Slow-Tight Binding Inhibition of Proteinase K by a Proteinaceous Inhibitor: Conformational Alterations Responsible for Conferring Irreversibility to the Enzyme-Inhibitor Complex

Jui Pandhare\textsuperscript{1,3}, Chandravanu Dash\textsuperscript{2,3}, Mala Rao\textsuperscript{3} and Vasanti Deshpande\textsuperscript{3}\textasteriskcentered

\textsuperscript{3}Division of Biochemical Sciences, National Chemical Laboratory, Pune-411 008, India

Present addresses: \textsuperscript{1}Metabolism and Cancer Susceptibility Section, \textsuperscript{2}HIV Drug Resistance Program, National Cancer Institute, Frederick, MD 21702, USA.

*Author to whom all correspondence should be addressed

Telephone: 91-20-589 3034, Fax: 91-20-588 4032, E-mail: vasanti@dalton.ncl.res.in

Running Title: Two Step Inhibition of Proteinase K by a Proteinaceous Inhibitor
ABSTRACT

The kinetics of slow-onset inhibition of Proteinase K by a proteinaceous alkaline protease inhibitor (API) from a *Streptomyces* sp. is presented. The kinetic analysis revealed competitive inhibition of Proteinase K by API with an IC$_{50}$ value $5.5\pm0.5 \times 10^{-5}$ M. The progress-curves were time-dependent consistent with a two-step slow-tight binding inhibition. The first step involved a rapid equilibrium for formation of reversible enzyme-inhibitor complex (EI) with $K_i$ value $5.2\pm0.6 \times 10^{-6}$ M. The EI complex isomerized to a stable complex (EI*) in the second step due to inhibitor induced conformational changes, with a rate constant $k_5$ $(9.2\pm1 \times 10^{-3}$ s$^{-1}$). The rate of dissociation of EI* ($k_6$) was slower $(4.5\pm0.5 \times 10^{-5}$ s$^{-1}$) indicating tight binding nature of the inhibitor. The overall inhibition constant $K_{i*}$ for two-step inhibition of Proteinase K by API was $2.5\pm0.3 \times 10^{-7}$ M. Time dependent dissociation of EI* revealed that the complex failed to dissociate after a time point and formed a conformationally altered, irreversible complex EI**. These conformational states of enzyme-inhibitor complexes were characterized by fluorescence spectroscopy. Tryptophanyl fluorescence of Proteinase K was quenched as a function of API concentration without any shift in the emission maximum indicating subtle conformational change in the enzyme, which is correlated to the isomerization of EI to EI*. Time dependent shift in the emission maxima of EI* revealed the induction of gross conformational changes, which can be correlated to the irreversible conformationally locked EI** complex. API binds to the active site of the enzyme as demonstrated by the abolished fluorescence of 5-iodoacetamidofluorescein labeled Proteinase K. The chemoaffinity labeling experiments lead us to hypothesize that the inactivation of Proteinase K is due to the interference in the electronic microenvironment and disruption of the hydrogen-bonding network between the catalytic triad and other residues involved in catalysis.
INTRODUCTION

An effervescence of research efforts has been expended in the design and synthesis of inhibitors of proteolytic enzymes not only to understand the active site structure and mechanism of these enzymes but also to help generating new therapeutic agents. Specific inhibitors of proteases have proved valuable in a number of applications ranging from mechanistic studies to possible therapeutic uses. Protein inhibitors of proteases are ubiquitously present in plants, animals and microorganisms (1). Fundamentally, proteinaceous inhibitors should serve as substrates for proteolysis rather than being their inhibitors. Elucidation of this paradox is the basis for the extensive research on structure-function relationship of proteinaceous inhibitors of proteases. The importance of proteolytic processes in the regulation of post-translational processing of precursor proteins and the involvement of proteases in intracellular protein metabolism and in various pathological processes have recently stimulated tremendous interest in studying the kinetic properties of naturally-occurring target oriented protease inhibitors.

Serine proteases are divided in trypsin-like and the subtilisin-like families, which have been independently evolved with a similar catalytic mechanism (2-6). The functional importance of catalytic triad and oxyanion hole in catalysis of serine proteases has been clearly established (7, 8). Proteinase K, the serine protease from subtilisin family is a highly active extracellular alkaline serine endopeptidase from *Tritirachium album* limber and named Proteinase K because of its ability to digest native keratin (9, 10). By virtue of its unusual stability at low concentrations of SDS and urea, Proteinase K has immense applications in basic and applied research. X-ray crystallographic studies have revealed that the catalytic triad of Proteinase K is formed by the residues His69, Asp39, Ser224, and a single free Cys73 residue is located below the functional His69 (11). During catalysis Ser224 functions as the primary nucleophile and His69 plays a dual role as proton acceptor and donor at different steps in the reaction. The Asp39 residue is known to participate in a complex hydrogen bond network with His69, thus bringing the His69 residue in the correct orientation to facilitate nucleophilic attack by Ser224. Determination of the mechanism of inhibition of this protease will provide better insights in understanding the mechanism of inhibition and will shed light on the protein-protein interactions at molecular level.
Serine proteases and their protein inhibitors have been the most intensively studied group of protein-protein complexes. An enormous number of known and partially characterized inhibitors of serine proteases from plants, animals and microorganisms have been documented and have been grouped in different families (1, 12). Among the proteinaceous inhibitors from microorganisms, the well-characterized inhibitors are from *Streptomyces* (13) and belong to the family: *Streptomyces* subtilisin inhibitor (SSI). The future development of these inhibitors for their potential application in therapeutics and biocontrol agents will undoubtedly depend on application of kinetic techniques that yield quantitative information about the behavior of the inhibitors. When the structure of inhibitor can be correlated with the true dissociation constants for their enzyme-inhibitor complexes, a systematic approach can be made towards the design of more effective inhibitors for a target enzyme using protein engineering. Delineating the inhibition mechanism and role of the reactive site residues of the inhibitors and understanding the binding efficiency will provide further insight for their potential application. Considering the physiological importance of the serine alkaline proteases and their role in various physiological and biotechnological processes, there is a lacuna in the studies on the kinetics of the mechanism of inhibition by their naturally occurring protein inhibitors.

Previously we have reported the isolation and purification of an alkaline protease inhibitor (API) from the extracellular culture filtrate of the *Streptomyces* sp. NCIM 5127 (14). The protein has been purified to homogeneity by ammonium sulfate precipitation, preparative polyacrylamide gel electrophoresis, and anion exchange chromatography. The biochemical characterization has revealed that API is a dimeric protein (*M*<sub>r</sub> 28kD) with five disulphide linkages. Chemical modification studies of API and its binding interaction with the alkaline protease from *Conidiobolus* sp. have revealed the presence of a tryptophan residue in the reactive site and a disulfide bond at or near the reactive site of the inhibitor (15). The biochemical and secondary structural analysis of the inhibitor have revealed that the API belongs to SSI family of inhibitors. Inhibitors belonging to the SSI family follow the standard mechanism of inhibition wherein the inhibitor acts as a highly specific substrate for limited proteolysis by the target enzyme (16). These inhibitors bind very tightly to enzyme in the manner of a good substrate and are cleaved very slowly. We present the first report of a proteinaceous alkaline protease inhibitor (API) exhibiting slow-tight binding inhibition against Proteinase K.
The steady state kinetics revealed a two-step inhibition mechanism and the conformational modes observed during the binding of inhibitor to the enzyme were conveniently monitored by fluorescence analysis. The mechanism of inactivation of Proteinase K by API was delineated and the kinetic parameters associated with the enzyme-inhibitor interaction were determined. The role of hydrogen bonding interactions in the inhibition mechanism of Proteinase K was deciphered by investigating the fluorescence of the IAF-labeled enzyme and a model for the mechanism of interactions has been proposed.
EXPERIMENTAL PROCEDURE

Materials

Purified Proteinase K from *Tritirachium album* limber, 5-Iodoacetamidofluorescein, and sAAPF-pNA were obtained from Sigma Chemical Co. All other chemicals used are of analytical grade.

Microorganism, Growth Conditions and Purification of API

The *Streptomyces* sp. was grown in liquid casein-starch medium for 96 h at 28°C on a rotary shaker at 200 rpm. The cells were separated by centrifugation and the cell free supernatant was checked for the presence of API. API was purified from the *Streptomyces* sp. as reported (14). Briefly, the extracellular culture filtrate containing API was concentrated by ultra filtration and purified by polyacrylamide gel electrophoresis using gel-X-ray film contact print technique. In the gel-X-ray film contact print technique, after electrophoretic resolution of the protein, a vertical strip of the gel was cut and incubated for 10 min in 0.1 M carbonate-bicarbonate buffer, pH 10.0 containing 0.5 mg/ml of the bacterial alkaline protease, subtilisin. The gel was overlaid on an equal sized X-ray film and the hydrolysis of gelatin was followed for 20 min at 37°C. The band corresponding to the inhibitory activity was excised and eluted by homogenization followed by vacuum filtration and purified further by DEAE cellulose chromatography.

Proteinase K Assay and Inhibition Kinetics

Proteolytic activity of Proteinase K was measured by assaying the enzyme activity using casein and the synthetic substrate sAAPF-pNA. Proteinase K (100 nM) was dissolved in Tris-HCl buffer, 0.05 M pH 8.5, containing 1mM CaCl₂. The reaction was initiated by the addition of 1 ml of casein (5 mg/ml) at 37°C for 30 min. The reaction was quenched by the addition of 2 ml of 5% acidified-TCA followed by incubating for 30 min at room temperature. The unhydrolyzed casein precipitate was separated by centrifugation and filtration through Whatmann No.1 filter paper. Absorbance of the TCA soluble products was measured at 280 nm. The enzymatic activity in the presence of the synthetic substrate is determined as described (17). One unit of protease activity was defined as the amount of enzyme that causes an increase of one absorbance unit at 280 nm.
nm for alkaline proteases. One protease inhibitor unit was defined as the amount of inhibitor that inhibits one unit of protease activity.

For initial kinetic analysis, the kinetic parameters for the substrate hydrolysis were determined by measuring the initial rate of enzymatic activity. The inhibition constant ($K_i$) was determined by Dixon (18) and Lineweaver-Burk's method. The $K_m$ value was also calculated from the double-reciprocal equation by fitting the data into Microcal Origin. For Lineweaver-Burk's analysis, Proteinase K (1 μM) was incubated with API at (1 μM) and (2.5 μM) and assayed at increased concentration of casein (1-10 mg/ml). In Dixon’s method proteolytic activity of Proteinase K (1 μM) was measured in the presence of (5 mg/ml) and (10 mg/ml) of casein, at concentrations of API ranging from 1-5 μM at 37°C for 30 min. The reciprocals of substrate hydrolysis (1/v) were plotted against the inhibitor concentration and the $K_i$ was determined by fitting the data using Microcal Origin.

For the progress curve analysis, assays were carried out in a 1 ml reaction mixture containing enzyme, substrate, and inhibitor at various concentrations. The reaction mixture contained Proteinase K (100 nM) in the required buffer and varying concentrations of API and casein (5 mg/ml). Reaction was initiated by the addition of Proteinase K at 37°C and the release of products was monitored at different time intervals at 280 nm. In each slow-binding inhibition experiment, five to six assays were performed with appropriate blanks. For the kinetic analysis and rate constant determinations, the assays were carried out in triplicates and the average value was considered throughout. Further details of the experiments are given in the respective figure legends.

**Evaluation of Kinetic Parameters**

Initial rate studies for reversible, competitive inhibition were analyzed as per Equation 1.

$$v = \frac{V_{\text{max}} S}{K_m (1 + I/K_i) + S}$$  \hspace{1cm} (1)

Where $K_m$ is the Michaelis constant, $V_{\text{max}}$ is the maximal catalytic rate at saturating substrate concentration [S], $K_i = (k_d/k_3)$ is the dissociation constant for the first reversible enzyme-inhibitor complex, and I is the inhibitor
concentration (19). The progress curves for the interactions between API and Proteinase K were analyzed using Equation 2 (20, 21).

\[ [P] = v_0 t + \frac{v_0 - v_s}{k} (1 - e^{-kt}) \]  \hspace{1cm} (2)

Where \([P]\) is the product concentration at any time \(t\), \(v_0\) and \(v_s\) are the initial and final steady-state rates, respectively, and \(k\) is the apparent first-order rate constant for the establishment of the final steady-state equilibrium. Corrections have been made for the reduction in the inhibitor concentration that occurs on formation of the enzyme inhibitor (EI) complex. This is because in case of tight binding inhibition, the concentration of EI is not negligible in comparison to the inhibitor concentration and the free inhibitor concentration is not equal to the added concentration of the inhibitor. The corrections of the variation of the steady-state velocity with the inhibitor concentrations were made according to Equation 3 and 4 as described by Morrison and Walsh (22).

\[ Q = \left( \frac{K_i' + I_t - E_t}{K_m + S} \right) + 4 \left( \frac{K_i' + I_t - E_t}{K_m + S} \right)^{1/2} \]  \hspace{1cm} (3)

\[ v_s = \frac{k_7 SQ}{2(K_m + S)} \]  \hspace{1cm} (4)

Where \(K_i' = K_i^* (1 + S/K_m)\), \(k_7\) rate constant for the product formation, \(I_t\) and \(E_t\) stands for total inhibitor and enzyme concentration, respectively.

The relationship between the rate constant of enzymatic reaction \(k\), and the kinetic constants for the association and dissociation of the enzyme and inhibitor was determined as per Equation 5.

\[ k = k_6 + k_5 \left( \frac{1 / K_i}{1 + (S / K_m) + (1 / K_i)} \right) \]  \hspace{1cm} (5)

The progress curves were analyzed by eqs. 2 and 5 using non-linear least-square parameter minimization to determine the best-fit values with the corrections for the tight binding inhibition. The overall inhibition constant is determined as given by Equation 6.
For the time-dependent inhibition, there exists a time range in the progress curves wherein formation of EI* is small. Within this time range it is possible to measure the effect of the inhibitor on \( v_0 \), i.e., to measure \( K_i \) directly. Values for \( K_i \) were obtained from Dixon analysis at a constant substrate concentration as described in Equation 7.

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}}S} \left(1 + \frac{1}{K_i}\right) \quad (7)
\]

The rate constant \( k_6 \), for the dissociation of the second enzyme-inhibitor complex was measured directly from the time-dependent inhibition. Concentrated Proteinase K and API were incubated in a reaction mixture to reach equilibrium, followed by large dilutions in assay mixtures containing near-saturating substrate. Proteinase K (1 mM) was pre-incubated with API (500 \( \mu \)M) in sodium-phosphate buffer, 0.05 M, pH 7.5. 5 \( \mu \)l of the pre-incubated sample was removed and diluted 5000-fold in the same buffer and assayed at 50°C using casein at (150 mg/ml) at different time intervals.

**Gel Filtration Analysis of the Enzyme-Inhibitor Complexes**

The quaternary structure of the enzyme-inhibitor complexes was monitored by size exclusion chromatography on Protein-Pak 300SW HPLC column (7.8 mm x 300 mm) using a Waters liquid chromatograph system. The column was equilibrated with potassium phosphate buffer 0.05 M, pH 7.5 before the analysis. For the formation of enzyme-inhibitor complex Proteinase K (1 mM) was pre-incubated with API (500 \( \mu \)M) in sodium-phosphate buffer, 0.05 M, pH 7.5. 5 \( \mu \)l of the pre-incubated sample was removed at 30 min and after 60 min and loaded on the system to analyze the conformational changes in the enzyme-inhibitor complex. The elution of the complexes was monitored by absorption at 280 nm.

\[
K_i^* = \frac{[E][I]}{[EI] + [EI^*]} = K_i \left( \frac{k_6}{k_5 + k_6} \right) \quad (6)
\]
**Fluorescence Analysis**

Fluorescence measurements were performed on a Perkin-Elmer LS50 Luminescence spectrometer. Tryptophanyl fluorescence was excited at 295 nm and the emission was recorded from 300-500 nm at 25°C. The slit widths on both the excitation and emission were set at 5 nm and the spectra were obtained at 50 nm/min. For inhibitor binding studies, Proteinase K (1 μM) was dissolved in sodium phosphate buffer, 0.05 M, pH 7.5. Titration of the enzyme with API was performed by adding different concentrations of the inhibitor to a fixed concentration of enzyme solution. For each inhibitor concentration on the titration curve a new enzyme solution was used. All the data on the titration curve were corrected for dilutions and the graphs were smoothened. The magnitude of the rapid fluorescence decrease ($F_0 - F$) occurring at each API concentration was computer fitted to the Equation 8, to determine the calculated value of $K_i$ and $\Delta F_{max}$ (23).

\[
(F_0 - F) = \frac{\Delta F_{max}}{1 + (K_i/[I])}
\]  

(8)

The first order rate constants $k_{obs}$ at each inhibitor concentration $[I]$ were fitted to Equation 9 for the determination of $k_5$ under the assumption that for a tight binding inhibitor $k_6$ can be considered negligible at the onset of the slow loss of fluorescence.

\[
k_{obs} = \frac{k_5[I]}{(K_i + [I])}
\]  

(9)

Time course of the protein fluorescence following the addition of inhibitor were measured for 10 min with excitation and emission wavelengths fixed at 295 and 335 nm, respectively, with data acquisition at 0.1 s intervals. Corrections for the inner filter effect were performed as described by Equation 10 (24).

\[
F_c = F \text{ antilog } [(A_{ex} + A_{em})/2]
\]  

(10)

Where $F_c$ and $F$ stand for the corrected and measured fluorescence intensities, respectively, and $A_{ex}$ and $A_{em}$ are the absorbances of the solution at the excitation and emission wavelengths, respectively. Background fluorescence from API and buffer were appropriately corrected.
Fluorescent Chemoaffinity-Labeling of Proteinase K and the Effect of API Binding on the 5-IAF Fluorescence

Proteinase K (1 mM) was modified by treatment with 100 fold molar excess of 5-iodoacetamidofluorescein (5-IAF) in 50 mM potassium phosphate buffer, pH 7.5 for 24 h in dark. At pH 7.5 5-IAF specifically reacts with free –SH groups. The labeled protein was purified through Sephadex G-10 and eluted with phosphate buffer. Fractions showing absorbance at 280 nm and 490 nm were pooled and concentrated by lyophilization. The fluorescence spectrum of 5-IAF labeled Proteinase K was monitored at an excitation wavelength of 490 nm and emission wavelength from 490-600 nm. The number of Cys residues labelled was determined by using the molar absorptivity for 5-IAF of 80,000 cm⁻¹ M⁻¹. The stoichiometry of the fluorescence labeling was determined to be 1:1 between 5-IAF and Proteinase K.
RESULTS

Kinetic Analysis of the Inhibition of Proteinase K by API

The alkaline protease inhibitor (API) was produced extracellularly by a Streptomyces sp., and has been characterized for its inhibitory activity towards the alkaline proteases (14). Initial inhibition kinetic assessments revealed that API is a competitive inhibitor of Proteinase K with an IC$_{50}$ (concentration of the inhibitor required for 50% inhibition of the enzyme) value of 5.5±0.5 x 10$^{-5}$ M (Fig. 1). The steady-state rate of proteolytic activity of Proteinase K reached rapidly in the absence of API, whereas, in its presence a time-dependent decrease in the rate as a function of the inhibitor concentration was observed. The progress curves in the presence of API revealed a time range where the initial rate of reaction was similar to that in the absence of the inhibitor, and does not deviate from linearity (Fig. 2), therefore conversion of EI to EI* was minimal. This time range for a low concentration of API was 8 min for Proteinase K, wherein all the classical competitive inhibition experiments were used to determine $K_i$ ($k_4/k_3$) value (Equation 5). The inhibition constant $K_i$ associated with the formation of the reversible enzyme inhibitor complex (EI) determined from the reciprocal equation was 5.2±0.6 x 10$^{-6}$ M, which was further corroborated by the Dixon method (Fig. 3). The apparent rate constant $k$, derived from the progress curves of Proteinase K when plotted versus the inhibitor concentration followed a biphasic hyperbolic function (Fig. 4), revealing a fast equilibrium precedes the formation of the final slow dissociating enzyme-inhibitor complex (EI*), indicating two-step, slow-tight inhibition mechanism. Indeed the data could be analyzed with equation 5 by non-linear regression analysis to obtain the best estimate of the overall inhibition constant $K_i^*$ (2.5±0.3 x 10$^{-7}$ M). In case of slow-tight binding inhibitors, since the EI* complex is stable and dissociates slowly, the rate constant $k_6$, for the conversion of EI* to EI was determined in an alternative method, by pre-incubating high concentrations of enzyme and inhibitor for sufficient time to allow the system to reach equilibrium. Further, large dilution of the enzyme-inhibitor complex into a relatively large volume of assay mixture containing saturating substrate concentration causes dissociation of the enzyme-inhibitor complex and dissociation constant can be determined by the regeneration of enzymatic activity. Under these conditions, $v_0$ and the effective inhibitor concentration are considered approximately equal to zero and the rate of activity regeneration will provide the $k_6$ value. The pre-
incubated (30 min) mixture of Proteinase K and API was diluted 5,000-fold into the assay mixture containing the substrate at saturating substrate concentration. By least-squares minimization of eq 2 to the data for recovery of enzymatic activity, the determined $k_6$ value was $4.5\pm0.5 \times 10^{-5} \text{s}^{-1}$ (Fig. 5), which clearly indicated a very slow dissociation of EI*. The final steady-state rate $v_s$ was determined from the control that was pre-incubated without the inhibitor. The value of the rate constant $k_5$, associated with the isomerization of EI to EI*, was $9.2\pm1 \times 10^{-3} \text{s}^{-1}$ as obtained from fits of eq 3 to the onset of inhibition data using the experimentally determined values of $K_i$ and $k_6$ (Table-I). The overall inhibition constant $K_{i*}$ is a function of $k_6/(k_5 + k_6)$ and is equal to the product of $K_i$ and this function. The $k_6$ value indicated a slower rate of dissociation of EI* and the half-life ($t_{1/2}$) for the reactivation of EI* determined from the $k_6$ value was $4.27\pm0.5 \text{ h}$, suggesting higher binding affinity of API towards Proteinase K. When the incubation time was more than 60 min, the inhibitor failed to dissociate from the complex, since there was no recovery in the enzymatic activity (Fig 5). This observation can be attributed to gross conformational changes in the EI* complex induced leading towards the formation of a conformationally locked irreversible enzyme-inhibitor complex (EI**). To characterize the EI** we have carried out the quaternary structural analysis by gel filtration chromatography on a HPLC system. As revealed by figure 6A, the complex formed after 30 min of equilibration showed a retention time of 16 min, which demonstrated the formation of EI* complex. However, when the equilibration time between the enzyme and inhibitor was more than 60 min (Figure 6B), the retention time of the complex was shifted to 15 min indicating a change in the conformation of the EI*. This difference in the retention time can be attributed to the formation of irreversible complex EI** due to gross conformational changes induced in the EI* complex. These conformational states of the enzyme inhibitor complex are further characterized by fluorescence spectroscopy. All our kinetic analysis for the slow-tight binding inhibition has been determined in the time frame before the formation of the irreversible complex.
Fluorescence Changes of Proteinase K due to Binding of API and the Dependence of Fluorescence on API Concentration and Time.

The kinetic analysis of Proteinase K inhibition by API revealed a two-step inhibition mechanism, where the EI complex isomerizes to a tightly bound, slow dissociating EI* complex. To investigate and correlate this isomerization to the conformational modes in the Proteinase K due to binding of API, we have analyzed the fluorescence emission spectra of the protease in the presence and absence of the inhibitor. Proteinase K exhibited an emission maxima (λ_max) at 335 nm, as a result of the radiative decay of the π - π* transition from the Trp residues (Fig. 7A). The binding of API resulted in a concentration dependent quenching of the fluorescence. Absence of any blue or red shift in λ_max negated drastic gross conformational changes in the three-dimensional structure of the enzyme due to inhibitor binding. Therefore, the fluorescence changes in Proteinase K due to API binding can be correlated to localized conformational changes in the protease. The subtle conformational changes induced during the isomerization of EI to EI* were monitored by analyzing the tryptophanyl fluorescence of the complexes as a function of time. Binding of API resulted in an exponential decay of the fluorescence intensity as indicated by a sharp decrease in the quantum yield of fluorescence followed by a slower decline to a stable value (Fig. 7B). Further, titration of API against Proteinase K revealed that the magnitude of the initial rapid fluorescence loss (F_0 – F) increased hyperbolically (Fig. 7C), which corroborated the two-step slow tight binding inhibition of Proteinase K by API. From the data in figure 7, the magnitude of the rapid fluorescence decrease at a specific API concentration was found to be close to the total fluorescence quenching, indicating that the EI and EI* complexes have the same intrinsic fluorescence. The K_i value determined for the magnitude of the rapid fluorescence decrease (F_0 – F) was 5.8 ± 0.6 x 10^{-5} M and the k_5 value determined from the data derived from the slow decrease in fluorescence was 9.5 ± 1 x 10^{-3} s^{-1}. These rate constants are in agreement with that obtained from the kinetic analysis, therefore, the initial rapid fluorescence decrease can be correlated to the formation of the reversible complex EI, while the slow, time dependent decrease reflected the accumulation of the tight bound slow dissociating complex EI*. 
The conformational modes observed in the EI* complex were found to be time dependent. After 60 min the fluorescence spectra of EI* revealed a shift in $\lambda_{\text{max}}$ indicating induction of gross conformational changes in the enzyme (Figure 7D). To examine whether the inhibitor can be released from the EI* after 60 min, the complex was allowed to dissociate. However, it failed to release the inhibitor, which revealed that the complex has undergone irreversible conformational changes induced by the interaction of the inhibitor. These observations revealed that the slow-tight binding EI* complex can release the inhibitor only when the complex has undergone the subtle conformational changes, however once the inhibitor is bound to the enzyme for a longer time, the complex changes into a conformationally locked complex (EI**).

**Effect of API Binding on the IAF Fluorescence of Proteinase K**

The Cys73 present in close proximity of the catalytic triad of Proteinase K has been exploited to probe the interaction of API with the active site residues by chemoaffinity labeling. We have differentially labeled Proteinase K using the fluorescent label 5-iodoacetamidofluorescein (5-IAF). 5-IAF is known to react specifically with the free -SH groups of cysteine residues of proteins (25). The interaction of API with the Cys73 and other residues at/near the active site of the enzyme was investigated by monitoring the changes in fluorescence of the IAF labeled Proteinase K upon API binding. The unlabeled enzyme did not show fluorescence when excited at 490 nm however the IAF labeled Proteinase K displays a $\lambda_{\text{max}}$ at 520 nm, which does not interfere with the Trp fluorescence of the protein. Therefore the changes in the emission spectra of IAF-Proteinase K will exclusively reflect the localized conformational/electrostatic changes in the active site of Proteinase K due to the binding of the inhibitor. The 1:1 labeling ratio between IAF and Proteinase K indicated the specific labeling of the free Cys73. As revealed from the emission spectrum of the complex (Fig. 8), binding of API to the IAF labeled Proteinase K resulted in a rapid quenching in the fluorescence yield. This revealed that API binds to the active site of the enzyme and the interference in the electronic environment of the active site residues of Proteinase K is due to the interaction of the reactive site residue of API resulting in the quenching of the fluorescence.
DISCUSSION

We present the first report of a slow-tight binding proteinaceous inhibitor (API) of the alkaline protease Proteinase K. API showed high potency against Proteinase K and its interaction with the enzyme indicated its “tight-binding” nature. The two-step inhibition mechanism was corroborated by the equilibrium binding studies and by the correlation of the kinetic data with the conformational changes induced in the enzyme-inhibitor complexes.

![Scheme I](image)

Where, E stands for free enzyme, I is free inhibitor, EI is a rapidly forming pre-equilibrium complex, and EI* is the final enzyme inhibitor complex. Alternately, E may undergo inter conversion into another form E* which binds to the inhibitor by a fast step, where $k_{cf}$ and $k_{cf}^{-1}$ stand for the rate constants for forward and backward reaction, respectively, for the conversion of the enzyme.

In a number of enzymatic reactions, the target enzyme does not interact with the competitive inhibitors as per competitive inhibition mechanism, rather display a slow-onset of inhibition. This type of inhibition is called slow-binding inhibition and the inhibitor is refereed to as slow-binding inhibitor (26-29). Slow binding enzyme inhibition, which refers to the establishment of the equilibria between enzyme, inhibitor, and enzyme-inhibitor complexes, occurs slowly on the steady-state time scale (30) and has been thoroughly reviewed (31-36). Enzyme-catalyzed reactions where the concentrations of the enzyme and
inhibitor are comparable, and the equilibria are set up rapidly are referred to as tight binding inhibition. Kinetically the slow-binding inhibition can be illustrated by three mechanisms (Scheme I). When an inhibitor has a low $K_i$ value and the concentration of I varies in the region of $K_i$, both $k_3I$ and $k_4$ values would be low (37, 38). Thus, a simple second-order interaction between enzyme and inhibitor, and low rates of association and dissociation would lead to slow-binding inhibition (Scheme Ia). Alternatively, a two-step model depicts the rapid formation of an initial collisional complex EI, which slowly isomerizes to form a tightly bound slow dissociating complex EI* (Scheme Ib). Slow binding inhibition can also arise due to an initial slow interconversion of the enzyme E into another form E*, which binds to the inhibitor by a fast step (Scheme Ic). Kinetically, these mechanisms can be differentiated by investigating the behavior if the enzyme-inhibitor system at varying concentrations of the inhibitor. Scheme Ia would predict that in the presence of substrate the initial rate of substrate hydrolysis will be independent of inhibitor concentrations since the concentration of EI would be significantly low. However, in Scheme Ib (slow-tight binding inhibition), the inhibitor will inhibit the enzyme competitively at the onset of the reaction, and at increasing concentration of inhibitor, the initial rate of substrate hydrolysis will decrease hyperbolically as a function of time. In tight binding inhibition corrections have to be made for the reduction in the inhibitor concentration that occurs on formation of the EI complex, since the concentration of EI is not negligible in comparison to the inhibitor concentration and the free inhibitor concentration is not equal to the added concentration of the inhibitor. An understanding of the basis of the isomerization of EI complex to EI* complex could lead to the design of structures that allow titration of the lifetime of an EI* complex. The future development of tight-binding inhibitors will undoubtedly depend on application of kinetic techniques that yield quantitative information about the behavior of the inhibitors. The kinetic analysis of Proteinase K inhibition provides a unique opportunity for the quantitative determination of these rates and affinities, which can be extended to other slow-tight binding inhibition systems.

Scheme I also describes two alternative models for the time-dependent inhibition. The mechanism in scheme Ia, where the binding of the inhibitor to the enzyme is slow and tight, but occurs in a single step is, eliminated based on the data of Table-I, because the inhibitor has measurable effect on the initial rates before
the onset of slow-tight binding inhibition of Proteinase K. Scheme Ic represents the inhibition model where
the inhibitor binds only to the free enzyme that has slowly adopted the transition-state configuration can also
be eliminated by the observed rates of onset of inhibition. As in most ground-state inhibitors, formation of EI
complex between Proteinase K and API was too rapid to be measured at steady-state kinetics and was likely
to be near diffusion control. However, the isomerization of EI to the second tightly bound enzyme inhibitor
complex EI*, was too slow and relatively independent of the stability of the EI. The $k_6$ values revealed very
slow dissociation of the EI* complex indicating a highly stable, non-dissociative nature of the second
complex. Therefore for slow-tight binding inhibition the major variable is $k_6$, the first-order rate constant
associated with the conversion of EI* to EI, and the apparent inhibitor constant $K_i^*$ depends on the ability of
the inhibitor to stabilize the EI*. The half-life as derived from the $k_6$ value indicated a longer half-life of the
EI*, which is an essential parameter for an inhibitor to have biotechnological applications. Our results for the
inactivation of Proteinase K therefore are consistent with the slow-tight binding mechanism as described in
Scheme Ib. However, the time dependent non-dissociative nature of the EI* complex and the formation of an
irreversible conformationally locked EI** complex revealed a new mechanism of inhibition and lead us to
propose a new model describing conversion of a slow-tight binding inhibitor to an irreversible inhibitor. We
propose that API behaves as a slow-tight binding inhibitor in accordance with Scheme Ib until the formation
of the EI*, however, as a function of time it acts like an irreversible suicidal type inhibitor (Scheme II),
essentially due to the conformational modes attended by the enzyme-inhibitor complex. These conformational
changes are mainly induced due to extended secondary interactions between the two protein molecules. The
size exclusion chromatography corroborated the changes in the conformational states of the enzyme-inhibitor
complexes. The gross conformational changes in the EI* leading towards the formation of EI** was reflected
in the changes of the retention times on the elution profile of the complexes. Scheme II describes the kinetics
and conformational modes of the proposed mechanism of inhibition of Proteinase K by API, where the rate
constant $k_{con}$ is associated with the irreversible conformational change in the EI*.

![Scheme II](image-url)
The two-step inhibition mechanism of Proteinase K by API was reflected in the fluorescence analysis of the enzyme-inhibitor complexes. The rate constants derived from the fluorescence analysis of the complexes corroborated the values derived from the kinetic analysis. Therefore, we propose that the initial rapid fluorescence loss reflected the formation of the reversible complex EI, whereas the subsequent slower decrease was correlated to the accumulation of the tightly bound complex EI*. Absence of any shift in the tryptophanyl fluorescence of the complexes negated any major alteration in the three-dimensional structure of Proteinase K due to the binding of API at EI* stage. The proteolytic activity decreased linearly with increasing concentrations of API yielding a stoichiometry close to 2:1 (also revealed by fluorescence) expected for the dimeric API, that binds two molecules of enzyme. The agreement of the rate constants concomitant with the fluorescence changes observed during the time-dependent inhibition, lead us to correlate the localized conformational changes in the enzyme-inhibitor complex to the isomerization of the EI to EI*. The shift in the emission maxima as a function of time in the EI* indicated induction of gross conformational changes in the complex due to the secondary interactions within the two protein molecules. These interactions are strong enough to form an irreversible complex of the enzyme-inhibitor from which the inhibitor failed to dissociate. This is the first report that a proteinaceous slow-tight binding inhibitor behaving as an irreversible suicidal type inhibitor as a function of time.

The crystal structure of subtilisin complexed with SSI has revealed the primary and secondary contact regions between the enzyme and inhibitor and the induction of global induced-fit movement on the inhibitor upon complexing with subtilisin (39). The main role of the “secondary contact region” is to support the reactive site loop or the primary contact region of the inhibitor during the interaction. API being a SSI family inhibitor and Proteinase K belonging to subtilisin family, we have exploited the interactions of SSI-subtilisin to interpret the interactions of API and Proteinase K at molecular level. 5-IAF has been utilized as a fluorescent probe to investigate the microenvironment of cysteine residues of proteins since it covalently interacts with the free –SH group. We have exploited the Cys73 residue of Proteinase K to decipher its interactions with API, since it is present near the critical His69 of the catalytic center. Interaction of API with
the IAF labeled Proteinase K indicated the binding of the inhibitor at/near the active site of the enzyme. The Trp residue of API has been reported to play a critical role in the mechanism of inhibition (15). We visualize that quenching of the IAF fluorescence of Cys73 of Proteinase K is a consequence of stacking effect of the π-π electrons of the Trp residue of API. In the EI complex, API binds to the active site of Proteinase K, where the Trp residue and other residues near the reactive site of API interfere with the hydrogen bonding network of the catalytic triad Ser224, His69, and Asp39. Due to the π-π stacking interaction of the Trp residue of API with the essential His69 of the enzyme the electronic environment of the catalytic triad of the active site gets disturbed. Further the hydrophobic interactions between the π-π electrons of the reactive Trp of API and the active site His69 of Proteinase K may interfere with the electron acceptor and donor function of His69 leading towards the inhibition of the enzyme (Figure 9). At the same time other residues at the reactive site of API can also form various weak atomic interactions (hydrogen bonding, van der Waal’s, etc) with the charged residues of the active site and the neighboring residues of the enzyme. The formation of a tight complex between the enzyme and inhibitor can be attributed to the secondary interactions between API and Proteinase K. The secondary structure analysis has revealed the presence of β-sheet in API. β-sheet structures are known to play a critical role during the secondary contacts between the SSI inhibitors and the target enzyme. These secondary β-sheet interactions along with the network of hydrogen bonding, non-ionic and other weak interactions interaction between the active site and reactive site residues probably are sufficient to induce substantial conformational changes in the EI* complex leading towards the formation of the irreversible EI** complex. These extensive interactions between the active site residues of Proteinase K and the reactive site residues of API are probably responsible to convert a slow-tight binding inhibitor into an irreversible suicidal inhibitor of Proteinase K.
REFERENCES


FIGURE LEGENDS

Figure 1. Proteinase K Inhibition by API
The proteolytic activity of the Proteinase K (100 nM) was determined in Tris-HCl buffer, 0.05 M pH 8.5, containing 1 mM CaCl₂ and the substrate in the presence of increasing concentrations of API. The percent inhibition of the protease activity was calculated from the residual enzymatic activity. The curve indicates the best fit for the percent inhibition data (average of triplicates) from where the IC₅₀ value was calculated.

Figure 2. Time Dependent Inhibition of Proteinase K as a Function of API Concentration
The reaction mixture contained Proteinase K (100 nM) in Tris-HCl buffer, 0.05 M pH 8.5, containing 1 mM CaCl₂, varying concentrations of API and casein (5 mg/ml). Reaction was initiated by the addition of Proteinase K at 37°C. The points represent the hydrolysis of substrate as a function of time and the lines are the best fits of data obtained from eq 2 and 5, with the corrections made as per the eq 3 and 4. Concentrations of API were 0 μM (■), 0.62 μM (○), 0.95 μM (▼), 1.55 μM (♦), and 3 μM (▲).

Figure 3. Initial Rate of Proteolytic Activity of Proteinase K in the Presence of API
Enzymatic activity of Proteinase K was estimated in Tris-HCl buffer, 0.05 M pH 8.5, containing 1 mM CaCl₂ and the hydrolysed products were estimated spectrophotometrically at 280 nm. A. Proteinase K (1 μM) was incubated without (■) or with the inhibitor at 1 μM (○) and 2.5 μM (▲) and assayed at increased concentration of casein (1-10 mg/ml) at 37°C for 30 min. The reciprocal of substrate hydrolysis (1/v) for each inhibitor concentration were plotted against the reciprocal of the substrate concentration. B. Proteinase K (1 μM) was assayed using casein at (○) 5 mg/ml and (■) 10 mg/ml with increasing concentrations of API at 37°C for 30 min. The reciprocal of substrate hydrolysis (1/v) were plotted against the reciprocal of the inhibitor concentration. The straight lines indicated the best fits for the data obtained by non-linear regression analysis and analyzed by Lineweaver-Burk’s reciprocal equation (A) and Dixon method (B), respectively.

Figure 4. Dependence of Proteinase K Inhibition on API Concentration
The rate constants k, were calculated from the progress curves for each API concentrations and plotted against the inhibitor concentrations. The solid line indicates the best fit of the data obtained.
Figure 5. Dissociation Rate Constant ($k_6$) for Proteinase K-API Complex

Proteinase K (1 mM) was pre-incubated without (■) or with API (0.5 mM) for 30 min (○) or for 60 min (▲) in sodium-phosphate buffer, 0.05 M, pH 7.5, at 37°C. At the specified times indicated by the points, 5 µl of the pre-incubated sample was removed, diluted 5000-fold in the same buffer, and was assayed for the proteolytic activity using casein (150 mg/ml). The rate constant associated with the regeneration of activity ($k_6$) was determined by estimating the absorption of the released products as described in the text.

Figure 6. Gel Filtration Analysis of the Proteinase K-API Complexes

Proteinase K (1 mM) was pre-incubated with API (0.5 mM) for 30 min (A) or for 60 min (B) in sodium-phosphate buffer, 0.05 M, pH 7.5, at 37°C. 5 µl of the pre-incubated sample was loaded on a Protein-Pak 300SW HPLC column. The quaternary structure of the enzyme-inhibitor complexes was analyzed by the changes in the retention time by monitoring the absorption at 280 nm. The retention time of the complex formed after 30 min was 16.5 whereas the complex had a retention time of 15 min when the equilibration time was more than 60 min.

Figure 7. Fluorescence Analysis of Interaction between Proteinase K and API

A. Steady state fluorescence emission spectra of Proteinase K as a function of API Concentration. Protein fluorescence was excited at 295 nm and emission was monitored from 300-400 nm at 25°C. Titration was performed by the addition of different concentrations of the inhibitor to a fixed concentration of enzyme. Proteinase K (1 µM) was dissolved in sodium-phosphate buffer, 0.05 M, pH 6.0, and the concentrations of API used were 0 µM (■), 0.62 µM (□), 1.24 µM (○), 2.48 µM (△), 3.72 µM (▲), 4.34 µM (△), 5.58 µM (◇), and 6.2 µM (▼).

B. Time Dependent Effect of API on the Intrinsic Fluorescence of Proteinase K. API was added to Proteinase K (1 µM) at the specified time (indicated by the arrow) and the fluorescence emission was monitored for 300 s, at a data acquisition time of 0.1 s. The excitation and emission wavelength were fixed at 295 and 335 nm, respectively. The data were the average of five scans with the correction for buffer and dilutions. The concentrations of API used were 0 µM (■), 2.48 µM (○), and 5.58 µM (▲).
C. Effect of API Concentration on the Tryptophanyl Fluorescence of Proteinase K. A specified concentration of Proteinase K (1 µM) was treated with increasing concentrations of API. The fluorescence was measured at 25°C (excitation 295 nm and emission 335 nm). Each measurement was repeated five times and the average values of the fluorescence intensity at 335 nm were recorded. Control experiments with the buffer and inhibitor were performed under identical conditions. The fluorescence changes (F - F₀) were plotted against the inhibitor concentrations. The resulting hyperbola indicates the best fit of the data obtained.

D. Time Dependent Conformational Changes in the Enzyme-Inhibitor Complex. Proteinase K and API were incubated for 60 min and the changes in the conformational states were monitored by fluorescence. Tryptophanyl fluorescence of Proteinase K (■) was quenched upon addition of API (●) without any shift, however when the incubation time is above 60 min (▲) there was a shift in the emission maximum by 5 nm, indicating induction of gross conformational changes in the enzyme-inhibitor complex. There was no change in the emission spectra once these conformational changes are induced after 90 min (▼) and 120 min (▲) indicating the irreversible nature of the changes in the enzyme-inhibitor complex.

Figure 8. Effects of API Binding on the IAF Fluorescence of Proteinase K.
Proteinase K (1 mM) was incubated with 50 fold molar excess of 5-iodoacetamidofluorescein (5-IAF) in 50 mM potassium phosphate buffer, pH 7.5 for 24 h in dark, and purified on Sephadex G-10 column. The fluorescence of unlabeled (■) and IAF-labeled (●) Proteinase K was measured at an excitation of 490 nm and the emission was measured from 490-600 nm. Addition of API to the reaction mixture containing the IAF labeled Proteinase K (▲) resulted in the quenching of the IAF fluorescence.

Figure 9. Stereo View Representation of the Inhibition Mechanism of Proteinase K by API.
A. The active site of Proteinase K contains the catalytic triad Ser224, His69, and Asp39. During catalysis Ser224 functions as the primary nucleophile and His69 acts as a proton acceptor and donor. The role of Asp39 is to bring the His69 residue in the correct orientation to facilitate nucleophilic attack by Ser224.

B. API binds to the active site of Proteinase K, where the molecular interaction of Trp residue present in the reactive site and other residues near the reactive site of API may interfere with the hydrogen bonding network
of the catalytic triad Ser224, His69, and Asp39 of Proteinase K. We also propose that due to the \( \pi-\pi \) stacking interaction of the Trp residue with the essential His69 of the enzyme, the electronic environment of the catalytic triad of the active site gets disturbed. These interactions between the reactive site residue of API and the active site residues of Proteinase K may impair the nucleophilic attack of Ser224 on His69 affecting the electron acceptor and donor function of His69 leading towards the inhibition of the enzyme.

**ABBREVIATIONS**

API, alkaline protease inhibitor from *Streptomyces* sp.; sAAPF-pNA, N-succinyl-L-Ala-Ala-Pro-Phe p-nitroanilide; 5-IAF, 5-iodoacetamidofluorescein; EI, reversible enzyme-inhibitor complex; EI*, isomer of the second enzyme-inhibitor complex; EI**, irreversible enzyme-inhibitor complex; SSI, *Streptomyces* subtilisin inhibitor.

**TABLE-I**

*Inhibition constants of API against Proteinase K*

Values of rate constants for Proteinase K inhibition by API were calculated from Scheme I. IC\(_{50}\) is from the inhibition profile, \( K_i \) was determined from the steady-state time range for the competitive inhibition. \( k_6 \) is calculated from the regeneration assay, \( K_i^{*} \) and \( k_5 \) were determined from the equations as described in the text.

<table>
<thead>
<tr>
<th>Inhibition constants</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC(_{50})</td>
<td>5.5 ± 0.5 x 10(^{-5}) M</td>
</tr>
<tr>
<td>( K_i )</td>
<td>5.2 ± 0.6 x 10(^{-5}) M</td>
</tr>
<tr>
<td>( K_i^{*} )</td>
<td>2.5 ± 0.3 x 10(^{-7}) M</td>
</tr>
<tr>
<td>( k_5 )</td>
<td>9.2 ± 1 x 10(^{-3}) s(^{-1})</td>
</tr>
<tr>
<td>( k_6 )</td>
<td>4.5 ± 0.5 x 10(^{-5}) s(^{-1})</td>
</tr>
<tr>
<td>( k_5/k_6 )</td>
<td>2.0 ± 0.2 x 10(^{2})</td>
</tr>
<tr>
<td>( t_{1/2} )</td>
<td>4.27 ± 0.5 h</td>
</tr>
</tbody>
</table>
Figure 1

![Graph showing inhibition percentage vs. API concentration](image-url)
Figure 2
Figure 3

A

B
Figure 4

![Graph showing the relationship between inhibitor concentration and rate. The x-axis represents inhibitor concentration in μM, ranging from 0 to 100. The y-axis represents rate in s⁻¹, ranging from 0.00 to 0.04. The graph shows a curve that increases with increasing inhibitor concentration, reaching a plateau at higher concentrations.]
Figure 5

![Graph showing the relationship between Regenerated Activity and Time. The x-axis represents Time [min] ranging from 0 to 200, and the y-axis represents Regenerated Activity [10^{-2}] ranging from 0 to 100.]
Figure 6

A

B
Figure 7

A

![Fluorescence Intensity vs. Wavelength](image1.png)

B

![Fluorescence Intensity vs. Time](image2.png)
Figure 7

C

D

Relative Fluorescence vs. Inhibitor [µM]

Fluorescence Intensity (A.U.) vs. Wavelength (nm)
Figure 8

Fluorescence Intensity (A.U.)

Wavelength (nm)
Figure 9

A

His69
Asp39
Ser224

His69
Asp39
Ser224

B

His69
Trp
Asp39
Ser22

His69
Trp
Asp39
Ser224
Slow-tight binding inhibition of proteinase K by a proteinaceous inhibitor: Conformational alterations responsible for conferring irreversibility to the enzyme-inhibitor complex

Jui Pandhare, Chandravanu Dash, Mala Rao and Vasanti V. Deshpande

*J. Biol. Chem.* published online September 24, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308976200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2003/09/24/jbc.M308976200.citation.full.html#ref-list-1