The Contribution of Arginine Residues within the P6–P1 Region of α₁-Antitrypsin to Its Reaction with Furin*

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A series of mutants incorporating furin recognition sequences within the P6–P1 region of the reactive site loop of α₁-antitrypsin were constructed. Variants containing different combinations of basic residues in the P1, P2, P4, and P6 positions replacing the wild type P9LEAIPMP1 sequence were evaluated for their capacity to establish SDS-resistant complexes with furin, to affect association rate constants (kₐnd and kₐss), or to inhibit furin-dependent proteolysis of a model precursor in vivo. Each variant abolished processing of pro-von Willebrand factor in transfected HEK293 cells. The kₐss of all variants were found to be similar (1.1–1.7 × 10⁶ M⁻¹ s⁻¹) except for one mutant, RERRRRR, which had a kₐss of 3.3 × 10⁶ M⁻¹ s⁻¹. However, the stoichiometry of inhibition varied with values ranging from 2.9 to >24, indicating rapid formation of the acyl-enzyme intermediate (high kₐnd). Moreover, those variants having high stoichiometry of inhibition values were accompanied by the rapid formation of cleaved forms of the inhibitors. The data suggest that the rate of conversion of the acyl-enzyme (EI) into the highly stable complex (EI⁺) was affected by replacement of specific residues within the reactive site loop. Taken together, the results reveal how furin recognition sequences within the context of the biochemical properties of serpins will play a role in the capacity of the protein to follow either the inhibitory or the substrate pathway.

Proteolytic processing is a ubiquitous process necessary for the production of biologically active polypeptides. Within the exocytic or endocytic pathway, a wide variety of precursors or proproteins of serum proteins, hormones, growth factors, cell surface receptors, extracellular matrix proteins, bacterial toxins, and viral coat proteins must undergo proteolytic cleavage to liberate their active moieties. Evidence accumulated over the past 10 years suggests that this event involves a family of calcium-dependent serine proteases called subtilisin-like-protein convertases (SPCs)¹ (1, 2). Among the seven members of this family, furin (SPC1, PACE) has been the most thoroughly studied (3). Furin is a ubiquitously expressed enzyme that processes precursor molecules within the trans-Golgi network/endosomal system. The wide variety of furin-cleaved proproteins highlights the crucial role of this enzyme in numerous cellular and pathological events.

The initial characterization of the enzymatic signature of furin spearheaded efforts in the design and conception of compounds aimed at inhibiting this convertase. One of the particularities of the specificity of furin is the requirement for arginine residues both at the P1 and P4 sites of the scissile bond, Arg-Naa-Xaa-Arg ↓, forming the minimal recognition site of the enzyme (4). Moreover, in more than 40 precursor proteins cleaved by furin, half possess a serine in the P1’ position. Site-directed or naturally occurring mutations clarified the role of the determinants that make up the specificity of furin. A set of sequence rules that govern the constitutive cleavage by this enzyme was put forward (5): (i) an Arg at P1 is essential; (ii) at least two of the three residues at positions P2, P4, and P6 must be Arg or Lys for efficient cleavage; the presence of basic residues at all three positions results in highest cleavage efficiency; and (iii) a hydrophobic/aliphatic amino acid at P1’ hinders cleavage.

The involvement of furin in various disease states incited investigators to use these sequence rules as a starting point for the design of inhibitory compounds. Initially, chloromethylketones were synthesized; these act as irreversible inhibitors (6) but have untested reactivities against other mammalian convertases. Peptidyl substrates based on the sequence of human proparathyroid hormone (7) or on the pro-segment of SPC3 were later shown to have inhibition constants toward furin in the micromolar to nanomolar range (8). Recent reports have demonstrated how a variety of templates ranging from simple polyarginine compounds to engineered eglin C proteins, natural subtilisin inhibitors, can also inhibit furin (9, 10).

Another class of inhibitors are serpins (serine protease inhibitors). Serpins are naturally occurring proteins that play a vital role in the regulation of serine protease activity. Although many serpins target serine proteases of the trypsin family, a recently described member of this family, PI8, has been shown to inhibit furin in vitro, by a rapid tight binding mechanism.

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1 The abbreviations used are: SPCs, mammalian subtilisin-like-protein convertase; AT, α₁-antitrypsin; AT-PDX, α₁-antitrypsin Portland; RSL, reactive site loop; SI, stoichiometry of inhibition; MAC, 4-methyl-7-amidocoumarin; serpin, serine protease inhibitor; vWF, von Willebrand factor; PAGE, polyacrylamide gel electrophoresis.
Furin Inhibition by α1-Antitrypsin Mutants

(11). This protein is an ovalbumin-type serpin that contains two furin recognition sequences (RNSRCKR142) in its reactive site loop (RLS). Conversely, using site-directed mutagenesis, the archetypal serpin, α1-antitrypsin (AT), has been modified to design a potent furin inhibitor (12). Modification of the reactive site of AT to accommodate the minimal furin recognition sequence (AIPM358 → RIPR358) yielded a molecule, AT-PDX, with a reported IC50 of 0.6 nM (13, 14). Moreover, AT-PDX showed specificity toward furin and was a poor inhibitor of other SPCs (14).

Serpins function by initially binding into the catalytic pocket of the target enzyme through their RSL. The recognition of the serpin by the protease is largely attributed to the P1–P1′ bond of the RSL. Subsequently, it is proposed that a refolding step occurs during which the RSL is inserted into the center of β-sheet A of the serpin to form a highly stable and kinetically trapped covalent serpin-proteinase complex called EI* (15). Although controversy persists as to the exact mechanism of this conformational change (16, 17), the recently solved crystallographic structure of the serpin-proteinase complex α1-antitrypsin-trypsin (17) describes, at the atomic level, how the RSL gets inserted into β-sheet A of the serpin. In agreement with the extreme stability of EI*, its dissociation into the active enzyme and the cleaved inhibitor is reported to be very slow (18). Accordingly, it is generally believed that the formation of the EI* is irreversible even though recent data argue for the reversibility of the EI* complex (19).

In the present study, we have introduced various elements of furin recognition motifs into the RSL of AT to improve the inhibitory activity of AT-PDX. The inhibitory characteristics of the resultant serpins toward furin were assayed in vitro and in vivo. We observed that some variants had improved association rates (kassoc) relative to AT-PDX. Moreover, we demonstrated that some variants had high stoichiometries of inhibition and readily dissociated from the enzyme as a cleaved form without, however, impairing their ability to inhibit furin-dependent processing of vWF in vivo. Based on the recent structural elucidation of the EI* complex of AT and trypsin (17), we propose that the rapid dissociation and the high stoichiometry of inhibition of the variants are caused by a significant increase in the Gibbs free energy of the transition state for the formation of the EI* complex. We further discuss the implications of our results in the context of possible molecular determinants that could control kassoc and whether a serpin follows the substrate (rapid dissociation) or inhibitory (slow dissociation) pathways.

EXPERIMENTAL PROCEDURES

Materials—DNA modification enzymes were obtained from APBiotech (Baie d’Urfe, Canada). All culture reagents were from Life Technologies, Inc. The Escherichia coli strain BL21 (CodonPlus) was obtained from Stratagene (La Jolla, CA). Phenylmethylsulfonyl fluoride and bovine serum albumin were obtained from Sigma. Aprotinin and ketone (dec-RVKR-CH2Cl) were purchased from Bachem Bioscience. AT-PDX was purchased from ApBioTech. All resulting cDNAs were sequenced and subcloned into pBlueScript-KS (+) as a EcoRI-SalI fragment. The amino acid sequences of the reactive site loops of all AT variants used in this study are presented in Fig. 1A.

Construction, Expression, and Purification of Wild Type and Variants of AT—Subcloning of the serpin variant constructs in pQE-31 has been described previously in detail (13). Briefly, all cDNAs were digested with BamHI and KpnI and subcloned into pQE-31. This procedure replaces the signal peptide of all ATs by a polyhistidine affinity tag; it also changes Glu267 → Thr, but these modifications do not affect the activity of this serpin (20). The purification of native serpin variants from total soluble cytoplasmic proteins in E. coli was performed as described previously (13) with modifications. All purification steps were carried out at 4 °C. The E. coli BL21 cells were grown at 30 °C in TB medium containing 200 μg/ml ampicillin to A600nm = 0.8. Isopropyl-β-D-thiogalactopyranoside was then added to a final concentration of 1 mM, and expression of recombinant protein occurred over the following 4.5 h. Recombinant serpins accumulated mainly as inactive soluble proteins (21). The cells from a 0.5–1.0-liter culture were harvested by centrifugation and resuspended in 2% initial culture volume in S buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10% glycerol, 20 mMimidazole, 15 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 0.5 mg/ml Pefabloc SC, and 1 mM EDTA) containing 1 mg/ml of lysozyme for 30 min. Triton X-100 (2% final) was then added to the lysate, and the mixture was incubated on ice for 30 min. Cell debris was removed by centrifugation at 11,000 × g for 25 min. Ni2+-nitrilotriacetic acid resin equilibrated with S buffer was added to the supernatant, and the slurry was stirred for 1 h. The slurry was washed twice with W buffer (50 mM sodium phosphate, pH 6.5, 500 mM NaCl, 30% glycerol, 0.5% Tween 20, 40 mMimidazole, 15 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin), and the bound proteins were eluted with E buffer (50 mM sodium phosphate, pH 6.5, 300 mM NaCl, 15 mM β-mercaptoethanol, 150 mM glycerol, 350 mMimidazole, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 0.1% polyethylene glycol; M = 8000). The fractions containing the recombinant serpins were identified by SDS-PAGE and pooled. This sample was further purified to Mono-Q HR5/5 anion exchange columns and sound proteins were eluted with a linear gradient of 120–250 mM NaCl in 10 mM sodium phosphate, pH 6.5. The protein concentration of each preparation was determined by Bradford assay using bovine serum albumin as a standard, and all preparations were normalized by densitometry of Coomassie Blue-stained SDS-PAGE gels.

General Kinetic Methods—Purified hfur714 (a shorter form of the human furin in which the transmembrane domain and cytoplasmic tail have been deleted) was produced as described previously (22). The concentration of catalytically active fur714 was determined by titration with the active site-directed irreversible inhibitor dec-RVKR-cmk as described previously (23). Enzymatic activity was determined by the cleavage of the fluorescent substrate boce-RVRR-MAC (excitation at 380 nm and emission at 460 nm). All enzymatic assays were carried out in kinetic buffer (100 mM Hepsa, pH 7.5, 1 mM CaCl2, 1 mM β-mercaptoethanol, and 500 μg/ml bovine serum albumin). The data were fitted to the hyperbolic Michaelis-Menten rate equation to determine values for Kα and kcat for boce-RVRR-MAC as described (22) and were found to be 21.8 μM and 5.5 s−1, respectively.

Determination of the Stoichiometry of Inhibition—Active site–titrated hfur714 was used to determine the molar ratio of the AT variants necessary for complete inhibition of the enzyme. The stoichiometry of inhibition (SI) values for the inhibition of hfur714 were determined by incubating different concentrations of AT variants in a total volume of 100 μl at 37 °C with a fixed concentration of hfur714 in kinetic buffer for 30 min. The residual amidolytic activity was determined by the addition of 100 μM boce-RVRR-MAC, and the reactions were stopped with 5 mM benzamidine for 30 min. The rate of substrate hydrolysis was plotted as a function of inhibitor concentration, and linear regression to the x axis was used to calculate the amount of inhibitor required to completely inhibit the enzyme. The SI was determined by regression analysis of the dependence of residual hfur714 activity on [I]/[E]0.

Slow Tight Binding Inhibition Kinetics—Slow tight binding inhibi-
tion experiments were conducted under pseudo-first order conditions. Each assay consisted of four to six reactions with varying amounts of serpin and a control reaction without inhibitor. The assays were started by the addition of a constant amount of enzyme (500 pmol) to a solution containing the fluorogenic substrate (final concentration, 100 μM) and the appropriate inhibitor concentration in kinetic buffer. Progress curves were performed under final concentrations of reactants of 500 μM hfur/714; 100 μM boc-RVRR-MAC; 10–80 nM AT-PDX or AT-EK1; 10–100 nM AT-EK2; 20–200 nM and AT-EK3; or 15–90 nM AT-EK4. Synchronous substrate hydrolysis was measured in separate experiments, and the background rate of substrate hydrolysis in the absence of enzyme was subtracted from the data prior to estimating rate constants. Considering an irreversible reaction \( (v_0 = 0, k_{\text{diss}} = 0) \) for each serpin variant, the collected data were determined by fitting to the integrated rate equation for slow tight binding inhibition (24).

\[
P = v_f + \left( v_0 - v_f \right) \frac{1 - e^{-kt}}{k}
\]

(Eq. 1)

The data were fit by nonlinear regression using Enzfitter (Biosoft, Ferguson, MO) to obtain values for the initial velocity \( (v_f) \), the steady-state velocity \( (v_0) \), and the apparent first order rate constant \( (k) \) for the establishment of steady-state equilibrium of the protease-inhibitor complex. The values obtained from nonlinear regression analysis were then used in various graphical transformations (24, 25) to obtain the inhibition and rate constants for interaction of the various serpin variants with furin.

Analysis of Complex Formation by SDS-PAGE—The ability of AT variants to form SDS-stable complexes with hfur/714 was assessed by incubating the variants (170 nM) with hfur/714 (24 nM) in 100 μl of kinetic buffer containing 20 μg/ml bovine serum albumin for 5 h at 37 °C. For analysis of complex formation kinetics, AT variants and hfur/714 were incubated as above for selected time intervals between 30 s and 30 min. The reaction mixtures were stopped with 5 μl EDTA, lyophilized, and boiled in Laemmli buffer under reducing conditions. Proteins were separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane, which was then blocked with 5% nonfat dry milk in TBS. The membrane was then sequentially incubated with anti-hfur/714 monoclonal antibody (MON-152) and anti-pan-His monoclonal antibody according to the manufacturer’s instructions (Qiagen) to detect the complexes, hfur/714, and His-tagged proteins, respectively. The membrane was developed using the ECL protocol (Amer sham). For each experiment, the residual enzymatic activity was determined by the addition of 100 μM boc-RVRR-MAC.

Immunoprecipitations—hEK293 cells were transfected either with a construct expressing pro-vWF or cotransfected with a construct expressing one of the various serpin variants using PugeNec (Roche Molecular Biochemicals), in accordance with the manufacturer’s instructions. A total of 3 μg of DNA for pro-vWF and 0.5 μg for each serpin variant, subcloned in pcDNA3, were transfected in 6-cm cultures plates. 24 h after transfection, the cells were washed with PBS and then metabolically labeled in Met/Cys–minimal essential medium containing 10% dialyzed fetal bovine serum, 2 mM l-glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 50 μg/ml [35S]Met/Cys (Expre’soS-S labeling mix; PerkinElmer Life Sciences) for 3 h; following this incubation, the medium was harvested. The medium was incubated with the anti-vWF polyclonal antibody (1:500, Dako) followed by antibody capture with protein A/G-agarose. Following centrifugation, the supernatant was recovered and incubated with anti-α1-antitrypsin monoclonal antibody (1:500) (Calbiochem) followed by incubation with protein A/G-agarose. Immunoprecipitates were resolved on 7% SDS-PAGE for vWF and 10% SDS-PAGE for serpin variants.

RESULTS

Design, Expression, and Purification of Recombinant α1-Antitrypsin Variants—Based on the previously demonstrated furin recognition sequence rules (5, 26), we designed, constructed by oligonucleotide-directed mutagenesis, expressed, and purified four structural variants of AT-PDX: AT-EK1, AT-EK2, AT-EK3, and AT-EK4 (shown in Fig. 1A). To mimic the proteolytic degradation of serpins containing arginine residues within the reactive site loops, each recombinant serpin was expressed as polyhistidine fusion proteins in the BL21 Codon-Plus E. coli strain. The serpin variants were produced as soluble proteins and were purified under native conditions from total cytoplasmic proteins in a two-step procedure using nickel affinity column chromatography and MonoQ fast protein liquid chromatography. Final yields varied between serpins (AT, 2.4 mg/liter; AT-PDX, 1.2 mg/liter; AT-EK1, 1.8 mg/liter; AT-EK2, 2.4 mg/liter; AT-EK3, 3.6 mg/liter; AT-EK4, 1.2 mg/liter) but were in the same range. Fig. 1A shows the analysis by SDS-PAGE of the purified recombinant wild type and AT variants migrating as 47-kDa proteins. All purified serpins were estimated to be >90% pure by densitometric analysis.

Kinetic Analysis of hfur/714 Inhibition by AT Variants—We evaluated the SI for each AT variant by titration experiments. The active titer of furin was determined using the active site-directed irreversible inhibitor dec-RVKR-cmk. Serpins are mechanism-based inhibitors and exhibit a branched reaction pathway (Scheme 1). According to this mechanism, serpin inhibitory activity is affected by the ratio between the rate constant for the substrate pathway \( (k_h) \), which generates cleaved, uncleaved serpin (I), and the rate constant for the inhibitory pathway \( (k_i) \), which generates the stable serpin-enzyme complex \( (E^I) \). This ratio is reflected in the stoichiometry of inhibition, SI [SI = (1 + k_h/k_i)]. The value of SI indicates the “efficiency” of a serpin for a given protease, which equates the number of moles of inhibitor required to completely inhibit 1 nM of a target protease. For cognate serpin-protease reactions, the reaction flow almost entirely toward stable complex formation, resulting in SI values close to 1. Table I shows that 1 mol of hfur/714 was inhibited by 3.6, 2.9, 13, and 24 mol of AT-PDX, AT-EK1, AT-EK4, and AT-EK3, respectively. This indicates that the reaction flow for AT-PDX and AT-EK1 with hfur/714 partitioned with slight preference to the pathway
Therefore, relative to AT-PDX and AT-EK1, AT-EK4 showed an induced a 4.5-fold increase in the SI value with hfur/714. This scheme describes the action mechanism of serpin action.

Scheme 1. Branched pathway for the suicide substrate inhibition mechanism of serpin action. This scheme describes the accepted minimal kinetic mechanism for the reaction of a serpin, I, with a protease, E, to form a stable, covalent inhibited complex, EI*. Initial formation of the noncovalent Michaelis complex, EI, is followed by acylation of the enzyme by the serpin to form the initial acyl-enzyme (first part of proteolytic process), EI'. The intermediate complex EI' can partition (branch point in the mechanism) to undergo a conformational rearrangement resulting, through the inhibitor pathway, in the formation of the final inhibited complex, EI*, or to undergo hydrolytic deacylation to form cleaved serpin (I') and release active enzyme through the substrate pathway. The final inhibited complex, EI*, slowly decomposes through a single pathway, resulting in the release of cleaved serpin and active enzyme with rate constant ke. According to the proposed kinetic scheme of Nash et al. (19), this inhibition mechanism incorporates the possibility that the steps with rate constants k2 and k4 might be reversible and that EI* can decompose either through direct hydrolysis (with rate constant ke) or by the reverse of its formation through reversion to EI'. The equilibrium dissociation constant of Michaelis complex (Ks) represents the affinity between inhibitor and enzyme.

### Table 1

| Site (P2–P4) | kcat | SI | kₐs⁻¹ |
|-------------|------|-----|-------|
| AT-PDX²     | 1.3×10⁶ ± 0.1 | 3.6 | 6.1×10⁶ ± 0.1 |
| AT-EK¹      | 1.4×10⁶ ± 0.4 | 2.9 | 8.7×10⁶ ± 0.3 |
| AT-EK²      | 1.7×10⁶ ± 0.1 ND | 7.9×10⁶ ± 0.5 |
| AT-EK³      | 3.3×10⁶ ± 0.2 | 1.7 | 3.3×10⁶ ± 0.9 |
| AT-EK⁴      | 1.1×10⁶ ± 0.7 | 0.1 | ND |

*The SI values were determined by titration as described under "Experimental Procedures." The multiplication of the values kₐs and SI give the rate constant kₐs, which represents the formation of the covalent acyl-enzyme intermediate, EI'. Under the experimental conditions of the progress-curve assays, the cleavage pathway is negligible because Iₐ ≈ SIE₁ (21).

**ND**, not determined because of instability of serpin variant–protease complexes.

Generating the cleaved form (k₃ > k₄). The effect of Lys as compared with Arg in the P2 position (AT-EK4 versus AT-EK1) induced a 4.5-fold increase in the SI value with hfur/714. Therefore, relative to AT-PDX and AT-EK1, AT-EK4 showed increased substrate behavior in its reaction with hfur/714 (k₃ > k₄), partitioning mainly to the cleaved form rather than to the irreversible inhibited complex. Substituting Arg at both P2 and P6 (AT-EK3) produced a serpin with properties toward furin that were similar to AT-EK4, namely an increase in the SI relative to AT-PDX. The SI value for AT-EK2 was not determined because the serpin-enzyme complex was too short-lived (see Fig. 14). Therefore, although the actual SI for AT-EK2 is likely to be very high, it was not possible to measure it directly because of the too rapid complex dissociation of the serpin-enzyme complex (kₑ → 0).

**Progress Curve Kinetics**—We examined whether AT variants possessed the time- and concentration-dependent characteristics of serpins, namely slow tight binding inhibition kinetics. Inhibition kinetic studies to measure the kinetic constants for each AT variant were performed at short interaction time periods (5 min) with the enzyme, because of the instability of the serpin-enzyme complex of certain variants. As previously shown (14), the inhibition of furin by AT-PDX obeyed typical slow tight binding inhibition kinetics as indicated by the bi-phasic plot, for which maximal inhibition was achieved more rapidly with increasing concentrations of AT-PDX. This same inhibition mechanism was also observed with AT-EK1 (shown by the inhibition progress curves of Fig. 2), AT-EK3, and AT-EK4 (data not shown). Despite their high SI values, the kinetic characterization of AT-EK3 and AT-EK4 was performed under conditions where Iₐ ≈ SIE₁ × Eο, such that the effect of the cleavage pathway on kₐs can be ignored (27). Fig. 2 also shows that the inhibition kinetics of AT-EK2 contrasted with the other variants. Indeed, the progress curves show that inhibition by AT-EK1 and AT-EK2 produced patterns that were similar at short times (<300 s), but at longer times only AT-EK1 attained a steady-state rate (νₑ = 0), typical of serpins.

Inhibition experiments with AT-EK2 instead exhibited a return of furin activity, which eventually paralleled the curve produced in absence of inhibitor.

The apparent second order association rate constants kₐs of each serpin variant for hfur/714 were determined by plotting log ([P]₀ – [P]ₜ) versus time, where [P]₀ = νₑkₑ and [P]ₜ is the fluorescence measured at various times for individual progress curves (data not shown) (28). kₐs values were calculated from the slope of the line, which is obtained by the following equation: kₐs = (0.432Iₚkₑ/[S]₀Kₑ), where the kₐs values for AT-PDX (1.3×10⁶ M⁻¹ s⁻¹), AT-EK1 (1.4×10⁶ M⁻¹ s⁻¹), AT-EK2 (1.7×10⁶ M⁻¹ s⁻¹), and AT-EK4 (1.1×10⁶ M⁻¹ s⁻¹) are the same order of magnitude, whereas AT-EK3 (3.3×10⁵ M⁻¹ s⁻¹) has a 4-fold decrease in association rate compared with AT-PDX. By
nonlinear regression analysis, the data show that the initial velocity ($v_0$) was inversely proportional to the concentration of each AT variant, suggesting that the type of interaction between furin and the serpin variants follows a two-step mechanism of inhibition (24).

Formation of SDS-resistant Serpin-hfur/714 Complexes—To more fully understand the relationship between serpin and furin activity, we examined the kinetics of inhibitor-enzyme complex formation over a period of 30 min. First, we evaluated the enzymatic behavior of furin following short incubation times with the variants. Fig. 3 shows that when AT-PDX, AT-EK1, or AT-EK4 was incubated with furin for 30 s, enzymatic activity was completely abolished, suggesting rapid complex formation. Activity was abolished for at least 5 h (data not shown). However, AT-EK2 and AT-EK3 showed only transient inhibition of furin activity (40 and 20% inhibition observed after 5 min, respectively) followed by a rapid and complete regeneration of activity. Therefore it is likely that the regeneration seen with AT-EK2 and AT-EK3 was due to the dissociation of the complex into free enzyme and cleaved serpin. Consequently, we examined the progression of serpin-enzyme complex formation over the same incubation times by immunoblot analysis.

A hallmark of the serpin-serine proteinase interaction is the formation of a complex that is not dissociated by heating and incubation in SDS. This stability is likely due to the presence of a covalent bond between the enzyme and the inhibitor analogous to the acyl-enzyme intermediate formed during peptide bond hydrolysis (29, 30). To determine whether AT-EK1, AT-EK2, AT-EK3, and AT-EK4 had the ability to form an SDS-stable complex with hfur/714, an excess of each serpin was incubated with the protease, and the heated/denatured samples were analyzed by immunoblot. Our conditions enabled us to immunodetect the covalent furin-serpin complex, free furin, and uncleaved or cleaved inhibitor on the same blot. The data in Fig. 4 illustrate how each variant was able to form the characteristic SDS-stable complex (EI* or EI) with furin, which migrated with an apparent molecular mass of ~200 kDa. Cleaved inhibitor, $I^c$ (43 kDa), uncleaved inhibitor (47 kDa), and free enzyme (E) (83 kDa) are also shown. Incubation of furin with AT-PDX (Fig. 4A), AT-EK1 (Fig. 4B), and AT-EK4 (Fig. 4E) resulted in the rapid (<30 s) formation of an SDS-stable complex, which is consistent with the inhibition mechanism of serpins. However, reactions with AT variants containing an Arg residue in the P6 position (AT-EK2 and AT-EK3) initially led to the formation of the complex, which, after a few minutes, resulted in the release of the cleaved form of the serpin (Fig. 4C and D). AT-EK2 rapidly formed a complex with furin for ~5 min before being released from the enzyme as a cleaved serpin ($I^c$) with a concomitant reappearance of free and active enzyme. Only a small percentage of AT-EK3 formed a complex with the enzyme (Fig. 4D), and the cleaved form, $I^c$, was produced much more rapidly (<30 s) than with AT-EK2. In this case, the results indicate a slow conversion of EI’ to EI*,
Previous data obtained from experiments designed to evaluate the substrate specificity of furin demonstrated that specific elements are crucial for interaction with and cleavage by furin such as the minimal furin recognition sequence, where arginine residues are present in the P1 and P4 positions of the precursor molecule. Indeed, the majority of furin-processed proproteins contain the consensus recognition site -Arg-Xaa-(Lys/Arg)-Arg- (3). However, certain precursors efficiently cleaved by furin, such as proalbumin (32), proprotein C (33), and parathyroid hormone (34), do not possess an arginine in P4 and thus must rely on other determinants for cleavage. In the case of proalbumin and proprotein C, Arg residues in the P2 and P6 positions could possibly contribute to the recognition process, whereas parathyroid hormone has Lys in P2, P3, and P6, which would compensate for the missing arginine. Models of the substrate-binding region of furin (35) and other reports based on the design of peptide and protein engineered substrates to characterize the sequence specificity of this enzyme (7, 36, 37) indicate that the number and localization of the basic residues found within substrates are important factors in recognition by furin subsites.

Based on the minimal recognition site necessary for efficient cleavage by furin, Arg-Xaa-Xaa-Arg, an AT variant (AT-Portland or AT-PDX) that contains this recognition motif has been described (12). This variant was shown to be a potent, highly selective inhibitor of the amidolytic activity of this convertase; in vitro (14) and in vivo studies have demonstrated its capacity to inhibit furin-dependent processing of numerous protein precursors. This efficient inhibition is due to the typical serpin behavior of the variant, i.e. its slow-tight and practically irreversible binding to furin (13, 14).

In this study, we have assessed the biochemical properties of AT variants containing various furin recognition motifs within the P6–P1 region of the serpin. It is firmly established that the nature of the P1 residue in the RSL of serpins plays a crucial role in determining serpin specificity for its target protease (38, 39). Although the P1 residue is crucial in primary binding with the protease active site, other residues beyond the P1–P1' peptide bond of RSL also appear to be important in regulating the interaction and inhibitory specificity between serpin and protease and in either promoting or restricting rapid inhibition and formation of serpin-protease complexes (40, 41). Modifying the minimal recognition signal of AT-PDX (LERIR) to AT-EK1 (LERIRR), AT-EK2 (RERIR), or AT-EK4 (LERIKK) did not greatly affect the association rate constant ($k_{on}$) of these variants before the fork of the branched suicide substrate pathway (Scheme 1). However, AT-EK3 (RERRR) exhibited a 5-fold decrease in $k_{on}$. The $k_{on}$ derived from the analysis of inhibition progress curve, is a measure of the overall rate of the transformation of the noncovalent Michaelis complex (EI) into the final inhibited complex, EI*.

Based on a model structure of AT-PDX (14), it has been proposed that the double Arg substitution at the P1 and P4 positions should have negligible effects on the overall conformation of the serpin because both side chains face the solvent and therefore do not participate in tertiary interactions. Conversely, it is possible that the incorporation of Arg residues at P2 and/or P6 and Lys residue at P2 could alter the extended β-strand conformation of the RSL (34), thereby affecting the interaction with the protease with a concomitant reduction of $k_{on}$ (multiplying $k_{on}$ by S1 gives $k_{on}$ = $k_{j}/K_{j}$, which corresponds to the true second order rate constant of EI* formation, i.e. the steps prior to the branch point; Ref. 42 and Scheme 1). Interestingly, we observe that $k_{on}$ remains rather constant (except for AT-EK4), suggesting that these basic residues in the RSL of AT do not seem to influence the overall structure be-

\[ \text{FIG. 5. Inhibition of maturation of provWF by different AT reactive site loop variants in cellulo. A, the ability of each AT variant to inhibit processing of provWF in hEK293 cells, hEK293 cells were cotransfected with provWF vector (lane 2) or transfected with 0.5 µg of constructs expressing the different AT reactive site loop variants (lanes 3-7). At 24 h after transfection, the cells were metabolically labeled with [35S]Met/Cys for 3 h, after which the medium was harvested. provWF proteins were immunoprecipitated with anti-vWF. Inhibition of provWF maturation was analyzed by 7% SDS-PAGE. Lane 1, pcDNA3 control; lane 2, provWF; lanes 3-7, AT-WT, AT-PDX, AT-EK1, AT-EK2, and AT-EK3, respectively. ProvWF, immature form of provWF; vWF, mature form of vWF. B, differential rate of generation of cleaved serpin. The serpins remaining in the medium were immunoprecipitated with anti-AT. The proteins were analyzed on 10% SDS-PAGE. Lanes 1-7, mock, vWF, AT-WT, AT-PDX, AT-EK1, AT-EK2, and AT-EK3, respectively. I, uncleaved serpin; F, cleaved serpin.} \]
tutions on the cycle shows the effects of the P6 Leu→Arg and P2 Pro→Arg substitutions on the k₉₉ with furin. The expression by each arrow represents the ratio of k₉₉ (Table 1) for specific mutants. If the mutational effects are independent of each other, the magnitude of change in k₉₉ should be equal on parallel sides, i.e., the effect of a mutation should be independent of the residues in the other positions. Double-mutant cycle analysis shows that the effects of the arginine residues substituted in AT on the rate of inhibition of furin are dependent of each other. This is reflected by the difference in the magnitude of the decrease of k₉₉ on parallel sides of the cycle (0.9 versus 5.2 and 0.8 versus 4.2). Note that the products of each cycle are similar (0.9 × 4.2 = 3.78, compared with 0.8 × 5.2 = 4.16). These values are also similar to that for the complete mutation of AT-PDX to AT-EK3 (k₉₉[AT-PDX]/k₉₉[AT-EK3] = 3.90).

between P'3 and P8 of the RSL or that the latter is able to accommodate the replacement to adopt alternative conformations permitting efficient molecular recognition with furin. The fact that the k₉₉ of AT-EK4 is increased is also quite informative and novel. Indeed, this indicates that either the rate constant kₑ is increased (more efficient molecular recognition process) or kₕ decreased (higher affinity between AT-EK4 and furin) when substituting Arg with Lys at this position. Because the two side chains should, a priori, bear the same charge, the explanation for such a result goes beyond a simple macroscopic electrostatic effect. We propose that the increase in k₉₉ is caused by the fact, that the Lys side chain is less bulky than the Arg side chain (165 versus 200 Å² of accessible surface area) (43). In agreement with our experimental observations is the report that the subsite S2 pocket of furin has a limited size and depth that leads to the conclusion that the prevalent electrostatic interaction originating from Lys would be preferred (35).

The effects of the mutations interpreted in terms of a double mutant cycle (44) as depicted in Fig. 6 clearly demonstrate cooperation of interactions between the P6 and P2 residues in the inhibition of furin. The mutational effects are nonadditive; both the P2 Pro→Arg and the P6 Leu→Arg substitution independently caused relatively small decreases in k₉₉. However, when combined there was a 5-fold cooperative effect that decreased the k₉₉ with furin. The possibility of long range interactions between P2 and P6 residues in the case of AT-EK3 that adversely affect the ability of the serpin to interact with furin, for example through subtle changes in the RSL structure, may be considered. In addition to the reported independent contributions of the S4 and S6 subsite of furin to substrate binding (37), our results suggest that the S6 and S2 subsites of the enzyme could interact with the substrate in a dependent manner. This would demonstrate the discriminatory flexibility of the subsites of the proteases in the recognition patterns and interaction specificity of several furin substrates (39) by the favorable effects of contact compensation or auxiliary interaction at P2 and P6 positions in absence of the optimal residues at either P1 or P4 (37).

Introduction of various furin recognition sequences within AT led to significant changes in the behavior of the serpin-enzyme reaction after the fork in the branched suicide-substrate pathway. Although the SI values observed for AT-PDX and AT-EK1 (3.6 and 2.9) variants indicate that kₑ and kₕ are similar in magnitude (more characteristic of serpins) (42), the inhibitory properties of AT-EK2 and AT-EK3 were profoundly altered when Arg was introduced in P6 position. Indeed, we observe a loss of complex (E*I) stability as revealed by the reversibility of inhibition (Fig. 4, C and D). Based on the x-ray structure of the AT-trypsin complex (17), we have investigated putative structural explanations for this phenomenon. As depicted in Fig. 7, the molecular environment surrounding Leu³⁵³ (P6) at the interface of the β-sheet A and helix bH is made up of residues with hydrophobic side chains as portrayed by the molecular surface of this hydrophobic pocket in Fig. 7 (C and D). Although the burial of the hydrophobic Leu in such an environment is expected to be very favorable, insertion of a bulkier and charged side chain is much less probable. Indeed, such an environment is not well suited to counterbalance the large loss of solvation free energy of the charged guanidino side chain (45) of Arg³⁵³ were it to be inserted as part of the formation of a complete s4A strand as seen in the crystal structure of AT complexed with trypsin (17). We observe that the P1, P2, and P4 positions are more solvent-exposed in the structure of the AT-trypsin complex than is the P6 (17), suggesting that a desolvation effect caused by the introduction of a Arg residues (or Lys) can be expected to be less severe than that observed at P6. Accordingly, we propose that the formation of a long-lived E*I with Arg residues in P6 is improbable or, in other words, that both the energy of the transition state and E*I are higher compared with WT (Fig. 8). Such a thermodynamic effect will lead to a reduction and/or an increase of rate constant kₑ and k₋, respectively, therefore favoring the substrate pathway as observed experimentally.

Because our results suggest that it is energetically improbable to form the final inhibited complex E*I*, in the case of AT-EK2, AT-EK3, and AT-EK4, we propose that the nature of high molecular weight complexes, as observed in Fig. 4C, are a form in which the P1 residue of the RSL loop is covalently bound with the catalytic serine of the proteasine to form a transiently stable acyl-enzyme intermediate complex. Accordingly, the protease is not at this point "crushed" against the body of the serpin as has been proposed for the interaction between AT and trypsin (17). This intermediate (Fig. 4C) might consist of a common species from which partitioning between inhibitor and substrate pathways occurs, although this event would take place late in the process of complex formation as recently reported (30).

We examined the effect of these variants in hEK293 on the processing of pro-vWF. It has been shown that this precursor is efficiently cleaved by furin (48), although it remains to be determined whether other SPCs participate in the biosynthesis of this glycoprotein involved in coagulation. Expression of the AT variants in these cells abolished processing of provWF as demonstrated by the absence of mature form. Because of the demonstrated selectivity of AT-PDX toward furin (14), we believe that the variants used in this study also target this convertase in hEK293 cells, although the properties of these variants to other SPCs remain to be evaluated. Interestingly, the biochemical behavior of the variants observed in vitro, i.e., partitioning toward the substrate pathway, was also observed in cellulo as demonstrated by secretion of the cleaved form in the medium of transfected cells.

In conclusion, our results demonstrate that introduction of different furin recognition sequences within the P6–P1 region of the RSL of AT affected the general behavior of the resulting serpins. We show that furin recognition motifs that contain the essential molecular determinants can increase the association
FIG. 7. Model of the hydrophobic area surrounding Leu$^{353}$ of RSL in the cleaved form of antitrypsin. A, view of the general structure of cleaved antitrypsin, based on the crystallographic atomic coordinates reported for antitrypsin-trypsin complex (17), showing the backbone and some residues of molecule in stick representation. B, enlargement of the area indicated by the rectangle in A. This view shows the residues of helix B (hB, yellow) and the peptide backbone of $\beta$-strand s3A, s4A, and s5A (white) that surround Leu$^{353}$ (pink) of the RSL. Side chains of the P8–P1 residues of the RSL are shown (green). The side view (C) and top view (D) of the hydrophobic pocket into which Leu$^{353}$ is inserted are shown. The models were based on the coordinates of Protein Data Bank file 1EZX.

![Diagram of hydrophobic area surrounding Leu$^{353}$ of RSL](image)

FIG. 8. Free energy qualitative profiles for the inhibition of furin by AT reactive site loop variants based on the mechanism depicted in Scheme 1. The curved lines indicate energy barriers that cannot be defined using the kinetic constants in Table I. Free energy profiles for the reaction of furin with AT variants, for the case where the predominant pathway for the reversibility of the covalent inhibited complex (under conditions where [serpin]$_{I}$ << $K_{c}$) is through reversion to EI$^{*}$ leading to the release via the substrate pathway of cleaved serpin (i.e. $k_{1}$, $k_{-1}$, and $k_{s} = k_{-s}$; see Scheme 1). Different energy barriers reflecting the transition state of complex formation EI$^{*}$ represent the reaction between the serpine-proteinase pairs: AT-WT- elastase (dotted line), AT-PDX or AT-EK1 with furin (broken line), AT-EK4, AT-EK3, or AT-EK2 (where $k_{s} \rightarrow \infty$) with furin (solid line). Under these assumptions (where $k_{1}$ is constant), variations in $k_{s}$ represent variations in the stability of the transition state, relative to the full insertion loop in EI$, for the conversion of EI$^{*}$ to EI$^{*}$ (see “Discussion”); and variations in $K_{c}$ reflect variations in the stability of the final inhibited complex, with respect to the $E + I$ ground state. Variations in $k_{s}^{\text{rate}}$ represent the rate of formation of the covalent acyl-enzyme intermediate EI$. The EI$^{*}$ ground states for each serpine-proteinase pair were arbitrarily defined because the ground state level is not established. Free energies were calculated from SI in Table I using the relation $\Delta G^{\circ} = -RT\ln(k_{s}^{\text{rate}}/k_{s}^{\text{rate}})$. rate $k_{s}^{\text{rate}}$ of AT for furin. In particular, the P6 residue was shown to be very critical for the formation of a long-lived serpin-proteinase complex. Indeed the replacement of the wild type Leu in P6 by an Arg prevents full loop insertion of the RSL into $\beta$-sheet as s4A and the formation of a stable, long-lived EI$^{*}$ complex as evidenced by the rapid release of the cleaved serpins. Accordingly, the data obtained from the study of these mutations indicate that an acyl-enzyme intermediate may be a common species from which the partitioning to inhibitor or substrate pathway occurs. It is interesting to note that our results parallel those recently observed on another protease inhibitor, eglin C, a member of the potato chymotrypsin inhibitor family of serine protease inhibitors (10). Indeed, eglin C variants where positions P1, P4, and P6 are replaced by Arg exhibited temporary furin inhibition followed by proteolytic cleavage with no significant variations in $k_{s}^{\text{rate}}$. Moreover, replacement of P2 residues in both inhibitors by Lys also eventually led to the cleavage of the proteins. Although it could be argued that interactions between furin and the P6 Arg side chain could be responsible for their substrate-like behavior toward furin, we believe that for serpin variants it is more the intrinsic property of the introduced Arg that will influence how the RSL gets inserted into the s4A sheet with the resulting effects of labile complex formation and ultimately their proteolysis.

Designing optimal furin recognition sites using existing inhibitors has been the focus of numerous studies. The result of these efforts leads us to believe that to produce efficacious furin inhibitors having subnanomolar affinities, additional determinants will have to be identified outside the immediate vicinity of the recognition pocket of the enzyme. Elucidation of these determinants may be realized by randomly replacing residues of serpins or other types of inhibitors within or outside their RSL and evaluating their resulting inhibitory properties. Other approaches such as affinity labeling and mapping of furin sites interacting with photoreactive peptides can also be considered. These ongoing strategies will yield crucial information to the conception of furin and other subtilisin-like precursor convertase inhibitors.

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