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Human Proteinase Inhibitor 9 (PI9) Is a Potent Inhibitor of Subtilisin A

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Serine proteinase inhibitors function as regulators of serine proteinase activity in a variety of physiological processes. Proteinase inhibitor 9 (PI9) is a 42 kDa member of the ovalbumin family of serpins that is expressed in placenta, lung, and cytotoxic lymphocytes. In this study, we have described the inhibitory mechanism of recombinant human PI9 towards the bacterial endoproteinase subtilisin A. PI9 inhibited the amidolytic activity of subtilisin A via a rapid, single step mechanism with an equilibrium inhibition constant of 3.6 pM and an overall second-order association rate constant of 2.4 $\times$ 10^6 $M^{-1}s^{-1}$, which is the strongest inhibitory mechanism of PI9 that has been described. The inhibitory action of PI9 towards subtilisin as a model proteinase may yield some indication of potential proteinases that may be regulated by PI9 *in vivo.* © 1997 **Academic Press**

The mammalian serine proteinase inhibitors, or serpins, are a superfamily of proteins that range from 40 to 60 kDa in molecular mass, resemble α_1-proteinase inhibitor in overall structure and include a number of homologous proteins such as α_1-antichymotrypsin, α_2-antiplasmin, antithrombin III, and plasminogen activator inhibitors 1 and 2 (1). Serpins regulate a wide variety of physiological processes that involve proteinase activity including, but not limited to, blood coagulation, complement activation, cell migration and differentiation, intracellular proteolysis, and tumor suppression (2). Serpins inhibit their target proteinases usually through a two-step process by forming a loose 1:1 stoichiometric complex with the active site of the proteinase, followed by isomerization to a tight complex which is, in most cases, resistant to denaturants (3). Serpins can also inhibit their target proteinases through a single-step process. Serpins are composed of three β-sheets surrounded by eight α-helices, and their proteinase-inhibitory specificity is determined by the P_1–P_1'[2] residues located within the reactive site domain (1, 4, 5). The reactive site domain 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, with the P_1–P_1' residues acting as a pseudosubstrate for the target proteinase (6). Unlike a typical substrate, the serpin has the ability to form a tight complex with the proteinase that may be essential for its inactivation (4, 7–9). Most serpins can interact with more than one proteinase *in vitro,* but the affinity of such interactions must be determined in order to suggest physiological relevance.

Ovalbumin represents the parent prototype of a unique family of serpins that lack a typical cleavable amino-terminal signal sequence, but have been found to reside intracellularly (10, 11), or both intracellularly and extracellularly (12-15). Therefore, it can be inferred that the functions of members of the ovalbumin family of serpins may not be strictly confined to the cytoplasm. The lack of N- and C-terminal extensions and the presence of a serine residue at the penultimate position in the amino acid sequence is also characteristic of the ovalbumin-type serpins. The serpins previously classified as members of the ovalbumin family are plasminogen activator inhibitor-2 (PAI-2)[3] (15), an

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[2] Residues within the reactive site loop are numbered analogous to substrates as follows: $P_n \cdots P_3$–P_2–P_1–P_1'–P_2'–$P_3' \cdots P_n'$, with cleavage occurring at the P_1–P_1' bond.

[3] Abbreviations: PAI-2, plasminogen activator inhibitor-2; EI, elastase inhibitor; SCCA, squamous cell carcinoma antigen; PI6, proteinase inhibitor 6; PI8, proteinase inhibitor 8; PI9, proteinase inhibitor 9; SCCA2, squamous cell carcinoma antigen 2; CrmA, cytokine response modifier A; kDa, kilodalton; *p*-NPGB, *p*-nitrophenyl-*p*-guanidinobenzoate; Suc-AAPF-*p*NA, Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide; DMF, dimethyl formamide; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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elastase inhibitor (EI) isolated from monocyte-like cells (16, 17), squamous cell carcinoma antigen (SCCA) (13), maspin (18), proteinase inhibitor 6 (PI6) (19-21), two serpins initially identified by this laboratory, proteinase inhibitor 8 (PI8) and proteinase inhibitor 9 (PI9) (22), squamous cell carcinoma antigen 2 (SCCA2) (23), and bomapin (24). PI6, PI8 and PI9 are unique among the mammalian ovalbumin-type serpins in that they contain a cysteine residue in the P_1' position within the reactive site domain, which is also present in the cowpox virus serpin CrmA (22). It has been demonstrated that individual mammalian ovalbumin-type serpins can inhibit a variety of prototypic serine proteinases (19, 20, 23) and, in some cases, can exhibit cross-class specificity and inhibit cysteine proteinases (25). Thus, serpins may function not only by distinctly different mechanisms using a variety of kinetic parameters to regulate proteolysis (26), but also by interacting with distinctly different types of proteinases. Although most ovalbumin-type serpins exhibit defined proteinase inhibitory activity, the true physiological targets of these serpins have not yet been identified.

PI9 is 42 kDa serpin composed of 376 amino acids whose transcript can be found in placenta and, to a lesser extent, lung tissues (22). PI9 is unique among members of the ovalbumin family of serpins, as it contains a glutamic acid residue in the P_1 position within the reactive site domain. Another serpin that has an acidic residue at this position is CrmA, which contains an aspartic acid residue. CrmA has been demonstrated to be an inhibitor of interleukin-1β converting enzyme (ICE) and granzyme B (27-30). PI9 has also been found in the cytosol of cytotoxic lymphocytes and has been demonstrated to be an inhibitor of granzyme B (11). In this study, we have performed a detailed kinetic analysis of the mechanism of inhibition of the *Bacillus subtilis* endoproteinase subtilisin A by PI9.

MATERIALS AND METHODS

Recombinant human PI9 was prepared as described (22). Subtilisin A was generously provided by Dr. Lene Adamczewski from Novo Nordisk A/S (Bagsvaerd, Denmark). Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-*p*NA) was obtained from Calbiochem-Novabiochem International (San Diego, CA). α_2-macroglobulin was purchased from Boehringer Mannheim (Indianapolis, IN). Porcine trypsin was obtained from Novo Nordisk (Bagsvaerd, Denmark). Hepes buffer, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), dimethyl formamide (DMF) and *p*-nitrophenyl-*p*-guanidinobenzoate (*p*-NPGB) were purchased from Sigma Chemical Co. (St. Louis, MO). UltraFit 3.0 software was purchased from Biosoft (Ferguson, MO).

General kinetic methods. The concentrations of catalytically active porcine trypsin was determined by titration with *p*-NPGB as previously described (31, 32). Active site-titrated trypsin was used to titrate α_2-macroglobulin, which was then used to determine the catalytically active concentration of subtilisin A. Active site-titrated subtilisin A was used to determine the amount of PI9 necessary for a 1:1 molar binding stoichiometry for the determination of kinetic constants. The reactive concentration of PI9 was measured by titration with subtilisin A by incubating increasing amounts of PI9 with enzyme (5 nM) in a total volume of 180 μl of 20 mM Hepes (pH 7.5)/0.15 M NaCl/0.01% BSA in individual wells of a microtitration plate previously blocked with buffer containing 0.1% BSA. The reactants were incubated for 30 min at 37 °C and residual amidolytic activity was measured by adding 20 μl of 2 mM Suc-AAPF-*p*NA in 0.1 M Hepes/0.5 M NaCl/20% DMSO for a final concentration of 0.2 mM and monitoring the monitoring ΔA_{405}/min using a UVmax microplate reader (Molecular Devices, Sunnyvale, CA). The data were used to plot the enzymatic rate of substrate hydrolysis as a function to the amount of PI9 added to the reaction well. Linear regression to the *x*-axis was used to calculate the precise amount of PI9 required to form a 1:1 stoichiometric complex. Enzyme-substrate catalytic constants were measured in 20 mM Hepes (pH 7.5)/0.15 M NaCl/0.01% BSA/2% DMSO/1% DMF at 25 °C. Data were fitted to the Michaelis-Menton equation using UltraFit software to determine values for K_m and k_{cat}. The catalytic constants for subtilisin A and the chromogenic substrate Suc-AAPF-*p*NA were $K_m = 0.195$ mM and $k_{cat} = 379\ s^{-1}$.

Slow-binding inhibition kinetics. Inhibition progress curves were obtained under pseudo-first-order conditions by incubating the reactants in 0.5 ml of the buffer used to determine the catalytic constants at 25 °C. Polystyrene cuvettes were previously blocked with 20 mM Hepes (pH 7.5)/0.15 M NaCl/0.1% BSA, and reactions were started by adding the enzyme to a solution containing the chromogenic substrate and appropriate inhibitor concentration. Reactions for each experiment were started within 30 s and the cuvettes were placed in a Beckman DU-65 spectrophotometer equipped with a six-cell cuvette holder to allow simultaneous monitoring of multiple reactions at 405 nm. The final concentrations of the reactants were 0.1 nM subtilisin A, 0.8 mM Suc-AAPF-*p*NA and 0.5, 1.0, 1.5, 2.0, and 2.5 nM PI9. 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 (33),

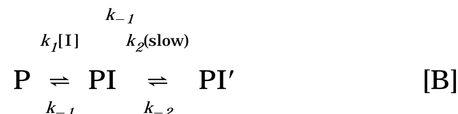
$$A = v_s t + (v_o - v_s)\frac{1 - e^{-k't}}{k'} + A_o, \qquad [1]$$

by nonlinear regression using UltraFit software to obtain values for the initial velocity (v_o), the steady-state velocity (v_s), the initial absorbance (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 (33-38) to obtain the inhibition and rate constants for the interaction of PI9 with subtilisin A.

RESULTS

Preliminary studies indicated that the interaction between PI9 and subtilisin A obeyed slow-binding inhibition kinetics, as the amidolytic activity of subtilisin A inhibited by PI9 attained steady-state equilibrium and the data were successfully fitted to Eq. 1. There are three mechanisms that can describe the slow onset of inhibition (34):

$$\mathrm{P} \underset{k_{-1}}{\overset{k_1[\mathrm{I}]}{\rightleftharpoons}} \mathrm{PI} \qquad [A]$$



$$\mathrm{P} \underset{k_{-1}}{\overset{k_1[\mathrm{I}]}{\rightleftharpoons}} \mathrm{PI} \underset{k_{-2}}{\overset{k_2(\mathrm{slow})}{\rightleftharpoons}} \mathrm{PI'} \qquad [B]$$

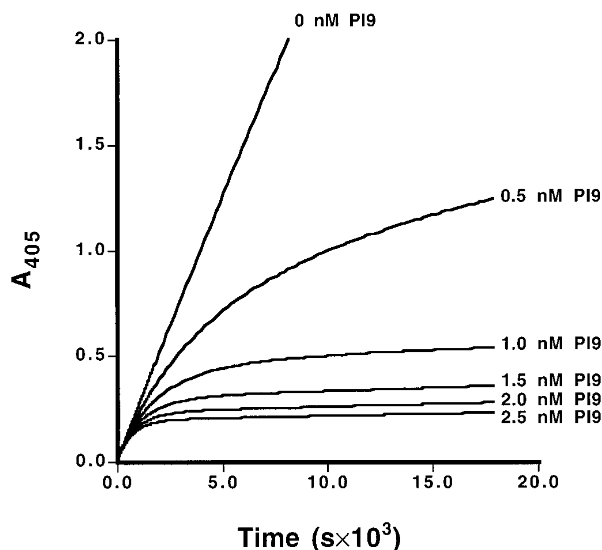


FIG. 1. Progress curves from slow-binding kinetics for the inhibition of subtilisin A by PI9. Subtilisin A (0.1 nM) was reacted with 0, 0.5, 1.0, 1.5, 2.0, and 2.5 nM PI9 in 20 mM Hepes (pH 7.5)/0.15 M NaCl/0.01% BSA/2% DMSO/1% DMF at 25 °C in the presence of 0.8 mM Suc-AAPF-*p*NA. The reactions were monitored continuously for 5 hours and the data were fitted to Eq. 1 to generate values for the variables v_o, v_s, A_o and k'.

$$P \underset{k_{-1}}{\overset{k_1(\text{slow})}{\rightleftharpoons}} P' \underset{k_{-2}}{\overset{k_2[1]}{\rightleftharpoons}} PI'. \qquad [C]$$

In mechanism A, the proteinase (P) binds to the inhibitor (I) in a single step to form an PI complex. In mechanism B, a loose PI complex is rapidly formed, followed by the slow isomerization to the tight PI′ complex. In mechanism C, the proteinase is assumed to exist in two forms, and active form P and an inactive form P′, which is accessible to the inhibitor. The isomerization of P to P′ is followed by the rapid formation of a tight PI′ complex. These different mechanisms can be distinguished by graphical transformations of the values obtained from the slow-binding kinetic approach (33). The kinetic characterization of the inhibition of subtilisin A by PI9 was performed with PI9 concentrations ranging from 5 to 25 times the molar concentration of subtilisin A. A family of inhibition progress curves representative of the reaction between subtilisin A and PI9 at its selected concentrations is shown in Fig. 1, and data obtained from the progress curves were fitted to Eq. 1 by nonlinear regression analysis. The results indicated that the initial velocity, v_o, was independent of the inhibitor concentration for each set of progress curves, suggesting that the inhibition of subtilisin A by PI9 follows a single-step mechanism described by mechanism A (33). This observation was confirmed by plotting v_{max}/v_o against the PI9 concentration, which had a slope of zero (data not shown). This eliminates the possibility that inhibition occurs according to mechanism B, since this mechanism requires that the initial velocity is dependent upon the inhibitor concentration. Furthermore, a plot of k' versus PI9 concentration demonstrated that k' is proportional to PI9 concentration, eliminating mechanism C (data not shown). Therefore, the data were treated in various ways to determine the kinetic constants for the inhibition of subtilisin A by PI9 according to mechanism A.

The apparent second-order association rate constant k_1 for the complex of PI9 and subtilisin A was calculated by fitting the data obtained from nonlinear regression analysis to the equation (33)

$$k' = k_{-1} + \frac{k_1[\text{I}]}{1 + \frac{[\text{S}]}{K_m}}, \qquad [2]$$

which predicts a linear relationship between k' and [PI9] (data not shown). From the slope of the line, k_1 was calculated to be 2.4 (±0.1) × 10^6 $M^{-1}s^{-1}$ (n = 5). Due to the degree of error present and its apparent small value, k_{-1} was not determined from the y-intercept of the line, but rather, directly from the slope of the line generated using the relationship $k' = k_{-1}(v_o/v_s)$ (data not shown) (33). The value of k_{-1} was calculated to be 7.9 (±0.1) × 10^{-6} s^{-1} (n = 5). This translates to a half-life of 1.02 days for the PI9-subtilisin A complex.

The equilibrium inhibition constant K_i was determined by plotting values of v_{max} and v_s obtained from nonlinear regression analysis against PI9 concentration according to the relationship $v_{max}/v_s = 1 + [\text{I}]/K_{iapp}$ and $K_i = K_{iapp}/(1+[\text{S}]/K_m)$. From the slope of the line generated (data not shown), K_i was calculated to be 3.6 (±0.1) pM (n = 5). Furthermore, the value of K_i determined from the relationship $K_i = k_{-1}/k_1$ was equal to 3.3 (±0.1) pM, in close agreement with the previously calculated value of K_i. In order to verify that the interaction of PI9 and subtilisin A occurs by mechanism A and to justify the use of a linear relationship to describe the relationship between k' and [PI9], a double-reciprocal plot of $1/(k'-k_{-1})$ versus 1/[PI9] was created (Fig. 2). The line did not cross the positive y-axis and passed through the origin, as expected for inhibition according to mechanism A (38). In addition, the interaction of PI9 and subtilisin A did not result in the formation of an SDS-stable complex as visualized by SDS-PAGE (data not shown). This observation is also consistent with inhibitory mechanism A.

DISCUSSION

In the present study, we have performed a detailed kinetic analysis of the inactivation of the *B. subtilis* endoproteinase subtilisin A by PI9, which occurred via a single-step mechanism. The equilibrium inhibition

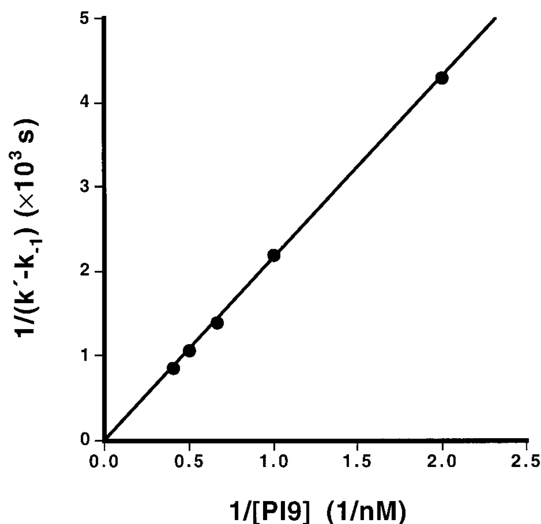


FIG. 2. $1/(k'-k_{-1})$ versus 1/[PI9] for the interaction of PI9 with subtilisin A. Values for k' were generated as described in the legend to Fig. 1. Values for k_{-1} were calculated from the slope of the line generated according to the relationship $k' = k_{-1}(v_o/v_s)$. The line does not cross the positive y-axis and passes through the origin, as described for a single-step binding mechanism according to mechanism A.

constant for the inactivation of subtilisin A by PI9 was 3.6 pM, indicating that PI9 is a potent inhibitor of this proteinase. The k_{assoc} for subtilisin A and PI9 was determined to be 2.4×10^6 $M^{-1}s^{-1}$, which exceeds the second-order association rate constants for the inhibition of plasma kallikrein by C1-inhibitor (6.9×10^4 $M^{-1}s^{-1}$) (39), factor Xa by PI6 (1.3×10^5 $M^{-1}s^{-1}$) (20), granzyme B by CrmA (2.9×10^5 $M^{-1}s^{-1}$) (30), as well as granzyme B by PI9 (1.7×10^6 $M^{-1}s^{-1}$) (11) and is comparable to the second-order association rate constant for the inhibition of trypsin by PI6 (20).

PI9 was unable to form an SDS-stable complex with subtilisin A, a proteinase that it rapidly and efficiently inactivates. The enzyme-inhibitor complex, as seen by SDS-PAGE, reflects a complex stabilized by an acyl-ester linkage between the active-site serine of the proteinase and the carbonyl of the P_1 residue of the cleaved serpin. However, evidence exists that acyl-ester linkage seen after treatment with SDS is most likely an artifact (2, 40, 41). In addition, PI9 appeared to be partially cleaved following incubation with subtilisin A. The inhibitory structure of serpins is conformationally unstable and the reactive site loop is highly susceptible to cleavage by proteinases that they do or do not inhibit. During the titration of PI9 with active site-titrated subtilisin A, it was observed that the PI9 preparation was not completely active on a basis of mass alone. The percent activity of PI9 was approximately 10% by titration with subtilisin A. This phenomenon was most likely not due to proteolytic inactivation of PI9, but rather, the disproportionate pathway that dictates the interaction of serpins with some proteinases. The exposed nature of the reactive site loop enables the serpin to act as either a substrate or an inhibitor for a proteinase, or both. This phenomenon has been observed for the interactions of α_1-antichymotrypsin and α_1-proteinase inhibitor with human mast cell chymase (42), C1-inhibitor with plasma kallikrein (43), and α_2-antiplasmin with trypsin and chymotrypsin (44) and is a recognized characteristic of some of the interactions that occur between proteinases and serpins. This suggests that several serpin molecules are required to inhibit a single proteinase molecule in order to overcome the inactivation of many of the serpin molecules during the reaction, and efficient inhibition can occur if the inhibitor is in excess of the enzyme. The presence of high levels of PI9 mRNA (22), as well as high levels of cytoplasmic PI9 antigen in placental tissue, as observed by immunohistochemical methods,[4] may be indicative of this phenomenon.

In this study, we have described the inhibitory mechanism of PI9 towards the amidolytic activity of subtilisin A. This interaction is currently the strongest inhibitory action described for PI9. The bacterial subtilisins are evolutionarily divergent from the trypsin-like serine proteinases (45) and are similar to the seven known mammalian dibasic processing endoproteinases, or convertases, of which furin is the prototype (for reviews see refs. 46–49). In separate experiments, PI9 was unable to inhibit the amidolytic activity of recombinant human furin, but it is entirely possible that PI9 does demonstrate inhibitory activity towards one of the other mammalian convertases.

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