

Chapter 2

Alpha-1 Antitrypsin: The Protein

Bethany Lussier and Andrew A. Wilson

Introduction

Alpha-1 antitrypsin (AAT), alternately referred to as alpha-1 proteinase inhibitor or alpha-1 protease inhibitor, is a member of the serine protease inhibitor (serpin) superfamily comprised of alpha-1 antichymotrypsin, C1 inhibitor, antithrombin, neuroserpin, and others [1, 2]. AAT is considered the major anti-elastase of the lower respiratory tract based on its unsurpassed ability to inhibit the serine protease, neutrophil elastase [3, 4]. In addition to its antiprotease activity, AAT has likewise been shown to have other biological effects, including the ability to modulate both inflammation and apoptosis [5, 6]. Mutant forms of AAT play a well-documented role in disease pathogenesis: misfolding of AAT protein, accumulation of misfolded protein polymers, and an associated decrease in secreted, functional monomers are known to cause clinical dysfunction and disease through both gain-of-function and loss-of-function mechanisms and are collectively known as “AAT deficiency.” In this section, we review the protein’s history, structural composition, regulation, and functional characteristics.

History and Classification

In 1963, the remarkable discovery was made by Laurell and Eriksson that serum protein electrophoreses of several individuals with severe obstructive lung disease of early onset lacked a band for alpha-1 globulin [1, 7, 8]. This missing electrophoretic band was later found to represent an inherited deficiency of a single protein with the

B. Lussier, M.D. (✉) • A.A. Wilson, M.D.
Boston University School of Medicine, 670 Albany St, 2nd Floor CReM, Boston,
MA 02118, USA
e-mail: Bethany.Lussier@bmc.org; awilson@bu.edu

capacity to inactivate serine proteases including trypsin, resulting in the designation “AAT” [8, 9]. Six years after its linkage to lung disease, Sharp and colleagues described an association between AAT deficiency and cirrhotic liver disease [10]. Since the mid-1970s, numerous AAT glycoforms have been documented by isoelectric focusing and used to identify deficiency states and phenotypes [11]. AAT glycoforms are classified based on differences in electrophoretic migration at acidic pH, with letters assigned to each variant in alphabetical order. Differences in this migration pattern depend primarily on the amino acid sequence, with heterogeneity of carbohydrate side chain modifications contributing to a lesser degree [9]. The most common normal AAT variant migrates a moderate distance and is designated “M,” whereas the most common deficiency allele migrates further under the same conditions and is designated “Z” [9]. An individual inheriting one allele encoding each of these two variants would be referred to as “PiMZ” under the protease inhibitor (“PI”) classification system. AAT is a pleiomorphic protein, with a large number of variants identified, including nine different glycoforms of the M-AAT protein (subtypes M0–M8) and at least six glycoforms of the Z-AAT mutant protein [3, 11–14].

Expression Pattern

AAT is a 52-kDa molecule produced primarily in liver hepatocytes and released directly into the bloodstream. The normal rate of synthesis is approximately 34 mg/kg/day leading to serum concentrations ranging from 150 to 350 mg/dL [3, 15]. While hepatocytes are the main source of circulating AAT, approximately 20 % of the total is produced by other AAT-producing cell types, a list that includes monocytes and macrophages, in addition to epithelial cells of the lung, kidney, intestine, pancreas, thymus, adrenal gland, ovary, testis, and corneum [16–24]. De novo synthesis has also been documented in some human cancers [25–28].

One-third of circulating AAT is degraded daily, and only a fraction of circulating AAT is transported into any given body compartment [3]. AAT is found in nearly all body fluids, including saliva, tears, breast milk, urine, and semen, with concentrations in bronchoalveolar lavage fluid reaching approximately 10 % of those in the circulation [15, 16, 23, 29–32]. AAT can likewise be found in feces with increased concentrations in the setting of inflammatory bowel disease [33, 34]. While local synthesis may contribute to body fluid or tissue AAT concentrations, it is generally believed that they are primarily determined by serum concentrations [15].

Transcription

The SERPINA1 gene encoding AAT is 12.2 kb in length and located on the long arm of human chromosome 14q31–32.3 [3, 35, 36]. The gene contains seven exons (designated Ia, Ib, Ic, II, III, IV, and V) and six introns [3, 37]. The 5′ untranslated

regions of AAT arise from the first three exons and a short 5' segment of the fourth exon. The AAT protein sequence is encoded by 1434 base pairs from the fourth exon (exon II) and together with exons III, IV, and V [3, 9, 37]. The active site of the mature protein, encoded by the seventh exon (exon V), contributes specificity to the functional domain of the inhibitor [3, 9]. The transcriptional start site, found in exon Ic in hepatocytes, varies with cell type, yielding a longer mRNA transcript in monocytes where it is encoded by exon Ia [3, 9, 18].

Regulation of the AAT gene is thought to be predominantly at the transcriptional level in association with a hepatocyte-specific promoter, with posttranslational regulation playing a lesser role [21]. Expression is tissue specific and directed by structural elements within a 750-nucleotide region upstream of the hepatocyte transcriptional start site in exon Ic that contains hepatocyte-specific and nonspecific enhancer elements [3, 9, 38, 39]. Trans-acting factors, including HNF-1, HNF-3, and others, are able to bind these regions and thereby contribute to AAT expression [3, 9, 24, 38, 40].

Intracellular Trafficking and Posttranslational Modification

The processing involved in normal biosynthesis of AAT is classical, with transcription of the mRNA precursor from the AAT gene followed by splicing and translocation to the rough endoplasmic reticulum where translation occurs. The newly synthesized polypeptide chain is secreted into the cisternae of the rough ER and undergoes cleavage of its signal peptide. There, the AAT precursor undergoes dolichol phosphate-linked glycosylation at three distinct asparagine residues (numbers 46, 83, and 247) as the protein takes on a three-dimensional conformation [9]. These glycosylations have functional significance, as they help maintain solubility and allow attachment of protein-processing enzymes [41]. Glucosidase-based trimming of two glucose units produces a monoglycosylated oligosaccharide that is recognized by molecular chaperones [41–44]. Conformationally mature glycoproteins are then released from the protein-folding machinery while incorrectly folded forms undergo additional processing. Released glycoproteins are transported to the Golgi in coatamer protein complex II vesicles en route to the Golgi complex [41]. Terminal modification of the carbohydrate side chains within the Golgi complex produces a mature 52-kDa AAT protein, resistant to enzymatic cleavage by endoglycosidase H, which is then secreted [9].

Protein Structure

AAT shares both structural and functional characteristics with other members of the serpin family including alpha-1-antichymotrypsin, C1 esterase inhibitor, alpha-2 antiplasmin, protein C inhibitor, and antithrombin III [3, 9]. The fully processed,

mature AAT protein is a 394-amino acid peptide, 52 kDa in size, that includes three carbohydrate side chains [9]. These asparagine-linked side chains are composed of N-acetylglucosamine, mannose, galactose, and sialic acid arranged as a core with two to three branching “antennae” [9]. In addition to their contribution to differences in electrophoretic migration of AAT protein used in protein phenotyping, AAT glycosylations contribute to protein stability and half-life in the circulation [11, 13]. They have likewise recently been demonstrated to contribute to the anti-inflammatory capacity of AAT protein through negative regulation of IL-8-induced neutrophil chemotaxis [45].

The crystal structure analysis of AAT’s three-dimensional conformation was described in 1984 by diffraction data and electron density mapping by Loebermann and colleagues [46]. Crystallographic analysis revealed a 6.7 nm × 3.2 nm globular protein with the three carbohydrate side chains on the outer surface, localized to one end. The AAT polypeptide chain is arranged into well-defined structural elements consisting of three beta-sheets (A–C) and nine alpha-helices (A–I), each formed by the first 150 residues. There are three internal salt bridges occurring within the molecule that have been implicated in folding and polymerization and subsequent deficiency states [9, 47, 48].

At the active site, a Met358–Ser359 bond is part of a highly stressed reactive center loop that if cleaved separates the two residues widely [9, 46] exposing the N-terminal region of the active site loop on two strands of beta-sheet A. It is this active site, designated “P1 residue”, which is responsible for the functional capacity of the inhibitor as well as its specificity [49]. This contribution is dramatically illustrated by the so-called “Pittsburgh” AAT variant, in which a single amino acid substitution at this active site (358 Met → Arg) results in synthesis of a protein that is structurally similar to antithrombin III, another serpin family member, with significant thrombin inhibitory capacity manifested as a clinically significant bleeding disorder [50].

Circulating AAT

AAT is ultimately secreted as a 52-kDa single-chain glycoprotein; an average concentration of AAT is 150–300 mg/dL in circulating plasma [51, 52] and has a half-life of approximately 5 days [3, 51, 52]. About one-third of the intravascular AAT pool is degraded daily with some potential increase resulting in the setting of inflammation [3, 53]. Based on its tertiary structure, it was previously believed that AAT primarily trafficked across cell membranes and into most tissues via passive diffusion [4]. However, recent studies have demonstrated that AAT uptake occurs primarily via clathrin-mediated uptake resulting in transcytosis across the endothelial cell layer [54–56]. Protein trafficking has potential significance in disease pathogenesis as a mechanism by which cigarette smoking might alter local tissue AAT concentrations [55].

In the most common deficiency state, circulating AAT levels are reduced to 10–15 % of normal [52]. The strong correlation of bodily fluid AAT levels with circulating levels means that lung AAT concentrations decrease in parallel with

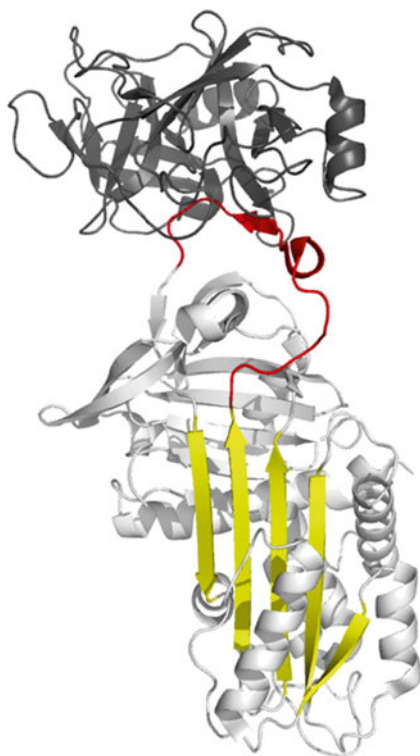
circulating levels in hereditary deficiency. While there is speculation that a gain-of-function mechanism, exerted either via extracellular, polymer-driven inflammation or direct cellular toxicity, could contribute to lung disease in AATD, alveolar destruction associated with disease has generally been considered to occur predominantly on a loss-of-function basis resulting from these low levels and their associated decrease in anti-elastase activity.

Protease Inactivation

AAT's most significant biological function is believed to be the inactivation of proteases, particularly in the lung. Although primarily recognized for its ability to neutralize neutrophil elastase (NE), AAT is also capable of inactivating other neutrophil proteases, including cathepsin G and proteinase 3 [57], as well as mast cell proteinase II [58]. It may also inhibit T-lymphocyte-derived serine esterases that are structurally similar to cathepsin G [59], though with lower binding efficacy [60].

NE is a highly active proteolytic enzyme formed in the promyelocytic phase of neutrophil maturation and stored in azurophilic granules for release along with other lysosomal oxidative enzymes upon neutrophil activation or death [61]. As depicted in Fig. 3.1, NE and other serine proteases recognize a 20-amino acid

Fig. 3.1 Structure of the alpha-1 antitrypsin–trypsin complex. Ribbon diagram of alpha-1 antitrypsin (in white, yellow, and red) in non-covalent complex with trypsin (in gray). The active site of trypsin fits the reactive center loop of alpha-1 antitrypsin (red). Alpha-1 antitrypsin's beta-sheet A is designated in yellow. Figure courtesy of Bibek Gooptu, based on publications by Elliott PR et al. (*Nat Struct Biol* 1996;3:676–681) and Dementiev A et al. (*Journal of Biological Chemistry* 2003;278:37881–37887), as well as PDB ID 1OPH



“pseudo-substrate” within the AAT molecule that includes the reactive center loop [62]. Upon binding, AAT forms a rapid, strong association with NE with binding strength several orders of magnitude greater than other serine proteases and an association constant of $9.7 \pm 0.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ [63]. The binding of NE to AAT and its subsequent inactivation shares characteristic features with other serpin–enzyme pairs. Upon binding, NE cleaves AAT’s reactive center loop, releasing stored potential energy and resulting in a conformational change in which NE is flipped to the opposite end of the AAT molecule. In the process, NE is distorted and catalytically inactivated [3, 48, 62, 64–66]. The resulting AAT–NE complex is then recognized by hepatic receptors and cleared from circulation [67].

The proteolytic capacity of normal AAT can be diminished in several circumstances. First, when exposed methionine residues are oxidized, the binding constant for serine proteases drops precipitously, significantly reducing functional protease activity [68–70]. Second, AAT can be cleaved and inactivated by the proteolytic activity of enzymes other than NE, including macrophage-derived metalloproteinase, neutrophil collagenases and gelatinases, and *Pseudomonas* elastase among others [60, 71].

Local and Systemic Fluctuations in AAT Secretion

Homeostatic mechanisms in response to tissue damage from infection or injury lead to characteristic changes in liver protein synthesis, known collectively as the acute phase response [72]. In this setting, IL-6-, TNF α -, and IL-1-driven STAT3 and NF- κ B p65 (RelA) signaling result in increased hepatic production of a group of so-called “acute phase reactant” proteins. AAT, together with c-reactive protein, ferritin, complement factors, and others, is significantly upregulated, while that of negative acute phase proteins including cortisol-binding globulin, transferrin, and albumin are decreased [73, 74].

Circulating blood AAT levels in the acute phase have been found to increase three- to fourfold [75]. In addition to the acute phase response, other inflammatory settings including pregnancy, trauma, surgery, malignancy, and treatment with oral contraceptives can also increase tissue AAT levels [76–80]. Finally, there is some evidence that circulating granulocytes could contribute to local AAT levels through the transcription, storage, and release of AAT following migration into tissues [45, 81, 82]. While some studies have demonstrated that AAT is stored in secretory vesicles within neutrophils [81, 82], more recent data suggests that approximately 80 % of neutrophil-associated AAT is localized to the cell membrane within lipid rafts, where it colocalizes with Fc γ RIIIb [45].

Anti-inflammatory and Anti-apoptotic Effects of AAT

In addition to its primary role in protease inactivation, AAT has been demonstrated to exert both anti-inflammatory and immunomodulatory effects in a variety of conditions and cell types, in some cases independent of its antiprotease activity. Effects

on activation and trafficking of multiple immune cell types have been documented, including neutrophils [45], mast cells [83], macrophages [84], and T cells [85]. Of these, the interactions between AAT and neutrophils have been most thoroughly evaluated. AAT inhibits neutrophil chemotaxis through two independent mechanisms: first, it forms a complex with IL-8, blocking its ability to bind CXCR1 and induce downstream AKT phosphorylation and subsequent cytoskeletal rearrangement that is necessary for neutrophil chemotaxis [45]. In a second mechanism, AAT blocks the ADAM-17/TACE-mediated shedding of Fc γ RIIIb in response to stimulation by soluble immune complexes [45], a process integral to neutrophil chemotaxis.

Studies of organ ischemia or cigarette smoke exposure have demonstrated attenuation of neutrophilic infiltration of the kidney, liver, and lung [86–89] as well as a reduction in cell death due to simulated myocardial infarction [90] in the setting of supplementation with exogenous AAT. Collectively, these studies have likewise demonstrated direct anti-apoptotic effects of AAT, as well as inhibition of caspases and anti-inflammatory effects characterized by attenuation of increases in local or systemic TNF α . Similarly, in animal lung transplant models, treatment with AAT reduced ischemic-reperfusion injury by inhibiting neutrophilic infiltration of the lung together with reductions in cytokines including IL-1 α , IL-4, IL-12p70, MCP-1, and TNF- α [91, 92].

Intriguingly, many of the anti-inflammatory properties of AAT appear to be independent of its antiprotease activity. Churg and colleagues demonstrated that oxidized AAT, lacking in antiproteolytic capacity, attenuated silica-induced increases in lung MCP-1 expression, MIP-2 α , activation of NF- κ B, and associated neutrophilic migration into the lung [93, 94]. More recently, Jonigk and colleagues demonstrated that a recombinant form of AAT lacking elastase inhibitory function exhibited anti-inflammatory properties in LPS-challenged mice (decreased infiltration of neutrophils, decreased BAL TNF- α and KC, decreased lung tissue expression of DDIT3 and XBP-1) as well as in freshly isolated human blood neutrophils (decreased TNF- α and IL-8 secretion *ex vivo*) [93, 94]. While antiprotease activity may not be necessary for AAT-mediated effects on inflammation, proper protein glycosylation does seem to be significant: recombinant AAT lacking glycosylations is unable to bind and modulate IL-8 and may lack other associated anti-inflammatory capacity [11, 45, 95]. This finding has significance since recombinant AAT protein, currently under consideration for clinical use as an inhaled therapy, lacks these glycosylations and therefore may not fully recapitulate the functional capabilities of the native human protein [45].

In addition to its effects on inflammation, AAT has been demonstrated to inhibit apoptosis through blockade of caspase activation in a variety of settings. Exogenously administered AAT has been found to prolong pancreatic islet allograft survival in mice, while related *in vitro* studies demonstrated its inhibition of TNF-induced apoptosis in murine insulinoma cells and associated abrogation of caspase-3 activation [96, 97]. Petrache and colleagues demonstrated that human AAT transgene expression effectively protects against apoptosis-induced emphysema caused by VEGF receptor blockade in mice [98]. This effect was associated with a decrease in

caspase-3 activity, a decrease in apoptosis, and a decrease in markers of oxidative stress. Additional work by the same group demonstrated that AAT and caspase-3 colocalize intracellularly and that AAT-mediated abrogation of caspase-3 activity requires an intact reactive center loop [5], in contrast to its anti-inflammatory effects as outlined above.

Finally, AAT may have additional properties as an inhibitor of HIV-1 infection. In vitro studies have demonstrated that AAT reduces HIV-1 infectivity and blocks HIV-1 production, an effect associated with suppression of the transcription factor NF- κ B. Additional work has subsequently demonstrated that this protective effect is exerted by the C-terminal 26-residue peptide fragment of AAT, also referred to as “VIRIP” or virus-inhibiting peptide, which blocks HIV-1 cell entry through binding and inhibition of the HIV gp41 fusion peptide [99]. Chemically synthesized variants of VIRIP have now been studied in patients and found to inhibit viral replication in HIV-infected individuals [100].

Together, these putative immunomodulatory and antimicrobial properties have driven research exploring potential novel therapeutic applications. Some examples include the use of AAT in the setting of inflammation and injury associated with diabetes mellitus, organ transplant rejection, reperfusion injury, asthma, and infectious diseases [77, 86, 91, 96, 100–106].

Summary

In summary, a great deal has been learned about this protein since its discovery 50 years ago, but many important questions remain. There is increasing awareness of a functional role for AAT that extends beyond its capacity to inactivate serine proteases, but the import of this role in persons with either normal or abnormal AAT genotypes/phenotypes is not yet well understood. Active research in this area, together with research examining protein trafficking within cells and across cell membranes, is likely to yield insights in the immediate future that will further our understanding of the role that this multifunctional protein plays in health and disease. Finally, it is anticipated that development of new, human cell-based models for studying AAT biology is likely to enhance the progress of research programs such as these in the years to come.

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