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Ntn-hydrolases unveiled

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Chapter 1

Introduction

Amidohydrolases

Peptides and proteins consist of amino acids linked together in a head-to-tail fashion as a result of the condensation reaction between an amine and a carboxylate group. The covalent bond that is formed between the amine and carboxylate is called *amide* or *peptide bond*. There are numerous enzymes capable of hydrolyzing amide bonds, which can roughly be divided into two classes (http://www.chem.qmul.ac.uk/iubmb/enzyme/), *peptide* and *non-peptide amidohydrolases* (E.C. 3.4 and E.C. 3.5, respectively). An enzyme family common to both peptide and non-peptide amidohydrolases is formed by the *serine hydrolases*; it makes use of a nucleophilic serine that attacks the scissile amide bond. A classic catalytic scaffold for serine-hydrolases consists of a *catalytic triad* comprising a nucleophilic serine, a histidine that functions as a general acid/base and an aspartate capable of stabilizing the positive charge that develops on the histidine. This catalytic triad was first observed in chymotrypsin by Blow *et al.* (1969), and similar catalytic triads were found later in other peptide hydrolases such as subtilisin (Alden *et al.*, 1970; Drenth *et al.*, 1971) and the α/β-hydrolase carboxypeptidase-II from wheat (Liao & Remington, 1990).

However, in the last 15 years a completely new peptide and non-peptide amido-hydrolase family was characterized, the *N-terminal nucleophile hydrolase* family, or Ntn-hydrolase family (Brannigan *et al.*, 1995). Both the serine proteases and Ntn-hydrolases make use of a hydroxyl nucleophile (or, in some Ntn-hydrolases, a thiol nucleophile) and are expressed as *zymogens*, or *precursors*, that need to mature to become fully active. However, there are some striking differences: firstly, Ntn-hydrolases completely lack the classic catalytic triad. Secondly, Ntn-hydrolase maturation is very different, in the sense that proteolysis is generally an intramolecular event and that one half of the cleaved scissile bond directly contributes to the active site of the enzyme.

The scope of this thesis is formed by structural studies of two different Ntn-hydrolases, *Penicillium chrysogenum* acyl-coenzyme A:isopenicillin N acyltransferase (AT; E.C. 2.3.1.164) and *Pseudomonas aeruginosa N*-acyl homoserine lactone acylase (PvdQ; E.C. 3.5.1.97). This introduction will focus on the unique features and the diversity of the Ntn-hydrolase superfamily.

The Ntn-hydrolase fold

Ntn-hydrolases are represented in all kingdoms of life and fulfill many important functions; from the degradation of misfolded proteins (Löwe *et al.*, 1995) to the biosynthesis of

nucleotides (Smith *et al.*, 1994). A common feature of Ntn-hydrolases is the presence of an N-terminal nucleophilic serine or cysteine. Furthermore, Ntn-hydrolases are the only amidohydrolases that can utilize a nucleophilic threonine (Seemüller *et al.*, 1995). Although biological context, primary structure and catalytic mechanism vary among Ntn-hydrolase family members, the 3-dimensional organization of the secondary structure elements in the catalytic core is strictly conserved.
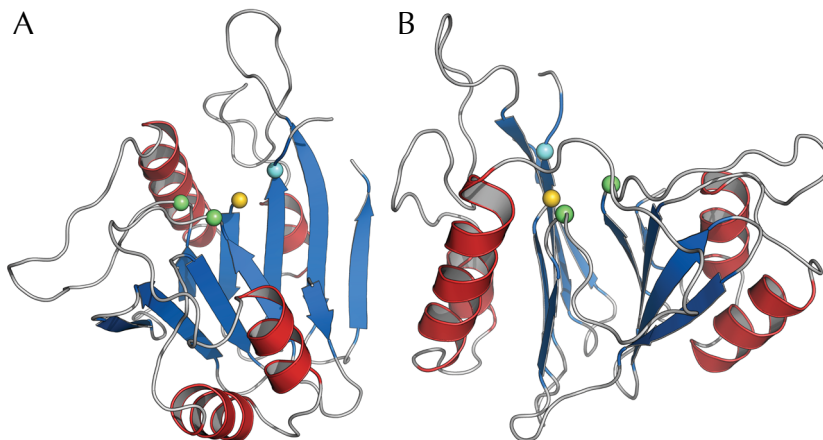

Figure 1: The structurally conserved core of the Ntn-hydrolases using AT as an example. The Ntn-hydrolase fold consists of two stacked anti-parallel β-sheets lined by α-helical bundles, (A) front and (B) side view. The location of the nucleophile, the nucleophile stabilizing residue and the oxyanion hole are indicated by yellow, cyan and green spheres, respectively.

Ntn-hydrolases have a typical fold with a structural core that consists of two central, stacked antiparallel β-sheets sandwiched between α-helical bundles (Brannigan *et al.*, 1995; Oinonen & Rouvinen, 2000), giving it a four-layered αββα-packing (Figure 1A/B). Although, the spatial configuration of the secondary structure elements in the core is highly conserved among Ntn-hydrolases, the order of these elements in the sequence may differ per family (for more information on this aspect, see the section on *The versatility of the Ntn-hydrolase scaffold*). Figure 2 shows the consensus topology of the Ntn-hydrolases based on the 3D-structures of AT and PvdQ; the N-terminal nucleophile is always located on β4. The structural variations between different Ntn-hydrolases are mainly found outside the structural core such as additional β-strands in the central sheets, inserts in loops that connect the secondary structure elements in the conserved core or the addition of whole domains. The next section will discuss the structures of different Ntn-hydrolases in more detail.
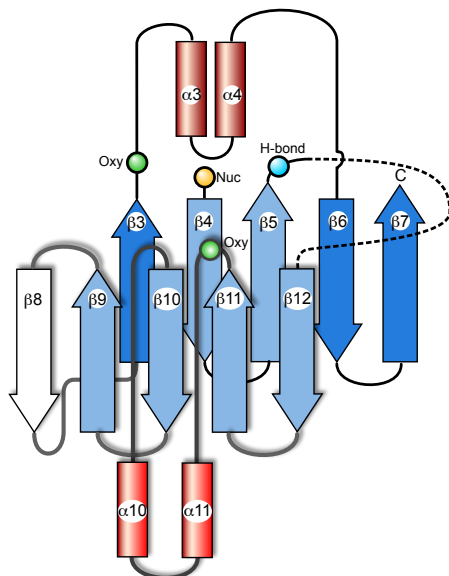
Figure 2: Topology diagram of the structurally conserved Ntn-hydrolase core. This figure also indicates the conserved location of the catalytic residues, based on AT and PvdQ. The oxyanion hole residue located on β11 is not present in all Ntn-hydrolases. In this family the conserved core is located within the β-subunit. Different shades of blue for β-strands and red for α-helices indicate the conservation region according to Oinonen *et al.* (2000). Light shades indicate secondary structures that are found in all Ntn-hydrolases, dark shades indicate elements that are less conserved. Numbering according to Oinonen *et al.* (2000)

The Ntn-hydrolase families

The SCOP database (Andreeva *et al.*, 2004) defines seven N-terminal nucleophile amidohydrolase families with a similar structural core, but lacking any discernible sequence similarity. The key representatives of these different Ntn-hydrolase families are the glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase or class II glutamine amidotransferase (GAT) (Smith *et al.*, 1994), penicillin V acylase (PVA) (Suresh *et al.*, 1999), penicillin G acylase (PGA) (Duggleby *et al.*, 1995), proteasome subunits (PS) (Löwe *et al.*, 1995), glycosylasparaginase (GA) (Oinonen *et al.*, 1995), γ-glutamyl transpeptidase (γG) (Okada *et al.*, 2006) and SPO2555-like (New York SGX Research Center for Structural Genomics, http://www.nysgxrc.org). Furthermore, SCOP defines three additional Ntn-hydrolase-like families, the archaeal IMP cyclohydrolase (PurO) (Kang *et al.*, 2007), *Streptomyces clavuligerus* ornithine acyltransferase (OAT) (Elkins *et al.*, 2005) and *Ochrobactrum anthropi* L-aminopeptidase D-Ala-esterase/amidase (DmpA) (Bompard-Gilles *et al.*, 2000). All these Ntn-hydrolase family representatives are shown in Figure 3, superposed using the secondary structure matching algorithm (Krissinel & Henrick, 2004) implemented in COOT (Emsley & Cowtan, 2004).
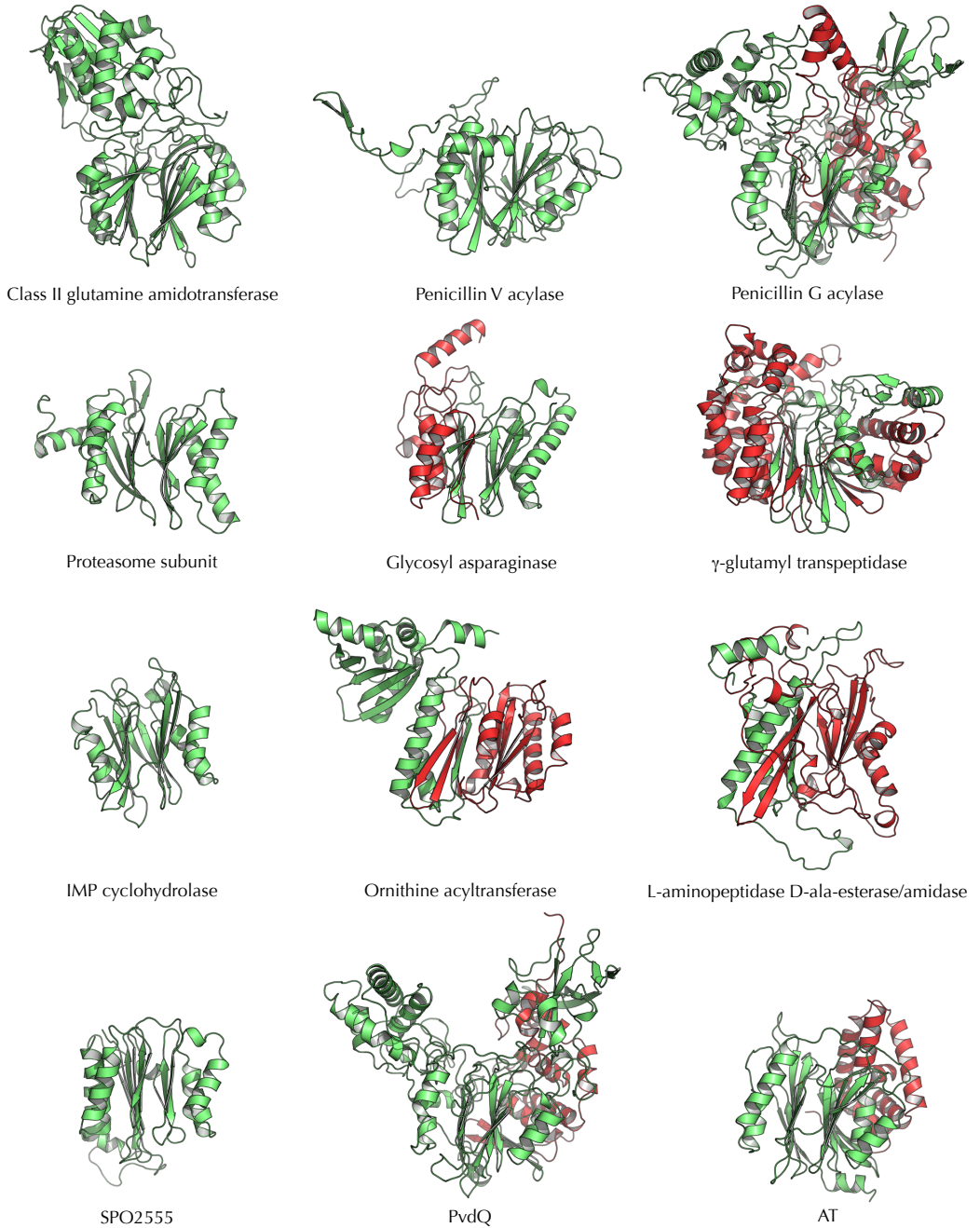
Figure 3: Ntn-hydrolases come in different shapes and sizes. This figure show the different families as defined in the SCOP database (Andreeva *et al.*, 2007). α-chains are red, β-chains are green. Furthermore, AT and PvdQ are also shown.

The SCOP classification is based on domain organization and the type of N-terminal nucleophile; the different classes are schematically represented in Figure 4. The IMP cyclohydrolase PurO forms an exception since it has a typical Ntn-hydrolase fold, but lacks the N-terminal nucleophile. In the other Ntn-hydrolases the N-terminal nucleophile is liberated after *post-translational modification*. Ntn-hydrolases show different forms of post-translational modification. The enzyme can be monomeric and lose its N-terminal formyl-methionine (certain class II glutamine amidotransferases) or short N-terminal peptide (PVA and PS). However, the mature form can also be heterodimeric, with post-translational modification resulting in the formation of two chains that stay associated (GA, γG, OAT and DmpA). Although OAT and DmpA have the typical αββα fold, the topology of the structural core is entirely different, even consisting of parallel β-strands. This indicates that these enzymes arose from a different ancestor (Cheng & Grishin, 2005) and therefore they are outside the scope of this thesis. In some Ntn-hydrolases two post-translational cleavage events take place, resulting in the excision of a *pro-segment* as often observed in the PGA family. The structural consequences of post-translational modification and the catalytic mechanism behind maturation will be discussed in more detail in the next section.
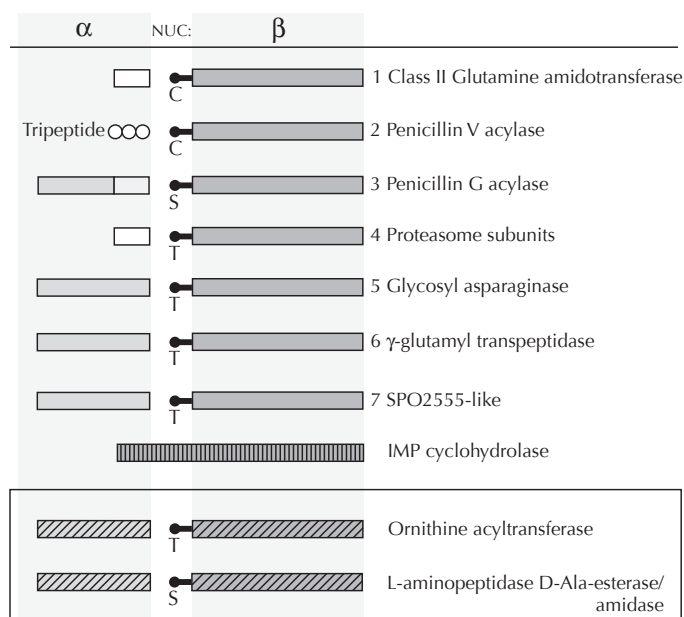

Figure 4: Schematic representation of the different Ntn-hydrolase families. The boxed enzymes have such a different topology that they probably arose from a different ancestor.

With respect to the enzymes discussed in this thesis, PvdQ belongs to the same Ntn-hydrolase family as PGA (Chapter 3). AT on the other hand is an α/β-heterodimeric cysteine-containing Ntn-hydrolase and can currently not be placed in any of the families (Chapter 4), similar to the 66.3 kDa mouse lysosomal protein (Lakomek *et al.*, 2009).

Precursor amide hydrolysis: Ntn-hydrolase activation

The structural core of the Ntn-hydrolases discussed in the previous sections carries the N-terminal nucleophile that fulfills a pivotal role in catalysis. The nucleophile is the N-terminal residue because a free α-amino group is absolutely essential for catalysis (Duggleby *et al.*, 1995). Upon cleavage of the scissile bond the generated α-amino group will act as the general base in catalysis; this will be discussed in more detail in the section on *Substrate amide hydrolysis: Ntn-hydrolase catalysis*. Additionally, in the precursor enzyme the part of the peptide chain that is N-terminal to the nucleophile occludes the entrance to the active site, but once the scissile bond has been cleaved, this part of the peptide folds out and the active site becomes accessible for a substrate molecule (see for example Figure 5A/B).
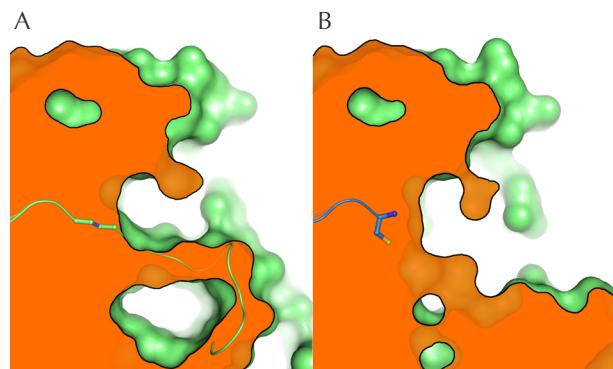
Figure 5: Solvent accessibility of the N-terminal nucleophile in precursor (A) and mature (B) AT. (A) Shows that the solvent can reach the scissile bond (blue), but it is buried too deeply to allow access of an external protease. (B) Once the precursor peptide is broken, the pro-peptide leaves and the active site becomes accessible for a substrate molecule.

Interestingly, the scissile peptide bond in the precursor enzyme that precedes the N-terminal nucleophilic residue is buried in the central core of stacked β-sheets, and can therefore not be reached by an external protease (Figure 5A). This indicates that precursor activation must result from an intramolecular cleavage event, which has also been demonstrated by biochemical experiments on for instance penicillin G acylase (Kasche *et al.*, 1999) and cephalosporin acylase (Kim *et al.*, 2006). Self-activation by intramolecular cleavage is very similar to *self splicing* (Kane *et al.*, 1990), which occurs in for example inteins, hedgehog proteins and pyruvoyl enzymes, a subject that has been comprehensively reviewed by *e.g.* Paulus (2000).

Proteolytic self activation proceeds through an N - O or N - S acyl shift

Biochemical experiments together with the elucidation of several Ntn-hydrolase precursor structures have shed light on the possible mechanism behind zymogen activation. In the precursor structures the scissile bond is flanked by the N-terminal nucleophile, which has a nucleophilic hydroxyl or thiol side chain; mutation of this serine, threonine or cysteine always results in the accumulation of enzyme in the precursor state. This suggests that the N-terminal nucleophilic residue is responsible for both substrate and precursor scissile bond hydrolysis. Likely, precursor activation proceeds via an *N - O* or *N - S acyl shift*, or *trans-esterification*, as result of a nucleophilic attack of the scissile bond by the N-terminal nucleophile side chain, similar to amide or ester bond hydrolysis in the substrates of *e.g.* α/β-hydrolases (Ollis *et al.*, 1992). Although such a (thio)ester precursor intermediate has never been observed directly in Ntn-hydrolases, biochemical data supports this mechanism. For example, a slowly processing mutant of *Flavobacterium meningosepticum* glycosylasparaginase (Guan *et al.*, 1996) shows a 150-fold decrease in precursor half-life upon addition of hydroxylamine (NH_2OH), a strong nucleophile that specifically reacts with (thio)ester intermediates (Lipmann & Tuttle, 1945). This indeed suggests that Ntn-hydrolase maturation in GA proceeds via an ester intermediate generated subsequent to a nucleophilic attack by the N-terminal nucleophilic threonine in GA. Furthermore, mutational analyses of catalytically important residues in *e.g.* GAT (Li *et al.*, 1999) and PvdQ (P. Nadal Jimenez, unpublished results) indicate that in addition to the N-terminal nucleophile other residues are also involved in autoproteolysis. This is probably due to the fact that scissile bond hydrolysis in precursor and substrate is similar, which allows for multipurpose use of catalytic elements, although the elements may perform different roles in each process (Chapter 4).

Figure 6 shows a general mechanism by which the scissile bond in Ntn-hydrolases is cleaved via an N - X acyl shift. It is initiated by deprotonation of the N-terminal nucleophilic hydroxyl or thiol group by a general base (Figure 6A), after which the nucleophile attacks the carbonyl carbon of the scissile bond (Figure 6B). The nucleophilic attack generates an *oxo-oxazolidine-* or *oxothiazolidine-anion transition state* (Figure 6C), stabilized by the oxyanion hole residues. The transition state will collapse as the amine acts as a leaving group, generating a (thio)ester intermediate (Figure 6D). Subsequently, the (thio)ester intermediate is hydrolyzed by an activated water molecule giving rise to the free N- and C-termini (not shown). Interestingly, there appears to be no universal mechanism of the deprotonation of the nucleophile; therefore, the next section will discuss

the proposed activation mechanisms of several Ntn-hydrolase representatives for which precursor structures have been solved.
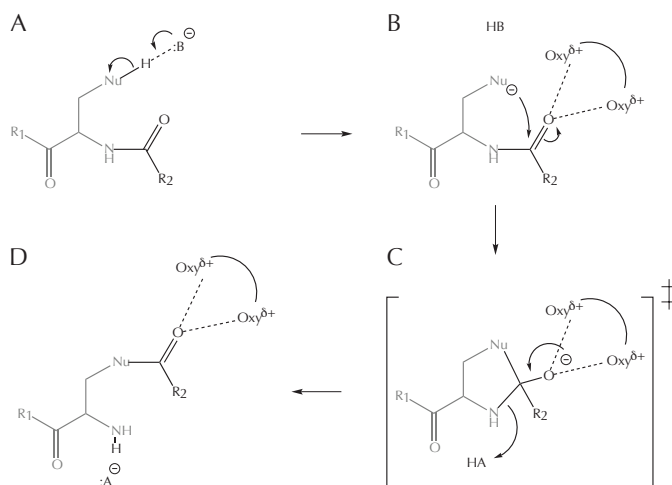

Figure 6: General mechanism of Ntn-hydrolase self-activation. (A) Nucleophile activation by a general base (**:B**). (B) Nucleophilic attack. (C) Transition state formation. The transition state will collapse upon protonation by a general acid (**HA**). (D) Upon transesterification a (thio)ester intermediate is formed.

A strained backbone conformation in precursor activation

Xu *et al.* (1999) were able to elucidate the first crystal structure of an Ntn-hydrolase precursor, a mutant of *Flavobacterium meningosepticum* glycosylasparaginase in complex with glycine, impaired in self-activation. Mammalian glycosylasparaginase (Oinonen *et al.*, 1995) is a lysosomal enzyme involved in the breakdown of glycoproteins, and dysfunctional GA in humans causes a rare, incurable lysosomal glycoprotein storage disease called aspartylglucosaminuria (Mononen *et al.*, 1993). Interestingly, this illness is often associated with mutations that affect the maturation of GA (Saarela *et al.*, 2001).

The three-dimensional structure of the slowly processing mutant of prokaryotic GA allowed the authors to propose a catalytic mechanism of the maturation reaction. The structure shows an unusual turn near the N-terminal nucleophile (Thr152) such that the side chain of the preceding residue (Asp151) comes in the vicinity (2.8 Å) of the threonine side chain (Figure 7). This unusual conformation, maintained by the aspartate (Qian *et al.*, 2003), allows Asp151 to deprotonate Thr152. This is followed by the nucleophilic attack on the carbonyl carbon of the scissile bond by the Thr152 hydroxylate, which results in formation of the oxo-oxazolidine-anion transition state. This transition state is stabilized by the oxyanion hole residues, which are a water molecule and the side chain of Thr170 (Figure 7). The α-amino leaving group is protonated by the Asp151 carboxyl moiety causing the collapse of the transition state into an ester intermediate. Next, the newly formed ester

intermediate is hydrolyzed by an activated water molecule, which causes the collapse of the intermediate into the free N- and C-termini.

The authors assign a pivotal role to Asp151 in autoproteolysis, which is supported by mutagenesis studies. For example, an Asp151Asn mutation results in GA arrested in the precursor state (Qian *et al.*, 2003); it is proposed that a highly strained backbone conformation is required to initiate maturation. Mutation of the equivalent aspartate in human Taspase1 also results in enzyme arrested in the precursor state (Khan *et al.*, 2005). However, these mutagenesis studies do not fully establish that Asp151 actually is the catalytic base. In human GA a mere structural role is proposed for the equivalent acidic residue while the catalytic base is proposed to be a water molecule (Saarela *et al.*, 2004). The involvement in catalysis of a basic water molecule has also been proposed for other Ntn-hydrolases. These enzymes often lack the acidic residue preceding the N-terminal nucleophile found in GA, for instance plant and *E. coli* asparaginase (Michalska *et al.*, 2006, 2008) and *E. coli* γ-glutamyltranspeptidase (Okada *et al.*, 2007). This latter type of mechanism will be discussed in more detail in the next section.
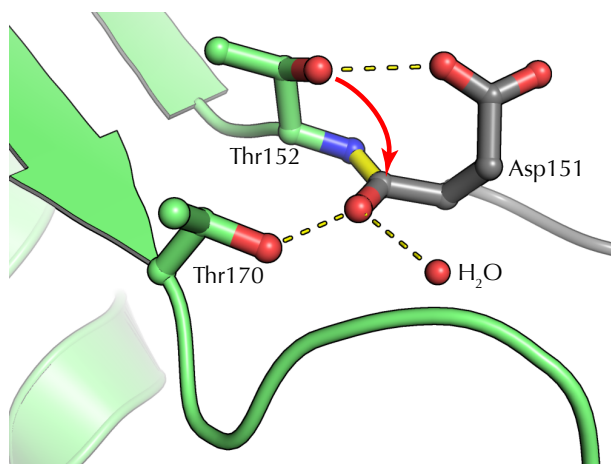
Figure 7: The autoproteolysis site in glycosylasparaginase. A highly strained backbone conformation is present near the scissile bond maintained by Asp151. Asp151 activates the Thr1 hydroxyl, which performs a nucleophilic attack on the scissile bond (red arrow). The transition state can be stabilized by a solvent molecule and Thr170.

A water molecule as a catalytic base

The self-activation mechanism proposed for GA is rather unique; a more common autoproteolysis mechanism among Ntn-hydrolases is observed in cephalosporin acylase (CA) (Kim *et al.*, 2000), which belongs to the same Ntn-hydrolase family as penicillin G acylase. Kim *et al.* (2002) were able to capture CA in the precursor state by mutating the N-terminal nucleophilic serine to an alanine. To their surprise no residue could be found in the vicinity of the scissile bond that could act as a general base, such as Asp151 in

GA. This supports the idea that the mechanism behind autoproteolysis in Ntn-hydrolases differs per family. The crystal structure shows the presence of a water molecule (Wat1) 3.3 Å away from the Cβ of Serβ1Ala in an unusual pseudo-tetrahedral coordination (Kim & Hol, 2001) held in place by the backbone carbonyl oxygen of Glnα168 and the backbone amide of Valβ70 (Kim *et al.*, 2002, 2003). Based on this observation the authors propose that the water molecule is a general base able to deprotonate the serine hydroxyl in the wild type precursor (Figure 8A). A mutation that causes the loss of this specific water molecule is blocked in the precursor state, even if the catalytic Serβ1 is present, indicating the importance of the water molecule (Yoon *et al.*, 2004). After proton transfer the Serβ1 hydroxylate can be stabilized by the backbone amide of Hisβ23. A similar organization of catalytic counterparts has been proposed for the maturation mechanism of a slowly processing mutant of penicillin G acylase (Hewitt *et al.*, 2000). The Serβ1 hydroxylate attacks the carbonyl carbon of the scissile bond, of which the transition state is stabilized by the backbone amide of Hisβ23. The transition state collapses into the ester intermediate upon protonation of the α-amino leaving group of Serβ1 by the Wat1 H_3O^+. Subsequently, Wat1 hydrolyzes the ester intermediate and the free termini are generated.
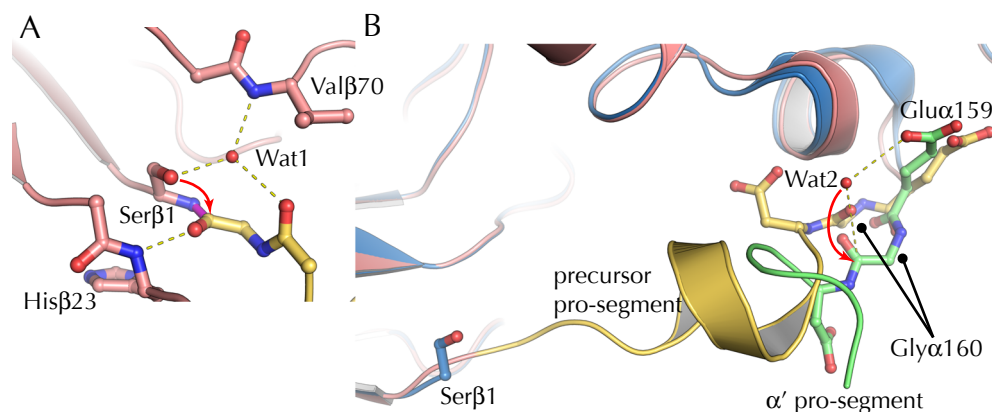
Figure 8: The two autoproteolysis sites in cephalosporin acylase. (A) Site 1. A tightly bound water molecule (Wat1) in a pseudo-tetrahedral coordination can accept a proton from the N-terminal nucleophilic serine, which performs a nucleophilic attack on the carbonyl carbon of the scissile bond (red arrow). The transition state can be stabilized by the backbone nitrogen of Hisβ23. (B) Site 2. Once the peptide bond in site 1 is broken, the strained pro-segment (yellow) relaxes (green), but it is still connected to the α-chain. In the relaxed conformation Gluα159 can activate a water molecule (Wat2), which attacks the Glyα160-Aspα161 scissile bond (red arrow).

After generating the α-amino group, the enzymes of the penicillin G acylase family of Ntn-hydrolases have not yet fully matured. CA, PGA and PvdQ all have a pro-segment that is still attached to the α-chain (denoted as α') after the initial autoproteolysis step. This pro-segment is removed by a second hydrolysis event to generate the fully mature species. Whether the second hydrolysis step is intra- or intermolecular remains a matter of debate. However, Kim *et al.* (2006) were able to show that a slowly processing mutant of CA was unable to fully mature upon the addition of completely functional enzyme, thus indicating that also the second hydrolysis step is intramolecular. The authors were able to arrest CA in the α'/β heterodimeric state and to solve its crystal structure. While the precursor structure shows a highly strained conformation of the pro-peptide, in the α'/β heterodimeric state its conformation has relaxed (Figure 8B). The relaxed conformation of the pro-segment is accompanied by a rearrangement of the residues around the second autoproteolysis site such that the side chain of Gluα159 can now activate a water molecule (Wat2) to attack the Glyα160-Aspα161 peptide bond (Figure 8B). Experiments have shown that maturation is impaired at low pH and in the presence of a carboxylate protease inhibitor, which is strong evidence that Gluα159 is involved in maturation (Kim *et al.*, 2006). Once the pro-segment is removed from the active site cleft the active site of the enzyme is fully accessible and can accept substrates for catalysis.

The role of the γ-turn in proteasome maturation

Although the proteasome also utilizes a water molecule as a catalytic base in autoproteolysis (Ditzel *et al.*, 1998), this enzyme displays an unusual catalytic coordination of the scissile peptide bond and, in contrast to the PGA family, loses its N-terminal chain. Therefore, proteasomal precursor activation is different from the previously mentioned mechanisms and will therefore be discussed separately.

The yeast 20S proteasome is a multi-subunit catalytic complex consisting of 28 subunits. Of the 28 subunits six are active Ntn-hydrolases, these include two copies of subunits β1, β2 and β5 each (Groll *et al.*, 1997). The active proteasome subunits are produced as precursors, and assembled into the proteasome. Spontaneous association of 28 proteasome subunits induces maturation of the 6 active subunits by removal of their pro-segments (Chen & Hochstrasser, 1996). Also in the proteasome the N-terminal nucleophilic residue, a threonine, is responsible for pro-segment removal and maturation. The introduction of a Thr1Ala mutation in β1 allowed Ditzel *et al.* (1998) to arrest this subunit in the precursor state; the mutant subunit was successfully integrated in the proteasome and crystallized.

The β1 subunit structure has a tight γ-turn N-terminal to the threonine, which is maintained by a hydrogen bond between the backbone oxygen of Leu-1 (the residue before Thr1) and the backbone amide of the N-terminal nucleophile. This sharp γ-turn allows a tight clustering of key autoproteolysis elements around the scissile bond, such as *e.g.* Lys33, the carbonyl oxygen of Leu-2, Ser129 and Wat1 (Figure 9). Due to the tight turn the Oγ atom of Thr1 is only 3.2 Å away from the carbonyl carbon of the scissile bond. The authors propose that Thr1 can donate its hydroxyl proton to a water molecule, which is located near the N-terminal nucleophile in both mature and precursor structures (Ditzel *et al.*, 1998; Groll *et al.*, 1997). The Oγ hydroxylate anion is stabilized by the positively charged Lys33 that is held in place by Asp17 and the backbone carbonyl oxygens of Leu-2 and Arg19. Subsequently, the hydroxylate performs a nucleophilic attack on the carbonyl carbon of the scissile bond, of which the transition state is stabilized by the oxyanion hole formed by a helical turn that harbors the backbone nitrogen and side chain of Ser129 and Gly130. Transesterification to the ester intermediate is initiated by the collapse of the transition state upon protonation of the α-amino leaving group by a solvent molecule. Finally, an activated water molecule attacks the ester intermediate, generating the free α-amino terminus.
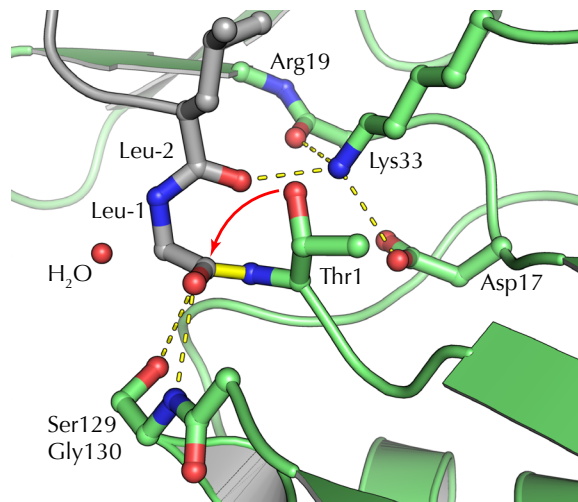
Figure 9: The autoproteolysis site in a proteasome subunit. A tight γ-turn is present near the N-terminal nucleophilic Thr1. The side-chain hydroxyl of Thr1 can be deprotonated by a solvent molecule, and the resulting negative charge is stabilized by the positive charge of Lys33. After that the Thr1 hydroxylate can perform a nucleophilic attack on the scissile bond (red arrow). The resulting transition state can be stabilized by Ser129 and Gly130.

Variations in Ntn-hydrolase autoproteolysis

From the previous sections it is evident that, even among similar enzymes, the mechanism of autoproteolysis is highly variable. Table 1 shows the conservation of the catalytic elements that are essential for autoproteolysis and their secondary structure location.

The **nucleophile** can be a serine, threonine or a cysteine. It is always located at the N-terminus of β4 (see Figure 2 for a topology diagram).

The **nucleophile stabilizing** residues are highly variable. In the different Ntn-hydrolases side-chains, backbone amides or even solvent molecules can participate in the stabilization of the nucleophilic state of the N-terminal nucleophile.

The **catalytic base**, which activates the serine or threonine nucleophile, is neither conserved. In cysteine-containing Ntn-hydrolases a catalytic base for thiol activation has thus far not even been found (Chapter 4; Lakomek *et al.*, 2009; Li *et al.*, 1999). Probably, due to the almost neutral pK_a of the cysteine side-chain, a catalytic base may even be unnecessary in enzymes with this nucleophile (Noren *et al.*, 2000).

The **oxyanion hole** is most strongly conserved, and is usually located on β5.

Table 1: List of proposed catalytic elements involved in autoproteolysis of Ntn-hydrolases with elucidated precursor structures: the proteasome subunits (PS), γ-glutamyl transpeptidase (γG), *E. coli* asparaginase (EcAIII), glycosylasparaginase (GA), cephalosporin- or glutaryl-7-amino cephalosporanic acid acylase (CA), penicillin G acylase (PGA) and acyl-coenzyme A:isopenicillin N acyltransferase (AT).

enzyme	PDB code	Ntn	Ntn-stabilizing		base		oxyanion hole	
			atom	SCSE	atom	SCSE	atom	SCSE
PS	1ryp	Thr1	Nζ Lys33	β12	H$_2$O	-	OγSer129	
							NSer129	β3
							NGly130	
γG	2e0w	Thr391	OγThr409	β5	H$_2$O	-	H$_2$O	
							OγThr409	β5
CA	1keh	Serβ1	NHisβ23	β5	H$_2$O	-	NHisβ23	β5
	1oqz							
EcAIII	3c17	Thr179	OγThr230	β11	OγThr230	β11	OγThr197	β5
			OγThr197	β5			NδAsn67	α4-β6 loop
GA	9gaf	Thr152	NA	NA	OδAsp151	β4	H$_2$O	
							OγThr170	β5
PGA	1e3a	Serβ1	NAlaβ68	β11	NA	-	NGlnβ23	β5
			NδAsnβ241	β3				
AT	-	Cys103	NAla168	β11	none	-	NAsp121	β5
			NδAsnβ246	β3				

SCSE secondary consensus structure element that harbors the mentioned residue(s)

The conservation of the oxyanion hole is in agreement with transition state stabilization being the primary determinant of scissile bond hydrolysis. Additionally, the oxyanion hole destabilizes the scissile bond by inducing a single bond character of the N - C peptide bond (Fodor *et al.*, 2006; Romanelli *et al.*, 2004) and a pyramidal configuration (Bürgi *et al.*, 1973; Esposito *et al.*, 2000; Marquart *et al.*, 1983), which reduces the stability of

the peptide bond and might provide the driving force behind spontaneous peptide bond hydrolysis (Johansson *et al.*, 2009). These processes only occur if the oxyanion hole is optimally oriented with respect to the scissile bond carbonyl oxygen. However, most precursor crystal structures lack such a strained scissile bond, which indicates that the structure of the scissile bond does not represent the actual pre-hydrolytic state, which might have led to the multitude of proposed mechanisms.

Substrate amide hydrolysis: Ntn-hydrolase catalysis

Once hydrolysis of the precursor scissile bond(s) has occurred, the enzyme is in the fully mature state. Autoproteolysis activates the enzyme by two means; firstly, departure of the occluding peptide chain opens up the ligand-binding site such that substrate can bind. Secondly, the generation of a new α-amino group completes the catalytic center. The unique catalytic mechanism of Ntn-hydrolases, which bears some resemblance to the autoproteolysis mechanism, and the pivotal catalytic role of the N-terminus will be discussed in this section.

The nucleophile, base and oxyanion hole

Duggleby *et al.* (1995) elucidated the structure of penicillin G acylase, the founding member of the Ntn-hydrolases, and proposed a catalytic mechanism that is generally applicable to all Ntn-hydrolases. After elucidating the crystal structure of PGA the authors were surprised that, unlike the histidine in the serine hydrolases, no suitable catalytic base was located near the N-terminal nucleophilic serine. Therefore, it was proposed that the α-amino group of the serine itself acts as the catalytic base. This rather unusual role of the α-amino group is supported by biochemical experiments that show deactivation of the enzyme upon specific carbamoylation of the α-amino group in CA (Lee *et al.*, 2000). Furthermore, the α-amino group needs to be neutral to accept a proton, which was confirmed by kinetic experiments (Duggleby *et al.*, 1995; Morillas *et al.*, 1999). Additionally, protonation of the α-amino group by performing substrate soaking experiments at a pH below the pK_a of the α-amino group allowed Kim and Hol (2001) to elucidate the crystal structure of CA in the substrate bound state. These are all strong indications that the α-amino group indeed acts as a general base, deprotonating its own hydroxyl or thiol group.
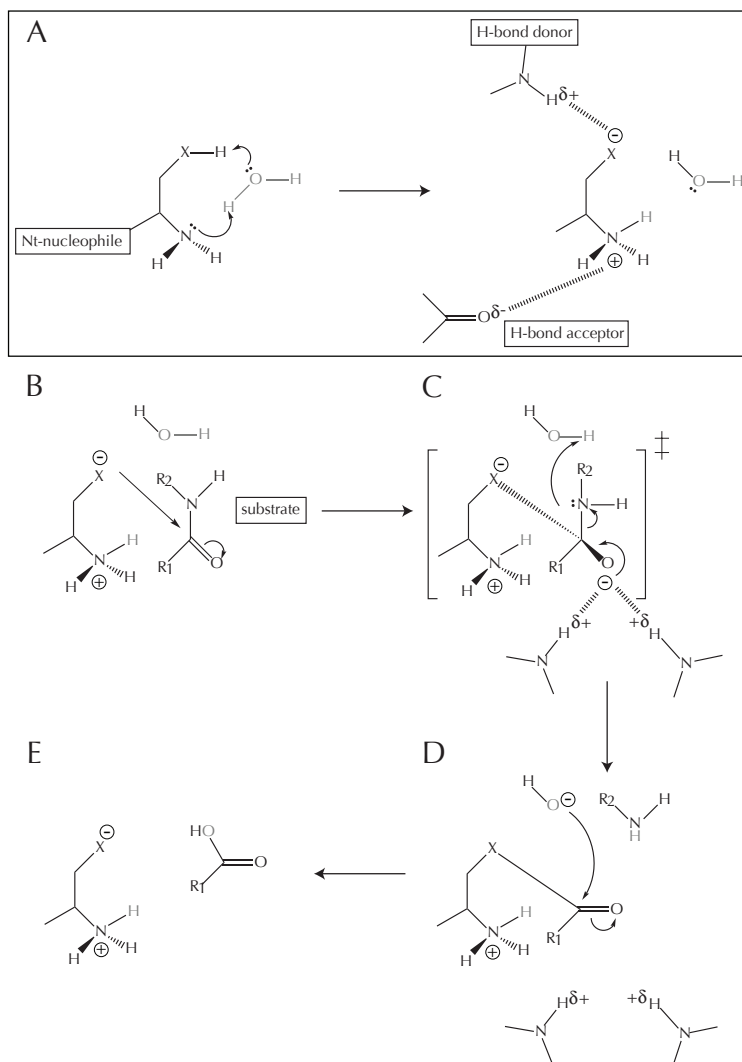
Figure 10: Mechanism of substrate hydrolysis by Ntn-hydrolases. (A) Activation of the N-terminal nucleophile by proton transfer from the hydroxyl to the α-amino group via a water molecule. (B) The hydroxylate or thiolate will attack the scissile bond generating a transition state, stabilized by the oxyanion hole (C). The α-amino leaving group is protonated by the conserved water molecule, causing the collapse of the transition state into a (thio)ester intermediate (D). The OH⁻ attacks the acyl-enzyme intermediate, resulting in the release of the product (E).

In the proposed catalytic mechanism the α-amino group is oriented by hydrogen bond acceptors, such that the lone-pair points towards the Oγ or Sγ proton of the nucleophile, allowing proton abstraction and transfer from the side chain to its own α-amino group. However, Duggleby *et al.* (1995) observed that the angle of approach of the Oγ proton is non-ideal and that, instead, the Oγ proton would be better relayed through a water

molecule that can be considered as a *virtual base* (the water molecule in Figure 10A). Although quantum mechanical computations (Chilov *et al.*, 2007) suggest that a water molecule is not a prerequisite for proton transfer, the water is always observed in all mature Ntn-hydrolase structures, suggesting that this water molecule is indeed involved in catalysis (*e.g.* Isupov *et al.*, 1996; Suresh *et al.*, 1999; Fritz-Wolf *et al.*, 2002; Guo *et al.*, 1998). Instead of a water molecule also a side-chain hydroxyl can act as a virtual base, which has been proposed for *Helicobacter pylori* γ-glutamyltranspeptidase (Boanca *et al.*, 2007) and *E. coli* asparaginase EcAIII (Michalska *et al.*, 2005).

Upon proton transfer the reactive charge-separated state is maintained by hydrogen bonding residues that stabilize the protonated amine, usually by side chain lone pairs, while the deprotonated side-chain nucleophile is stabilized by a backbone amide nitrogen atom (Figure 10A). Once the reactive state of the N-terminal nucleophile has been generated, the hydroxylate or thiolate moiety performs a nucleophilic attack on the carbonyl carbon of the scissile amide or ester bond in the substrate (Figure 10B). The nucleophilic attack results in the formation of a tetrahedral transition state, which is stabilized by the oxyanion hole (Figure 10C). The location and the composition of the oxyanion hole are highly conserved. The oxyanion hole usually consists of a backbone amide and the amide or hydroxyl group of an amino acid side chain. The oxyanion hole is vital for catalysis. For example, mutation of the oxyanion hole residue Asnβ241 to an Ala in penicillin G acylase results in inactive enzyme, which allowed capturing of the enzyme in the substrate-bound state (Alkema *et al.*, 2000; McVey *et al.*, 2001). Upon protonation of the scissile amide by the conserved water, the amine product is the leaving group, resulting in the formation of the acyl-enzyme intermediate (Figure 10D). Subsequently, the hydroxide attacks the carbonyl carbon of the newly formed (thio)ester intermediate, proceeding through a similar tetrahedral transition state stabilized by the oxyanion hole (not shown), resulting in the release of the acid product (Figure 10E).

The list of catalytically important residues of several Ntn-hydrolase families can be found in Table 2, which includes the proteasasome (Groll *et al.*, 1997), γ-glutamyl transpeptidase (Okada *et al.*, 2006), cephalosporin acylase Kim & Hol (2001), glycosyl asparaginase (Oinonen *et al.*, 1995; Tikkanen *et al.*, 1996), glutamine amidotransferase (Kim *et al.*, 1996), penicillin G acylase (Duggleby *et al.*, 1995; Alkema *et al.*, 2000; McVey *et al.*, 2001), acyl coenzyme A:isopenicillin N acyltransferase (Chapter 4) and PvdQ (Chapter 3). The catalytic elements involved in substrate hydrolysis vary among the different Ntn-hydrolase family members. However, the localization of these elements in the structural

core is very much conserved. This is in contrast to the mechanism of autoproteolysis discussed in the previous section, in which the localization of the catalytic elements varies widely. Interestingly, the residues involved in autoproteolysis are also often involved in catalysis, but they perform different roles (*e.g.* Chapter 4).

Table 2: The catalytic elements involved in substrate hydrolysis and their topological localization. The proteasasome (PS), γ-glutamyl transpeptidase (γG), cephalosporin acylase (CA), glycosyl asparaginase (GA), glutamine amidotransferase (GAT), penicillin G acylase (PGA), AT and PvdQ.

enzyme	PDB code	Ntn	Ntn-stabilizing		"virtual base"	oxyanion hole	
			atom	SCSE		atom	SCSE
PS	2f16	Thr1	NζLys33	β12	H$_2$O	NGly47	β11
γG	2dbw	Thr391	OγThr409	β5	H$_2$O	NGly483	
						NGly484	β11
CA	1jvz	Serβ1	N/NδHisβ23	β5	H$_2$O	NValβ70	β11
			OδAsnβ244	β3		NδAsnβ244	β3
human GA	1apz	Thr183	N/OγThr201	β5	H$_2$O	OγThr234	
						NGly235	β11
E. coli GAT	1ecg	Cys1	OArg26	α4/β6	NA	NδAsn101	
			NTyr73	β5		NGly102	β11
PGA	1fxv	Serβ1	N/OεGlnβ23	β5	H$_2$O	NAlaβ68	β11
			OδAsnβ241	β3		NδAsnβ241	β3
AT	-	Cys103	N/OδAsp121	β5	H$_2$O	NAla168	β11
			OδAsn246	β3		NδAsn246	β3
PvdQ	-	Serβ1	N/NδHisβ23	β5	H$_2$O	NAlaβ168	β11
			OδAsnβ269	β3		NδAsnβ269	β3

SCSE secondary consensus structure element that harbors the mentioned residue(s)

Versatility of the Ntn-hydrolase scaffold

The Ntn-hydrolase families are a highly versatile class of enzymes. Not only their properties such as substrate preference and functioning in a variety of biological processes are diverse, but also the connectivity of the structurally conserved core is variable. This section will discuss the versatility of the Ntn-hydrolase fold. Unfortunately, the implications of structural variations on the architecture of the ligand-binding site and substrate specificity have to be left outside of the scope in this chapter. Although the ligand-binding site is always found on top of the β-strands opposite to the N-terminal nucleophile, between β10 and β11, the residues that line the pocket lie outside of the structurally conserved core, making structural comparisons very difficult.

To investigate the relationship between the Ntn-hydrolase families, in principle amino acid sequence comparisons of the different Ntn-hydrolases could be useful. However, while Ntn-hydrolases have maintained their fold, their sequences have evolved beyond any recognizable similarity. Therefore, the 3D-structures themselves are used in the comparisons. Figures 3 and 4 show the variability of the domain organization and the N-terminal nucleophile. Furthermore, the previous sections have already shown the variability of the catalytic mechanisms of autoproteolysis and substrate conversion. These are probably examples of divergent evolution, in which a common ancestor has changed in the course of evolution by acquiring different extensions of the polypeptide chain and developing different functions. This section will discuss the structural variations of the Ntn-hydrolases.

Structural variability of the Ntn-hydrolases

One could envision that an ancestral Ntn-hydrolase would have had a simple fold with an active site that does not need to activate itself by autoproteolysis. The archaeal IMP cyclohydrolase PurO (Saridakis *et al.*, 2002; Kang *et al.*, 2007) could resemble such an ancient Ntn-hydrolase fold. Disregarding its homo-oligomeric state, PurO has the smallest and simplest Ntn-hydrolase fold known so far (Figure 3), consisting of only one single peptide chain. Although PurO has a canonical $\alpha\beta\beta\alpha$ Ntn-hydrolase fold, it lacks the N-terminal nucleophile (Saridakis *et al.*, 2002), and its catalytic residues and catalytic mechanism are also entirely different (Kang *et al.*, 2007). However, the location of its active site is fully conserved, as indicated by the superposition of the N-terminal nucleophile of Ntn-hydrolases with Arg5 of PurO, which is involved substrate recognition.

Figure 11 shows the changes from a hypothetical common ancestor that could have led to the different Ntn-hydrolase families. The first step would be the generation of an N-terminal nucleophile, which can either be liberated by an external enzyme, *e.g.* the removal of the leading formyl-methionine in the GAT family member *E. coli* GLMS (Isupov *et al.*, 1996) or by autoproteolysis, *e.g.* PS (Ditzel *et al.*, 1998). Due to removal of the N-terminal fragment, enzymes such as PS, SPO2555-like and PVA consist of one single chain, and represent the least complex Ntn-hydrolases. Other, more complex Ntn-hydrolases contain additional functional domains, such as the C-terminal phosphoribosyl transferase domain in GAT (Li *et al.*, 1999), or have extensions N-terminal to the nucleophile such that the N-terminal nucleophile becomes embedded in the amino-acid sequence. In the latter case, liberation of the N-terminal nucleophile leads to an α/β heterodimer as observed in PGA, GA and γG, in which the α-chain remains associated with the chain that carries the N-terminal nucleophile. Both chains are tightly interwoven, forming one single Ntn-hydrolase

domain, similar to the monomeric Ntn-hydrolases.

Interestingly, not only the N-terminal nucleophile itself and its localization in the primary structure are variable, but also the arrangement of secondary structure elements may differ. PurO, PS, PVA, PGA, AT and PvdQ share the same topology as displayed in Figure 2A/B, while GAT, GA and γG show a different connectivity of secondary structure elements, but a conserved 3-dimensional arrangement of these elements. In the sequence of GAT α4, β6 and β7 are placed behind the N-terminal β-strand that bears the nucleophile (Figure 11-1), while in GA and γG β3, α3, α4, β6 and β7 are part of the α-chain (Figure 11-2), while the rest of the core is part of the β-chain. These different topologies indicate that the Ntn-hydrolase fold allows for shuffling of secondary structure elements, while maintaining the strictly conserved fold.
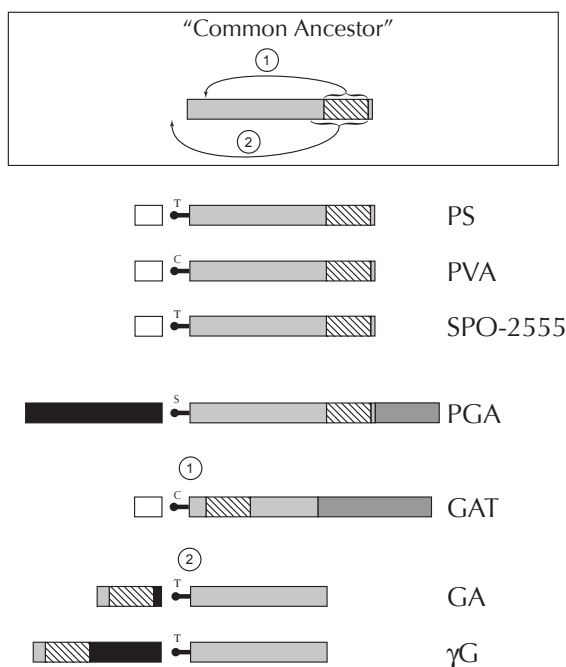

Figure 11: Events that might have caused the diversification of the Ntn-hydrolases throughout evolution. A common ancestor (light grey), *e.g.* PurO, might have developed an N-terminal nucleophile, resulting in an enzyme such as PVA. Structural additions (dark grey) have resulted in *e.g.* GAT. The addition of domains, caused the enzyme to be heterodimeric (black), for instance AT. Other diversifications might have occurred on the primary structure level by shuffling events (1) and (2), *e.g.* γG.

Autoproteolysis in other proteins

The observation that autoproteolysis is a general mechanism by which Ntn-hydrolases activate themselves is not entirely unique. As already mentioned in the beginning of this chapter, many enzyme families are able to cleave one of their own peptide bonds, and thereby alter their structure, which introduces a novel functionality that can be used in various biological processes. Proteins that utilize autoproteolysis for activation

are structurally and functionally unrelated, but for convenience proteins displaying autoproteolytic activity can be divided in three functional classes: I) enzymes, II) structural proteins and III) response proteins.

For example, class I (poly)proteins carry an internal pepsin, chymotrypsin, trypsin or papain protease, which can release itself as well as other proteins in the peptide-chain that are associated with *e.g.* virus build-up, toxin release or cell maintenance. This class includes for example HIV-1 protease (Louis *et al.*, 1994), hepatitis NS3 (Yao *et al.*, 1999), cholera toxin MARTX (Prochazkova *et al.*, 2009) and the cell household enzyme calpain (Moldoveanu *et al.*, 2002). Of the class I enzymes the pyruvoyl proteins will be discussed in a little more detail in the next paragraph as their self-activation is different from the other enzymes and lacks a classic internal protease.

Class II proteins have an autoproteolytic activity that results in a catalytically inactive product that contributes to the build-up of cellular structures. This class includes proteins such as enterogenic *E. coli* EscU that is involved in the formation of the type three secretion apparatus (Zarivach *et al.*, 2008), Nup98, which forms part of the structure of the nuclear pore complex in eukarya (Hodel *et al.*, 2002; Rosenblum & Blobel, 1999) and the SEA-protein, which coats lung and intestine epithelial cells with a mucus layer (Macao *et al.*, 2006). The class II Sindbis virus capsid protein and the inteins will be discussed in the next paragraph.

Class III self-cleaving proteins are involved in cell-signaling, in which an autoproteolytic event acts as a signal. This class includes for example the eukaryotic death domain protein PIDD involved in apoptosis (Tinel *et al.*, 2007), certain G protein-coupled receptors such as EMR2 (Lin *et al.*, 2004) and the E. coli LexA repressor involved in DNA damage response, which upon self-cleavage dissociates from the DNA such that DNA-repair genes can be transcribed (Luo *et al.*, 2001). The class III hedgehog protein will be discussed in more detail in the next section.

Autoproteolysis in these three different classes utilizes various catalytic elements such as the familiar Ser or Cys proteases, a Ser-Lys catalytic diad (LexA), a His-Phe/Leu-Ser motif (Nup98, EMR2 and PIDD), an Asn-Thr-Pro-His motif (EscU) and a Hint-domain (hedgehog- and intein proteins). Except for LexA, EscU and the classical Ser/Cys-containing proteases autoproteolysis proceeds through an N - O(S) acyl-shift backbone rearrangement initiated by attack of the nucleophile on its preceding peptide bond. For some examples of these different classes the result of autoproteolysis is displayed in Figure 12, and their autoproteolytic mechanism and *in vivo* functioning will be discussed in the next section.

More information on the autoproteolysis mechanisms that are not discussed in the next section can be found in the aforementioned references.

Class I. An example of an enzyme that has to cleave itself to become catalytically active, is given by the pyruvoyl enzymes such as aspartate decarboxylase (Albert *et al.*, 1998). In this latter enzyme an N - O acyl shift takes place, induced by a Ser, which results in an ester intermediate, similar to Ntn-hydrolases. However, in pyruvoyl enzymes the formation of the ester intermediate is not followed by hydrolysis by water, but by an elimination reaction in which the carboxylate-group of the N-terminal domain departs, resulting in the formation of a C-terminal dehydroalanine. Subsequently, this electrophilic moiety is attacked by a water molecule, which yields a pyruvate-modified enzyme (Figure 12). The pyruvate group forms the catalytic center, which forms a Schiff-base with a substrate and directly stimulates the departure of carbon dioxide. The pyruvoyl enzymes form an excellent example of self-cleaving proteins that utilize an intramolecular autoproteolytic step in order to become an active, mature enzyme. Comparison of the autoproteolytic- and catalytic mechanism shows that the same residues are involved in both mechanisms (Lee & Suh, 2004; Schmitzberger *et al.*, 2003), similar to various Ntn-hydrolases as discussed in *e.g.* Hewitt *et al.* (2000) and Chapter 4.
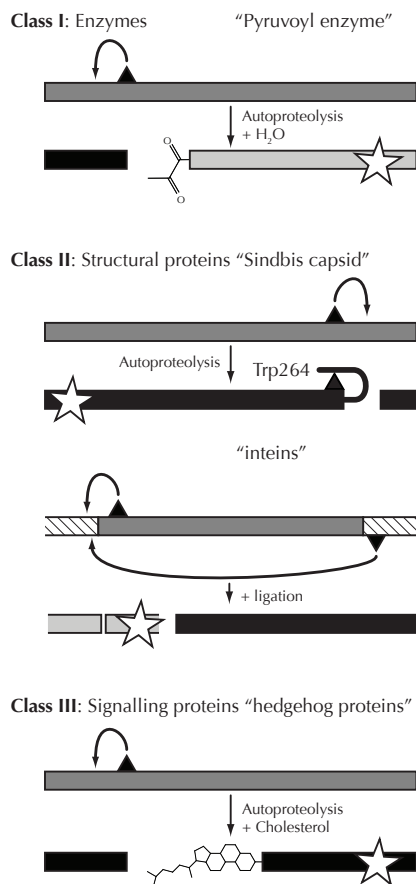

Figure 12: Different protein classes display autoproteolysis. A triangle corresponds to the autoproteolytic center and the arrow indicates the nucleophilic attack. The white star indicates the subunit that has a biological function after autoproteolysis.

Class II. The Sindbis virus nucleocapsid core is formed by a very intriguing self-cleaving protein (Choi *et al.*, 1991). In order to release the Sindbis core protein (SCP) from the viral polyprotein, the SCP has a built-in catalytically active chymotrypsin-like protein, which utilizes a classical Ser-His-Asp catalytic triad that cleaves at its own C-terminus. However,

after autoproteolysis the C-terminal Trp264 remains tightly associated with the active center (Figure 12), such that the enzyme can catalyze only one reaction. Once the enzyme has committed catalytic suicide, it is reused by the virus as a structural coat protein. The SCP protein is an example of a protein family that has an intrinsic enzymatic activity that alters the structure of the protein in some way. However, during autoproteolysis this enzymatic activity is sacrificed in order to participate in the build-up of larger biological structures.

Inteins are typical self-cleaving proteins, which have been intensively studied and have found applications in *e.g.* protein engineering (Noren *et al.*, 2000; Cheriyan & Perler, 2009). Although inteins do not directly contribute to the build-up of larger structures such as the aforementioned SCP, they are structurally very important. Inteins can be found embedded in a host protein, and the intein activity assures the excision of the intein guest protein without any deleterious effects on the structure and function of the host protein. The protein-level self-excising activity of the inteins is reminiscent of mRNA splicing. Therefore, inteins can be regarded as protein-introns, which display protein-splicing activity. During splicing the intein ligates the N- and C-terminal part of the host protein, or exteins, back together (Paulus, 2000). An example of an intein is the yeast PI-SceI homing endonuclease, which displays two activities (Duan *et al.*, 1997). Firstly, the intein escapes the ATPase host protein and ligates the exteins together to assure that the ATPase can function normally. Secondly, the intein carries internal endonuclease activity, which can introduce a double-stranded nick in the DNA of an intein-less site, resulting in self-replication through the host genome by homologous recombination repair activity (Stoddard, 2005). Therefore, the intein-coding DNA has been called mobile selfish DNA (Duan *et al.*, 1997). Splicing activity is the result of two intramolecular catalytic events at both extein-intein interfaces (Figure 12), which are close together in the 3-dimensional structure. First a nucleophilic Cys, which is located at the N-terminus of the intein, performs a nucleophilic attack on its own peptide bond initiating an N - S acyl shift. After that, another Cys, which is located at the N-terminus of the C-terminal extein, attacks the newly formed thioester intermediate, resulting in a branched intermediate, which quickly rearranges into the free intein and the ligated exteins (Poland *et al.*, 2000). Although PI-SceI utilizes two nucleophilic Cys residues, other inteins contain a nucleophilic Ser or Thr instead or combinations (Noren *et al.*, 2000), similar to Ntn-hydrolases. Furthermore, the environments of both nucleophiles are conserved among different inteins. Once the intein has excised itself it does not display any catalytic activity.

Class III Hedgehog proteins are a class of cell-signaling proteins that utilize autoproteolysis in a way similar to pyruvoyl enzymes (Hall *et al.*, 1997). However, the fold of the hedgehog proteins is very different, and is very similar to the inteins. In contrast to the inteins, hedgehog proteins do not display splicing activity, but show only one autoproteolytic event, initiated by a Cys. Similar to the pyruvoyl enzymes, the *Drosophila* hedgehog protein undergoes an N - S acyl shift induced by the Cys located on the N-terminus of the C-terminal domain. However, similar to the pyruvoyl proteins, the thioester intermediate is not hydrolyzed by water, but it is attacked by a cholesterol molecule (Figure 12). The cholesterol-modified N-terminal domain quickly dissociates from the C-terminal domain and associates with the cell membrane acting as a signal involved in tissue differentiation during *Drosophila* development. Once the hedgehog proteins have matured, the C-terminal domain, which harbors an N-terminal nucleophile does no longer fulfill any enzymatic role.

Although many of the aforementioned proteins have a nucleophilic residue (LexA and SEA), or even an N-terminal nucleophile after autoproteolysis (hedgehog proteins, inteins, NPC, EMR2 and PIDD), none of them display any enzymatic activity as mature proteins, except for the class I self-cleaving proteins. This means that in class II and III self-cleaving proteins autoproteolysis immediately results in the loss of catalytic activity. Compared to the class I enzymes only the pyruvoyl enzymes and the Ntn-hydrolases undergo self-cleavage, such that the autoproteolytic site becomes the active site in the mature species. However, the pyruvoyl enzymes have acquired a prosthetic group during autoproteolysis, and as a result a novel catalytic tool. Therefore, the Ntn-hydrolases are the only self-cleaving enzymes that manage to efficiently reuse their entire autoproteolytic center as a mature enzyme.

Variability of the autoproteolysis mechanism

The high variability of the Ntn-hydrolase fold, even in the structurally conserved core, indicates that the Ntn-hydrolase scaffold is evolutionarily adaptable and allows for changes. Nevertheless, the catalytic mechanism of all mature Ntn-hydrolases can be described by one general mechanism utilizing similarly coordinated catalytic elements (Table 2). Furthermore, Ntn-hydrolases manage to fully reuse their autoproteolysis site as catalytic site in the mature enzyme (see *e.g.* Chapter 4), in contrast to the other self-cleaving proteins. These observations make it all the more surprising that no general mechanism for autoproteolysis has been proposed yet for these enzymes (see the section on *Precursor amide hydrolysis: Ntn-hydrolase activation* and Table 1). While Ntn-hydrolases have two

autoproteolytic elements in common, a nucleophile and an oxyanion hole, the proposed mechanisms of autoproteolysis differ in the proposed base. Actually, there is no data available that a common catalytic base even exists in autoproteolysis.

Nevertheless, we cannot exclude that, instead of a base, intrinsic strain in the vicinity of the scissile bond in autoproteolysis is common to all Ntn-hydrolases, in combination with a well-coordinated nucleophile and an oxyanion hole. For example, the self-cleaving SEA domain of the mucosins (Macao *et al.*, 2006) is proposed to undergo spontaneous autoproteolysis due to a strained scissile bond in combination with a nucleophilic attack of the GS peptide bond by the serine side-chain in the GSVVV motif, located at the N-terminus of a β-strand. Mutation of the nucleophilic Ser to an Ala prevents autoproteolysis, similar to what has been observed in the Ntn-hydrolases. Although the SEA protein and its fold bear no resemblance to the Ntn-hydrolases, its proposed maturation manner is similar to the mechanism proposed for GA, in which also a strained conformation drives autoproteolysis. In GA, Asp151 maintains this strained conformation and mutation of Asp151 in GA resulted in enzyme impaired in autoproteolysis due to loss of strain in the vicinity of the scissile bond (Qian *et al.*, 2003). Also the precursor structures of inteins reveal the presence of a strained scissile peptide bond, such as an N-terminal *cis*-peptide (Klabunde *et al.*, 1998) or both N- and C-terminal strained *trans*-peptides (Poland *et al.*, 2000). Furthermore, distorted ω- or φ/ψ-angles have been found in several Ntn-hydrolase precursor structures, such as in the scissile bond of PS, γG and PGA, and in the pro-segment of PGA, CA and PS. Therefore, strain could provide a substantial part of the driving force in autoproteolysis. However, not all precursor structures of Ntn-hydrolases clearly show the presence of a distorted backbone conformation in the vicinity of the scissile bond, *e.g.* AT and EcAIII.

Another important determinant for autoproteolysis is the polarization of the scissile bond by the oxyanion hole. Polarization of the scissile bond can increase the single bond character of the N - C peptide bond, which makes the scissile bond more susceptible to a nucleophilic attack (see the section on *variations in Ntn-hydrolase autoproteolysis*). Regretfully, without atomic resolution data it is not possible to observe scissile bond polarization directly in a precursor crystal structure. However, a strongly polarized amide nitrogen and weakened N - C bond have been observed by NMR in the GyrA intein precursor (Romanelli *et al.*, 2004). Leading those authors to suggest a "ground-state destabilization model", in which polarization of the scissile bond drives autoproteolysis together with pro-segment strain.

From the observations given above, it is clear that pro-segment strain and polarization of the scissile bond do not unambiguously explain autoproteolysis in all Ntn-hydrolases. Even though the crystal structures of quite a few Ntn-hydrolases have been elucidated, they all seem to differ in maturation mechanism, as well as in structural organization and substrate preference. Therefore, the structural investigations of Ntn-hydrolases remain of interest. By now the Ntn-hydrolase class contains 7 families (Andreeva *et al.*, 2004), all representing different enzymes with various functions. Considering their variability, the (structural) studies of these enzymes will add to their general understanding. The enzymes that will be discussed in detail in the following chapters of this thesis are the quorum-quenching *N*-acyl homoserine lactone acylase PvdQ (Chapters 2 and 3) and the acyl coenzyme A:isopenicillin N acyltransferase AT involved in penicillin biosynthesis (Chapter 4). These enzymes are two new Ntn-hydrolase representatives, again different from the known Ntn-hydrolases in maturation manner, structural organization and substrate preference.