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Chapter 79

Aminopeptidase N

Databanks

MEROPS name: aminopeptidase N
MEROPS classification: clan MA, subclan MA(E), family M1, peptidase M01.001
IUBMB: EC 3.4.11.2 (BRENDA)
Species distribution: subkingdom Metazoa
Reference sequence from: *Homo sapiens* (UniProt: P15144)

Name and History

The early history of ***aminopeptidase N*** (***AP-N***) relates to its role as ***Cys-Gly dipeptidase*** or ***cysteinyl-glycinase*** when Binkley and colleagues proposed that this peptidase activity present in apparently purified RNA preparations contributed to polypeptide biosynthesis by acting in reverse in a sequential fashion [1,2]. In 1957, Giorgio Semenza used newly developed chromatographic procedures to purify, and demonstrate unequivocally, that cysteinyl-glycinase was a protein distinct from Binkley's RNA preparation [3] and subsequently developed a convenient assay procedure [4]. The enzyme has also been referred to in its earlier days as ***aminopeptidase M*** (for microsomal or membrane aminopeptidase), reflecting its tight association with a microsomal membrane fraction in pig kidney from which it was first purified by Pfleiderer & Celliers [5]. The use of the name aminopeptidase M is still occasionally seen today in the literature and the enzyme has also been confused with the cytosolic 'leucine aminopeptidase' because of their overlapping substrate specificities and similar tissue distributions. In 1980 it was suggested that the enzyme should be renamed ***aminopeptidase N***, reflecting its preference for action on neutral amino acids [6] and that is the commonly recognized terminology today. The name ***membrane alanyl aminopeptidase*** (***m-AAP***) was introduced at some point to clarify the nature and localization of the enzyme and to distinguish it from its cytosolic counterpart. The enzyme is widespread but is particularly abundant in the brush border membranes of kidney, small intestine and placenta and is also rich in liver. Much of the original characterization of AP-N was performed on the renal or intestinal enzymes. However, the presence of the enzyme in brain has attracted substantial interest since the discovery that it can participate in the hydrolysis and inactivation of the enkephalins by hydrolysis of the Tyr1-Gly2 bond [7,8]. AP-N also turns out to be identical with the human ***cluster differentiation antigen CD13*** expressed on the surface of myeloid progenitors, monocytes, granulocytes and myeloid leukemia cells [9]. AP-N is also expressed on stem cells [10]. Soluble AP-N is found in plasma and urine [11,12], presumably derived by shedding of the membrane-bound enzyme but the mechanism of release has not been characterized.

Activity and Specificity

AP-N has a broad substrate specificity removing N-terminal amino acids (Xaa↓Xbb-) from almost all unsubstituted oligopeptides and from an amide or arylamide. It has usually been assayed with derivatives of alanine, *e.g.* Ala-NHMec or the $NHPhNO_2$ or NNap derivatives, because Ala is the most favored residue. Leu-NHMec and other bulky hydrophobic amino acid derivatives are also good substrates but leucinamide is poorly hydrolyzed. For aminoacyl derivatives, the favored order is reported to be Ala, Phe, Tyr, Leu, Arg, Thr, Trp, Lys, Ser, Asp, His and Val. Pro- and α- or γ-Glu-derivatives are very slowly attacked. When a prolyl residue is preceded by a bulky hydrophobic residue, *e.g.* Leu, Tyr or Trp, unusual secondary reactions can occasionally arise such that the Xaa-Pro combination is released as an intact dipeptide (see, for example, McDonald & Barrett [13]). Dipeptides are readily hydrolyzed, *e.g.* Cys-Gly, as in the original studies on this activity [3,4]. Subsite interactions are important and hence chain length greatly affects the rates, although precise rules governing specificity have not been defined. The specific recognition by AP-N of the N-terminal amino acid of its substrate appears to involve a critical glutamate residue in the active site (Glu350) [14].

The pH optimum is around 7.0 although the optimum can rise to 9.0 as the substrate concentration is increased. However, the K_m is lowest in the pH range 7.0–7.5. Metal chelating agents are effective inhibitors, consistent with the metallopeptidase nature of the enzyme, and

sulfhydryl reagents are without effect. A comparison of the effects of a range of metallopeptidase inhibitors on membrane aminopeptidases has been carried out by Tieku & Hooper [15]. Amastatin (originally described as an inhibitor of glutamyl aminopeptidase (aminopeptidase A) (Chapter 82) is also a very effective inhibitor of AP-N, with an increase in potency when preincubated with the enzyme, the K_i value decreasing from 20 μM to 20 nM, *i.e.* it is a slow, tight-binding inhibitor which involves a conformational change in the enzyme–inhibitor complex and the kinetics of this reaction have been examined in detail in [16]. Probestin is also a potent inhibitor with a reported I_{50} of 50 nM [15]. Bestatin is also a well-recognized inhibitor of AP-N although considerably less potent than amastatin or probestin [15,17]. Actinonin ($I_{50} = 2$ μM) can be considered a relatively specific inhibitor of AP-N compared with other membrane aminopeptidases [15]. The enzyme is only very weakly inhibited by puromycin (see *Distinguishing Features*, below). Based on such inhibitory data, a selective enzyme assay for AP-N has been devised [18]. A range of potent and selective inhibitors of AP-N have been described based on derivatives of 3-amino-2-tetralone [19], some of which exhibit K_i values in the nanomolar range. The proposed mode of binding of these compounds is as bidentate ligands with the amino and carbonyl functions coordinating to the active-site zinc. Highly potent and selective AP-N inhibitors have been designed which utilize phosphinic compounds that mimic the transition state of substrates of the enzyme [20]. Prodrugs of dual inhibitors of the two enkephalin-degrading enzymes, AP-N and neprilysin (Chapter 127), also based on a phosphinic acid design, are highly effective as anti-nociceptive compounds [21]. Numerous other warheads for selective inhibition of AP-N have been developed, for example, lysine-ureido derivatives [22], or amino-benzosuberone [23]. A dual inhibitor of AP-N and dipeptidyl peptidase IV (Chapter 745) has potential as a therapeutic in central nervous system inflammation being effective in animal models of autoimmune encephalomyelitis [24]. Other families of AP-N inhibitors have been thoroughly reviewed in [25,26].

Structural Chemistry

AP-N is a type II integral membrane protein located on the plasma membrane as an ectoenzyme. The pI is approximately 5. The native enzyme exists as a homodimer of subunit M_r 140 000–150 000 in most species [27], although it is reported to be monomeric in the rabbit [6]. It is heavily glycosylated with carbohydrate accounting for at least 20% of the mass of the protein. The polypeptide chain is susceptible to proteolysis, generating two fragments of M_r approximately 90 000 and 45 000 that have been referred to in the earlier literature as β- and γ-subunits respectively (the intact chain being the α-subunit). This artifact of preparation led to the suggestion that the native enzyme may be a trimer [28].

The enzyme was originally cloned from a human intestinal cDNA library [29] and subsequently from rat [30,31] and rabbit kidney [32]. The rat enzyme comprises a 966 amino acid polypeptide with a small cytoplasmic domain, a 24 amino acid hydrophobic segment close to the N-terminus which serves as the membrane anchor region and the bulk of the polypeptide chain including the active site present as an ectodomain. The sequence includes nine potential N-linked glycosylation sites and a typical zinc-binding motif (His-Glu-Xaa-Xaa-His) in which the two closely spaced histidines represent two of the zinc ligands. The third zinc ligand is a glutamate and the protein contains one Zn^{2+} per subunit. Chemical modification experiments have been used to identify arginyl, histidyl, tyrosyl and aspartyl/glutamyl residues at the active site [33]. The structure of mammalian AP-N has not yet been solved although it is known for the *Escherichia coli* enzyme [34,35], which shows 44% similarity with the human enzyme and this structure has been used for the rational design of human AP-N inhibitors. The *Lactococcus lactis pepN* gene encodes an aminopeptidase homologous to AP-N with almost 30% identity between the bacterial and mammalian proteins and with particularly high conservation around the active-site region [36].

Preparation

In the kidney, AP-N represents as much as 8% of the brush border membrane protein, thereby providing a convenient and abundant source to initiate purification. It was first isolated from pig kidney as 'cysteinyl-glycinase' [3] and subsequently as an aminopeptidase [5,37]. The protein can be purified in either hydrophilic or amphipathic form by proteinase (trypsin, papain) treatment or detergent solubilization respectively. Conventional chromatographic procedures can then be used to isolate the enzyme (*e.g.* Feracci & Maroux [6]). The pig small intestinal AP-N has also been purified by immunoadsorbent chromatography [38]. A 130 kDa glycoprotein purified from pig kidney brush border membranes by affinity chromatography on immobilized 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate (SITS) followed by concanavalin A-Sepharose, turned out serendipitously to be AP-N [39], suggesting that the protein possesses an anion-binding site. This procedure provides a convenient purification method for the enzyme, which represents the major concanavalin A-binding protein in brush border membranes. AP-N in the larval midgut cell membranes of the silkworm, *Bombyx mori*, is partially sensitive to release by phosphatidylinositol-specific phospholipase C,

suggesting that in this species the enzyme may be anchored through a glycolipid anchor rather than a transmembrane domain [40].

Biological Aspects

AP-N is widely distributed among species and tissues although it is of greatest abundance in brush border membranes of the kidney, mucosal cells of the small intestine and in the liver. It is also present in the lung where it is identical to the p146 type II alveolar epithelial cell antigen [41] and is located on endothelial cells in blood vessels. On polarized epithelial cells, AP-N is localized to the apical domain and is targeted there through an apical sorting signal thought to be located in the catalytic head group region of the protein [42]. In the kidney, AP-N contributes to the extracellular catabolism of glutathione (43). The cysteinyl-glycine generated during the catabolism of glutathione by γ-glutamyltranspeptidase is hydrolyzed by the two ectoenzymes AP-N and membrane dipeptidase (Chapter 377) contributing approximately equally [44]. In the intestine, the enzyme functions in the final stages of protein and peptide digestion.

A detailed localization of the enzyme has been carried out in the brain because of its potential involvement in terminating the actions of certain neuropeptides, especially the enkephalins [45–47] and angiotensin III, which is a key brain regulator of vasopressin release [48]. In addition to being present on endothelial cells and synaptic membranes, AP-N is found on astrocytes and pericytes [47,49]. It is abundant in the choroid plexus and can therefore also serve to prevent access to the brain of potentially damaging circulating peptides. On vascular cells, AP-N may serve to metabolize certain vasoactive peptides [50]. An important location of AP-N is in hematopoietic cells, where it is referred to as CD13 [9]. Here, its expression is restricted primarily to myeloid cells, but it is also found on antigen-presenting cells, melanoma cells and lymphocytes. On granulocytes it may cooperate with neprilysin (Chapter 127) to downregulate responses to chemotactic factors such as formyl-Met-Leu-Phe [51]. More generally in the immune system it may serve to inactivate certain cytokines [27,52,53]. The immunopotentiating and reported antitumor activities of bestatin may relate to inhibition of AP-N [54]. Reduction in expression or activity of AP-N results in inhibition of growth of T cells, probably through a mechanism involving glycogen synthase kinase-3 [55]. AP-N appears to regulate the cycle-dependent bioavailability of interleukin-8 in the endometrium and its activity is, in turn, regulated by estrogen [56]. In general, AP-N expression is dysregulated in various cancers, mainly solid tumors, and in inflammatory diseases. Its roles in the immune system and correlation with neoplastic transformation are summarized in Riemann *et al.* [27]. Consistent with its role in cancers, AP-N itself has been implicated in angiogenesis [57] and cell motility, and is upregulated in gastric cancer [58] and is a poor prognostic factor in colon cancer [59,60]. The expression of AP-N has also been explored in human prostate and hepatic cancer for which the enzyme may be a valuable histological marker [61–63]. AP-N has also been proposed as a therapeutic target in human cancer stem cells [64]. The potential of AP-N as a target in cancer therapy has been thoroughly reviewed in Wickström *et al.* [65].

The human AP-N gene, which occupies 35 kb, is localized on chromosome 15 [66,67]. Separate promoters control transcription of the human gene in myeloid and intestinal epithelial cells [68]. The pig AP-N gene has been cloned and localized to porcine chromosome 7 [69,70].

A novel feature of AP-N is its ability to serve as a receptor for certain viruses, especially coronavirus 229E, an RNA virus that causes upper respiratory tract infections in humans [71]. Mutagenesis studies suggest that the virus-binding site lies close to the active-site region, although enzyme activity is not essential for virus binding. Human AP-N also appears to mediate human cytomegalovirus infection although, again, enzyme activity is not essential for infection [72]. Another coronavirus, transmissible gastroenteritis virus, which causes a fatal diarrhea in newborn pigs, uses intestinal AP-N as its receptor [73] and peptides identified by phage display which interact with porcine AP-N can inhibit the viral infection [74]. AP-N appears to be the major receptor for the CryIAc toxin of *Bacillus thuringiensis* in *Lymantria dispar* (gypsy moth) [75,76]. At the cell surface AP-N also associates with the 14-3-3σ protein (stratifin) hence regulating matrix metalloproteinase-1 expression in epithelial-stromal cellular communication [77].

AP-N is synthesized in a fully active form. Substance P and bradykinin, which are not substrates for AP-N, have been reported as natural inhibitors of the enzyme with K_i values in the low micromolar range [78]. However, it is unlikely that they play any physiological role in regulating enzyme activity and the enzyme is therefore probably essentially unregulated at the surface of cells. One study has, however, shown that oxidoreductase-mediated modulation of the thiol status of the cell-surface markedly affects the activity of AP-N [79] and the enzyme is upregulated in response to hypoxia [57]. Expression of AP-N may vary during cell growth and differentiation and certain cytokines, *e.g.* interleukin-4 and interferon γ, can upregulate levels of AP-N mRNA and protein [27]. In endothelial morphogenesis AP-N is a transcriptional target of Ras signaling [80].

The development of an AP-N knockout mouse has allowed study of the physiological properties of the

enzyme. The AP-N null mice develop normally with no apparent physiological or histological abnormalities but they demonstrated an impaired angiogenic response under pathological conditions which is consistent with an angiogenic-promoting role of AP-N in human cancers [81]. Mutations, polymorphisms and alternative splicing of the *AP-N* gene seen in leukaemias can cause marked changes in the trafficking and location of the AP-N protein affecting its function [82,83].

Distinguishing Features

AP-N can be distinguished from the cytosolic leucine aminopeptidase by its membrane association and its poor hydrolysis of leucinamide (see above). It can be distinguished from another aminopeptidase in brain (cytosol alanyl aminopeptidase; aminopeptidase PS; Chapter 86) capable of hydrolyzing the enkephalins by its relative insensitivity to puromycin ($K_i = 78$ mM compared with 1 mM for the puromycin-sensitive activity). Actinonin is a relatively selective inhibitor. The dipeptidase activity of AP-N can be distinguished from that of the mammalian membrane dipeptidase (Chapter 377) by the sensitivity of the latter to cilastatin [84].

Related Peptidases

Several mammalian aminopeptidases with homology to AP-N have been cloned, including the cytosol alanyl aminopeptidase (Chapter 86), which has been implicated in cell growth and viability [85], and human placental leucine aminopeptidase/oxytocinase (Chapter 84), which is also a type II integral membrane protein and may play a role in the degradation of oxytocin and vasopressin [86]. The major protein present in GLUT4 vesicles in fat and muscle tissues is a glycoprotein of M_r 160 000 that has structural homology to AP-N and exhibits aminopeptidase activity *in vitro* [87]. The cytosolic leukotriene A_4 hydrolase (Chapter 96) also has aminopeptidase activity and belongs to the AP-N family [88].

Further Reading

For reviews, see Bauvois & Dauzonne [25], Sjöström *et al.* [89] and Mina-Osorio [90].

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