is a slow step [12]. The enzyme has one or sets of active sites where the catalysis occurs, and can not work faster when all of these active sites are occupied by substrate. The enzyme therefore achieves its highest initial velocity, designated V_{max} , and the turnover rate does not increase with addition of more substrate.

3. $E_{\text{tot}} = E_{\text{free}} + ES$
4. $V_0 = V_{\text{max}} \cdot [S] / K_m + [S]$
 - when $[S] > K_m$ then $[E]_{\text{tot}} \approx [ES]$ and $V_o = V_{\text{max}} = k_2 \cdot [ES]$
 - when $[S] = K_m$ then $[E]_{\text{tot}} = \frac{1}{2} [ES] + \frac{1}{2} [E]_{\text{free}}$ and $V_o = \frac{1}{2} (k_2 \cdot [ES])$
 - when $[S] < K_m$ then $[E]_{\text{tot}} \approx [E]_{\text{free}}$ and $V_o = (k_2/K_m) \cdot [E] \cdot [S]$

Equation 3, 4: Relationship between enzyme-forms (1) and initial velocity equation (2). E_{tot} is total enzyme amount, E_{free} is unbound (free) enzyme and ES is enzyme-substrate complex. V_0 is the initial velocity, V_{max} is the maximal velocity denominator, $[S]$ is substrate concentration, K_m is the Michaelis-Menten equilibrium constant.

The Michaelis - Menten constant, K_m , is the substrate concentration for which the reaction achieves half of the V_{max} [13]. According to the following equation, this entity will be low in cases where the enzyme has a high affinity for the substrate or stated differently; ES formation (k_1) is higher than the rate of ES dissociation ($k_{-1} + k_2$). This constant is independent of enzyme amount since it is usually in shortage compared to the substrate. The reaction might have several intermediate steps, govern by thermodynamics, but ultimately the rate limiting step will be the slowest reaction step. The K_{cat} relates the V_{max} to $[E]_{\text{tot}}$. The specificity constant is a ratio between substrate binding (K_m) and subsequent turnover to product (K_{cat}). The specificity constant describes the full reaction scheme, incorporating the rate constants for all the steps in the forward reaction. It can be seen as a measure of how efficiently an enzyme traverses a given substrate along the reaction pathway. This constant relates only to one substrate. Because the constants reflect both affinity (K_m) and catalytic ability (K_{cat}), it is useful for comparing different enzymes against each other, or a comparable measure of the adaptation of different substrates to the same enzyme.

5. $K_m = (k_{-1} + k_2) / k_1$
6. $K_{\text{cat}} = V_{\text{max}} / [E]_{\text{tot}}$
7. $K_{\text{sp}} = K_{\text{cat}} / K_m$

Equation 5, 6, 7: Equilibrium constants describing enzyme properties. K_m is the Michaelis-Menten constant, K_{cat} is the equilibrium constant for the catalytic step and K_{sp} is a fraction with numerator K_{cat} and denominator K_m .

This constant relates only to one substrate. Because the constants reflect both affinity (K_m) and catalytic ability (K_{cat}), it is useful for comparing different enzymes against each other, or serve as measure of the adaptation of different substrates to the same enzyme. The reaction rates, which are kinetic properties, can be used to find equilibrium constants of a given reaction, as for instance seen with equation 5, which subsequently enables us to calculate approximate values on thermodynamic quantities, such as Gibbs free energy (see equation 10).

1.2 Thermodynamics of Catalysis

All reactions are systems that imposes work on the surroundings [14]. Molecules destined to react with each other move around at different speeds dependent on their energy, and they collide with each other and the container. For a reaction to progress favourably collisions between reactants are necessary, and the collision angle, the relative translational energy, the internal (particularly vibration) energy will all determine the chance that the collision will produce a product molecule. Reactions have (a) short-lived intermediate(s) with a higher energy than the reactants creating an energy-barrier to cross. Molecules not able to achieve the activation energy will not traverse the energy-barrier and will not progress to product. Thus, a spontaneous reaction, meaning that it releases energy during progression, might still be stagnant or have a long reaction time if it is unable to cross this barrier. Catalysts, such as enzymes, lower this transition state energy barrier (see figure 3). The enzyme might do this by for instance offer proximity and aid orientation between two reacting molecules, donate a surface of electrostatic charges or a stabilising hydrophobic environment, form covalent intermediates with the substrate or even quantum tunnelling. The catalyst will return back to its initial state after catalysis. Reducing the activation energy leads to more product since more reactant molecules have enough energy to traverse the new energy barrier (figure 3 top). The enzyme thereby accelerates the time it takes to reach equilibrium by stabilising this transition state. Gibbs (free) energies (ΔG) are state functions that express the spontaneity of a chemical process in terms of enthalpy and entropy changes under conditions of constant temperature and pressure and is convenient for evaluating among other in which direction a reaction goes under experimental conditions (see equations 8-10).

$$8. \Delta G(p,T) = U + pV - T\Delta S$$

$$9. \Delta G(p,T) = \Delta H - T\Delta S$$

$$10. \Delta G(p,T) = -RT \cdot \ln K$$

Equation 8-10: Gibbs free energy $\Delta(G(p,T))$ as a function where p is pressure, T is absolute temperature, U is the internal energy of the system, V is volume, and S is entropy while H is the enthalpy of the system. R is the gas constant and K is the equilibrium constant.

The enzyme-catalysed reaction has several energy barriers to cross; in binding the substrate among other because it costs energy to de-solvate the substrate in entering the enzyme cavity (figure 3 bottom, step 1), activating and reorienting the substrate (step 2) and final release of product from the enzyme cavity (figure 3 bottom, step 3).

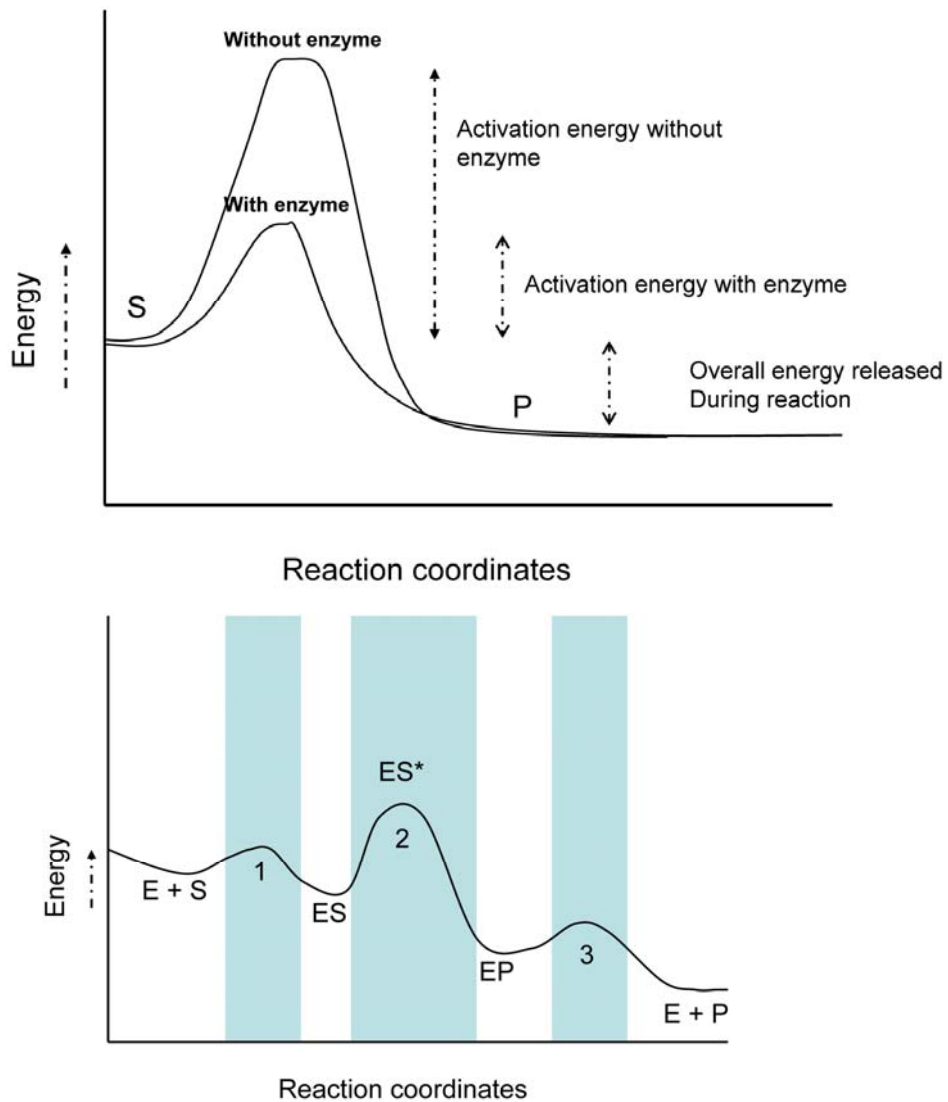


Figure 3: Diagram of a reaction without or with enzyme, showing the energy niveau with reaction coordinates (top). The reactants S, substrate, is transformed into product (P), and their respective internal energies are the same despite the potential different reaction pathways (enzyme-catalysed versus un-catalysed). Diagram of a reaction with enzyme, showing the energy niveau with reaction coordinates (bottom). Here is shown a more detailed view of the energy-barriers for the different steps in an enzyme-catalysed reaction. Area of interest in kinetics, marked in blue, and is dependent on the reaction pathway between the different molecules involved in each reaction step (1-3).

1.3 Breaking peptide bonds with water

The peptide bond is metastable and will uncatalysed break apart in water [15]. However, this process is slow. Hydrolytic enzymes, such as metalloendopeptidases (MePs) are able to break such bonds in peptides or proteins [16]. Proteases can be endopeptidases, which means that they cleave internal peptide bonds in substrates, or exopeptidases, which cleave terminal peptide bonds [16]. Exopeptidases can be further subdivided into aminopeptidases and carboxypeptidases [16]. The MePs are endopeptidases that are active at neutral pH [17, 18], but some can also have other substrate-dependent preferred pH-ranges [17], have a broader pH-range of activity [19] or be active at a lower pH [20]. MePs distinguish themselves from other endopeptidases by their dependence on metal ions as cofactors. The fact that some catalytically important amino acids are conserved among these enzymes suggests the evolution of a shared catalytic mechanism. Discoveries on individual enzymes might be transferable to other enzymes, and for instance thermolysin has been a prototype enzyme for other MePs [21]. Despite a wealth of information, there is so far no consensus on catalytic mechanism, but several suggestions agree at some points while diverge at others. Recruitment and positioning of a varying number (1-3) of water molecules is vital [22, 23]. One of these water molecules interacts with the zinc-ion and forms hydrogen bonds with a glutamate residue adjacent to the first histidine in the zinc-binding motif (see figure 4). This has been supported by x-ray structures as well as inactive mutants of this glutamate [24]. Crabbe et al. found the same for three mutant forms of proMMP-2 [25]. The proposed reaction mechanism by Matthew et al. is shown in Figure 3 [26]. The zinc-ion is now pentacoordinated with three interactions tethering it to the enzyme. These three interactions are either all histidines for the metcincins or two histidines and a glutamate for the gluzincins (see figure 6). In an intermediate, the glutamate has removed the hydrogen completely, and donated it to the amine-group. An oxygen atom of the carboxyl-group interacts with the donated hydrogen atom through hydrogen bonding. There are also hydrogen bond interactions formed between the substrate peptide and amino acids in the enzyme that acts to stable the transition form (aa₁ and aa₂). In the M4 family, aa₁ is a tyrosine (Tyr¹⁵⁷ in TLN) while aa₂ is a histidine (His²³¹ in TLN). The histidine is further stabilised by an aspartate (Asp²²⁶ in TLN). These amino acids have analogues in other

MePs, for instance Neprilysin [27-31]. The peptide bond is broken, resulting in the release of an amine and formation of an enzyme-carboxylate complex. The carboxylate is expelled from the active site with the introduction of a new water molecule.

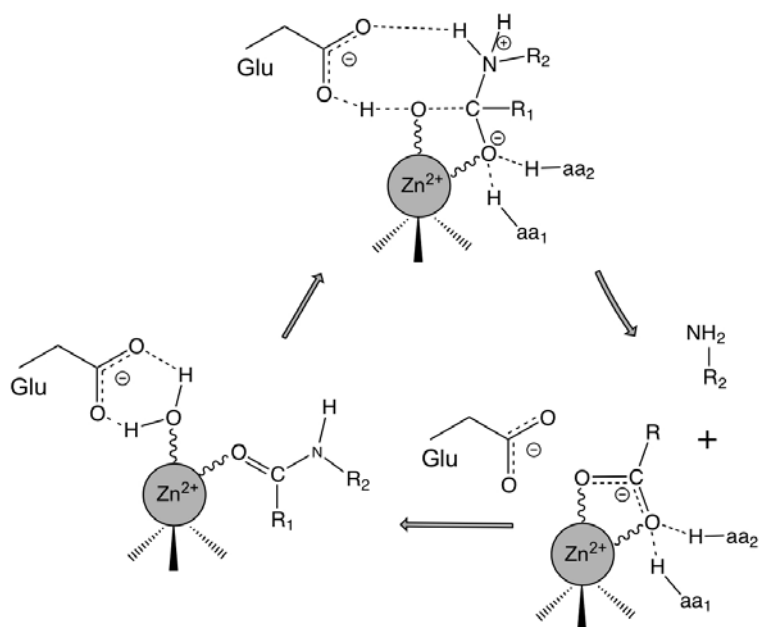


Figure 4: Proposed catalytic mechanism of peptide bond cleavage. The illustration shows the catalytic zinc-ion interacting both with a water molecule (that also interacts with a glutamate residue) and a peptide (bottom left), the water acts as a nucleophile and the peptide as electrophile (top) and the Enzyme-Carboxylate complex and amine product. The illustration is made with ChemBioDraw Ultra version 12, and is inspired by [26].

Enzymes can undergo conformational changes necessary for catalysis [32, 33]. There are some suggestions that TLN and related bacterial enzymes go through dynamic movements in the hinge-region that connects the C-terminal domain to the N-terminal domain [34]. These movements lead to a closer interaction of some of the amino acids in the active site as they are contributed by each domain.

1.4 Binding Substrates

Enzymes initiate binding to their substrate before catalysis, and it was first believed that the active site was complimentary shaped to the substrate (lock and key model [35]). It was later suggested that a perfect complimentary fit would create a co-stabilisation halting catalysis altogether, and a “*induced fit*”-model was suggested instead [36, 37]. Protein structure flexibility might also be used as an active means of gaining selectivity [37-40]. The binding energy needs to be strong enough to support a complex, but at the same time weak enough to catalyse the reaction and for the complex to dissociate [36]. This is supported by a balance between enthalpy- and entropy- terms. Enzymes themselves are stabilised by the effect of many (mostly weak) interactions between amino acids in the range of 30-65 KJ/mole, which is equivalent to only a few weak hydrogen bonds [41]. Thus, only a few intermolecular interactions between substrate and enzyme might have great impact on the stability of the complex. Hydrophobic and electrostatic interactions are inter-molecular non-covalent forces in the interaction [42-44], but also covalent bonds between enzyme and substrate can be formed and broken during catalysis [5, 45]. Hydration of non-hydrophobic interactions and inter-peptide attractive effects (hydrogen bonding, salt bridges, and classical van der Waals 6-12 type interactions) are of general stabilising nature, whereas for instance desolvation of the substrate and enzyme binding sites are energy-costly and serve as barriers promoting the destabilisation of the complex.

1.5 Substrate specificity

An enzyme might act on a few [46] or several substrates [47], depending on how unique the substrate properties that is recognised by the enzyme are [48, 49]. The ability to select preferred substrates resides in specificity. One of the factors contributing to specificity is making favourable interactions between the enzyme binding pockets, and corresponding residues in the substrate [50]. A binding-pocket is usually a cavity on the enzyme that attracts a certain charge or hydrophobic nature on the substrate-residues to complement its own charges inside the pocket. The architecture of the pocket can combine a largely hydrophobic surface with a charged bottom. This helps to focus the charge or the pocket might have asymmetrical charge distributions throughout the surface. The volume of the pocket is a result of the

structure, which is constantly vibrating and moving. Pockets are also often formed by flexible loop regions. Amino acid-residues lining the pocket may be directed inwards or outwards thus affecting the volume (Figure 5).

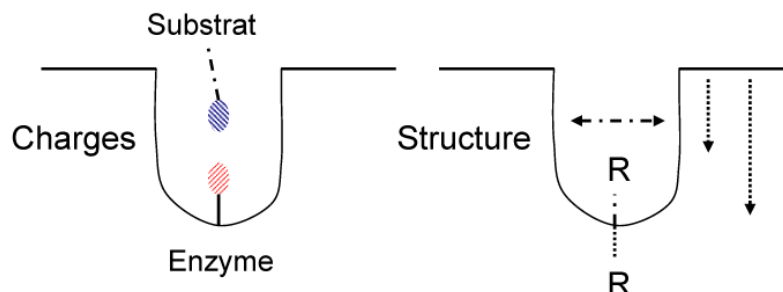


Figure 5: Substrate-binding pocket. The illustration shows an enzyme pocket (left) with one charge (red) and a substrate with the opposite charge (blue). The enzyme pocket (right) has a structure where the volume is dependent on the depth and width. An amino acid residue might be directed inwards so that the residue forms the bottom or might be directed outwards. The amino acid backbone will determine the bottom of the pocket if the residue is directed outwards creating a larger volume.

There are usually several sub-pockets close to the catalytic triad [51], generally extending from S3 to S4' in MePs [52]. The three dimensional structure creates a fold so that pockets can be side-by-side on one side along an imaginary horizontal axis on the substrate or on opposite sides of this axis. Subsites are numbered S1–Sn upwards towards the N terminus of the substrate (non-primed sites), and S1'–Sn' towards the C terminus (primed sites), beginning from the sites on each side of the scissile bond [53], and the substrate residues they accommodate are numbered P1–P_n, and P1'–P_n', respectively (figure 6).

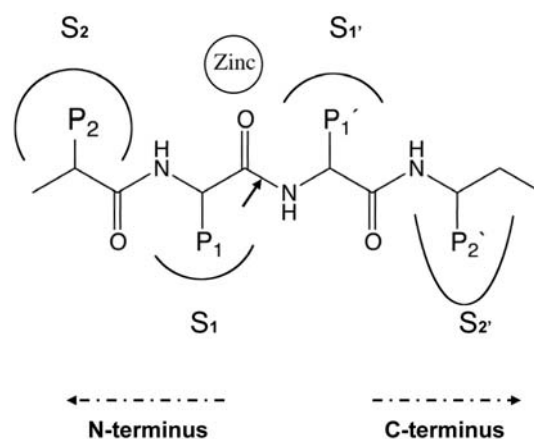


Figure 6: Substrate-binding pocket nomenclature. The illustration shows an enzyme active site with substrate. Subsites are numbered S1–S2 upwards towards the N terminus of the substrate (non-primed sites), and S1'–S2' towards the C terminus of the substrate (primed sites). Primed or non-primed sites are on opposite ends of a vertical axis of the scissile bond. The arrow points to the scissile bond.

A substrate might interact perfectly with all sub-pockets creating a strong interaction, or marginally with several pockets still with enough additive strength for binding. A few (or one) sub-pocket(s) being dominant might also be a possibility. It is also possible that some pockets interact negatively with the substrate, and then the other sub-pockets might compensate for the negative interactions of this pocket (securing substrate binding) or they might not, in which case the putative substrate is not bound and not a substrate anymore. Uniting sub-pockets is thus a molecular mechanism that may create specificity in some cases, while promiscuity in others. For instance, MMP-2 have been investigated with peptide libraries in order to map molecular recognition [54]. Small amino acids, such as alanine and glycine, were preferred in P1, but also asparagine and aspartate was accepted. Leucine in P1' (46 %) forms the predominant molecular specificity of MMP-2 along with other large hydrophobic residues. In P2', the MMP-2 preferences included basic amino acids, notably demethylated lysine formed during library preparation, and large hydrophobic residues. The P3' preferences included the small amino acids alanine, glycine and serine. This shows adaptation in individual pockets. However, the amino acid composition of the substrates seldom (fewer than 5 %) had optimal residue-interactions at all sub-sites. A given substrate would thereby create a number of optimal fits together with non-

optimal ones (neutral or potentially negative), which might help to explain the cleavage-site promiscuity of the enzyme – and inferred on the MMPs as a group. Substrate specificity is also regulated by overall structure of the active site, or even the global protein structure. MMPs and Thermolysin, have an exposed shallow active site cleft that enables them to catalyse break-down of both smaller and larger substrates [55]. In contrast, the neprilysin family are biased favouring smaller peptides instead of proteins [56]. An enzyme might also combine two catalytic processes in order to broaden substrate reportoar, such as the MMPs that have an additional triple helical activity that enables them to cleave bigger and more complex substrates [57].

1.5.1 Catalytic chamber

Neprilysin, NEP-like enzymes and Insulysin have a catalytic chamber that can encapsulate their substrate and for this reason they are referred to as cryptases [56]. The smaller entry port into the chamber contributes to substrate specificity by excluding larger proteins from gaining access to the active site (figure 7). The substrate selectivity is also determined by the size and charge distribution of the enzymes crypt as well as the conformational flexibility of substrates [56]. The NEP-like enzymes cleave amyloid - β peptides of 36-43 amino acids that are known to aggregate and form abnormal deposits in the brain of Alzheimers patients [56, 58]. The protein fold adapted by these peptides in the deposits are shared by for instance prions, which are disease-associated misfolded proteins [59]. The aggregates also possess an internal motif of four amino acids, FAED, which is similar to the putative TGF- β active site, WSXD. This similarity might explain why TGF- β is a substrate for these enzymes. The motif might also transfer TGF- β activity onto the amyloid- β proteins [60]. This illustrates that size and sequence-recognition in the substrate is important for specificity.

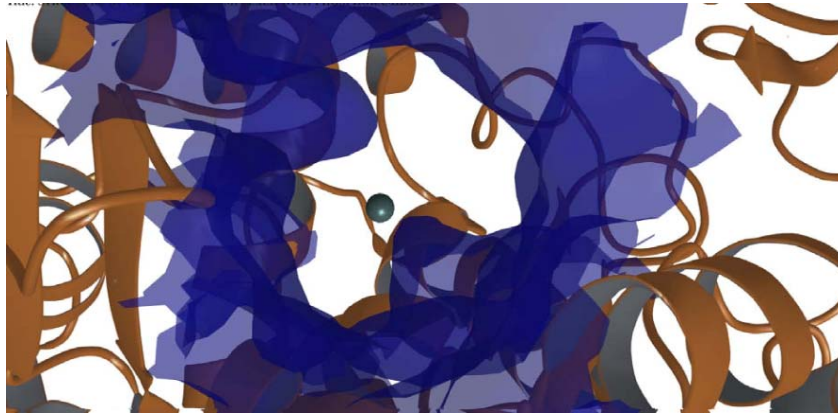


Figure 7: Illustration of restricted entry into the catalytic of NEP-1. The protein is coloured in gold and the zinc-ion is silver-coloured. A blue surface is generated that lines the borders of the entry into the active site (residues not specified). The illustration is made within Maestro (Schrödinger).

1.5.2 Triple helical hydrolysis

Some of the MePs are able to cleave substrates that are fibres [61-64]. Fibres consist of peptides or proteins, which have polymerised into longer chains of protein subunits. The collagen fibre consists of three α -chains of approximately 1000 residues with repeating Gly- X (often Pro)- Y (often HYP), which adopts a left-handed poly-Pro II like helix [63-65]. Three of these α -chains intertwine to form a right-handed superhelix, and the structural complexity makes it resistant to proteolysis by most enzymes [63, 65-67]. Several MMPs have been shown to possess collagenolytic activity [68, 69]. The ability of each enzyme to efficiently hydrolyze the triple helix is varying, and the gelatinases are weaker collagenolytic enzymes than for instance MMP-1 [61]. Ability to cope with thermal substrate stability has been shown to be important for catalysis [69, 70], while also substrate residues might facilitate local unwinding [71]. Enzymes also need regions outside of the active site for their affinity towards high-molecular-weight substrates, which are sometimes referred to as exosites. The helix needs to be unwound, and work on a catalytically inactive MMP-1 showed that the helicase activity was not dependent on the catalytic glutamate [72]. The enzyme can also move on the larger substrate [73, 74]. MMP-2 and -9 apparently required their inserts of FnII-like modules to hydrolyze elastin [75], collagen [76-79], and THP mimics of collagens [80]. MMP-2 has a collagenase activity, and the catalytic and hemopexin domains were shown sufficient to cleave rat type I collagen [81]. A later study found that collagen cleavage was reduced without the fibronectin

repeats, and found indications that MMP-14 and MMP-2 bind collagen at a different site than MMP-11 and MMP-8 [79]. MMP-9 has also been found to cleave triple helical collagens [82]. The formation of fibers exemplifies that increased structure complexity of the substrate also can lead to substrate specificity.

1.6 Families of Metalloproteases

It is estimated that about 2 % of the human genome constitute proteases [83, 84]. These are broadly grouped in five main types depending on the amino acid residues important for catalysis [85]. The largest class recruit a metal-ion as a prosthetic group [85]. The metalloproteases are among the most structurally and functionally diverse types of enzymes, but share a number of functional properties such as pH optimum near neutral, inhibition by chelating agents and resistance to inactivation by other reagents. 80 families are so far annotated in the MEROPS database [86, 87]. The largest category of the metalloproteases, and also metalloproteins in general, tether a catalytic zinc-ion to their active site. In general the zinc-binding is usually performed by three amino acid residues, these are either His, Glu, Asp or Lys residues as they are known metal-binding residues. The HEXXH motif is relatively common among all proteins, but is a shared and defining characteristic among the majority of zinc-metalloproteases. This motif is often “abXHEbbHbc”, where “a” is most often a valine or threonine, “b” is an uncharged residue while “c” is a hydrophobic residue. The metalloenzymes with this motif belongs to the MA clan (MA). MA includes several enzyme families of pharmacological or biotechnological interest, which all require one catalytic metal ion that in most cases is zinc. Thereby, the clan (together with the ME clan) differs from other clans (MF, MG, MH, MN and MQ) that coordinate two metal ions acting in collaboration on the substrate in order to complete the catalytic process [88]. MA(M) and MA(E) are both in MA, and are commonly referred to as metzcincins and gluzincins respectively (see figure 8).

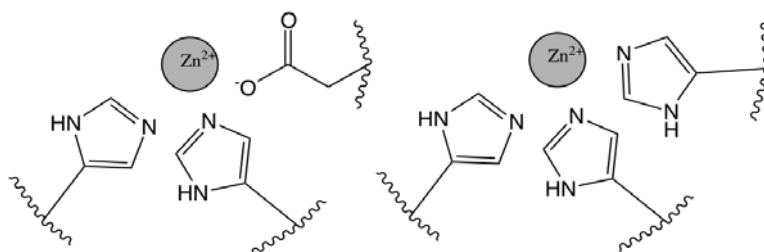


Figure 8: Illustration of the catalytic triad interaction with zinc-ion in MA (E) (left) and MA (M) (right). The illustration is made with ChemBioDraw Ultra version 12.

The MA(E) enzymes all have a glutamic acid as one of the zinc-tethering residues together with two histidines, while the MA(M) has three histidines. The MA (M) designation is based on a conserved methionine downstream of the catalytic triad that is incorporated in a structural fold - the «met-turn» [89].

1.6.1 Activation mechanism

MePs are sometimes produced as inactive precursor enzymes [90, 91]. Activation of these precursors then becomes an important posttranslational regulation mechanism. MMPs exemplifies this, and it was early believed that the MMPs were kept inactive by inhibitors and that they were activated as a consequence of inhibitor-removal from the enzyme-inhibitor complex, either through proteolysis or dissociation of the inhibitor [92-94]. Studies on human fibroblast collagenase (HFC) found that all modes of activation tested lead to dissociation of the cysteine residue (Cys⁷⁵) from the zinc atom with following exposure of the active site [93, 94]. It was then proposed that this residue in the propeptide domain was oriented towards the catalytic zinc-ion in the inactive enzyme, thereby blocking the access to the active site, and that upon activation this interaction was broken [93, 94]. The interaction can be broken by dislodging the N-terminus, where the cysteine is found, so that a water-molecule can enter and initiate autolysis or by enzyme-cleavage (figure 9) inside the bait region to expose the catalytic zinc-ion. The partially active enzyme can remove parts of its N-terminus, where the cysteine residue is located, in an inter-autoactivation reaction thus acquiring full activity. This lead to the idea of a “cysteine switch” since the cysteine residue is “off” when the protein is active and “on” when the protein is in its inactive

form. Later studies found that this cysteine residue is conserved in most known MMPs, and these are probably all activated in this manner [94, 95]. The cysteine-switch has for some enzymes not been sufficient for activation [96] and there are alternative pathways [97]. There are also novel MMPs discovered without the classical cysteine switch [98, 99]. In pseudolysin, the propeptide is cleaved in the periplasm but remains associated with the mature peptide and this non-covalent complex is inactive. It has recently been suggested that the propeptide contains a peptidase inhibitor domain known as 'pepSY' [100]. The N-terminal propeptides are autocatalytically removed, and it has been shown that this propeptide-part assist in folding of the protein [101]. Enzymes can be dependent on other factors, such as salt, in the activation process as for instance seen for thermolysin [102-105].

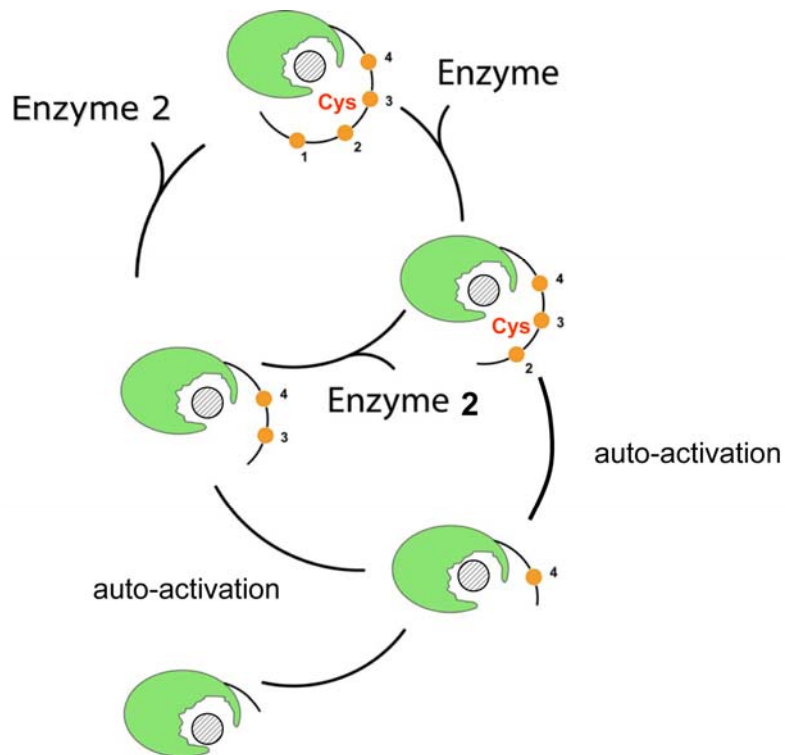


Figure 9: Activation of a precursor MMP. Aspects of the illustration such as cleavage and activation by another enzyme and/or auto-activation may apply to other MePs. The zinc-ion in the precursor enzyme is blocked from interacting with water by a peptide extension, allowing a cysteine-residue to replace the water molecule in interacting with the zinc-ion. After an enzyme cleaves in this region, the catalytic water molecule enters the active site and the precursor gains partial activity, even though the cysteine residue is still present, and can activate itself further. Now the cysteine-residue is removed and full activity is gained. An Enzyme might cleave in collaboration with a second enzyme (enzyme 2) or enzyme 2 might cleave the initial precursor form independent of the other enzyme.

1.6.2 M4 the Thermolysin family

Eubacterial endopeptidases that are secreted by both gram-positive and gram-negative bacteria with similarity to thermolysin are found in the M4 family [106]. Most members of the family degrade extracellular proteins and peptides for bacterial nutrition, especially prior to sporulation, but they can also be used as virulence factors [21]. A small subset of amino acids dictates substrate specificity among M4-proteins [107]. Most members of this family have a preference for cleavage of Xaa+Yaa, in which Xaa hydrophobic residue and Yaa is Leu, Phe, Ile, or Val and primary for an aromatic residue in P1' [108, 109]. There are so far 24 family members, with at least an experimentally determined structure for five according to the MEROPS database . There are 125 hits in the PDB-database for thermolysin alone, but individual hits should be carefully reviewed to make sure that all are thermolysin structures. The enzymes have a two-domain structure with the active site between the domains [110]. The N-terminal domain includes a distinctive six-strand beta sheet with two helices, one of which carries the zinc-tethering catalytic triad. This N-terminal domain is similar in other families (for instance M10) while the C-terminal domain is unique for the family, is predominantly helical and carries the third zinc ligand involved in stability. Some of the thermolysin structures are co-crystals with inhibitors, address structural changes in solvent or radiation-response or represent the enzyme alone. Msp protease from *Legionella* may have a role in the virulence of Legionaire's disease [111]. Vibriolysin from *Vibrio cholerae* is a haemagglutinin that has been shown to affect intracellular tight junctions by degrading occludin [112].

Thermolysin

Thermolysin (TLN) is a neutral metalloproteinase enzyme with a molecular weight of 34.6 KDa initially discovered to be secreted by *Bacillus thermolyticus* Rokko in 1962 [21]. TLN degrades extracellular proteins and peptides that are subsequently taken up by the bacteria as nutrition. The bacteria are an extremophile, meaning that it thrives in high temperature environments, necessitating temperature-stable enzymes [21]. TLN exemplify this by retaining half of its activity following incubation at 86.9 degrees for 30 minutes [113, 114], and shows no signs of any major conformational changes associated with denaturising until at least 70 degrees. In 1972, thermolysin became the first metalloprotease for which a structure could be determined by

experimental methods [110]. The enzyme was found to consist of two roughly spherical domains separated by a deep cleft. The N-terminal domain consists mostly of beta-sheets, while the C-terminal domain is dominated by alpha helices. The authors had to re-examine the electron density maps on the original thermolysin structure, assumed to represent free enzyme, and found that it probably contain a dipeptide in the active site and instead correspond to an enzyme-inhibitor or enzyme – substrate complex [34]. Thermolysin hydrolyses specifically peptide bonds on the amino side of large hydrophobic residues [26, 115, 116], and may therefore also used in partial fragmentation of proteins prior to Mass spectrometric analysis [117]. The enzyme has been used in the location of disulfide bridges in molecules resistant to other proteases [118]. Thermolysin is also used to catalyse the formation of precursor molecules of aspartame [119], which is an artificial sweetener, and is included in washing liquids for degrading protein stains on clothes. Less of the bitter-tasting by-products are formed during aspartame-synthesis catalyzed by thermolysin. Thermolysin is also widely used in reverse hydrolysis reaction forming peptide bonds between supplied precursor molecules, has uses in prion disease diagnosis [120] and has also aided discovery of putative inhibitors towards other enzymes for which it shares some active site features, for instance Neprilysin and MMPs [121].

Pseudolysin

Pseudolysin is 33 KDa large enzyme with an isoelectric point of 5.5 [122, 123] with the first structures resolved by x-ray in 1991 [124, 125]. There are currently three structures deposited in the PDB-database (accession numbers: 3DBK, 1U4G, 1EZM), and the enzyme is similar to thermolysin both by sequence, structure and partially in substrate preferences. Pseudolysin has a more open substrate-binding cleft compared to thermolysin and contains four cysteine residues, which form disulfide bonds with their nearest neighbours and has one calcium ion-binding site, while thermolysin bind four calcium ions and has no cysteine residues [21]. Pseudolysin seems to first have been cloned and expressed in 1987 [126], but the elastase cleaving properties of *Pseudomonas aeruginosa* isolates had already been known for more than 20 years before this time [127]. Studies included works on the strain isolates themselves [128] as well as investigations with partially purified protein extracts [129]. There are several site-directed mutant produced that verify residues involved in either catalysis [130-134] and/or substrate-binding. There are several substrates found for this enzyme

[135]. Pseudolysin is the major virulence factor of *Pseudomonas aeruginosa* septicaemia, causing tissue damage (especially in the lung where elastin is degraded) and may compromise the immune system by degradation of immunoglobulins, complement components and serpins.

1.6.3 M10A the MMP-1 family

The matrix metalloproteinases constitute the M10A family according to the MEROPS database [136]. The founder enzyme is MMP-1, or trivial name interstitial collagenase-1, that was first found present and active in tadpoles undergoing metamorphosis [137, 138]. The enzyme was found involved in collagen metabolism, and a human orthologue enzyme was first identified in rheumatoid synovium. There are at present 24 counted family members, who includes two connected identical genes of MMP-23, or 23 non-redundant members [139]. The zinc-binding motif in this family consists of the consensus sequence HEXGHXXH and a conserved methionine eight residues downstream this sequence that forms a «Met-turn» [140]. MMP-1 can be dissected into a signal peptide region, a prodomain, a catalytic domain, a linker sequence followed by a C-terminal domain homologues to hemopexin and vitronectin (hemopexin domain) [141, 142]. These features are all found in individual family members, except that MMP-7 and MMP-26 are truncated without the hemopexin domain and others may have additional domains. These additional domains are for instance three fibronectin type II domains found in the active site of MMP-2 and MMP-9, or stalk regions and transmembrane domain seen in MT1-MMP [142, 143]. Combined the family members can cleave several static extracellular components such as collagen that normally are very resistant to turnover [144, 145], and they have recently been found to have broader activities acting on for instance cytokines and participate in regulating other signalling molecules [146-167]. There are also suggested intracellular locations and substrates for some of the MMPs [168]. The individual family members can be regulated on a transcriptional level, by compartmentalisation, regulation of access to their substrate and by native inhibitors. The TIMPs, tissue inhibitors of MMPs, are one example of native inhibitors of these enzymes. MMPs seem to touch almost every aspect of mammalian biology, and there are more than 1,000 new publications each year focusing on MMPs [137].

Gelatinases

Two M10A family members have been found active towards denatured collagens, and these are MMP-2 (also known as gelatinase A) and MMP-9 (also known as gelatinase B) [61, 143]. Three fibronectin repeats inserted in the active site separates these enzymes from other MMPs [77, 78, 169, 170]. Variations in the substrate specificity of the gelatinases suggest that they have different sub-site preferences [171]. MMP-2 remains the most widely expressed MMP, and is produced constitutively by cells in culture as well as in tissue environments, while MMP-9 has an induced expression pattern in fewer cell types [172-175]. Both gelatinases are found in plasma, while MMP-9 also in the saliva. Human proMMP-9 is predicted to be slightly larger than proMMP-2 (~76 KDa versus 72 KDa), which in part is explained by a longer hinge-region. SDS-PAGE indicates a size of 92 KDa for proMMP-9 (105 KDa in mouse), and the extra mass has been shown to be due to N- and O- linked glycosylation [175]. Both gelatinases are inhibited by zinc-chelators, among their unique features is the binding of their proforms to TIMP-1 (gelatinase B) and TIMP-2 (gelatinase A) [175, 176]. They are differently regulated at the transcriptional and extracellular level, and may sometimes differently impact on the same disease [177].

MMP-2

MMP-2 was the first of the M10A gelatinases to be discovered [178], and is constitutively expressed by many cell types [172]. MMPs are often inducible and tightly regulated on a transcriptional level, and the constitute expression has been linked to several differences (compared with other MMPs) in elements in the promoter-region [172]. The protein activity has been suggested to be regulated on a posttranslational level by; secreting, compartmentalisation, activation and inhibition [179, 180]. Because of the known involvement of MMP-2 in pathological disease [181], there have been several attempts to find good inhibitor drugs specifically inhibiting this protein (inhibitor-chapter in this thesis). The enzyme has been crystallised [95] . Unlike the collagenases (MMP1 and MMP8), which uses the hemopexin-like domain to bind and position substrate for cleavage at the active catalytic domain, MMP-2 uses the C-terminal domain to bind cell surface attachment sites and for activation [68].

MMP-9

MMP-9 is the second member of the matrix metalloproteinase family that is recognised to be a gelatinase, and was found in 1974 [182]. The number “9”-designation was given after a MMP meeting in 1989 [143]. The proteolytic activity was investigated using different sources [183, 184], and it was found to migrate slower than MMP-2 in a gelatine zymography. The size was found to be ~90-110 KDa. Human proMMP-9 is predicted to be slightly larger than proMMP-2 (~76 KDa versus 72 KDa), which in part is explained by a longer hinge-region, but extra mass has been shown to be due to N- and O- linked glycosylation [185-187]. Cancer-associated glycoforms of the enzyme has been found [188] and also shown to influence on binding-strength to some substrates [189]. MMP-9 contains all the typical domain structures of the MMPs, but like MMP-2 has three fibronectin repeats in the active site. These repeats have sequence differences. The hinge-region is longer and more flexible in MMP-9 than MMP-2 because of a unique collagen V like domain found here. Gen expression is regulated so that the enzyme is normally expressed by trophoblasts, osteoclasts, neutrophils and macrophages, but the expression is inducible [190, 191] and the enzyme has been found expressed in [192-195]- and contributing to- several pathologies [196-202]. There are several substrates, either cytokines or larger ECM components [203].

MMP-14

MMP-14 was the first membrane-tethered MMP to be discovered, and has since then received a lot of research attention [204]. The enzyme has also been found to act as an important *in vivo* - activator of other MMPs [204]. MT1-MMP is shown to form a ternary complex with TIMP-2 and proMMP-2, which can lead to activation of MMP-2 [205]. It was first proposed that the complex creates an intermediate active MMP-2, which gains partial activity and subsequently mature to the fully active form [205]. In a supplementary activation pathway, proposed by Lafleur et al., TIMP-2 aids in the maturation of the intermediate, but is not essential for the formation of the intermediate [206]. The discovery of the MMP-14 activation pathway paved the way for findings on other membrane tethered MMPs in the activation of proMMP-2 [207]. MMP-14, has shown to infer malignancy to non-tumorigenic as well as tumorigenic

cell lines, frequently expressed by many types of cancer cells [208], is the only MMP that in knock-out mice causes lethality and has been found necessary for tumour dissemination in an genetically induced cancer model [209, 210]. The enzyme is apart of cell membrane, which enables it to be a focal point for enzyme activity at the edges of podosomes and invadopodia [211, 212]. The extracellular part of the enzyme, harbouring the active site, can be shedded [213]. The enzyme can also be shuttled inwards via the actin filament, accumulate in centrosomal compartment and cleave pericentrin [214-216]. Pericentrin is an integral component of the centrosome that serves as a multifunctional scaffold for anchoring numerous proteins and protein complexes, and is likely important to normal functioning of the centrosomes [217]. The cleavage of pericentrin by MMP-14 is suggested to cause chromosomal instability, aneuploidy and subsequent tumorigenicity [214-216]

1.6.4 M13 the Neprilysin family

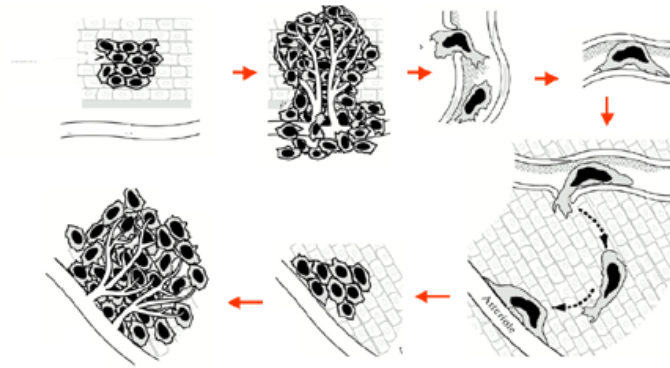
Neprilysin is a mammalian cell surface ecto-metallopeptidase that belongs to the MA clan, and also serves as the prototype for the M13 protein family [218, 219]. The human family currently consist of around 8 known members (NEP-1, NEP-2, ECE-1, ECE-2, PEX, XCE, DINE, KELL), and Except for ECE-1, all members of this family have first been discovered through their sequence homology with NEP and thereafter found to be catalysts [219]. The neprilysin family are interesting drug targets because of their involvement in pathologies [220-246], but also have other important functions [247]. Neprilysin can act on enkephalin [248], which are endorphins as they bind to the body's opioid receptors, and enkephalinases are thus potential analgesic drug targets. The family has also many other substrates [249]. The protein database contained in august 2010 several structures of individual neprilysin family members that can be instrumental for further modeling studies, and therefore are examined here. There are seven entries in the PDB-database for neprilysin (entries; 2QPJ, 2YVC, 1Y8J, 1R1H, 1R1I, 1R1J, 1DMT) with a resolution of 1.95 – 2.60 Å. All, except 1Y8J, includes a co-crystallized inhibitor among other phosphoramidon. There is so far only a single structure for human ECE-1, complexed with phosphoramidon, with a resolution of 2.38 Å (entry; 3DWB).

2. Pathological roles for MePs

Enzymes, as biocatalysts, do work together to establish and maintain homeostasis in a living organism [250]. There are several control mechanisms that secure that individual enzyme are not over-active or under-active (thesis-chapters 1.61 and 4.4.2). Enzymes not properly regulated will build-up (if over-active) product molecules or accumulate substrate (if under-active), and the organisms dependent on the enzyme will divert from the normal homeostasis. The organisms will now show symptoms and signs of disease. The study of a disease looking into mechanism and processes that are dys-regulated is referred to as pathogenesis. It is important to note that dys-regulation of a given enzyme can form basis for different types of diseases (thesis chapter 1.6), but they can also work together to promote a disease. This chapter will focus on tumor metastasis and bacterial invasion and sepsis. Extracellular matrix degradation and blood vessel regulation are vital steps that are explored in more depth.

2.1 Tumor metastasis

The process where cancer cells spread from the primary tumour and form secondary tumours in another organ is referred to as metastasis [251]. In contrast to the primary tumour, that often can be surgically removed, secondary tumours are harder to eradicate surgically and/or by localised irradiation. Metastasis is therefore the principle cause of death for most cancer patients [252, 253]. The metastatic process is a multistep process [253], as illustrated in Figure 10, and each step in this process is tightly regulated. Initially, the metastatic cancer cells must be able to detach from the primary tumour and penetrate into the circulation [251].



Figur 10: Illustration showing different steps in tumour metastasis. The illustration was found online (<http://molpath.ucsd.edu/faculty/Yang.shtml>). Permission of used was granted by associate professor Yang Jing, University of California, San Diego.

Vital in this invasion process is the down regulation of adhesion molecules that tether the cancer cell to its neighbours. Furthermore, the cells must cross the basal lamina and the endothelial lining and penetrate into the blood or lymphatic vessel. In the circulation the cancer cells will survive for shorter or longer periods before they enter a microenvironment expressing the corresponding adhesion molecules, and then the opposite sequence of reactions can take place (extravasations). The cell penetrates the vessel wall and establishes a tumour in the new organ by proliferation. Both the escape from the primary tumour and colonisation of the remote site are complex processes and are crucial for metastases being referred to as an inefficient process. Only a very low amount of the tumour cells detaching from the primary tumour are supposed to form a metastatic lesion [254]. Metastasis is therefore a complicated process involving a variety of different proteins at each step.

2.2 Bacterial invasion and sepsis

Bacteria invade body parts or tissues to secure self-sustainment or for improving living conditions causing local tissue injury and potentially disease [255, 256]. Helping agents such as enzymes and their substrate products are called virulence factors [257]. Numerous metalloenzymes aid the bacteria in this process and they may be recruited for direct or indirect degradation of connective tissue (thesis-chapters 2.3 and 1.6.2). *Pseudomonas aeruginosa* is for instance an opportunistic pathogen that can cause fatal infection in susceptible hosts [258]. *Pseudomonas* damages the host tissue directly and is also able to increase the mucus viscosity to make it less elastic and less transportable [259]. Adhesins enables the bacterium to adhere among other to host cells and colonize lung or wounded areas [256]. Like many other human pathogens, the microbe can inject toxic proteins directly from its own cytoplasm into the cytoplasm belonging to a host cell [260]. Pseudolysin is probably responsible for the destruction of arterial elastic laminae in the vasculitis observed in cases of *Pseudomonas* septicaemia [261]. Septicaemia is when viable bacteria enters into the blood system, and is commonly referred to as blood poisoning. The presence of a pathogen, such as bacteria, may provoke a systemic inflammatory response throughout the body, which is known as sepsis [262, 263]. Although this is a systemic response, it might be triggered by local presence of pathogens in the blood, urine, lungs, skin, or other tissues. Self-preservation is then also mediated by interfering with the hosts defence mechanisms. Pseudolysin and several other proteases from different pathogenic bacteria have been found to cleave plasma proteins such as immunoglobulins [264-266], complements factors [267, 268] and cytokines that are immunactive. The effect on the host cells are also up-regulation of cytokines and chemokines, affected cytoskeleton as well as other signal glitches rendering the immune cell unable to move towards and engulf bacteria. Severe sepsis is the accumulation of negative effects from the systemic inflammatory response, the infection, as well as potential organ dysfunction.

2.3 Extracellular Matrix Degradation

Cells in the body are surrounded by a thick layer of different macromolecules called the extracellular matrix (ECM) [269]. The ECM includes the interstitial matrix and the basement membrane in addition to defining the local environment of individual or communities of cells [269]. This matrix is composed of diverse types of molecules outside the cells acting as a compression buffer against various stresses imposed on cells, connecting cells and functions as a reservoir for signalling molecules. The matrix itself is composed of proteoglycans (heparin sulphate, chondroitin sulphate and keratin sulphate), non-proteoglycan polysaccharide (hyaluronic acid), fibers (collagen and elastin) and also fibronectin and laminin [270]. Extracellular matrix turn-over is however needed both in normal homeostasis and pathologies necessitating recruitment of proteolytic enzymes for this job. Traditionally the MMPs are recognized as ECM re-modelling enzyme, and some claim that they collectively cleave everything in the ECM, but ECM re-modelling is not performed by these enzymes alone [63, 270]. Hydrolysis of elastic fibers and other components of the ECM likely occur via both extracellular and intracellular means [63]. Altered regulation of these enzymes and subsequent remodelling of the ECM are hallmarks of several pathologies [271-273]. Many signalling molecules, previously tethered to the ECM, are also released and activated in these processes, while product fragments from the partial degradation of extracellular matrix components, recently termed Matrikines, can also become active transmitters of signals in close-by cells [274-279]. The weakened infrastructure, after ECM degradation, enables cells to migrate from their normal environment, for instance in cancer metastasis, as well as gain entry to blood and lymphatic system by breaching through basement membranes lining these organs. The extracellular fragments have been shown to act as chemotactic agents [280, 281], change cell behaviour [282] and affect host interaction for invading microbes and tumours. Most bacterial proteinases have a weak degradative activity against collagen, and bacteria might recruit collagenases from the host [283]. Fibroblasts are found locally at these sites already before infection, while cells such as neutrophils and macrophages are recruited when bacteria are present. These cells secrete several enzymes such as proMMP-8, proMMP-1 and proMMP-9. MMP-8 and MMP-1 cleave native triple helical type I collagen producing fragments that can denature and subsequently be cleaved by MMP-9 [283]. These MMPs were purified from different cell-types and their activation both by different bacterial proteinases and homologues from different bacterial species was examined [283]. Their results indicated that vibrio proteinase and pseudolysin showed strong activation of proMMP-9, and highlighted a potential

mechanism were bacterial enzymes can indirectly create ECM destruction and tissue damage by affecting host enzymes.

2.4 Blood-vessel regulation

The blood vessels are tubes that transport blood around in the different parts of the body [284]. The blood vessel diameter is mostly regulated by contraction or relaxation of the smooth muscle tissue in around the vessel [285]. The tissue responds to several factors that are broadly divided into vasodilators or vasoconstrictors [286]. The vasodilators decrease contraction in this tissue causing increased vessel diameter and increased flow of blood as the resistance or blood pressure decreases. Sympatic nerves or surrounding endothelial cells can regulate the vessel by transmitting paracrine signals onto the tissue [287-289]. Bradykinin (BK) is a nine amino acid long peptide belonging to the kinin group[290]. This peptide causes among other blood vessels to dilate [291]. BK is thought to act as a local janus-faced hormone, exerting both unwanted- and beneficial physiological effects [291]. This fact makes it's regulation by local kininases, enzymes cleaving kinins, in the blood or on the endothelial cells important to understand. BK is in the body first generated from cleavage of kininogens by a serine protease named Kallikrein. BK can then activate B2 receptors leading for instance to VGF production. Several zinc-metalloproteases from separate families are capable of converting Bradykinin into inactive metabolites. Des-Arg9-BK functions as an activating ligand for B1 type receptor, and can be metabolised further by ACE and APP. ECE-1 cleaves bradykinin, and also produce a vasoconstrictor, endothelin, and therefore the turnover of bradykinin to inactive metabolites instigates a synergistic vasoconstricting effect. *Pseudomonas a.* has also been found to cause a deregulation of the bradykinin-pathway that subsequently leads to improved dissemination into the blood system [292, 293]. The first authors proposed that this action was due to the dilatory actions of Bradykinin and that the induced vessel leakage may serve as entry port for the bacteria into the blood network. Other bacteria seem also to affect kinins so to induce and exploit vascular leakage [294, 295].

3. Drug-aided treatment

The body tries to control any changes in its natural homeostasis induced by a foreign intruder, for instance aided by the innate and adaptive immune defence [296-298], but might be unable to do so and outside help in the form of drug intervention might be necessary [299]. The simplest philosophy in drug targeting is that there should be one factor that causes or greatly contributes to the disease of interest, which then can be targeted [300], which in many cases is an over-simplified assumption [301]. Once a factor, such as an enzyme, is found promoting a process, which is enhanced in the disease, intervention aims to interrupt the continued promotion by the enzyme to this process. This means not to kill the process entirely, but more killing the excess work of the enzyme leading to disease, which stated differently; is adjusting the homeostasis back to normal. The body might be able to reverse the effects caused by the enzyme, but killing the process will not automatically reverse the damages already caused by the enzyme target. Time of intervention then becomes of importance. It is useful to learn from studies looking into pathogenesis in order to find single factors that contribute more than others in a malignant process. MePs are diverse governing global processes (such as cancer invasion), small specific processes and are present in normal as well as pathological environments (thesis-chapter 1.6). MePs are checkpoints for potential intervention [302], and some have been attempted targeted for more than 50 years [51, 303]. The targeting can be direct interaction with the enzyme or targeting the regulation of the enzyme [51]. Problems in finding drugs that can be marketed indicates that it exist barriers that needs to be over-come or by-passed before we have useable drugs [304]. Developing working animal models replicating the disease, and even finding the correct disease to target can be difficult for MePs [305]. Branches of systems biology are devoted to mapping substrate preferences for MePs, most notably the bigger “degradomics” profiling of the MMPs [166, 306], while others attempt to categorize phenotypic effects of drugs [147, 307-310]. Such studies increase understanding of the targets roles in that environment and may give clues to structural adaptations towards selected substrates. Databases of phenotypic effects of drugs can also be used to give clues about potential off-target enzyme partners, and systemise similar side effects of unrelated drugs [147, 307]. These databases might be able to propose additional targets for existing drugs, often implicated in different therapeutic categories. They are also particularly useful in

those cases where side-effects are due to inhibition of structurally distant enzymes, because these would normally need more experimental indications for us to believe in. Drugs with similar binding profiles tend to cause similar side-effects [147, 307]. Finding one enzyme promoting a process, which is enhanced in the disease, the aim of an inhibitor is to discontinue the ongoing promotion by the enzyme to this process. In a given disease, one enzyme/target can contribute in different ways. Processes that govern cancer (or bacterial invasion) can be either found in several cancers or be specific for one cancer-type. It is naturally difficult to predict and anticipate adverse effects without a complete knowledge of the substrates and regulation mechanisms of the target.

3.1 M4

Thermolysin itself seems not to be implicated in any human disease as the bacteria that produces it lives in a very thermophilic environment. However, thermolysin has acted as a model for other thermolysin-like enzymes that are causative of human disease (see thesis 1.6.2 and chapter on thermolysin). Secreted virulence factors from pathogens such as Legionella [311], Listeria, Clostridium, Staphylococcus, Pseudomonas and Vibrio belong to the M4 protein family [261]. Pseudomonas a. is considered a dangerous pathogen as it is naturally resistant to many classical antibiotics [312]. Antibiotic resistance is an old phenomena [313], but use and misuse of antibiotics might have contributed to creation of a niche for tolerant pathogens [314, 315]. Currently, antibiotics target a small selection of global processes essential for bacterial survival, and therefore a process-change induced to tolerate one drug would potentially impair the effect of other drugs too. Multidrug-resistant pathogens (MDRP) are associated with increased treatment expenses and might lead to poorer outcome [316-319]. Pharmaceutical companies has over a time period not focused in finding and developing new drugs increasing further the need to address MDR and exploration of novel targets [320-323]. Therapeutic inhibition of several M4 enzymes in those diseases that they are involved is believed to be such novel targets.

3.2 M10A

The M10A enzymes are collectively and individually implicated in many diseases [324], and for this reason are seen as drug targets [325, 326]. Drug development programs were launched in the 1980-90s mostly focusing on metastasis and angiogenesis [327]. Trials on drugs were discontinued mainly because of induced side-effects [327, 328]. The number of marketed M10A drugs today can be counted on a single hand, which is a low number compared to the amounts of inhibitors currently available [304]. As it seems that MMPs touch almost every aspect of mammalian biology, it is natural that they themselves have pro-disease as well as anti-disease effects, which necessitates that MMPs implicated in a disease are subdivided into anti-target and target respectively [329, 330]. An anti-target in one disease might be a target in another disease. Naturally, the drug needs to hit a single or several targets, while have no or little interactions with anti-targets. Studies then need to validate what are targets and anti-targets. A process can be extracted in order to see how each enzyme contributes. The vast amount (and still increasing) number of substrates for each enzyme, with partial redundancy (two enzymes share the same substrate) not only create difficulties in both designing a drug but also problems with how, where and when to administer a MMP-targeting drug.

3.3 M13

The M13 enzymes have been attempted targeted in several diseases [331], mostly connected somehow to the blood environment and regulation [58, 332-338]. In some of the diseases, the target has been located in the brain, and therefore the blood-brain barrier needed to be crossed [339]. Several of the inhibitors with nanomolar affinities for NEP and APN have been shown to completely inhibit enkephalin degradation *in vitro* and *in vivo* [340-342]. The location of the target(s) forces the drugs to cross the blood-brain barrier, and several of the early inhibitors have a high water-solubility preventing them from reaching their target(s). Highly potent and selective inhibitors targeting each enzyme were covalently linked by a disulfide bond in order to expose their lipophilic nature and allow them to cross this barrier [339]. The bond uniting them is relatively stable in plasma, but is rapidly degraded in the brain environment [339]. Targeting of this family is not only inhibition, but also activation [343]. Attention has been given to the “Endothelin axis”, which then aim to target one or several of the components found collaborating in cell signalling [338]. In cardiovascular disease,

there has been an interest of making inhibitors with affinity towards two or three enzymes [344-350]. Dual targeting of NEP-1 and ECE-1 has been shown to have synergistic effect on blood pressure regulation [339]. One of the reasons for this is probably that ECE-1 is inhibited from producing the vasoconstrictor (Endothelin) and also from degrading the vasodilator Bradykinin. NEP-1 is also inhibited from degrading Bradykinin together with other vasodilators (Endorphin-1 and -2). An undesired effect in targeting NEP-1 is that the enzyme normally degrades endothelin and acts as an antagonist of ECE-1 regarding this substrate.

4. Inhibition

Enzyme inhibition is an important part of understanding any enzyme [351]. Here, a reaction between a molecule and an enzyme that blocks the action of the enzyme, either temporarily or permanently is studied. This process occurs in the natural world all the time, and it has a number of applications [351]. The molecule itself is called an inhibitor, and can be used as a drug. Here it is important to note the difference between a “drug” and an “inhibitor” [352]. The drug can be an inhibitor that has been more extensively tested in several models, and is safe enough to use in a given disease treatment regime either on plants, humans or other animals. So, many inhibitors are characterised in the intention of developing it into a drug [353]. However, an inhibitor might be very potent, meaning that it effectively reduces the enzyme activity at low concentration, but may be unfit to use as a drug. These inhibitors are still useful in biology studies. Specific inhibitors can for instance be administered to cells in order to turn off or modulate signalling pathways as long as they are tolerated by the cells. Metabolites in the Krebs cycle (or any other metabolic pathway) were also better understood by administering inhibitors of the different enzymes in the pathway [354]. The continued developments of reductionist models (such as cell cultures and FRET assays) as well as interest in isolating metabolites from other species clearly advocate applications for inhibitors in studying enzymes in a biological context [355, 356]. They can also be used as control mechanisms in synthesis processes dependent on enzymes [357]. Enzymes can also be seen on a mechanistic level using inhibitors to explore surfaces, charge distributions or investigation into how collisions and catalysis of a substrate in a solvent occurs, which might have unknown or unrecognized applications. The active site of several MePs and other ligand binding

pockets are ideally suited for high-affinity interactions with drug-like inhibitors [358].

4.1 Activity towards isolated enzymes

The potential inhibition of an enzyme by small molecular compounds can be explored in systems with isolated enzymes. Among the benefits of using isolated enzymes is that it might be easier to investigate casualities that might otherwise be harder to isolate and study in cell or tissue environments that are more complex [359]. In return it might be that findings on isolated enzymes are not applicable and inferable to more complex systems because important factors are eliminated [359]. It is also important to note that enzymes inside cells are often found in highly concentrated multi-protein plasma environments. An enzyme inhibition assay can be dissected into; an enzyme component, the buffer environment, the inhibitor and a method for detection and evaluating inhibition. The enzymes themselves are sometimes hard to obtain in the needed quantities [360]. Therefore, biological systems that can synthesise a protein encoded by a gene of interest are needed. This process is called engineered protein expression, and the protein is named a recombinant protein. Hosts, with few exceptions, will translate the codons into the correct amino acids making the native primary protein sequence [361]. Enzymes provided by the host perform the modifications of the protein, and non-functional proteins can arise if the host lack these enzymes, or read the instructions in the primary sequence differently from the native host [362]. This is something we should have in mind since most of our studied enzymes are recombinant mammalian enzymes produced and isolated from among other bacterial hosts (manuscript 1: methods and material section). Membrane-tethered enzymes are particularly difficult to study as isolated enzymes without the ability to truncate and remove the catalytic- site from the overall body of the enzyme, which would normally incorporate the protein in the lipid-bilayer of the cell membrane [363-365]. The amino acid sections that allow the protein to traverse the lipid-bilayer is hydrophobic, and might create solubility problems [364]. The activity of isolated enzymes can be measured in FRET-based, fluorescence resonance energy transfer assays [366]. Inhibition by a putative ligand is usually inferred from interruption of enzyme activity in an assay such as a FRET-based assay [366, 367]. Here, energy is transferred between two chromophores [366]. A donor chromophore may transfer energy to an acceptor chromophore (in proximity, typically less than

10 nm) through non-radioactive dipole-dipole coupling. The donor is excited by an external light energy source, and ideally little energy should be transferred out of the system for us to detect in the energy transfer onto the acceptor chromophore. In an enzyme assay, the two chromophores can be linked to a protein sequence that is recognized by one or several proteases [366, 368, 369]. The length of the protein sequence and incorporation of several elements recognized by different enzymes allows the same synthetic substrate to be used to assay enzymes of different families and classes [368]. Proteolytic enzymes that recognize this sequence will cleave and separate the two chromophores. Two molecules are now produced creating distance between the donor-acceptor chromophore-pair, and the energy that was transferred to the acceptor can be transferred to a detector instead. The energy measures will be proportional to the number of product molecules generated, which is related to the amount of active enzyme. The activity of the ligand will be connected to its ability to interrupt in this process [369].

4.2 Inhibition types

Inhibitor molecules act on enzymes in different ways [370, 371]. Most often inhibitors that covalently attaches to the enzyme are irreversible, while inhibitors that mediate interactions through hydrophobic and ionic bonds can associate or dissociate reversibly. However, there are grey areas where an inhibitor associates so hard, and hardly dissociates, that it is irreversible, but is still viewed as binding reversibly (although tightly), and there are cases where covalent-linkages inactivating an enzyme are removed thus restoring enzyme activity. The stability of covalent bonds is relative, and esters are among the least stable covalent bonds in aqueous solution. There are four main types of inhibition; competitive, non-competitive (or mixed), uncompetitive and suicide or mechanism-based inactivation.

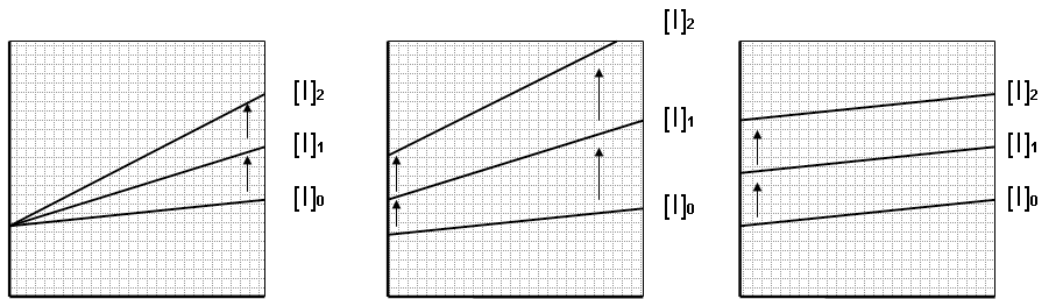


Figure 11: Graphs illustrating competitive (first left), mixed or non-competitive inhibition (middle) and uncompetitive inhibition (far right). The graphs are also called lineweaver-Burke plots, where the x-axis is $1/[S]$ and Y-axis is $1/V_0$. $[I]_x$ are inhibitor concentrations where $[I]_0 < [I]_1 < [I]_2$.

The signpost of competitive inhibition is that inhibition effect is lowered or removed by addition of more substrate (figure 11). Often this is because they compete for occupation of the same binding site, but also allosteric binding to distant sites might lead to enzyme changes so that once one is bound, that excludes the other from binding. Increased concentration of substrate (low $1/[S]$), on a lineweaver-Burke plot, will null the effect of inhibition and lines for enzyme activity with or without inhibitor all intersect at the Y-axis. This also implies that the inhibitor only binds to one form, free enzyme, and not substrate-bound enzyme. The signpost of non-competitive inhibition is that inhibition effect is not lowered or removed by addition of more substrate, and that the inhibitor binds with same strength to free or substrate-bound enzyme (or that substrate binds equally well to free or inhibitor-bound enzyme). Mixed inhibition is signified by an inhibitor that binds to both enzyme forms, albeit with different affinities. These two mechanisms have lines intersecting on the x-axis (non-competitive) or in second quadrant (mixed) on a lineweaver-Burke plot (figure 12). Uncompetitive means that the inhibitor will only bind to an enzyme-substrate complex, interpreted from parallel lines on a Lineweaver-Burke plot (figure 11).

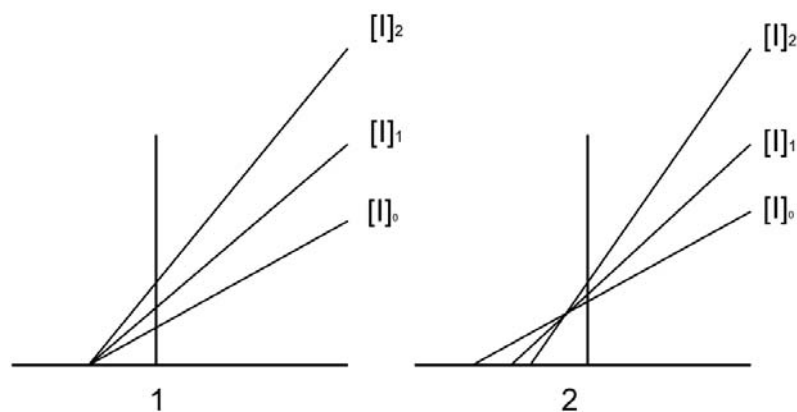


Figure 12: Graphs of non-competitive and mixed inhibition (left, right). These are lineweaver-Burke plots (X-axis = $1/[S]$, Y-axis = $1/V_o$) $[I]_x$ are inhibitor concentrations were $[I]_0 < [I]_1 < [I]_2$.

4.3 Interpretation of Inhibition

The potency of a putative inhibitor needs to be evaluated quantitatively somehow. Naturally this is often related to the wanted trait of the compound – inhibition of enzyme function. Different amounts of an inhibitor can be added to an enzyme assay creating a dose-response curve. The half maximal inhibitory concentration (IC_{50}) is then the amount of a compound such that the enzyme activity is lowered by half [372]. This value is found with the substrate concentration as a parameter (fixed), while the inhibitor concentration is varied (see figure 17), and is a much used value for comparing small or larger sets of putative inhibitors for an enzyme. The IC_{50} -value is more accurately determined the more $[I]$ tested, and it is good practice to include $[I]$ -values both above (reducing the activity by more than half) and below (reducing the activity by less than half) than the anticipated IC_{50} -value. The IC_{50} -value, for a non-tight-binding inhibitor, is dependent on the $[S]$ in the experiment, and is an affinity measure, assuming that the inhibitor binds to enzyme, but can tell nothing about what the inhibitor has affinity towards, meaning if either free or bound enzyme (or both) is targeted by the inhibitor. Another numerical value describing inhibition, K_I , is the equilibrium constant for the dissociation of the E-I complex. Half of the amount of E-I complexes are found dissociated when $[I] = K_I$, and is therefore an approximation of the affinity between these components. The value is found by making variables of both $[S]$ and $[I]$, and can then be thought of as collecting IC_{50} -values at different $[S]$. The relationship between IC_{50} and K_I can be calculated, but is

dependent on the inhibition type to explain the cause of inhibition – if one enzyme form (either free or bound enzyme) or two enzyme forms (both free and bound enzyme forms) are targeted by the inhibitor. The IC_{50} will move towards been equal to K_I for small values of $[S]$, twice of the K_I when $[S] = K_m$ and increase for larger values of $[S]$, while the opposite is true for an uncompetitive inhibitor.

$$11. IC_{50} = K_i \cdot (1 + [S]/K_m)$$

$$12. IC_{50} = ([S] + K_m) / ((K_m/K_i) + ([S]/K'_i))$$

Equations 11, 12: IC_{50} -calculations using equilibrium constants. Equation 11 is for competitive inhibitors, while equation 12 is for mixed inhibitors. IC_{50} is the inhibition constant where the activity is reduced by 50 %, K_i is the equilibrium constant between inhibitor and free enzyme, $[S]$ is substrate concentration, K_m is the Michaelis-Menten constant, K'_i is the equilibrium constant between inhibitor and enzyme-substrate complex.

4.4 Exploring binding surfaces

Substrate-binding precedes catalysis, and the low energy involved in stabilisation of an E-S complex, and also the fact that the substrate may not be perfectly adapted for the enzyme (rather than the transition state), opts for possibilities of interruption of this step. An inhibitor can create a stable complex with the enzyme, much like the lock and key analogy. Potential targeting areas can be inferred from the structure on enzymes alone or from interfaces between enzymes and other ligands (presented thesis-chapter 5.1.1). Enzyme-substrate complexes are very useful in this regards, but are difficult to experimentally determine [373]. The crystal structure of an IDE-Bradykinin complex (PDB-entry: 3CWW) draws some attention in this thesis as the fluorometric assay utilized is a FRET coupled site-directed sequence resembling bradykinin [374]. The amino acids involved in the recognition of this substrate in the co-crystal can then be natural amino acid-interaction partners for synthetic ligands. Knowledge can also be extrapolated onto similar enzymes as insulysin share similarities with other metalloproteases [56]. Binding surfaces between inhibitors and enzymes are also relevant in this regards [375]. A separation of native versus natural inhibitors can be made. Native inhibitors can be seen as those produced by the system and are locally available to regulate the activity of “*in vivo*”-targets. Natural inhibitors

are native inhibitors of some systems, but may be extracted from this environment and introduced to inhibit enzymes of other systems which it is not native to. The large size of interacting surfaces might give clues to surface targets, and it also gives information on structural adaptations brought forwards by evolution that scientists can copy or improve by smaller-molecular ligands. In the following, some of the most important natural/native inhibitors will be presented for the enzymes worked on in this thesis. It is important to note that some of the inhibitors act globally on many enzymes family (for instance Phosphoramidon and α -Macroglobulin) while others are more specifically targeting one family or individual family members (for instance TIMPs). Larger inhibitors (including peptide-based) are very important as predecessor for small-molecular compounds, and is therefore included here as well.

4.4.1 Phosphoramidon

Phosphoramidon is a chemical compound derived from cultures of *Streptomyces tanashiensis*, which acts as a tight-binding but unselective inhibitor toward several MePs [376-379]. This compound potentially acted as a regulator of the hosts own MePs, but also as an antibiotic compound fighting off other microorganisms, and it has been influential for inhibitor design targeting similar proteins. Phosphoramidon has been co-crystallised with several family members in the MA clan with the same monodentate antagonisation of the catalytic zinc-ion by the inhibitor [380]. The inhibitor might then stabilise loops and parts of the protein scaffold so that crystallisation is possible. Phosphoramidon has also been used to down-regulate activity of phosphoramidon-sensitive peptidases in biological models to verify the contributions of such enzymes [303, 306].

4.4.2 TIMPs

Proteins of the TIMP, Tissue inhibitors of metalloproteinases, family act as native inhibitors of the MMPs [375, 381-387]. Four members are found so far, and these are often abundant in most tissues and body fluids. Overall topology differs considerably, in spite of 40 % sequence identity [388]. In diseases these are found down-regulated or show an altered ratio with its target. Restoring the normal balance has been a therapeutic strategy, but TIMPs can for instance also promote cancer initiation themselves. These proteins bind tight to their target, in the pM-range, and each of the TIMPs act unselectively on several MMPs. TIMPs can also be selective towards some

of their targets, and in the case of TIMP-2-mediated proMMP-2 activation give information about other protein-protein interaction surfaces [389]. Studying interactions between TIMPs and interacting protein partners can thereby reveal information about epitopes on the enzymes that can be target by other means (such as small-molecular inhibitors) [390, 391]. TIMPs can also be modified to gain selectivity and used as inhibitors [392, 393]. The N-terminal subdomain exhibits an OB-fold, and seems to be vital for the inhibitory actions of individual TIMPs towards their respective MMP-target [140, 375].

4.4.3 α -macroglobulin

Alpha-2-Macroglobulin is a large plasma protein abundant in the blood [394]. The immature inhibitor has a bait region of about 35 amino acids, which includes several sequence-recognition sites of various enzymes. Proteolytic cleavage in the bait region induces a conformational change that entraps the endopeptidase [395]. In the blood, the resulting proteinase- α 2M complex is in turn recognized by macrophage receptors and cleared from the system. The bait-region in this mechanism-based inhibitor has been manipulated by researchers attempting to create selectivity by manipulating numbers and sequences of cleavage sites. Interacting sites in the proteinase- α 2M complex has also been studied and compared with similar natural mechanism-based inhibitors present in various tissues and environments as potential drug/inhibitor targeting sites [396, 397].

4.5 Small Molecular Ligands

Small molecular ligands, which are often organic molecules, that binds to a protein or peptide and affecting their activity can have therapeutic value or other uses. Key interactions between protein-proteins (natural inhibitors) can be mimicked and potentially improved [398]. These molecules also might avoid being degraded, contrary to administered peptide or proteins, and prolong their availability in the body, avoid some side-effects (but potentially gain others). These might also be more cost effective and easier to model due to size and degree of complexity. Ligands can be made to target several areas of an enzyme that are either enzyme specific or more general trends.

4.5.1 The Active-site

The active site can be said to be that part of an enzyme where substrates bind and undergo a chemical reaction. This will always include the catalytic triad, which are those amino acids that are involved in catalytic transformation. For enzymes that have many different substrates, and of very different sizes, it becomes a bit unclear what interacting sites constitutes apart of the active site and what are not. In the following, the active site will include the catalytic triad, the prosthetic zinc-ion as well as sub-pockets. The Zinc-ion with valence number two is an intermediate acid in Pearson's Hard and Soft Acid and Bases, which preferentially binds to hard ligands [399, 400]. However, when held by three nitrogen donors from histidine-residues, the fourth coordination site on the tetrahedral Zinc favours softer ligands [399-401]. Strong interactions with the zinc-ion will create un-selectivity, while interacts with sub-pockets gain selectivity. Ligands often have a ZBG (zinc-binding group) coupled to a backbone that will support interactions with sub-pockets [304]. Regarding the MMPs, the S1'-subpocket have been seen as the pocket vital for substrate specificity [54, 402], but this view is contemporary and might shift according to better understanding of all sub-pockets involvement in substrate-binding. Selective MMP-13 inhibitors that act non-competitively and non-chelating by positioning deep inside the S1'-pocket have been described [403], while similar inhibitors have been found and speculated to have similar binding-site.

4.5.2 Hydroxamates

Hydroxamates are essential growth factors, which functions mostly as iron-binders, named siderophores, aiding the microbe to scavenge essential iron from the exterior environment and transport it into the cell [404-407]. Hydroxamate-groups have therefore been incorporated in different antagonists targeting the active sites of metalloenzymes. The widespread use of hydroxamates in MMPis is partly due to the fact that Hydroxamate- affinity columns was traditionally used in purification of MMPs [408, 409]. Comparative studies on compounds differing only in their ZBGs also indicated hydroxamic acids to have the best in vitro potencies toward members of this family [409]. Targeting MMP-3, the hydroxamate in isolation has been shown to have a K_d of 17 mM and an IC_{50} of 25 mM, indicating that a backbone with several

favourable interactions with the active site facilitates a further tight binding [409, 410]. Batimastat and marimastat are two well-known MMPIs belonging to the succinic class of hydroxamate-based inhibitors [411]. These mostly differ in that marimastat is more bioavailable. Both of them had an inhibition in the nanomolar range, and batimastat was the first of this class to enter clinical trials. Batimastat had a poor bioavailability, and failed the clinical trials due to side effects such as muscular-skeleton problems. The hydroxamates have been associated with toxicity as well [412]. Reverse Hydroxamate inhibitors have better pharmacokinetic properties than their hydroxamate counterparts [413].

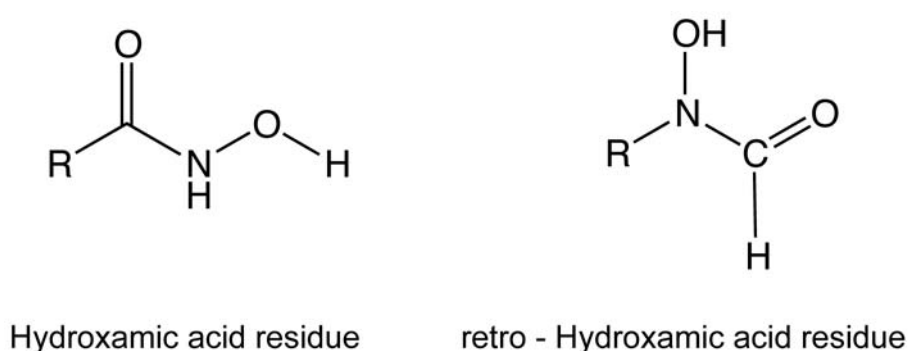


Figure 13: The illustration shows the atomic constituents of the hydroxamic acid (left) and the retro-hydroxamic acid (right). The illustration is made with ChemBioDraw Ultra version 12.

4.5.3 Non-Hydroxamates

Inhibitors with a ZBG-group different from the hydroxamates, and retrohydroxamates, which targets the catalytic zinc-ion with polar or charged groups is often referred to as non-hydroxamates in the field of MePs inhibition [414, 415]. This group of inhibitors is diverse both in ZBG and overall scaffold making it hard to find statements that are valid for all cases. However, several of the non-hydroxamates have been investigated to address the two main problems associated with the majority of hydroxamates; specificity and bioavailability [416]. Specificity could sometimes be gained by weaker interaction with the catalytic zinc and with more importance put on interactions with sub-pockets and overall target topology. There are several examples of non-hydroxamates with a ZBG consisting of sulfhydryl, carboxylic, and phosphonic-moities [417]. PAC-1, and its derivatives, and the isatin 1,2,3 triazoles are

also non-hydroxamates according to our own findings. Many of the ZBGs are inspired by natural inhibition mechanisms seen in molecular switches [416, 418-420] or from exploration into interfaces of native inhibitors and their targets [421-423] .

4.5.4 Mechanism-based Inhibitors

Mechanism-based inhibitors have been an idea in the wake of the promiscuous bidentate zinc-coordination seen in many inhibitors [424, 425]. There are different types of mechanisms that can be used to gain selectivity. One approach is to create a pro-drug that is inert and needs to be activated. The switch itself is often coupled to the enzyme-target or features in the local environment of the disease-cause so to potentially gain selectivity and potency. Features of the prodrug might also create better availability and/or might affect stability [426]. Partial cleavage of the pro-inhibitor, which is the case for α -macroglobulin, can be an activating switch, but also other means can be used to activate the prodrug. Some cancers are found to have elevated levels of reactive oxygen species (ROS), which then can be used to activate ROS-triggerable prodrugs targeting MMPs [427]. The inhibitor can also have chemical groups that react with the enzyme target, for instance by creating covalent bonds to residues that are essential for catalysis. One inhibitor, SB-3CT, acts as a selective inhibitor for the gelatinases [428]. The inhibitor binds slowly to the catalytic zinc-ion, mediated by non-covalent interactions with the enzyme [428-433]. The glutamate involved in catalysis then goes to nucleophilic attack on the thiirane ring, which results in a covalent bond between the glutamate and the inhibitor and a sulphuric ion interacting with the catalytic zinc. Thiiranes were tested against their oxirane-counterparts, which replaces the sulphur-ion with oxygen, which did not have the same activity highlighting the importance of the sulphur-atom. Functional groups were added in order to combat solubility issues as well as better interaction with the deep S1'-pocket of MMP-2. The inhibitor could become a promising drug as it showed inhibition of MMP-9 in an animal study on transient focal ischemia, rescuing both Laminin from proteolysis and neuron from apoptosis [434] and also has an antimetastatic properties on liver cells [435].

4.5.5 Exosites

Regions outside the catalytic site that contribute with interacting surfaces for larger substrates, other catalytic functions or mediate other protein-protein dockings are interesting to target [436, 437]. On enzymes, these elements are often more individualised because they are subject to a different conservational pressure than the active site, and might be purposely different to allow for instance interactions with other sets of protein partners. The interest in exo-site is growing, and efforts are made gradually to understand which exosites are involved in what processes [436, 438-443], and mapping residues that then defines those exosites in these. Non-small-molecular-inhibitors phosphonate coupled peptide transition state analogue, were more selective towards the gelatinases than other MMPs compared [444, 445], and it was proposed that the size of the inhibitor allowed it to interfere in exo-site dependent processes. P713 is a peptide with high identity with a segment of the human $\alpha 1(I)$ pro-collagen sequence could inhibit the gelatinolytic process mediated by MMP-2 [446]. Seven compounds (out of ~17000) has also been suggested to interact with specific residues characteristic for MMP-13 hemopexin domain using a cheminformatics-based approaches including sequence comparisons in the hemopexin-region, virtual screening, docking and assessment of drug-like qualities [447]. These inhibitors remain to be tested in additional assays other than “*in silico*”-based. The authors also commented on that during their study the number of MMP sequences in the NCBI Entrez protein public database almost doubled, and that analysis of the sequences revealed that the number of residues unique to MMP-13 reduced from 34 to only 10, whereas the number of putative functional residues (HCR-13_{pf}) reduced from 5 to 3.

5. Free energy calculations

A molecular system goes through chemical changes in an attempt to minimize its internal energy and to increase its entropy (chapter 9 and 10 [448]). The most important function attempting to describe such systems is Gibbs free energy equation. The free energy can be calculated from the equilibrium dissociation constant K_I , which then approximates the affinity between enzyme and the inhibitor. For inhibitor design, this is valuable as it can be a theoretical approach for examining individual contributions in an E-I complex, or also compare smaller or larger sets of inhibitors before testing in the laboratory. There are many computational approaches that attempt on calculating free energies, which can be somewhat divided according to how many system variables are included and how complex they interpret these. Accuracy of the estimates naturally depends on what is included as part of the model. Theoretical protein systems are viewed as molecular mechanical systems, among other neglecting the spin contributions from electrons. This is an inherent simplification that is found in most of the systems available. MM approaches range in complexity, but clear borders can not be drawn [449]. MM-PBSA, LIE and docking and scoring are examples of different MM approaches [449]. Accuracy of the estimates naturally depends on what is included as part of the model, and sometimes the approaches are finally tuned towards certain test-sets and thereby biased in their assessments. Still, Theoretical interpretation on ligand binding, like docking and scoring, may contribute with molecular insight into the binding process and are therefore implemented for instance in virtual screens or correlated with experimental findings in persuite of new drug or ligand leads [450-452].

5.1 Modelling the system

It is important for us to understand what our model consists of and also more about the limitations and potential errors in predictions that might arise. Many times one purposely simplify the model so to make it easier to accommodate screening processes with accepted thresholds for errors, while other times there are errors associated with the methods that are so hard to avoid that they are accepted. The model can be limited to only the enzyme and ligand, or include to varying degrees both interactions with the surrounding (solvation) and also dynamic movements. The latter are also dependent on the protein structures available.

5.1.1 Protein structures

For many proteins the structures have been experimentally determined, mostly using NMR or X-ray crystallisation methods, and efforts are made to accelerate the determination rate of protein structure [453, 454]. The architectural design of the protein is then found as coordinates of atoms, which can be deposited in databases. Inhibitors can be co-crystallised with the target, and true positions under those experimental conditions can then be found. Such co-crystals form the basis for knowledge-based inhibitor design [455]. These co-crystals might have crystal-induced artifacts that might influence the scoring parameterisation and subsequent performance [456, 457]. Theoretical assumptions on placement of inhibitor can also be done on a determined target structure as well [455]. Then, the theoretical calculations are very dependent on the coordinates of the structure, which can be referred to as structure-based inhibitor design. Theoretical calculations might also impose changes on the experimentally determined structure, and it is therefore important to bear these changes in mind when assessing the model itself and its predictive power. Going some steps backwards, we note that purified high-concentrated protein is packed together in an orderly fashion in X-ray crystallisation methods, which may create artifacts. The x-rays may impact on the structure during data collection [458-460]. The method attempts on creating an image of the protein using low temperatures limiting conformational sampling and potential protein instability [457]. There are some attempts on using higher temperatures in the x-ray collection phase so to get more information on conformational sampling [461]. Resolution is also a varying factor, where some structures have been found with high resolution and other with low. The resolution can be improved in the theoretical

model, by among other utilising assumptions on expected bond lengths and connecting atom-partners. Environmental conditions such as salt and pH might be higher or lower than normal among other to stabilise the protein, for instance proteins with self-catalytic actions might be kept inactive this way. The state of the protein under other pHs can be theoretically modelled thus altering properties of the experimental structure. Often static assumptions on K_a -values of amino acids are used in this process potentially neglecting imposed alterations by the surrounding amino acids on some residues K_a -values. Structures of the target enzyme co-crystallised with another inhibitor has many uses. By itself, the inhibitor can point to structural adaptations to mimic, but it can also be replaced with another inhibitor of interest. Then, it is important to bear in mind that the structure might have been structurally moulded towards the co-crystallised inhibitor and may be structurally biased towards similar inhibitor-scaffolds.

5.1.2 Force fields

The motions of electrons and nuclei in a molecular system are described by the Schrödinger equation (chapter 7 [462]). In molecular modelling (MM), a force field can then be viewed as an attempt to solve this equation for a given molecular system empirically with experimental data and approximations. A quantum mechanical (QM) solution in contrast would rely on theoretical calculations only. The Born-Oppenheimer approximation, detaching the movement of electrons from the nuclei as they have a much smaller mass and higher velocities, treats the nucleus as an unmoving object while only considering the single electrons effect on the other electrons. This assumption allows a transition from QM into molecular mechanics (in between we find “ab initio” QM and Semi-Empirical QM). MM moves toward Newtonian physics viewing molecules as a collection of masses centred at the nuclei (atoms) that are connected by springs (bonds). The molecular mechanics energy (E_{MM}) is expressed as the sum of different energy contributions seen in equations 13-15 (chapter 2 [463]).

$$13. E_{MM} = E_{local} + E_{Nonlocal} (+ E_{Miscellaneous})$$

$$14. E_{local} = E_{Bond} + E_{Angle} + E_{dihedral\ angle}$$

$$15. E_{Nonlocal} = E_{vdW} + E_{cc}$$

Equations 13-15: Force field equations (chapter 2 [463]). E_{MM} is the molecular mechanics (force field) approximated solution to the Schrödinger equation. Local energies (E_{local}) are attributed to formation of covalent bonds (E_{Bond}), the angle between 3 given atoms (E_{Angle}) and from the dihedral angle ($E_{dihedral\ angle}$). The nonlocal energies describes electrostatic (E_{cc} , columbic contributions) and van der Waals interactions (E_{vdW}). $E_{Miscellaneous}$ includes parameters to adjust for deviations between theoretical values and experimental findings.

The nonlocal energies describes electrostatic (columbic contributions) and van der Waals interactions. These forces can have effects exceeding the local ones. However, they can have tremendous local effects too, that needs to be implemented either in the local or nonlocal energy term. Due to among other their far reaching nature, the nonlocal energies are the most difficult to account well for in a MM simulation. MM needs information gathered from experimentally derived crystal structures, NMR and vibration and microwave spectroscopy. At present, there are many different algorithms for generating a force field, which can have different parameters that affect the E_{local} and $E_{Nonlocal}$. The different algorithms can implement quantum mechanical considerations to different degree, and they can have other applications to account for limitations as well as other factors that can not be implemented in the former expressions ($E_{micellaneous}$).

5.1.3 Energy minimisation

Molecules can adopt many distinct structural arrangements, which correspond to different energies (chapter 3 [463], chapter 10 [462]). These energies can be graphically represented as energy landscapes, and valleys would then represent minimum values. Minimums will have smaller energies relative to their neighbours (local), but still might not be the overall minimum (global). Molecules would more often adopt the conformations corresponding to minimums as these are more stable. Energy minimisation of a molecule is usually performed to arrive at these more populated states in the landscape, and thereby the quality of the model in subsequent work is improved (chapter 3 [463], chapter 10 [462]). The force field expression (E_{MM}), remembering that we might have approximated or neglected entropy effects, is what is minimized. Small errors in the bond length, affecting E_{bond} , will contribute the most to errors in E_{MM} . For the computer, performing a minimization is not a trivial matter. The methods can be divided into first-order, only requiring an expression for the energy, or second-order, requiring an energy expression and its derivatives. Steepest descent and conjugate gradients belong to the first – order category. In steepest descent the derivative of the energy function guides moving towards energy minimums. The derivative, will be defined as the slope of the energy function. The longest steps are taken farther away from the minimum, distance from x_0 to x_1 longer than x_1 to x_2 , so it slows down towards the minimum. Problems arise in circumstances where the derivative function moved towards zero because the energy function flattens out without been a minimum. In the three dimensional energy landscape, searches might not follow a straight line but instead move shift direction to cover more area in the landscape. For the second-derivative methods, such as Newton Raphson, the first derivative of the energy function will dictate the direction of the vector, while the second-derivative gives the curvature of the direction vector (chapter 3 [463], chapter 10 [462]). The second-derivative methods are less reliable far from minimums while faster and more reliable closer to a minimum. Effort is made to combine the first and second order methods because of their opposing strengths. There are different considerations a modeller should bear in mind when it comes to the minimization process. The process can be initiated with different starter energies to avoid that the algorithm is stranded in a point that might not represent a minimum. Different algorithms and/or force fields might also be used.

5.1.4 Ion-charges

The strength of the direct interactions between Ligand and Enzyme are often over-rated due to the difficulties with assigning proper charges onto the metal-ion [464]. The net charge of the metal might be higher in the model since it is difficult to correctly assess how the charge might be affected by the protein environment as well as distributed onto nearby amino acids. Quantum mechanical approaches on the interacting site might be a way to address this, but come at a computational cost [465].

5.1.5 Water-problems

Water molecules might for instance actively take part in a catalytic mechanism [464-466], and thus their removal is detrimental if the structure will be used in catalytic mechanism simulation. The same might be for water molecules kept and hydrogenated. The size of the enzyme most often creates a cavity with a unique environment. Hydration of non-polar groups and van der Waals interactions between the same groups generally defines the hydrophobic interactions. Destabilisation of protein structure is associated with a negative free energy and is a result of hydration of polar and aromatic groups. The negative free energy from hydration of polar and aromatic residues is much higher than the positive Gibbs free energy from hydration of nonpolar groups and the net hydration effect is thus destabilised at moderate and low temperatures. Therefore, we need to be aware of and also report our subjective choices even for an automated preparation process. Enzyme-ligand formation is composed of two processes; desolvation of the ligand while binding and binding at the binding site. A designed model system not accounting for desolvation often prefers a ligand A over ligand B given that ligand A has a 9 kcal/mol desolvation barrier and a 21 kcal/mol binding contribution, while ligand B with 3 kcal/mol desolvation barrier and 18 kcal/mol binding contribution. This is simply because the complex nature of a buffer makes it difficult to include. Enzymes might also have associations between water and the binding sites, which also need to be desolvated [467]. Ligands interacting with the active site zinc of metalloproteases needs to expel the recruited water molecule at a thermodynamic cost. This is also true for water associated with other binding pockets, for instance water associated within collagen substrate-pockets.

5.2 Docking

Fitting a ligand into the binding cavity of the receptor is referred to as docking [468]. Early day docking was limited to treating both the receptor and ligand as static structures. There are several approaches for performing docking, but it normally consists of two steps – finding the binding mode and scoring of these [353]. A small-molecule has probably many binding modes inside the bigger enzyme, so normally the algorithm is also asked in this process to make a preliminary sorting. The algorithm finds the binding modes based on evaluation of physical and chemical complementarity. There are of course many algorithms, which incorporate empirical-, force-field- or hybrid- notions on the system [469-475]. They also give different emphasis on contributions in the interface – physical or chemical complementarity. The algorithms are thus biased in their output dependent on their ability to- and how- they can interpret the input. The development of docking goes in many directions simultaneously, but two main branches seems to be working on improving calculations while the other explores ways of turning the static system into a more dynamic system. “induced fit” for instance tries to account for structural changes in the protein induced by the ligand thus creating a better fit and subsequently a different evaluation of the molecule [476, 477]. One of the ways to perform “induced fit” docking is to first do a preliminary docking and then investigate changes in that local environment that would improve the fit. If such a potential is found, the protein structure would be altered and the ligand would be re-oriented before the last scoring. With Figure 14, we can somewhat illustrate the problem that any algorithm encounter – what contributes to the binding? We see that the phenyl-group is physically very complementary to the pocket. We also see that this pocket constitute different charges (illustrated with colours), and the ligand residues that are oppositely charged are placed next to them. Novel docking and scoring tools are continuously developed to improve “*in silico*” high throughput screening. An existing protein crystal with an identical or structurally similar inhibitor located at the binding-site can be used as a guide for the docking as well as for evaluating the ability to find correct poses [478]. Improvements in scoring can be done on training sets. Training sets are sets of compounds mixing a small set of active ligands with inactive decoy molecules.

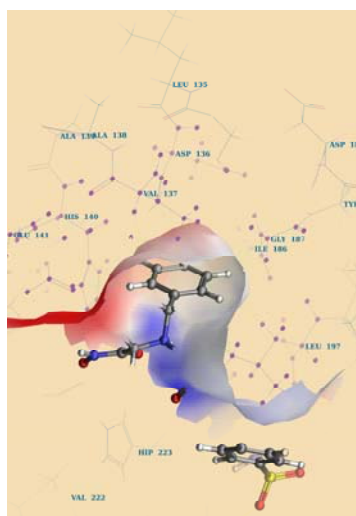


Figure 14: A small-molecular ligand docket inside one sub-pocket of Pseudolysin. The illustration is made within Maestro (Schrödinger).

5.2.1 Docking with Glide

Glide (grid-based ligand docking with energetics) is a docking program [426, 479]. In grid-based docking one defines a three dimensional space around the area one believes that the ligand will bind, and surface cavities on the receptor are identified. These potential binding sites are mapped for descriptors, such as hydrogen-bond donors and acceptors, metal ions and hydrophobic groups. Glide uses an empirical hierarchical-based approach for finding poses [426, 479]. Each ligand is dissected into a *core* region and *rotamer groups* (Figure 15). The core is what remains when each terminus of the ligand is severed at the “last” rotatable bond, while rotamer groups are attached to the core by rotatable bonds, but does not contain additional rotatable bonds. Carbon and nitrogen end groups terminated with hydrogen are not considered rotatable because their conformational variation is of little significance. Tautomeric states, ionic states and other descriptive properties are defined for each ligand before the docking [380].

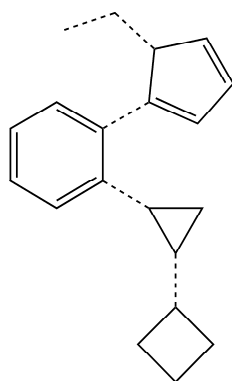


Figure 15: Core-region and rotamer-groups. The illustration shows a molecule where rotatable bonds (rotamers) are stripped, while cyclic-regions (cores) are shown in lines. The illustration shows 4 core-regions attached with 5 rotatable bonds. The illustration is made with ChemBioDraw Ultra version 12.

During docking ligand conformations are evaluated and potentially eliminated as the pose search progresses and becomes computationally more demanding. Glide can incorporate so called constraints in one or several of the steps that will affect the outcome [426, 479]. A constraint is a ligand-receptor interaction (for instance a shared hydrogen-bond) or a ligand-feature (for instance a ligand core) that is specified by the user as essential or important before the evaluation of poses begins. Docked poses that fail to meet the requirements are rejected. Another final more elaborate scoring is performed on the top poses, and therefore the use of two different scoring functions in the separate steps are advocated [353].

5.3 Free energy perturbation

Perturbation theory is one of the oldest and most useful general techniques in applied mathematics [480]. Perturbation can mean to introduce several small changes in a physical system and thereby gradually altering it. The alchemic transformation is described by the numbers of intermediates generated and how these intermediates are different from each other. A term for the potential energy for the different conformations is created during the molecular dynamics (MD) simulation.

$$16. U_I = \lambda_1 U_A + \lambda_2 U_B$$

Equations 16: U_I is the potential energy of the intermediate and U_A and U_B are the two extremes, while λ_1 and λ_2 are ratio parameters. A coupling parameter λ can be the summation of the two (λ_1 and λ_2), which will add up to 1.

The ensemble amount, which is the size of the collection of intermediates, is dictated by the rate at which λ_1 and λ_2 is changed. The changes between the intermediates can be made smaller (or “softer”) the larger the ensemble grows, which also would increase accuracy as sample size increases. The free energy can be calculated as a difference between two defined states (A and B) [481, 482].

5.4 Linear interaction energy

Linear interaction energy (LIE) - approach is a more recent method that can be used to find the free energy differences between ligand-enzyme complexes [482, 483]. The forces that contribute to ligand-binding are expressed as polar and non-polar contributions. Solvation free energies of non-polar molecules depend approximately on linear measures of physical characteristics, which enable an estimation of these forces by scaling of average Lennard Jones energies. Empirically fitted coefficients are found (α and β). An additional constant, γ , may be required to reproduce absolute binding free energies for instance if constant terms are affected by the protein environment, for instance hydrophobicity of the binding site. This coefficient will be specific for a given protein but conserved on any series of ligands studied on that protein. Molecular dynamics simulations are carried out on each ligand in two different surroundings (water or in a protein (with cofactors)-water solvent). The MD then enables us to find the convergent thermal averages of the molecules, which forms

the positional basis for finding interactive forces among atoms and ultimately leads to the calculation of ΔG_{bind} . LIE spends more computational effort on physically relevant structures than on unphysical intermediates as with alchemical FEP [481]. LIE can be used to estimate free energy differences for compounds with bigger differences because of the emphasis of the solvent-solute interaction energies over the internal transformation process itself, while still deriving information from the force-field. It also seems robust for use with different force fields on the same ligands. Due to the fact that it is a recently developed method its validity has not yet been tested for a great number of systems.

Summary of manuscript 1

Background and aim:

PAC-1 has recently been shown to interact strongly with free zinc-ion, and has been a lead ligand scaffold for further drug testing by Hergenrother et al. Isatin,1,2,3 triazoles is a different inhibitor scaffold with many applications. Since MePs are depended on a catalytic zinc-ion, we set out to test the potency of these compounds towards several families belonging to the MA clan. Targeting these enzymes has either a direct pharmaceutical application or a biotechnological one, as evident from the thesis-introduction.

Methods

Numerical values of inhibition (IC_{50} and K_I) were found with a fluorescence-based FRET-assay. A single-cuvette fluorescence-recorder was used with a recording time either of 1 minute or 10-15 minutes as specified in the manuscript. Two different substrates were used. Relevant enzyme-structures deposited in the Protein Database (PDB) were collected and the inhibitors were docked in the different enzymes using a Grid-based approach.

Results and Conclusion

The ligands would inhibit the different targets within a low to high μM -range, and thus acted unselectively. Structural modification of the putative zinc-targeting group of PAC-1 reduced or deleted inhibitory strength. A more in depth examination of inhibition found that some of the compounds inhibited activated MMP-9 through a mixed inhibition mechanism, while had a competitive inhibition mechanism towards MT1-MMP. The reason for this is still unknown to us. We suggested a binding mode, based on docking, for all the compounds, and found that they could adopt several energetically similar poses in each active site. The inhibitors in low μM -range, interacted with the S1'-pocket of the MMPs. Structural changes on Isatin 1,2,3 triazoles extending the P1' was also shown to increase inhibition strength.

Summary of manuscript 2

Background and aim:

Therapeutic inhibition of several M4 enzymes in those diseases that they are involved is believed to be novel targets for future antibiotics. Targeting these enzymes has either a direct pharmaceutical application or a biotechnological one, as commented on in the thesis-introduction. Hydroxamates and retrohydroxamates are potent inhibitors of the MePs, expected to both target the catalytic zinc-ion and form favourable hydrogen-bond interactions inside the active sites. Due to their expected interactions with the catalytic-ion, several hydroxamate-derivatives were synthesised for evaluation as inhibitors of Thermolysin and Pseudolysin.

Methods

Numerical values of inhibition, IC_{50} , were found with a fluorescence-based FRET-assay. Measurements with either a single-cuvette fluorescence-recorder or a plate reader were used with pre-incubation and recording time as specified in the manuscript. Two different substrates were used. The inhibitors were docked in the different enzymes using a Grid-based approach (Glide).

Results and Conclusion

Data analysis of IC_{50} -values collected with the bradykinin-like substrate showed that 5 out of 8 synthesised compounds displayed increased inhibition strength towards pseudolysin relative to thermolysin. Two compounds had the same value towards both enzymes, while two other had increased inhibition of thermolysin compared with pseudolysin. It was difficult to find a relative correlation between experimental results and modelling scoring, meaning docking scores or free energy calculations in LIE. The molecular modelling could be used to locate amino acids within 4 Å of individual compounds, and also explore important sub-site interactions. Strong interactions with amino acids in the S_2' -pocket for ML25 and LM2 seems to be the main reason that they are quite strong pseudolysin inhibitors. Docking into Thermolysin, indicated the same, but also showed additional interactions with the S_1 -pocket.

Summary of manuscript 3

Background and aim:

The enzymes in manuscript 2 were now attempted targeted with a different set of hydroxamates. These compounds now contain different backbone elements, compared among themselves and with those in manuscript 2, that are anticipated to interact with the substrate sub-pockets of Thermolysin and Pseudolysin.

Methods

The methods described in manuscript 2 were also used in manuscript 3. LIE calculations on MD simulations were performed.

Results and Conclusion

Data analysis of IC₅₀-values collected with the bradykinin-like substrate showed that 7 out of 8 compounds displayed increased inhibition strength towards pseudolysin relative to thermolysin. One inhibited thermolysin better than pseudolysin. The strongest binders for both thermolysin and pseudolysin were SM10 and SM12, which both poses two ring systems. The largest and most electron rich ring system occupy the S₁ subpocket which is larger and more hydrophilic than the S₂' subpocket. The present information is useful for designing new thermolysin and pseudolysin inhibitors.

Discussion

There are several aspects of the work that could be debated in this section. However, this section will mostly be directed towards methods and data analysis. Exploration of these inhibitors as potential drugs towards MePs should be, in my opinion, left for the future. I hope that differences between “inhibitor” and “drug” were somewhat apparent to the reader in the introduction. “Scavenging inhibitors” is found here in the discussion more because it argues about the strategy of the work. The section could, but will not, investigate deeply into docking and LIE as methods despite the fact that this section is methods-oriented. I hope again that it was apparent to the reader that choices in parameters affect the outcome, and several methods and algorithms could be discussed potentially derailing the discussion with details.

6. Scavenging inhibitors

The word scavenging may refer to a search through refuse for salvageable material, and the work in the thesis may be seen as scavenging since all the inhibitors had other intended uses. That does not exclude that these uses can not co-exist and be utilised, but it affect the future of these inhibitors. In article 1, the target were originally procaspase-3 [484], which is a pro-enzyme in the apoptotic signalling pathway. The first small molecule found to directly activate procaspase-3 was discovered in 2006, and was given the name PAC-1 (**procaspase-3 activator-1**) [484, 485]. Many compounds target early or intermediates in the signalling cascade, and therefore their effects might be absent in those cancers with deeper-downstream signalling errors. Most apoptotic signalling pathways affect the activation of procaspase-3 enabling active caspase-3 to hydrolyse many protein substrates. Thus, affecting the activation of procaspase-3 has potential in cancer intervention. The recent discovery of PAC-1, combined with the potency (relative to our findings) and also the fact that it targets a downstream target creating less side-effects, will contribute to the authors probably focusing on this biological target. The same can be seen from publications by our collaborators [486]. However, our future publication seems to be the first attempt to investigate inhibition properties of PAC-1, and derivatives, toward any metallo-endopeptidase, so this might lead to more targets described for PAC-1 in the future. It will then be interesting to see if the new targets will be novel enough to spark an interest in synthesising PAC-1 derivatives for those new targets, and not just remain

off-target/side-effects parallel to the original use. The inhibitors used and described in manuscript 2 and 3 were given to us by two international collaborators [304, 487]. Selective targeting of MMP family members has been an aim for both these groups. However, they have also explored new targets for some inhibitors [487]. Our collaborators at the Pisa University have a broad competence in chemistry (synthesis and analysis), biological assays, and modelling, with several international collaborators. It might then be that they already have enough synthesis work planned for their original targets, and would be reluctant to perform synthesis only dedicated to our new targets. Then we are left with the scavenging role; only characterising their future designed MMPIs or TACE inhibitors, towards our targets (if they share their future endeavours with us). That does not mean that our findings with those inhibitors might not be interesting and lead to future publications. It just means that our modelling will be descriptive, and that potential prospective modelling predictions performed will not be followed up.

7. Isolated Enzymes

As stated in the introduction, our assays are dissected into; an enzyme component, a buffer environment and a way to detect and analyse strength and mechanism of the inhibitor. In article 1, several truncated recombinant Membrane-tethered enzymes, for instance MMP-14 and NEP-1, was used, and it is important to be aware of that these might act differently due to posttranslational modifications or the truncations made. MMP-9 and MMP-2 was activated by a proteolytic enzyme (MMP-9), trypsin, or an inter-autoactive process (MMP-2) (assuming that no trace contamination of other proteolytic enzymes is active in the bought sample). The activator can affect the binding and catalysis towards some substrates depending on cleavage site and what is still left on the N-terminal site on the enzyme.

8. Quenching

Quenching refers to any process which decreases the fluorescence intensity of a given substance [488]. The FRET assay is a quenching mechanism since an energy donor is coupled to an acceptor. The donor reaches an excited state when light of absorbing energy is introduced, and this energy is transferred to the acceptor. The quenching is dependent on the donor – acceptor distance (r), falling off at a rate of $1/r^6$ and on the donor-acceptor spectral overlap and the relative orientation of the donor and acceptor transition dipole moments [488]. Enzyme cleavage between the donor and acceptor lowers the internal quenching, and light energy is now recorded by a detector. This is how we could connect enzyme activity to intensity of light production over time, while inhibition was inferred from the ability to lower light production by a supposed enzyme-interact. There are especially two causes, which we should be aware of, of quenching that might occur after the enzyme has cleaved the FRET substrate. The FRET-substrate might be at a concentration so that despite the enzyme-cleavage, intended to create distance between acceptor and donor, the donor will still have a non-covalently bonded neighbouring acceptor “stealing” the light energy that should have been recorded by the detector. This is partly the reason why high substrate concentrations were avoided among other in finding some of the experimental kinetic values. Quenching could also be caused by the small molecular compounds tested as inhibitors (figure 16), especially aromatic ring structures at those light intensities in the experiments (300-400 nm). This quenching would be interpreted as part of the enzyme-inhibition, unless further investigated. However, quenching is most likely to occur with high concentration of the inhibitor [488]. Thus a poor inhibitor is more likely to be evaluated as a better inhibitor, but not a potential tight inhibitor since influence of quenching is, which was mistaken as true inhibition of the enzyme, lost at low concentrations.

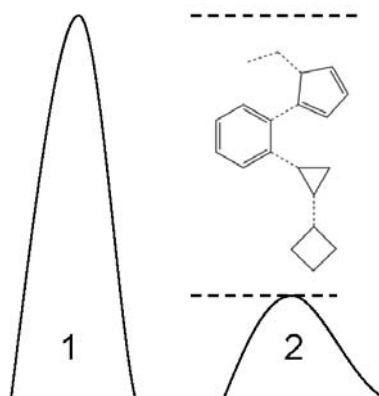


Figure 16: Quenching illustration. The illustration shows an absorption peak (1) and the corresponding peak where a quenching molecule is added (2). The quenching molecule absorbs energy equivalent to the difference between peak 1 and peak 2.

9. Inhibition: Data collection

IC₅₀-values were estimated based on plots of collected data on rates with or without different concentrations of inhibitor. The mode of operandum was different in manuscript 1 compared with manuscript 2 and 3. This because a single cuvette – recorder was used in manuscript 1. The single-cuvette-recorder is time-consuming since measurements are performed subsequently instead of in parallel. Analysis of the first measurement with an arbitrary inhibitor-concentration can also pinpoint other concentration to test. Thus it is possible to narrow the range of inhibitor-concentration to include the most vital parts of the activity curve (ranging from no-inhibition to more than 50 % inhibition). The plate reader (used in manuscript 2 and 3) analysis several samples, meaning inhibitor concentrations, simultaneously. In manuscript 2 and 3 a wider range of inhibitor concentrations were tested, which often would range over several log-values (figure 17, right).

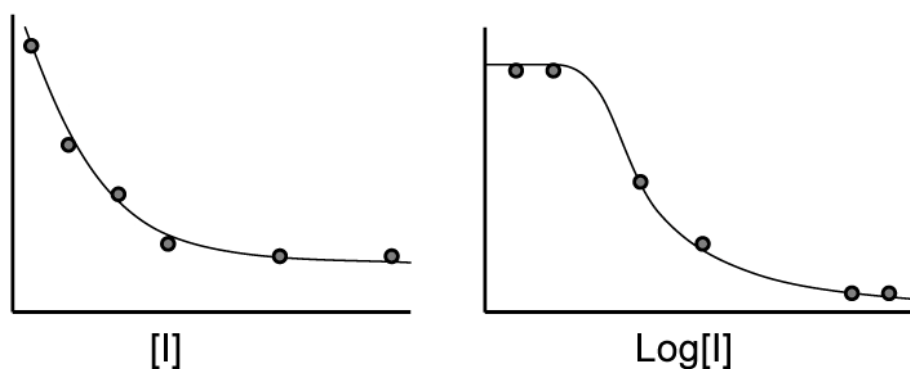


Figure 17: IC₅₀-measurements. The illustration shows two graphs (Y-axis V_1/V_0 (rate with inhibitor versus rate void of inhibitor)) where the left has all the inhibitor concentration in one log (left), while the other (right) has inhibitor concentrations over a range of log-values. One log will then be a narrow inhibitor concentration-range, while a range of log-values includes a wider range of concentrations.

10. Parameter assumptions in assay design

The enzyme assays utilised in the work incorporate several parameters, constants in the experiments, and variables. In the phase where an assay protocol is established, potential influences of the parameters should be tested. However, sometimes these tests are labour-intensive, and assumptions are made instead. The buffer composition is one of the factors that will influence assay results. In manuscript 1, HEPES was used instead of Tris-HCl because of the range of buffering capacity, we have utilised it before and also that it should contain less Zn^{2+} that might influence the inhibition capacity of our compounds. The inhibitors were all dissolved in 100 % DMSO. It might be that some (or all of the inhibitors) could be dissolved in a lower % of DMSO. This would in turn reduce the amount of DMSO present during data collection. An experiment to address DMSO effect on MMP-9 was performed, which found that DMSO reduces the enzyme activity (results not shown), potentially by denaturation or by interfering with optimal enzyme working conditions. It was also assumed that it would have a similar effect on MT1-MMP and the other enzymes. Therefore, DMSO was parameterised according to a dose that would allow us to replace it with larger concentrations of inhibitors during IC₅₀-collection, but still retain a good measurable enzyme activity. DMSO was thus included at 5 % in all our experiments based on the initial testing on MMP-9. The alternative would be not to account for the added DMSO, but then it might have been that the inhibitors would

have been (wrongly) assumed to perform better simply because of activity reduction due to the addition of DMSO with the inhibitor. The addition of DMSO with the inhibitor is larger the more inhibitor is added to the solution. Small amounts of Brij35 were added to the buffer because it has been shown to stabilise the gelatinases [489]. It was then included for all enzymes to make the buffer conditions as similar as possible. Brij35 may influence the catalytic activity and inhibition of proteinases [490]. Enzyme was added just before measuring, and therefore inhibitor and substrate competes immediately for enzyme-binding. The need to pre-incubate the inhibitors with enzyme without the presence of substrate, which is necessary for slow tight-binding inhibitors, was again tested only with MMP-9 and assumed for the other enzymes (results not shown). It might be that a given inhibitor can act as a tight-binding inhibitor on one enzyme and not require pre-incubation for inhibition of another one. It also might be that similar looking inhibitors act differently, again affecting the correctness of our assumptions. High amounts of CaCl_2 were included while assaying, and we especially wanted to test if Ca^{2+} was similar enough to Zn^{2+} so that it would act as a competitive inhibitor-target for our inhibitors. Again MMP-9 was used to test this. Due to how active MMP-9 had been purified, CaCl_2 could be lowered to 333 μM in the assay. We established both a new K_m -value and a new IC_{50} -value on PAC-1, and found no larger difference between the results with or without CaCl_2 thus concluding that Ca^{2+} did not act as a competitive target for the inhibitor. Here an assumption is made that PAC-1 can represent all the inhibitors, which might be correct, partly correct (it might represent only the PAC-1 derivatives but not the Isatin 1,2,3 triazoles) or no inference should be made (PAC-1 only represents itself, having different qualities from all the other inhibitors). A second assumption is that MMP-9 is a good model for the other enzymes. This is probably less true since the different enzymes are found to respond to Ca^{2+} differently. However, CaCl_2 was parameterised at 10 mM to make the protocol more uniform for all the enzymes.

11. Kinetic Interpretations

The paper is based on measured constants. Naturally it is important to evaluate the accuracy of the measurements and discuss the use of them throughout the article. The substrates were mostly chosen because of solubility and that we had worked with them in the laboratory before. Two different substrates had to be used simply because neither was suitable for use for all enzymes. Authors designing the synthetic bradykinin-substrate suggested that it was unsuitable for MMPs [374]. The K_m -values for the enzymes toward their respective substrate was experimentally determined in duplicates (manuscript 1, table 1). Obtaining K_m -values was important because the value provides an approximate quantitative measure of E-S complementarity in binding, and is found from initial rates measurements over a range of $[S]$. In our experiments, we got a standard deviation of 30-40 %. It goes without saying that a 40 % standard deviation points to low accuracy. The low accuracy of the K_m will also have ripple effects on the determination of inhibition constants and the correlation between different types of constants. Table 2 presents the IC_{50} -values determined under a discrete $[S]$, and $[S]/K_m$ was attempted equal among some of the enzymes so to make the parameters similar between enzymes. A high standard deviation for K_m translates into low accuracy also for $[S]/K_m$. The K_m -value is also implemented in the correlations between IC_{50} -values and K_I -values as illustrated also leading to lower accuracy in the inference on this correlation. Even though we might be able to measure our values accurately, there are potential experimental biases that we need to be aware of. The K_m -value is found from initial rates measurements over a range of $[S]$, and the choice and range of $[S]$ s will create biases. Using $[S]$ exclusively $< K_m$ or $> K_m$ will then result in accumulation of values on the two linear sections of the Michaelis-Menten graph [491]. This will represent the phases where $[S]$ is most limiting or non-limiting to the reaction respectively, and it will be difficult to infer the complete graph from either side. To avoid such biases a range going from $(0.3 - 3) \cdot k_m$ (or even better $(0.1 - 10) \cdot K_m$) is suggested including 6-7 individual measurements [491]. The individual measurements should be scattered evenly on the graph or cover the three sections (linear-phase, curvature, second-linear phase, figure 18).

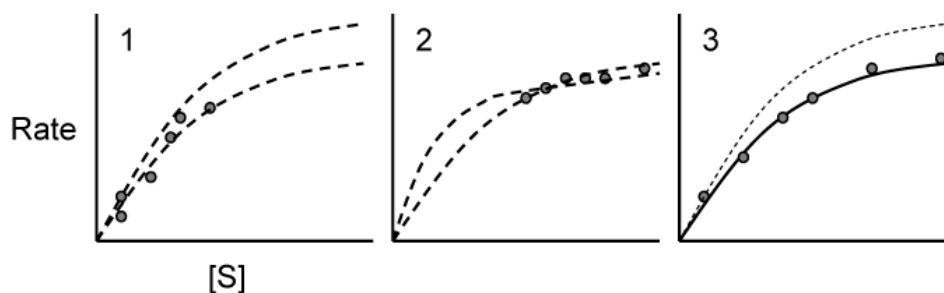
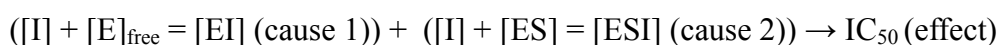
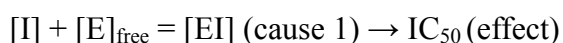


Figure 18: Kinetic interpretation of a MM plot. Plot 1 illustrates an experimental design with majority of points below putative K_m , while plot 2 has majority of points higher than putative K_m . As attempted explained in text and shown in illustration, these experimental designs will create uncertainty in overall graph appearance and thus is unfit to find K_m and V_{max} . Plot 3 has a good range in scatter of measurements, and it will be more likely to select one graph (line) instead of another (stripped line).

We feared creating quenching at $[S] > 10 \mu\text{M}$, and did not include values of $[S]$ over this threshold. A bias was thus created due to this for those enzymes with an expected K_m -value above this threshold (for instance thermolysin, Pseudolysin and MMP-14, Table 1), where all the values are gathered $< K_m$. NEP-1 and MMP-9 are less biased as it was possible to collect both above and below the expected K_m -value. The high K_m for MMP-14 creates a bias in the data set compared with that of MMP-9, which affect results in table 3.

12. Causality I

There is a cause and effect relationship (also referred to as causality) between two events where one follows the first and is dependent on the occurrence of the first. In manuscript 1, we have studied causality in different ways. A putative effector/inhibitor was added to interfere with the natural process of an enzyme, and a good regression relationship was found between the addition of inhibitor and lowered enzyme activity. The IC_{50} is a value measuring the effect that one inhibitor has on a given system, in our case the FRET-based system. We would for instance expect to half the MMP-9 activity with 46 μ M PAC-1, final concentration under same experimental conditions, using the information collected in table 2. Expecting to find a physical interaction between enzyme and inhibitor, would allow the cause to either be that the inhibitor binds to one or both forms of the enzyme available ($[E]_{free}$ and $[ES]$).



Kinetic examination on a smaller inhibitor set allowed us to differentiate the two plausible cause explanations (Table 3) for those inhibitors. The physical interaction between enzyme and inhibitor was explored with theoretical calculations, docking. The interaction surface is found based on defined criteria's optimising hydrophobic, hydrophilic, hydrogen-bonds and most often utilising surface cavities and prosthetic groups as potential mediators of interaction (we will not discuss the criteria's). Cause 1 is easier to explore for us because we have access to PDB-entries representing $[E]_{free}$ and we can compare our findings with literature - PAC-1 can be expected to target the zinc because it chelates free zinc and a binding mode is suggested. Cause 2 is very difficult to explore in any theoretical model, but especially docking because the protein is treated as very static. As explored in the introduction, these enzymes go through many dynamic movements to perform binding and catalysis of a substrate. There are also differences in movement dependent on the substrate, and we would not know how well the FRET-based system mimics a natural substrate. We would therefore not know what literature to compare to and incorporate in our model if we would be able to create a trustable model of $[ES]$. The interaction in ESI would also be difficult to trust because now we have no expected binding sites. However, key

amino acids in this new interaction site could be mutated in the enzyme and tested both in the FRET-based assay and the theoretical model. This requires a lot of extra work on something that might not be useful anyway. From a rational drug design perspective, we would like to dissect and isolate any key interacting sites and potentially modify and enhance them. The opposite is true for neutral or negative features that repel interaction. These compounds were expected to target the catalytic zinc, and therefore we would have that $[E]_{\text{free}}$ can be separated into catalytic zinc & overall topology. The enzymes that we have been working on have to “hidden” their active site differently from the exterior environment, so overall topology could mean global protein structures that determine accessibility to the active site, which potentially exclude a perfectly adapted inhibitor from entering into the active site in the first place. However, it seems that inhibitors gain entry into the active site inferred from the small differences in effect for a single inhibitor targeting enzymes with different active-site accessibility (Table 2). Thus, overall topology will be used to refer to structural features close to the zinc-ion (such as sub-pockets). The different scaffolds of the inhibitors allowed us to somewhat explore the individual contributions of these two enzyme features. Modification of the alcohol-group in PAC-1, which is suspected of zinc-interaction, led to decreased inhibition potency; replacement with a hydrogen-atom lead to no inhibition (PAC-1a) while other replacements (keeping the oxygen) decreased inhibition several folds. Comparing the inhibitors, we were allowed to conclude;

- 1) Overall topology – interactions alone were not enough for inhibition (no inhibition with PAC-1a)
- 2) Zinc-interaction (or groups suspected of zinc-interaction) was important for inhibition.

Since the zinc-group alone (without the backbone) was not included in the study, further weighing of importance of the zinc-interaction could not be postulated. The same also for finding out what is the actual zinc-interaction group;

- Is only the alcohol-group interacting with the catalytic zinc (monodentate-binding)?
- Is the alcohol group in combination other groups on the inhibitor (such as the ketone or electron-rich nitrogen or the aromatic side-chain) interacting with the catalytic zinc?
- Can the receptor determine if both or one of them is used?

The scaffold based on Isatin 1,2,3 triazole incorporated larger structural and chemical differences and the putative zinc-interaction group was different from PAC-1. As a group these inhibitors seemed to have a lower inhibition strength compared to PAC-1 and PAC-1 analogues (except PAC-1a, 250D and 246C). Docking revealed that scaffold differences altered the groups targeting the zinc-ion, and further sub-grouping of this scaffold was necessary. We found an interesting cause – effect relationship in ISD-1, TZ-5, TZ-8, where the longer P1'-chain (cause-factors) created a correlating effect. A similar approach to causality was done in these papers as in paper 1, but the cause-analysis was somewhat different. The ZBG is the same for all the inhibitors, and it is viewed as a strong zinc-binding group. However, there are some contradictory results to be found in the literature as presented in the introduction. In manuscript 2 and 3, the near overlapping orientation of the ZBG-domain in the inhibitors allowed us to focus more on overall topology differences as a stronger contributor to inhibition strength than we could in manuscript 1. Moreover, a stronger computational examination was also performed accounting for both movement of the receptor as well as incorporation of water. The more reliable computations allowed us to further analyse contribution that causes the effects on individual receptor-inhibitor interactions.

Future Perspectives

We have in our manuscripts characterised sets of ligands, using a combination of experimental work and theoretical modelling. There are many possibilities left for us to continue with. We could continue a long time with the same inhibitors, trying to correlate new experiments with new modelling. The docking in manuscript 3 could for instance be followed up with MD simulations and LIE. In the laboratory, we could alter pH-conditions during measurements. The later would give us indications on how the equilibrium constants (K_I and K_m) are dependent on pH. We could also try to change ionic states of the ligands and active sites in our theoretical model accordingly. This might enable us to dissect and alter vital residues (either on the enzyme or on the ligand) in the enzyme-ligand interface. However, ligand binding is dependent on mutual interactions between ligand and enzyme, which are both affected by the pH. It would therefore be difficult to combine the correct protonic states in the modelling. The same is also valid for trying to construct a more accurate model of the E-S-I complex in manuscript 1. We would rather like to move from describing these ligands into predicting new ligands to synthesis. Some of the tested compounds could serve as lead fragments for suggesting substituents and/or additions to the structures by the use of modelling, and then get the most interesting synthesised. For such a purpose, our present results are very helpful. . We have found structure-activity relationships, have a broad fundament for testing both selectivity and inhibition strength (both between families (inter) and inside families (intra)) and finally our results are that our inhibitors are weak inhibitors instead of strong inhibitors (suggesting possibilities for improvements). It is worth mentioning that co-crystals between inhibitors and enzymes could be sought. We have earlier made co-crystal between an inhibitor and thermolysin (PDB-entries: 2WIO, 2WHZ, unpublished paper [492]). We have in our modelling found differences in preferred binding mode between families so that a pose inside thermolysin might not represent that of a M10A enzyme. However, a co-crystal with thermolysin might give information that can be applied on pseudolysin or other M4 members. The work was done in the tumour biology group, which works on cells and animals, and the inhibitors could be tested on different cell types. We would also like to improve the selectivity among the enzymes before testing on cells.

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