Serpin-like properties of α1-antitrypsin Portland towards furin convertase

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Abstract Recent studies have demonstrated that a serpin variant, α_1-antitrypsin Portland (AT-PDX), can inhibit the mammalian convertase furin. Here, we examine the mechanism by which this inhibition takes place. We find that furin, which does not belong to the trypsin-like serine protease family, the usual targets of serpins, forms an SDS-heat denaturation-resistant complex with AT-PDX both in vitro and in vivo. AT-PDX inhibited furin with an association rate constant (k_{ass}) of 1.5×10^6 M^{-1} s^{-1} which is similar to k_{ass} values reported for serpins with trypsin-like enzymes. These results illustrate that AT can be modified to act essentially as a suicide inhibitor of furin, an enzyme of the subtilase superfamily of serine proteases.

Key words: α_1-Antitrypsin; Serpin; Protease inhibitor; Furin; Subtilase; Mechanism-based inhibition

1. Introduction

The serpin (serine protease inhibitor) superfamily includes more than 60 proteins that can be subdivided into two major groups, one being inhibitory, the other non-inhibitory. The inhibitory serpins are single chain polypeptides of around 400 amino acids involved in modulating proteolytic activity in processes such as inflammation, coagulation, fibrinolysis, cell differentiation, complement activation and in pathologies such as Alzheimer's [1–4]. Like many inhibitors of the 'standard mechanism' class, which includes the ovomucoid third domain, Kazal and Kunitz inhibitor groups, serpins present a reactive site loop (RSL) which binds to the active-site cleft of the protease in a substrate-like manner. The structure of the RSL of standard mechanism inhibitors does not change between the free and bound forms, and furthermore the same structure is exhibited by inhibitors from different families in the absence of any sequence similarity. The stability of the complex formed with standard mechanism inhibitors relies on topological and charge complementarity between the RSL and the protease active site which favors the formation of a Michaelis complex-like structure; thus standard mechanism inhibitors behave as transition state analogs. In contrast, the RSL of serpins is extremely flexible and assumes several different conformations, many of which are not immediately suitable for binding to the active site of the protease [5–8].

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Abbreviations: Serpin, serine protease inhibitor; RSL, reactive site loop; AT, α_1-antitrypsin; htAT-PDX, histidine-tagged α_1-antitrypsin Portland; Da, dalton; PAGE, polyacrylamide gel electrophoresis; MCA, 4-methylcoumaryl-7-amide; IPTG, isopropyl-β-D-thiogalactopyranoside

Changing the RSL or the reactive site of serpins is one approach to the development of protein-based inhibitors. Recently this strategy has led to the development of an engineered serpin mutant, α_1-antitrypsin Portland (AT-PDX), which efficiently inhibited furin [9], a member of the mammalian convertases. Among these proteolytic enzymes that are responsible for the processing of precursor proteins [10], furin is certainly the best characterized [11]. This trans-Golgi membrane-associated, calcium-dependent endoprotease that cleaves on the C-terminal side of the recognition sequence Arg-X-X-Arg [12], is involved in proteolysis of many precursor molecules in the constitutive secretory pathway. The potential role of furin and other convertases in disease states [13] has prompted investigators to identify and characterize inhibitors in order to control production of biologically active peptides.

Considering that furin belongs to the kexin family of the subtilase superfamily [14], a serine protease family that has evolved independently of the trypsin family of serine proteases, we reasoned that thoroughly investigating the mechanism of inhibition of a serpin variant in this context was particularly compelling. Thus, we examined whether AT-PDX exhibited typical serpin-like properties with respect to its inhibition of furin. Formation of an indissociable AT-PDX/furin complex that completely abolished the enzyme's activity suggests that the archetype serpin, α_1-antitrypsin, may be easily modified to inhibit members of the subtilase family and that such modifications need not affect its regular behavior.

2. Materials and methods

2.1. Materials

The fluorogenic substrate, boc-Arg-Val-Arg-Arg-4-methylcoumaryl-7-amide (RVRR-MCA) and the furin inhibitor, decanoyl-Arg-Val-Lys-Arg chloromethylketone (dec-RVKR-cmk), were purchased from Bachem Bioscience Inc. Papain, protease-free bovine serum albumin (BSA), and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma; aprotinin was from Boehringer-Mannheim; plasma purified antitrypsin (AT) from Calbiochem. The bacterial expression vector pQE-31 and Ni^{2+}-NTA affinity resin was obtained from Qiagen. Protein concentration was determined by Lowry assay using BSA as a standard. The protective antigen (PA) of anthrax was a generous gift from Drs. S. Leppla and K. Klimpel (NIH, Bethesda, MD). Monoclonal antibodies (MON-148 and MON-152) were generously provided by Dr. Wim Van de Ven (University of Leuven). The cDNAs and vaccinia virus recombinants for AT-PDX and AT-Pitt were generous gifts from Dr. Gary Thomas (Vollum Institute) and Hedral Corp. (Portland, OR).

2.2. Construction, expression and purification of histidine-tagged AT-PDX in Escherichia coli

ATs were expressed as polyhistidine fusion proteins using the prokaryotic expression vector pQE-31 and the Qiaexpressionist system (Qiagen) according to the manufacturers' instructions with slight modifications. *Bam*HI-*Kpn*I cDNAs encoding ATs without their sig-

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nal peptide were inserted into pQE-31 that replaces the signal peptide with a polyhistidine affinity tag at the N-terminus. The ATs were expressed in the JM109 *E. coli* strain. Up to one liter of culture was grown at 37°C to an $O.D._{600}$ of 0.7–0.9 upon which induction was carried out using 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and growth continued for a further 5 h at 30°C. The production and native purification of ATs from total cytoplasmic proteins was then performed. Cells were harvested, resuspended in 30 ml of S buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 1 mM PMSF, 5 μg/ml aprotinin and 5 mM β-mercaptoethanol) containing 1 mg/ml of lysozyme and sonicated. Triton X-100 was then added to the cell lysate at a final concentration of 0.1%. Cell debris was removed by centrifugation at 11 000×*g* for 20 min and the supernatant was added onto Ni^{2+}-NTA resin previously equilibrated with S buffer. After stirring at 4°C for 1 h, the resin was washed twice and centrifuged. The resin was resuspended in 20 ml of W buffer (50 mM sodium phosphate, pH 6.0, 300 mM NaCl, 10% glycerol, 1 mM PMSF, 1 μg/ml aprotinin, 5 mM β-mercaptoethanol), loaded onto a column and proteins were eluted with E buffer (50 mM sodium phosphate, pH 4.5, 50 mM KCl, 5 mM β-mercaptoethanol); the pH of the eluted samples was immediately adjusted to 7.4. The eluate was applied to a Mono Q HR 5/5 fast protein liquid chromatography (FPLC) anion exchange column and proteins were eluted with a 100–250 mM NaCl gradient in 10 mM sodium phosphate (pH 6.5). The purity of ATs was assessed by SDS-PAGE and AT-containing fractions were aliquoted and stored in 0.1% polyethylene glycol at −80°C after snap-freezing in liquid nitrogen.

2.3. Analysis of reaction products by SDS-PAGE

The susceptibility to proteolysis of bacterially expressed htAT-PDX was confirmed by mobility of the protein (or protein fragments) on SDS-PAGE after incubation with furin or papain. Furin was produced as a secreted, soluble form (sfurin) and purified as previously described [12,15]. The RSL of htAT-PDX was cleaved by incubation with papain in P buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0) for 30 min at 37°C [16]. AT-PDX was also incubated with furin for increasing times in 100 mM HEPES, 1 mM $CaCl_2$, 1 mM β-mercaptoethanol at 30°C. The cleavage reactions were stopped by adding Laemmli buffer and the cleavage products were assessed by SDS-PAGE. To avoid the complete degradation of the protein by papain, the reaction conditions that resulted in the initial cleavage event were optimized by analyzing cleavages with different papain concentrations.

2.4. Kinetic assays and determination of inhibition parameters

Progress curves were used to estimate the parameters for the inhibition of furin by AT-PDX. All assays were performed at 30°C in I buffer (100 mM HEPES, pH 7.5, 1 mM $CaCl_2$, 1 mM β-mercaptoethanol, 500 mg/ml BSA) and 60–100 μM RVRR-MCA. Fluorescence (380 nm excitation, 460 nm emission) was measured on a Hitachi, model F-2000 spectrofluorometer. Each progress curve experiment consisted of 6–9 assays, one in the absence of serpin and others with AT-PDX at concentrations ranging from 3–15 nM; assays were initiated by the addition of furin at a final concentration of 86 pM. The data were fit to an equation describing reversible slow tight-binding inhibition as previously described assuming irreversibility [17], and the apparent association rate constant derived corrected for the presence of competing substrate by the relationship $k_{ass} = k_{app}(1+S/K_m)$. A cuvette containing only substrate was included as a control and the background rate of substrate conversion in the absence of enzyme was subtracted from the data prior to estimating rate constants.

2.5. Analysis of enzyme/inhibitor complex

The nature of the complex between AT-PDX and furin was examined in vitro and in vivo. For in vitro analysis, AT-PDX (530 nM) was incubated with furin (350 pM) for 60 min in I buffer at 30°C; the complex was incubated with Laemmli buffer (with or without DTT) and either boiled for 3 min or kept at room temperature. Proteins were resolved on 8% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked and then incubated with a monoclonal anti-furin antibody (MON-148) and developed with the ECL protocol (Amersham). For in vivo analysis, COS-7 cells grown on 60-mm dishes were co-infected with wild type (WT) and recombinant vaccinia virus expressing AT-PDX or human furin for 1 h at 37°C as previously described [18]. Four hours post-infection, cells were starved for 1 h in MEM medium lacking cysteine/methionine containing 10% dialyzed FBS and were pulse-labeled with 100 μCi of [^{35}S]Cys/Met labeling mix (Amersham) for 2 h. Cells were lysed in mRIPA buffer and half the cellular extract was immunoprecipitated with a polyclonal antibody against α_1-antitrypsin (Calbiochem), the other half with an anti-furin monoclonal antibody, MON-152, as previously described [19]. Proteins were resolved on 8% SDS-PAGE.

3. Results

3.1. Bacterial expression, purification and inhibitory properties of histidine tagged AT-PDX

In order to produce adequate quantities for a thorough characterization of AT-PDX, we expressed histidine tagged AT-PDX (htAT-PDX) in *E. coli* using a procedure developed by Bischoff et al. [20] with modifications. The cDNAs encoding AT-PDX or AT-Pitt were sub-cloned into pQE-31 which enables insertion of 6 histidine residues at the N-terminal of the gene of interest. It had previously been reported that the temperature at which the cultures were grown affected the solubility and activity of expressed AT recombinants [21]. We noticed that htAT-PDX accumulated mainly as an active

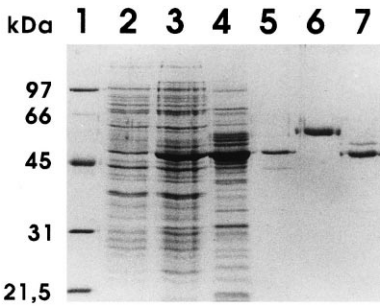


Fig. 1. Expression and purification of histidine-tagged AT-PDX from bacteria. Recombinant htAT-PDX was purified from IPTG-induced bacterial cell extracts using Ni^{2+}-NTA affinity resin and FPLC. Lane 1: molecular weight markers; lane 2: uninduced bacterial cell extract; lane 3: htAT-PDX induction with 2 mM IPTG; lane 4: elution of htAT-PDX from Ni^{2+} affinity column; lane 5: FPLC purification of htAT-PDX following nickel affinity resin step; lane 6: plasma purified AT; lane 7: AT deglycosylated with PNGase F.

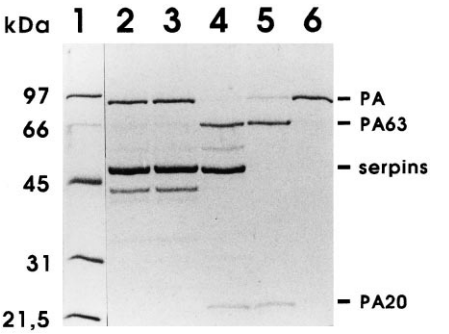


Fig. 2. AT-PDX inhibits furin-dependent proteolysis of PA in vitro. Furin (86 pM) was pre-incubated with 1.4 μM htAT-PDX for 1 min (lane 2) or 1 h (lane 3), with 1.4 μM htAT-Pitt for 4 h (lane 4) or without serpin (lane 5) before the addition of substrate (0.80 μM PA) in 30 μl final volume. The assays were stopped after 1 h with Laemmli buffer and boiled. Lane 1: molecular weight markers; lane 6: PA alone.

soluble form when bacteria were grown at 30°C, while most of htAT-PDX accumulated in an inactive form in inclusion bodies if cells were grown at 37°C (data not shown). Fig. 1 shows that following induction with IPTG, a major band of 47 kDa was produced (lane 3). Purification on the nickel column (lane 4) enriched our preparation and ultimately FPLC chromatography yielded 90% pure htAT-PDX (lane 5). For comparison, commercially available, glycosylated wild-type AT has a molecular weight of 52 kDa (lane 6) which upon deglycosylation with PNGase F yielded a protein of 47 kDa (lane 7). Up to 3 mg of non-glycosylated htAT-PDX was produced from 1 l of culture media.

To determine whether this preparation conserved its inhibitory properties toward furin, we initially compared $K_{0.5}$ values. Bacterially expressed htAT-PDX conserved its property at inhibiting furin's capacity to process a synthetic substrate in a manner that was indistinguishable from AT-PDX produced in eucaryotic cells [9]. Indeed, the $K_{0.5}$ of 0.013 μg/ml obtained with htAT-PDX from bacterial sources is similar to the $K_{0.5}$ of 0.030 μg/ml observed with AT-PDX produced in BSC-40 cells by a recombinant vaccinia virus [9] (data not shown). In addition, we verified whether this preparation efficiently impeded cleavage of protective antigen (PA) a previously characterized substrate for furin [12,22]. As shown in Fig. 2, when PA (83 kDa, lane 6) is incubated with furin, two cleavage products of 63 kDa and 20 kDa appear after 60 min (lane 5). Proteolysis was completely inhibited when furin was pre-incubated with htAT-PDX for 1 min or 1 h (lanes 2, 3, respectively). Incubation of furin with a similar quantity of htAT-Pitt did not affect furin's capacity to cleave PA (lane 4). These results demonstrate that lack of glycosylation due to the bacterial expression system used and presence of 6 histidine residues at the N-terminal of AT-PDX did not decrease its inhibitory properties toward furin. We therefore used this preparation for further characterization.

3.2. *Proteolysis of htAT-PDX by furin does not produce peptide fragments*

Because htAT-PDX has a furin recognition sequence at its reactive site, we initially verified whether incubation of htAT-PDX with furin yielded free proteolytic fragments; this would

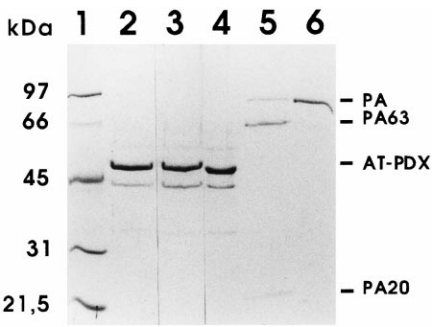


Fig. 3. Inhibition of furin by htAT-PDX does not result in release of serpin-derived fragments. Furin (86 pM) was incubated with 1.4 μM of htAT-PDX for 30 min (lane 2) or 24 h (lane 3). The susceptibility to release htAT-PDX-derived fragments was assessed by incubating htAT-PDX with 0.0001 U of papain for 30 min (lane 4). Furin viability over long incubation times was verified by pre-incubating the enzyme 8 h before the addition of 0.80 μM of PA for 1 h (lane 5). The assays were stopped with Laemmli buffer, boiled and resolved on 10% SDS-PAGE. Lane 1: molecular weight markers; lane 6: PA alone.

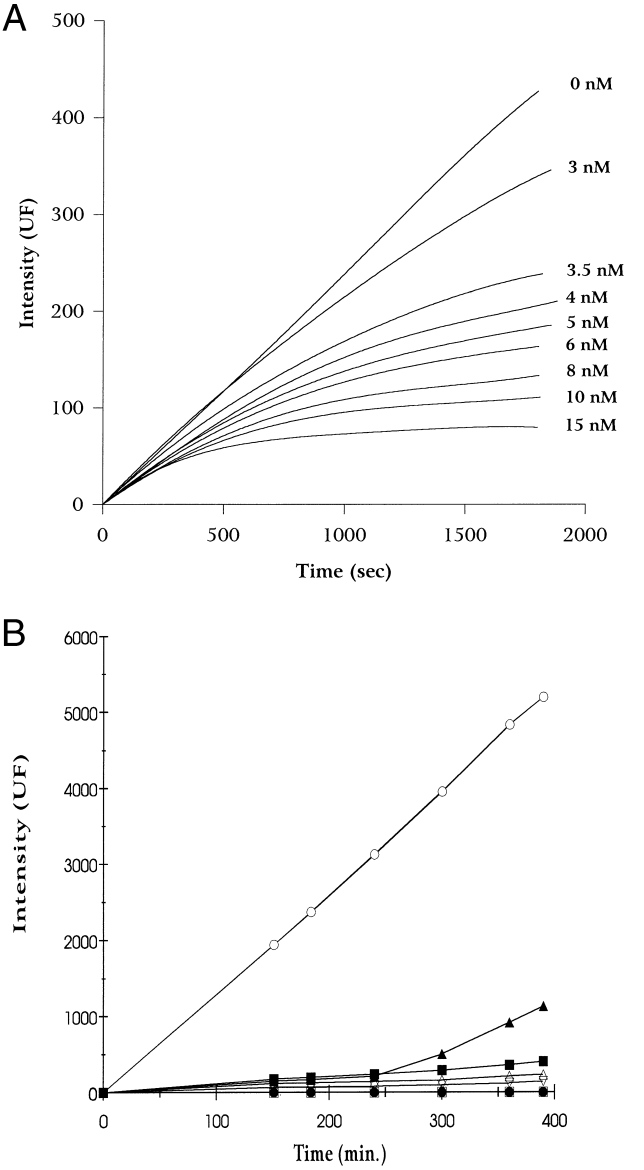


Fig. 4. Kinetic analysis of furin inhibition by AT-PDX. A: Progress curves for the inhibition of furin by htAT-PDX. Assays were performed as described in Section 2 with 100 μM boc-Arg-Val-Arg-Arg-MCA, 86 pM of furin and increasing concentrations of htAT-PDX at 0, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10 and 15 nM, respectively. B: Irreversibility of the enzyme-inhibitor complex. Furin (86 pM) was preincubated 1 h without inhibitor (○), with 0.85 μM htAT-PDX (● and □), with 10 μM (■ and ▲) or 50 μM (△ and ▽) decanoyl-Arg-Val-Lys-Arg-chloromethylketone for 1 h, diluted 40-fold and 100 μM boc-Arg-Val-Arg-Arg-MCA was added to measure remaining enzymatic activity. Two independent assays are shown for each inhibition condition.

have led to the possibility of a competitive based inhibitory mechanism. If furin cleaved at the P1–P1′ bond (Arg^{358}–Ser^{359}) and released its proteolytic fragments in a substrate-like manner, the main chain of the serpin should be lowered by 3–4 kDa. Fig. 3 shows that prolonged incubation (up to 24 h) of furin with htAT-PDX did not diminish its apparent molecular weight or yield proteolytic fragments (lanes 2, 3). Furin's enzymatic activity was maintained for at least 8 h since addition of PA at that time resulted in proteolysis of PA (lane 5). However, to ensure that htAT-PDX was suscep-

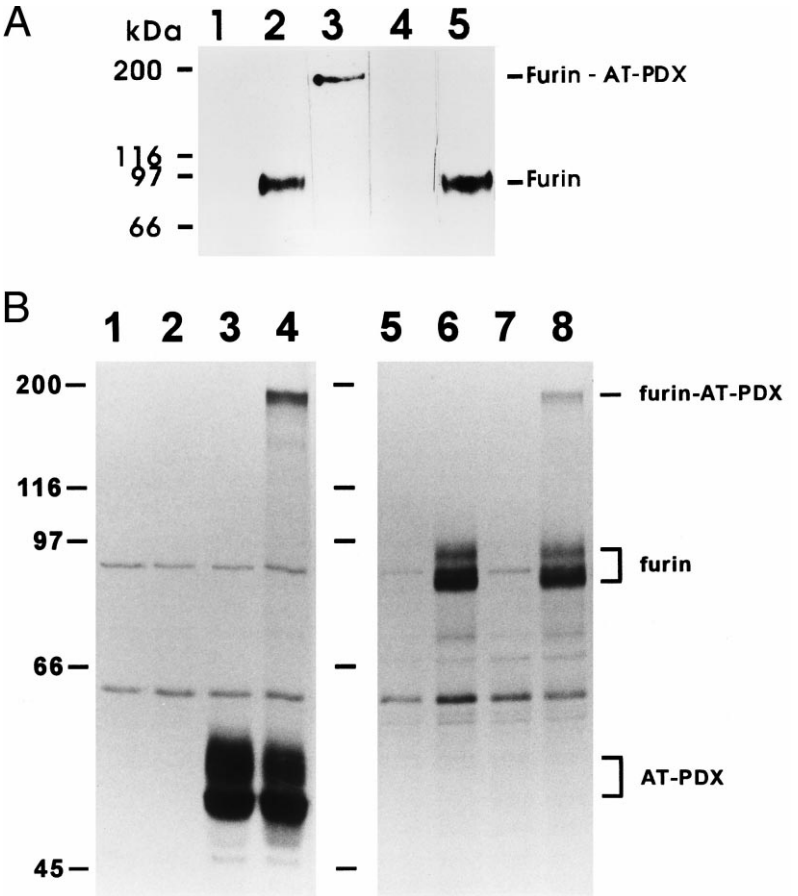


Fig. 5. AT-PDX forms a heat- and SDS-resistant complex with furin. A: 530 nM of AT-PDX (lanes 1 and 3) or htAT-Pitt (lanes 4 and 5) were incubated without (lanes 2 and 4) or with 86 pM of furin (lanes 2, 3 and 5) for 1 h. Western blotting was performed with monoclonal antibody MON-148 for detection of furin immunoreactivity. B: Formation of AT-PDX-furin complex in vivo. COS-7 cells were infected with recombinant vaccinia virus expressing native furin (m.o.i. = 2, lanes 2, 4, 6 and 8) and/or AT-PDX (m.o.i. = 4, lanes 3, 4 and 7, 8). Multiplicity of infection was adjusted to 6 with wild-type vaccinia virus in all lanes. Metabolic labeling with [^{35}S] and immunoprecipitation were carried out with anti-α_1-antitrypsin (lanes 1–4) or anti-furin MON-152 (lanes 5–8) as described in Section 2. AT-PDX-furin complex is indicated. Molecular weight markers are shown on the left.

tible to cleavage, we incubated it with papain. It had been demonstrated that papain, a cysteine proteinase, efficiently cleaves AT at Phe^{352}–Leu^{353} to produce a slightly smaller fragment [16]. As shown in Fig. 3 (lane 4), htAT-PDX is fragmented by papain, producing a polypeptide of 46 kDa. Although a smaller molecular weight form was expected, it is possible that the N-terminal histidine residues affected the migration in a way that loss of the C-terminal did not significantly affect its apparent molecular weight. The susceptibility of htAT-PDX to be cleaved by papain resulting in the detection of a smaller fragment and its resistance to release a fragment upon proteolysis by furin demonstrated that the local structure of the RSL was probably maintained but that the behavior of the serpin towards furin was quite different than towards papain.

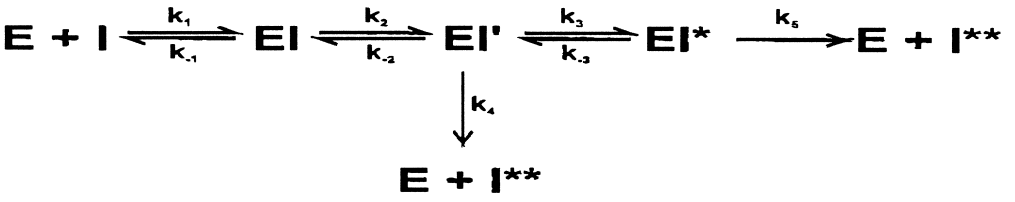
3.3. Inhibition of furin by AT-PDX is irreversible

To further characterize the nature of the inhibition of furin by htAT-PDX, kinetic parameters were determined by progress-curve kinetics; representative data sets are shown in Fig. 4A,B.

As illustrated, increasing levels of AT-PDX enabled steady-state equilibrium to be attained more readily. By non-linear regression, we calculated an association rate constant (k_{ass}) of $1.6 \pm 0.2 \times 10^6$ M^{-1} s^{-1} ($n = 7$) which is similar to the k_{ass} observed for inhibition of thrombin by protease nexin 1 (1.5×10^6 M^{-1} s^{-1}) or by AT variants where the P1 position is substituted with Arg (5.2×10^5 M^{-1} s^{-1}) [23]. We also verified if longer incubation times coupled to a dilution of the incubation reaction would result in a return of furin's enzymatic activity (Fig. 4B). This experiment was performed because mutations in the RSL of serpins could lead to the formation of a tight but reversible bond between the inhibitor and the enzyme [34]. We did not observe a return of activity after 400 min of incubation suggesting the irreversible nature of the interaction. Inhibition by the irreversible inhibitor peptidyl-chloromethylketone revealed no furin activity over the course of the incubation.

3.4. The htAT-PDX/furin complex is resistant to denaturation and heat

An interesting property of inhibitory serpins is their propensity to form a complex with the target proteinase that is resistant to heat and SDS treatment [1]. To characterize the type of interaction between htAT-PDX and furin, we performed Western blot analysis following SDS-PAGE of samples containing AT-PDX and furin using a monoclonal antibody that recognizes the N-terminus of active furin. Fig. 5A



Scheme 1.

shows that the soluble form of furin (sfurin) migrates as a protein of 83 kDa (lane 2) in accordance with the previously reported M_r [24]; the antibody did not recognize htAT-PDX (lane 1). When sfurin was pre-incubated with htAT-PDX, boiled in Laemmli buffer and resolved on SDS-PAGE, furin immunoreactivity was detected as a band of approximately 140 kDa (lane 3). This would correspond to the apparent M_r obtained from a complex of htAT-PDX (47 kDa) and sfurin (83 kDa). We did not observe formation of this complex when sfurin was pre-incubated with the htAT-Pitt variant (lane 5). Time-course experiments of complex formation revealed that the 140-kDa complex can be detected after only 1 min of incubation (results not shown). Thus, the AT-PDX/sfurin complex is resistant to denaturing conditions (SDS and heat), a property shared with the wild-type AT/elastase complex.

To examine whether this complex could be formed in vivo we expressed AT-PDX and native furin in COS-7 cells using vaccinia virus recombinants (Fig. 5B). Pulse labeling experiments show that after 2 h AT-PDX is immunoprecipitated as two broad bands of 52 and 56 kDa (lane 3), suggesting the glycosylated nature of the protein. Immunoprecipitations using the furin-specific antibody, MON-152, in cells expressing native furin revealed a series of bands at 96, 91 and 87 kDa representing different zymogen and active forms of furin (lane 6). We then used either the AT or the furin specific antibodies to immunoprecipitate solubilized proteins from cells expressing both AT-PDX and furin. The results show that a proportion of the expressed AT-PDX interacts with furin to produce the high molecular weight 190-kDa complex (lanes 4 and 8). The reasons why not more of the complex is detected may be, in part, due to the fact that only the enzymatically active and fully glycosylated form of furin could interact with AT-PDX; other immunodetected bands representing the zymogen or immature forms of the enzyme may not interact with the serpin. The inefficacy of the antibody to efficiently recognize the serpin/enzyme complex due to a distortion of the protease may be another reason for the relatively low levels of detected complex.

4. Discussion

It has been established that serpins operate as 'mechanism-based' or 'suicide substrate' inhibitors, and as such are the only known proteinaceous inhibitors known to do so. In this mechanism (Scheme 1), after the formation of an initial Michaelis complex (EI) follows the formation of an intermediate complex (EI′), after which the reaction partitions between two alternative and competing pathways; one which leads to a stable inhibitor-enzyme complex (EI*) and the other to the release of free, cleaved, inactive inhibitor (I**) and active enzyme (E). Once the EI* complex is formed, it can slowly break down either by a combination of pathways k_{-3} and k_4 or by an alternative pathway, k_5. The nature of mechanism based inhibition implies that inhibitor is depleted by the catalytic pathway. The assays done in this work were done under conditions where such depletion was negligible.

More specifically, formation of the complex between a serpin and a serine proteinase requires four distinct processes: (1) recognition and interaction between the P1 position of the serpin and the S1 binding site of the enzyme along with subsite interactions (in our case the P4 Arg of AT-PDX); (2) cleavage of the P1–P1′ bond and formation of a covalent acyl–enzyme bond or tetrahedral intermediate; (3) refolding of the serpin by insertion of strand 4A into beta sheet A; and (4) very slow deacylation to release the enzyme.

Antitrypsin and its variants have been extensively used as model serpins to elucidate the mechanism by which serpins inhibit serine proteases [5,6,25,26]. However, the majority of enzymes with which these variants interacted belong to the trypsin family of serine proteases. Human furin belongs to peptidase family S8, also known as the subtilase family, and has a very different architecture than elastase, the physiological target of native AT. Presently, unlike enzymes of the trypsin family, there exists no 'natural' inhibitor to this class of proteinase. The first reported inhibition of furin was performed using a peptidyl-chloromethyl ketone (decanoyl-Arg-Glu-Lys-Arg-chloromethylketone) to abolish production of gp120, the envelope glycoprotein of HIV [27]. This peptide-based strategy was employed to inhibit furin or convertase-mediated processing of other precursors [15,28–31]. Conversely, it was also observed that protein-based inhibitors, for example a variant of the Kazal inhibitor ovomucoid third domain, could affect convertase activity [32]. By focusing on known serine protease inhibitors and with the knowledge of furin's enzymatic specificity, the engineered antitrypsin variant AT-PDX was produced. This protein, in which the reactive site AIPM of AT was replaced by the furin recognition site RIPR, exhibited potent inhibitory properties towards furin [9]. However, the mechanism by which the protein abolished the activity of convertase remained unknown.

Based on these results, we initially hypothesized that AT-PDX acted as a competitive inhibitor towards furin and AT's original serpin properties were compromised. This is because interfering with the mobility of the RSL by modifying residues on the N-terminal side of the P1–P1′ bond [33] has been shown to diminish or abolish the serpin's ability to irreversibly inhibit its cognate proteinase [34]. Moreover, recent results have shown that the P6–P2 region of the reactive site of AT is important for rapid inhibition and formation of stable serpin-proteinase complexes [35]. Interaction between AT-PDX and furin resulted in the formation of an irreversible complex resistant to heat and denaturation. Therefore, replacement of the P1 and P4 residues in AT-PDX did not alter the serpin's

capacity to form the high molecular weight complex in vitro or in vivo. To our knowledge, this is the first time that an AT variant demonstrates serpin-like properties towards a serine protease of the mammalian subtilase family. Recently, reports have shown that wild-type AT can inhibit and form a complex with a bacterial enzyme, subtilisin Carlsberg [36], while other studies have demonstrated how the tobacco hornworm *Manduca sexta* can produce, through alternative exon usage, a variety of serpins, one of which (serpin 1B) also inhibited subtilisin Carlsberg [37]. Moreover, it has been observed that proteins having high sequence similarities to serpins have the potential to inhibit proteinases belonging to other classes of endoproteases. For example, the demonstration by Mathialagan and Hansen [38] that ovine uterine serpin, OvUS-1, could specifically bind to pepsin, an aspartic proteinase, provided evidence of a serpin with 'crossover' activity. The CrmA protein from cowpox virus that attenuates the enzymatic activity of FLICE, a cysteine proteinase, is another example of a serpin with 'cross-class' interactions [39].

Recent reports have described how AT-PDX has been used to control production of biologically active peptides by inhibiting furin in various expression systems. Heterologous expression of the serpin suppressed the stretch-induced hypertrophic growth of cardiocytes as well as the processing of brain-type natriuretic peptides [40], controlled growth of pancreatic beta cells [41] and inhibited processing of viral glycoprotein precursors [42]. Here, we have investigated the mechanism by which such inhibition takes place. With the continued pharmacological interest in the development of enzyme inhibitors, it is to be expected that other serpin variants will be engineered or discovered that selectively recognize other mammalian convertases. However, both in vivo and in vitro assays will be needed to assess serpin specificity and physiological relevance. It is well described that, depending on the type of assay used, contrasting results have been obtained when the inhibitory profiles of AT variants were evaluated [43–45], but progress towards delineating such selectivity is forthcoming. The pattern of interaction that we have described herein should be helpful in determining the inhibitory features of such molecules.

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