Inhibition of Soluble Recombinant Furin by Human Proteinase Inhibitor 8*

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Furin is a ubiquitous prototypical mammalian kexin/subtilisin-like endoproteinase that is involved in the proteolytic processing of a variety of proteins in the exocytic and endocytic pathways, with cleavage occurring at the C terminus of the minimal consensus furin recognition sequence Arg-Xaa-Xaa-Arg. In this study, human proteinase inhibitor 8 (PI8), a widely expressed 45-kDa ovalbumin-type serpin that contains two sequences homologous to the minimal sequence for recognition by furin in its reactive site loop, was tested for its ability to inhibit a recombinant soluble form of human furin. PI8 formed an SDS-stable complex with furin and inhibited its amidolytic activity via a two-step mechanism with a \( k_{\text{on}} \) of 6.5 \( \times 10^6 \) M\(^{-1}\) s\(^{-1}\) and an overall \( K_i \) of 53.8 pM. Thus, PI8 inhibits furin in a rapid, tight binding manner that is characteristic of physiological serpin-proteinase interactions. PI8 is not only the first human ovalbumin-type serpin to demonstrate inhibitory activity toward furin, but it is also the first significant inhibitor of furin identified that is not a serpin reactive site loop mutant, either naturally occurring or engineered.

The mammalian serine proteinase inhibitors, or serpins, are a superfamily of proteins that regulate proteolytic events in a wide variety of physiological processes including but not limited to blood coagulation, viral and parasite pathogenicity, intracellular proteolysis, and tumor suppression (1). Serpins inhibit their target proteinases by forming a 1:1 stoichiometric complex with the active site of the proteinase, which is in most cases resistant to denaturants (2). Serpins are composed of three \( \beta \)-sheets surrounded by eight \( \alpha \)-helices and a reactive site domain that is highly divergent among serpin family members and exists as a stressed loop with a canonical conformation that confers the optimal conformation for high affinity association with the substrate binding cleft of the cognate proteinase (3–6).

1. Residues within the reactive site loop are numbered analogous to substrates as follows: \( P_{-n}, \ldots, P_{-2}, P_{-1}, P_{+1}, -P_{-1}, -P_{+2}, \ldots, P_{+n} \), with cleavage occurring at the \( P_{n}-P_{n+1} \) bond.

2. The abbreviations used are: PAI-2, plasminogen activator inhibitor-2; SCCA, squamous cell carcinoma antigen; PI, proteinase inhibitor; HIV-1, human immunodeficiency virus-1; pERTKR-MCA, Pyr-Arg-Thr-Lys-Arg4-methylcoumaryl-7-amide; PAGE, polyacrylamide gel electrophoresis; \( \alpha_1 \)-AT, \( \alpha_1 \)-antitrypsin; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

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1851
we demonstrate that PI8 inhibits furin in a rapid, tight binding manner that is characteristic of physiological serpin-protease interactions.

**EXPERIMENTAL PROCEDURES**

**General Kinetic Methods**—Recombinant human furin was prepared from a vaccinia virus construct (20). Recombinant human PI8 was prepared as described (21). The $K_m$ for furin and the fluorogenic substrate Pyr-Arg-Thr-Lys-Arg-4-methylcoumaryl-7-amide (pERTKR-MCA, Bachem) as well as the catalytically active concentration of furin were determined as described previously (23). The $K_m$ for furin and pERTKR-MCA was determined to be 3.2 μM. Active site-titrated furin was used to determine the amount of PI8 needed for a 1:1 molar binding stoichiometry for the determination of kinetic constants. Furin (1.25 nm) was mixed with increasing amounts of PI8 in a total volume of 100 μl in 100 mM HEPES (pH 7.5) containing 0.5% Triton X-100 and 1 mM CaCl$_2$. Reactants were incubated for 30 min at 37 °C, and pERTKR-MCA was added to a final concentration of 50 μM. The enzymatically released 7-amido-4-methylcoumarin was then detected at 25 °C using an SLM Instruments SLM-8000 spectrofluorimeter with an excitation wavelength of 370 nm and an emission wavelength of 460 nm. The data were used to plot the enzymatic rate of substrate hydrolysis against the amount of PI8 used in the reaction. Linear regression to the stoichiometry with furin.

**Slow Binding Inhibition Kinetics**—Inhibition progress curves were obtained under pseudo-first order conditions by incubating the reactants in 0.3 ml of the same buffer used for the titration of PI8. Reactions were started by the addition of enzyme to a solution containing the fluorogenic substrate and the appropriate inhibitor concentration. Reactions for each experiment were started within 30 s, and the enzymatic production of 7-amido-4-methylcoumarin was detected as described earlier. The final concentrations of the reactants were 2 nM furin, 100 μM pERTKR-MCA, and 4, 8, 12, and 16 nM PI8. Spontaneous substrate hydrolysis was measured in separate experiments and determined to be negligible. The reactions were allowed to proceed until steady-state velocity was attained, and the data were fitted to the integrated rate equation for slow binding inhibition (24)

$$A = v_f + (v_o - v_f) \frac{1 - e^{-k't}}{k'} + A_o \quad \text{(Eq. 1)}$$

by nonlinear regression using UltraFit 3.0 software (Biosoft) to obtain values for the initial velocity ($v_o$), the steady-state velocity ($v_f$), the initial fluorescence ($A_o$), and the apparent first order rate constant ($k'$) for the establishment of steady-state equilibrium of the proteinase-inhibitor complex. The data obtained from nonlinear regression analysis were then used in various graphical transformations (25–29) to obtain the inhibition and rate constants for the interactions of PI8 with recombinant human furin.

**Detection of SDS-Stable Furin-PI8 Complexes**—Antibodies against recombinant human PI8 were generated in rabbits (30), and the IgG fraction was purified by protein A-Sepharose column chromatography. Furin and PI8 were incubated for 15 min at 37 °C, and the reaction mixtures were subsequently subjected to 10% SDS-PAGE under reducing conditions (31) and electrophoretically transferred to a nitrocellulose membrane in 10 mM CAPS (pH 11) buffer containing 10% methanol. The membrane was blocked with 1% nonfat dry milk in Tris-buffered saline containing 0.02% azide, and complexes were detected by incubating the membranes with rabbit anti-PI8 IgG, followed by incubation with $^{125}$I-labeled protein A and autoradiography.

**RESULTS**

**Inhibition of Human Furin by PI8**—Preliminary studies indicated that the interaction between furin and PI8 obeyed slow binding inhibition kinetics, as the amidoelastic activity of furin inhibited by PI8 attained steady-state equilibrium and the data were successfully fitted to Equation 1. On average, ten PI8 molecules were required to form a stable inhibitory complex with one molecule of furin, as determined by titration. The kinetic characterization of the inhibition of furin by PI8 was performed using PI8 concentrations ranging from two to eight times the molar concentration of furin. A family of inhibition progress curves representative of the interaction between furin and PI8 at the chosen PI8 concentrations is shown in Fig. 1. As expected, steady-state equilibrium was achieved more readily as the concentration of PI8 in the reaction mixture increased. Data obtained from the inhibition progress curves were fitted to Equation 1 by nonlinear regression analysis, and the results indicated that the initial velocity, $v_o$, was inversely proportional to the concentration of PI8 for each set of progress curves. This suggests that the slow onset of the inhibition of furin by PI8 follows the two-step mechanism

$$P \frac{k_1[I]}{k_1} \frac{k_2(\text{slow})}{k_2} \frac{\text{PI}}{k_1}$$

where a loose proteinase-inhibitor (PI) complex is rapidly formed, followed by a slow isomerization to the tight PI’ complex (25). This observation was confirmed by plotting $v_{\text{max}}/v_o$ against the PI8 concentration, which indicated a linear relationship with a positive slope (data not shown). The $K_i$ for the formation of the initial loose complex was calculated from the slope of the line using the relationship $v_{\text{max}}/v_o = K_m[I]/[S][K_i + (1 + K_m[S])]$, from which $K_i$ was calculated to be $6.5 \pm 0.1 \times 10^{-5}$ M$^{-1}$ s$^{-1}$ (n = 4). In addition, the apparent first order rate constant $k'$ was found to increase as PI8 concentration increases, which is consistent with the proposed mechanism.

The overall second order association rate constant $k_{\text{assoc}}$ was determined by plotting $\log([P]_o - [P]_i)$ as a function of time, where $[P]_o = v_o/k'$ and $[P]_i$ is the fluorescence measured at various times between 0 and 20 min for individual progress curves (data not shown) (28). The slope of the lines obtained is equal to $-0.43[I][k_{\text{assoc}}/1 + [S][K_i]$, from which $k_{\text{assoc}}$ was calculated to be $6.5 \pm 0.1 \times 10^{-5}$ M$^{-1}$ s$^{-1}$ (n = 4). The overall inhibition constant $K_i$ was determined from plots of both $v_{\text{max}}/v_o$ and $(v_o - v_f)/v_o$ versus PI8 concentration (data not shown). The slopes of these plots are equal to $K_i/[S][K_i']$, from which $K_i'$ was calculated to be $53.8 \pm 10.4$ pm (n = 4). To determine the rate constant for the reverse isomerization step $k_{-2}$ of the furin-PI8 tight complex, a plot of $k'$ against $v_o/v_f$ was generated. This plot was linear (data not shown), and $k_{-2}$
shown) to the hyperbolic equation (24) $k$ suggested earlier (29). The $y$ values between $k_2$ and to justify the use of a hyperbola to describe the relationship interaction of furin and PI8 occurs by the suggested mechanism used to determine a value for $k_2$ as described for a two-step binding mechanism.

Values for $k_2$ from Equations 1 and 2 that is linear and crosses the positive $y$ axis at a point approximately equal to $1/k_2$, as described for a two-step binding mechanism.

$$
\frac{1}{[\text{PI8}]} = \frac{[1]}{k} + \frac{[\text{PI8}]}{K_i}
$$

(Eq. 2)

by nonlinear regression analysis. Using this method, $k_2$ was estimated to be $3.3 \pm 0.2 \times 10^{-3} \text{ s}^{-1} (\eta = 4)$. To verify that the interaction of furin and PI8 occurs by the suggested mechanism and to justify the use of a hyperbola to describe the relationship between $k'$ and [PI8], a double-reciprocal plot of $1/(k' - k_2)$ versus $1/[\text{PI8}]$ was generated (Fig. 2) using the values obtained from Equations 1 and 2 that is linear and crosses the positive $y$ axis at a point approximately equal to $1/k_2$ for the mechanism suggested earlier (29). The $y$ intercept of the plot in Fig. 2 was used to determine a value for $k_2$ of $2.1 \pm 0.8 \times 10^{-3} \text{ s}^{-1} (n = 4)$, which is reasonably close to the value of $k_2$ determined by Equation 2. More importantly, the plot in Fig. 2 justifies the manipulation of data and determination of kinetic constants according to the suggested mechanism.

Formation of a PI8-Furin SDS-Stable Complex—Complex formation between furin and PI8 was visualized by Western blotting using rabbit anti-human PI8 IgG. As shown in Fig. 3, formation of a PI8-Furin SDS-stable complex was allowed to form, subsequently boiled, reduced, and subjected to 10% SDS-PAGE and immunoblotting with rabbit anti-human PI8 IgG. Lane 1, furin; lane 2, furin + PI8; lane 3, PI8.

**DISCUSSION**

In the present study, we have performed a detailed kinetic analysis of the inactivation of human furin by PI8. PI8 inhibited furin via a two-step mechanism characterized by the rapid formation of an initial loose complex followed by a slow isomerization to a tight, stable complex that was visualized by SDS-PAGE followed by Western blotting. The apparent molecular mass of the complex was approximately 225 kDa, which is significantly higher than the predicted mass of 140 kDa. Although the reason for the anomalously high molecular mass of the complex is unknown and will require further investigation, the aberrant migration may arise either from aggregation of furin-PI8 complexes or incomplete denaturation of the complex by SDS. It is unlikely, however, that this apparently higher molecular mass occurs as a result of an alternative binding stoichiometry, because furin has only one catalytic center and PI8 has only one reactive site loop to facilitate the interaction. A second immunoreactive species migrating at approximately 42 kDa was seen in lane 2 of Fig. 3, which most likely represents PI8 cleaved as a result of the interaction with furin. The overall inhibition constant for the inactivation of furin by PI8 was 53.8 nM, indicating that PI8 is a potent inhibitor of this protease. The initial loose complex of furin and PI8 had a $K_i$ of 7.2 nM, which is similar to the $K_i$ values of 8 and 6.6 nM for the inhibition of plasmin and chymotrypsin by $\alpha_2$-antiplasmin, respectively (26). The furin-PI8 loose complex is converted to the tight complex at a rate of $3.3 \times 10^{-3} \text{ s}^{-1}$, which is comparable with the rates reported for chymotrypsin-$\alpha_2$-antiplasmin and plasmin-$\alpha_2$-antiplasmin complexes (26). In addition, the $k_{assoc}$ for furin and PI8 was determined to be $6.5 \times 10^{5} \text{ M}^{-1} \text{ s}^{-1}$, which was lower than the rate of inhibition of subtilisin A by PI8 ($1.2 \times 10^{6} \text{ M}^{-1} \text{ s}^{-1}$) (21) but exceeded the rates of inhibition of plasma kallikrein by C1-inhibitor ($6.9 \times 10^{5} \text{ M}^{-1} \text{ s}^{-1}$) (32) and human thrombin and coagulation factor Xa by PI8 ($1.0 \times 10^{6} \text{ M}^{-1} \text{ s}^{-1}$ and $7.5 \times 10^{5} \text{ M}^{-1} \text{ s}^{-1}$, respectively) (21), as well as the rate of inhibition of granzyme B by CrmA ($2.9 \times 10^{5} \text{ M}^{-1} \text{ s}^{-1}$) (33). These comparisons indicate that the kinetic constants for the inhibition of furin by PI8 are of physiological significance. Furthermore, PI8 is the only ovalbumin-type serpin, as well as the only naturally occurring intracellular human serpin not associated with a disease state, demonstrated to be a significant inhibitor of furin. Previously, only peptide chloromethylketones and an $\alpha_1$-antitrypsin ($\alpha_1$-AT) variant have been described as significant inhibitors of furin. $\alpha_1$-AT Portland is an engineered variant of $\alpha_1$-AT Pittsburgh (34) that carries an additional Ala$^{255}$ $\rightarrow$ Arg mutation in its reactive site domain to provide the minimal consensus sequence for efficient recognition and processing by furin (35). PI8 contains the sequence Arg$^{386}$-Asn$^{387}$-Ser$^{388}$-Arg$^{389}$ at the P$_4$-P$_1$ positions in the reactive site domain that, based upon sequence alignment, is presumably recognized by the substrate.
binding cleft of furin in this interaction. Interestingly, P18 contains a second sequence Arg<sup>339</sup>-Cys<sup>340</sup>-Ser<sup>341</sup>-Arg<sup>342</sup> at the P<sub>1</sub>–P<sub>4</sub> positions in the reactive site domain, which may be involved in the interaction of P18 with furin or another mammalian convertase. The precise sequence in the P18 reactive site domain involved in the interaction between furin and P18 is presently unknown.

In order for P18 to inhibit furin in vivo, P18 must presumably enter the secretory pathway. The ovalbumin-type serpins PAI-2, SCCA, and maspin each lack a cleavable N-terminal signal sequence, but all can be found extracellularly. PAI-2, SCCA, and maspin each lack a cleavable N-terminal signal sequence, but all can be found extracellularly. PAI-2, as well as two forms of P18 mRNA, also seen with yeast invertase and human gelsolin, it is not unreasonable to suggest that a fraction of the P18 synthesized may be secreted and interact with furin under normal conditions or in response to specific stimuli. Therefore, P18 may regulate the activity of furin and, in turn, such events as pro-protein processing and virus infectivity by its secretion, either through facultative polypeptide translocation facilitated by hydrophobic interactions or by alternative transcriptional initiation to produce mRNA encoding P18 that contains a cleavable N-terminal signal sequence.

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