Slow-Tight Binding Inhibition of Xylanase by an Aspartic Protease Inhibitor

KINETIC PARAMETERS AND CONFORMATIONAL CHANGES THAT DETERMINE THE AFFINITY AND SELECTIVITY OF THE BIFUNCTIONAL NATURE OF THE INHIBITOR*

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The first report of slow-tight inhibition of xylanase by a bifunctional inhibitor alkalo-thermophilic Bacillus inhibitor (ATBI), from an extremophilic Bacillus sp. is described. ATBI inhibits aspartic protease (Dash, C., and Rao, M. (2001) J. Biol. Chem., 276, 2487–2493) and xylanase (Xyl I) from a Thermomonospora sp. The steady-state kinetics revealed time-dependent competitive inhibition of Xyl I by ATBI, consistent with two-step inhibition mechanism. The inhibition followed a rapid equilibrium step to form a reversible enzyme-inhibitor complex (EI), which isomerizes to the second enzyme-inhibitor complex (EI*), which dissociated at a very slow rate. The rate constants determined for the isomerization of EI to EI*, and the dissociation of EI* were $13 \pm 1 \times 10^{-6} s^{-1}$ and $5 \pm 0.5 \times 10^{-6} s^{-1}$, respectively. The $K_i$ value for the formation of EI complex was $2.5 \pm 0.5 \mu M$, whereas the overall inhibition constant $K_i$ was $7 \pm 1 \text{nm}$. The conformational changes induced in Xyl I by ATBI were monitored by fluorescence spectroscopy and the rate constants derived were in agreement with the kinetic data. Thus, the conformational alterations were correlated to the isomerization of EI to EI*. ATBI binds to the active site of the enzyme and disturbs the native interaction between the histidine and lysine, as demonstrated by the abolished isoindole fluorescence of o-phthalaldehyde (OPTA)-labeled Xyl I. Our results revealed that the inactivation of Xyl I is due to the disruption of the hydrogen-bonding network between the essential histidine and other residues involved in catalysis and a model depicting the probable interaction between ATBI or OPTA with Xyl I has been proposed.

In recent years, considerable efforts have been expended in the design and synthesis of glycosidase inhibitors, not only to understand about the active site structures and mechanisms of these interesting enzymes but also in generating new therapeutic agents. Specific inhibitors of glycosidases have proved valuable in a number of applications ranging from mechanistic studies (1, 2) to possible therapeutic uses such as control of blood glucose levels (3), viral infectivity through interference with normal glycosylation of coat proteins (4), against cancer, bacterial infections, and as insecticides (5). A number of naturally occurring reversible glycosidase inhibitors such as nojirimycin, castanospermine, swainsonine, and acarbose have been reported (1). Another class of inhibitors is the synthetic analogues of sugars containing reactive groups such as epoxides, isothiocyanates, and $\alpha$-halocarbons (1, 6). There are reports of mechanism-based inhibitors such as conduritol epoxides (7), the quinone methide-generating glycosides (8), and the glycosylmethyl triazenes (9).

Xylanases (1,4-$\beta$)-xylan xylanohydrolases) are glycosidases that catalyze the hydrolytic cleavage of $\beta$-1,4-linked polymers of $\alpha$-xylose (10). They have raised enormous interest in the past decade in view of their application in clarification of juices and wines, conversion of renewable biomass into liquid fuels and in development of environmentally sound pre-bleaching processes in the paper and pulp industry (11). Although extensive studies have been carried out on the industrial applications of xylanases, there is a paucity of reports on their molecular enzymology and clinical implications. Recently, glycosidases have been studied with a clinical perspective of locating enzyme-allergens (12). Some of these enzymes, including xylanases and cellulases have been found to cause occupational and non-occupational allergies, such as respiratory and irritant contact dermatitis. Therefore, from the biomedical point of view, inhibitors of this class of enzymes will have tremendous importance in the near future. In addition, inhibition of cellulolytic and hemicellulolytic enzymes have potential applications to prevent the degradation of wood and cloth by the action of the hydrolytic enzymes present in the gut of termites.

The three-dimensional structures of family 10 xylanases (13, 14) have revealed the extended substrate binding cleft in which the surface residues are linked by an extensive hydrogen bonding network. The clefts form deep grooves consistent with their endo-mode action and comprise a series of subsites, each one capable of binding a xylose moiety (15). The active site of xylanase contains two essential catalytic groups, one playing the role of acid/base and the other functioning as a nucleophile (16). These two groups have been identified as carboxyl groups and a covalent intermediate is formed, which undergoes hydrolysis to afford hemiacetal with net retention of anomeric stereochemistry (17). The transition states leading to and from the covalent intermediate have substantial oxacarbonium ion character, as indicated by kinetic isotope effects and by the effects of electron-withdrawing substituents on the sugar ring upon reaction rate (2, 17). Analysis of active site amino acids that play an important role in substrate binding and in catalysis has been greatly facilitated by solving the crystal structure of family 10 xylanases covalently linked to mechanism-based celllobioyl and xylobioyl inhibitors (18). To gain further insight into the details of the hydrolytic mechanism of glycosidases, specific inhibitors are necessary, which can act as mechanistic and structural probes. A diverse array of extremely
Slow-Tight Binding Inhibition of Xylanase

Fig. 1. Inhibition of Xyl I by ATBI. The xylanolytic activity of the purified Xyl I (2 μM) was determined in the presence of increasing concentrations of ATBI. The percent inhibition of the xylanase activity was calculated from the residual enzymatic activity. The sigmoidal curve indicates the best fit for the percent inhibition data (average of triplicates) obtained, and the IC_{50} value was calculated from the graph.

Fig. 2. Time course of inhibition of Xyl I by ATBI. The reaction mixture contained Xyl I (50 nm) in sodium-phosphate buffer, 0.05 M, pH 6.0, and varying concentrations of ATBI and xylan (10 mg/ml). The reaction was initiated by the addition of Xyl I at 50 °C. The points represent the hydrolysis of substrate as a function of time, and the lines indicate the best fits of data obtained from Equations 2 and 5, with the corrections made as per Equations 3 and 4. The concentrations of ATBI were 0.465 μM (■), 0.62 μM (●), 0.95 μM (▲), 1.55 μM (▼), and 3 μM (▲).

In this report, we present slow-tight binding inhibition of the thermostable xylanase (Xyl I)1 from a Thermomonospora sp. by a bifunctional inhibitor ATBI, isolated from an extremophilic Bacillus sp. 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 Xyl I by ATBI was delineated by monitoring the isindole fluorescence of the o-phthalaldehyde (OPTA)-labeled enzyme and a model for the probable interactions have been proposed.

EXPERIMENTAL PROCEDURES

Materials—Oat spelt xylan, dinitsroisalicylic acid, and o-phthalaldehyde (OPTA) were obtained from Sigma Chemical Co., St. Louis, MO. Sephadex A-50 and Sephacyr S-200 were obtained from Amersham Biosciences, Inc., Sweden. All other chemicals used were of analytical grade.

Microorganisms and Growth Conditions—Thermomonospora sp. producing Xyl I is an alkalothermophilic actinomycete having optimum growth at pH 9 and 50 °C. It was isolated from self-heating compost from the Barabanki district of Uttar Pradesh, India (20). The ATBI producing extremophilic Bacillus sp. was isolated in this laboratory from the soil sample of a hot spring at Vajreswari, Maharashtra, India (21). The optimal growth condition of the Bacillus sp. is pH 10 and 50 °C. For the production of the inhibitor, the Bacillus sp. was grown in a liquid medium containing soya meal (2%) and other nutrients (the pH of the medium was adjusted by the addition of sterile 10% sodium carbonate).

Purification of ATBI—The inhibitor was purified from the extracellular culture filtrate of the Bacillus sp. as described previously (23). Briefly, about 1000 ml of the extracellular culture filtrate was treated with 65 g of activated charcoal, and the supernatant was subjected to membrane filtration using Amicon UM10 (M, cut-off 10,000) and UM2 (M, cut-off 2000). The resulting clear filtrate was concentrated by lyophilization and loaded onto a prepacked Ultipore Lichrosorb RP-18 (LKB) column. The fractions detected at 210 nm were eluted on a linear gradient of 0–50% acetonitrile and water containing 0.01% trifluoroacetic acid, and the active fractions showing the inhibitory activity were found to be homogeneous.

Purification of Xyl I—The Thermomonospora sp. was grown at 50 °C for 96 h for the production of Xyl I. The enzyme was purified to homogeneity from the extracellular culture filtrate by fractional ammonium sulfate precipitation (35–55%), DEAE-Sephadex ion exchange chromatography and Sephacyr S-200 gel filtration chromatography (24).

Xylanase Assay and Inhibition Kinetics—The xylanase assay was carried out in phosphate buffer, 0.05 M, pH 6.0, by mixing a specified concentration of the enzyme with 0.5 ml of oat spelt xylan (10 mg/ml) in a reaction mixture of 1 ml and incubating at 50 °C for 30 min. The reducing sugar released was determined by the dinitrosalicylic acid method (25). One unit of xylanase activity was defined as the amount of enzyme that produced 1 μmol xylose equivalent per min using oat-spelt xylan as the substrate under assay conditions. Protein concentration was determined according to the method of Bradford (26) using bovine serum albumin as the standard.

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 method (27) and also by the Lineweaver-Burk’s equation. The K_{in} value was also calculated from the double-reciprocal equation by fitting the data into the computer software Microlab Origin. For the Lineweaver-Burk’s analysis Xyl I (2 μM) was incubated with ATBI at 1 μM and 2.5 μM and assayed at increased concentration of xylan (1–10 mg/ml) at 50 °C for 30 min. The reciprocals of substrate hydrolysis (1/v) for each inhibitor concentration were plotted against the reciprocals of the substrate concentrations, and the K_i was determined by fitting the resulting data. In Dixon’s method, xylanolytic activity of Xyl I (2 μM) was measured in the presence of 5 and 10 mg/ml xylan, at concentrations of ATBI ranging from 0.5 to 5 μM at 50 °C for 30 min. The reciprocals of substrate hydrolysis (1/v) were plotted against the inhibitor concentration and the K_{in} was determined by fitting the data using Microlab Origin.

For the progress curve analysis, assays were carried out in a reaction mixture of 1 ml containing enzyme, substrate, and inhibitor at various concentrations. The reaction mixture contained Xyl I (50 nm) in sodium-phosphate buffer, 0.05 M, pH 6.0, and varying concentrations of ATBI (0.5–3 μM) and xylan (10 mg/ml). Reaction was initiated by the addition of Xyl I at 50 °C, and the release of products were monitored at different time intervals by estimating the reducing sugar at 540 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 according to Equation 1,

\[
V = \frac{V_{max}S}{K_{m}(1 + [E]I) + S}
\]

where K_{m} is the Michaelis constant, V_{max} is the maximal catalytic rate at saturating substrate concentration [S], K_{I} = (k_{II}/k_{I}) is the dissociation constant for the first reversible enzyme-inhibitor complex, and I is the
inhibitor concentration (28). The progress curves for the interactions between ATBI and Xyl I were analyzed using Equation 2 (29, 30),

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

(Eq. 2)

where \([P]\) is the product concentration at any time \(t\), \(v_0\) and \(v_i\) 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. As a prerequisite for tight binding inhibitors, 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 the 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 Equations 3 and 4 as described by Morrison and Walsh (31),

\[ v_t = \frac{k_5 SQ}{2(k_5 + S)} \]  

(Eq. 3)

\[ Q = \left(\frac{[K_i'] + I - E_i}{k_i'}\right)^2 - \left(\frac{[K_i'] + I - E_i}{k_i'}\right) \]  

(Eq. 4)

where \(K_i' = K_i + [S]/K_i\), \(k_i\) is the rate constant for the product formation, and \(I\) and \(E_i\) 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 of the enzyme and inhibitor was determined as per Equation 5,

\[ k = k_o + k_4 I \left(\frac{1}{1 + (S/K_i)}\right) \]  

(Eq. 5)

The progress curves were analyzed by Equations 2 and 5 using nonlinear 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,

\[ K_i^* = \left(\frac{[EI]}{[E]_c + [EI]}\right) = K_i \left(\frac{k_o}{k_i + k_4}\right) \]  

(Eq. 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 directly measure the effect of the inhibitor on \(v_t\), 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,

\[ \frac{1}{v} = \frac{1}{V_{max}} \frac{K_i^*}{V_{max} S + (1/K_i)} \]  

(Eq. 7)

The rate constant \(k_o\), for the dissociation of the second enzyme-inhibitor complex was measured directly from the time-dependent inhibition. Concentrated Xyl I and ATBI were incubated in a reaction mixture to reach equilibrium, followed by large dilutions in assay mixtures containing near-saturating substrate. Xyl I (2 mM) was preincubated with equimolar concentrations of ATBI for 120 min in sodium phosphate buffer, 0.05 M, pH 6.0. 5 μl of the preincubated sample was removed, diluted 5000-fold in the same buffer, and assayed at 50 °C using xylan at (150 mg/ml) at different time intervals.

Fluorescence Analysis—Fluorescence measurements were performed on a PerkinElmer Life Sciences LS50 luminescence spectrometer connected to a Julabo F20 water bath. Protein fluorescence was excited at 295 nm, and the emission was recorded from 300 to 500 nm at 25 °C. The slit widths on both the excitation and emission were set at 5 nm, and the emission wavelength was recorded from 300 to 500 nm at 25 °C.

For inhibitor binding studies, Xyl I (2 mM) was dissolved in sodium phosphate buffer, 0.05 M, pH 6.0. Titration of the enzyme with ATBI was performed by the addition of 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 smoothed. The magnitude of the rapid fluorescence decrease \((F_0 - F)\) occurring at each ATBI concentration was computer-fitted to the Equation 8, to determine the calculated value of \(K_i\) and \(\Delta F_{\text{max}}\) (32),

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

(Eq. 8)

The first order rate constants for the slow loss of fluorescence, \(k_{obs}\), at each inhibitor concentration \([I]\) were computer-fitted to Equation 9 (32), for the determination of \(k_i\) under the assumption that for a tight binding inhibitor, \(k_i\) can be considered negligible at the onset of the slow loss of fluorescence,

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

(Eq. 9)

The time course of the protein fluorescence following the addition of inhibitor was measured for 10 min with excitation and emission wavelengths fixed at 295 and 340 nm, respectively, with data acquisition at 0.1-s intervals. Corrections for the inner filter effect were performed as described by Equation 10 (33),

\[ F = F_c \text{ antilog } (\ln A + A_{obs}/2) \]  

(Eq. 10)

where \(F_c\) and \(F\) stand for the corrected and measured fluorescence intensities, respectively, and \(A_c\) and \(A_{obs}\) are the absorbances of the solution at the excitation and emission wavelengths, respectively. Background buffer spectra were subtracted to remove the contribution from Raman scattering.

Effect of ATBI on the Isoindole Fluorescence of OPTA-labeled Xyl I—Fresh OPTA solution was prepared in methanol for each experiment. The modification was carried out by incubating Xyl I (2 μM) in 1 ml of 0.05 M sodium phosphate buffer, pH 6, with 50 μM OPTA at 25 °C. Methanol had no effect on the activity of the enzyme and was always less than 2% (v/v). The formation of Xyl-I isoindole derivative was followed spectrofluorometrically by monitoring the increase in fluorescence with the excitation wavelength fixed at 338 nm. To monitor the effect of ATBI on the isoindole fluorescence of Xyl I, the enzyme was preincubated with ATBI (5 μM) for 15 min, and then OPTA was added and the formation of isoindole derivative was monitored as described above.

RESULTS

Kinetic Analysis of the Inhibition of Xyl I—The aspartic protease inhibitor (ATBI) was produced extracellularly by an extremophilic Bacillus sp. and was characterized for its inhibitory activity toward HIV-1 protease, pepstatin, and the protease from Aspergillus saitoi (23, 34, 35). The bifunctional nature of ATBI was established by its potency toward Xyl I, the xylanase purified from the Thermomonospora sp. Xyl I, a member of family 10 xylanase is highly thermostable with half-lives of 86, 30, and 15 min at 80, 90, and 100 °C, respectively, and is stable in an expansive pH range of 5–10 with more than 75% residual activity. Initial kinetic assessments revealed that ATBI is a competitive inhibitor of Xyl I with an IC50 value of 6.5 ± 0.5 μM (Fig. 1). In the absence of ATBI, the steady-state rate of xylanolytic activity of Xyl I reached rapidly whereas, in its presence a time-dependent decrease in the rate as a function of the inhibitor concentration was observed. Examination of the progress curves revealed a time range where the initial rate of reaction did not deviate from linearity (Fig. 2), and the conversion of EI to E* was minimal. For a low concentration of ATBI, this time range was 8 min, within which classic competitive inhibition experiments were used to determine the \(K_i^*\) values (Equation 5). The value of the inhibition rate constant \(K_i\), associated with the formation of the reversible enzyme-inhibitor complex (EI) determined from the fits of data to the reciprocal equation was 2.5 ± 0.5 μM (Fig. 3), which was corroborated by the Dixon method (data not shown). The apparent rate constant \(k_o\), derived from the progress curves, when plotted versus the inhibitor concentration, followed a hyperbolic function (Fig. 4), thus revealing a fast equilibrium that precedes the formation of the final slow dissociating enzyme-inhibitor complex (E*I) and indicating a two-step, slow-tight inhibition mechanism (Scheme 1). Indeed, the data could be fitted to Equation 5 by non-linear regression analysis, which yielded the best estimate of the overall inhibition constant \(K_i^*\) of 7 ± 1 nM.

In an alternative method, the rate constant \(k_o\), for the conversion of E*I to EI, was determined by preincubating high concentrations of enzyme and inhibitor for sufficient time to
allow the system to reach equilibrium. 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 thus regeneration of enzymatic activity. Under these conditions, \( v_0 \) and the effective inhibitor concentration can be considered approximately equal to zero and the rate of activity regeneration will provide the \( k_I \) value. After preincubating Xyl I with ATBI, the enzyme-inhibitor mixture was diluted 5000-fold into the assay mixture containing the substrate at 50 °C. By least-squares minimization of Equation 2 to the data for recovery of enzymatic activity, the determined \( k_I \) value was 5 ± 0.5 \( \times 10^{-6} \) s\(^{-1}\) (Fig. 5), which clearly indicated a very slow dissociation of EI\(^*\). The final steady-state rate, \( v_{\text{ss}} \), was determined from the control that was preincubated without the inhibitor. The value of the rate constant \( k_{\text{f}} \), associated with the isomerization of EI to EI\(^*\), was 13 ± 1 \( \times 10^{-6} \) s\(^{-1}\) as obtained from fits of Equation 6 to the onset of inhibition data using the experimentally determined values of \( K_I \) and \( k_5 \) (Table I). The overall inhibition constant \( K_I \) is a function of \( k_5/(k_5 + k_6) \) and is equal to the product of \( K_I \) and this function. The \( k_5 \) value indicated a slower rate of dissociation of EI\(^*\) complex and the half-life \( t_{1/2} \) for the reactivation of EI\(^*\) as determined from \( k_6 \) values was 38.5 ± 4 \( \times 10^3 \) h, suggesting higher binding affinity of ATBI toward Xyl I, 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. \( E \) may undergo inter conversion into another form \( E^*\), which binds to the inhibitor by a fast step, where \( k_{\text{cd}} \) and \( k_{\text{dc}} \) stand for the rate constants for forward and backward reaction, respectively, for the conversion of the enzyme.

Scheme I 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 have measurable effect on the initial rates before the onset of slow-tight binding inhibition. 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. Our foregoing results for the inactivation of Xyl I were therefore consistent with the slow-tight binding mechanism as described in Scheme Ib.
where the $E_1$ complex isomerizes to a tightly bound, slow dissociating $E_1^*$ complex. This isomerization is a consequence of the conformational changes induced in Xyl I due to the binding of ATBI. The tryptophanyl fluorescence of Xyl I exhibited an emission maxima ($\lambda_{\text{max}}$) at $339 \text{ nm}$, as a result of the radiative decay of the $n-\pi^*$ transition from the Trp residues (Fig. 6). The binding of ATBI resulted in a concentration-dependent quenching of the fluorescence with saturation reaching at or above $6 \mu \text{M} \text{ ATBI}$ (Fig. 6, inset). The absence of blue or red shift in $\lambda_{\text{max}}$ negated any drastic gross conformational changes in the three-dimension structure of the enzyme due to inhibitor binding. The subtle conformational changes induced during the isomerization of $E_1$ to $E_1^*$ was monitored by analyzing the tryptophanyl fluorescence of the complexes as a function of time. Binding of ATBI resulted 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. 7). Furthermore, titration of ATBI against Xyl I revealed that the magnitude of the initial rapid fluorescence loss ($F_0 - F$) increased in a saturation-type manner (Fig. 8), which corroborated the two-step slow-tight binding inhibition of Xyl I by ATBI. From the data in Fig. 8, the magnitude of the rapid fluorescence decrease at a specific ATBI concentration was found to be close to the total fluorescence quenching observed Fig. 6, indicating that the $E_1$ and $E_1^*$ complexes have the same intrinsic fluorescence. The value of $K_r$ determined by fitting the data for the magnitude of the rapid fluorescence decrease ($F_0 - F$) was $2.6 \pm 0.5 \mu \text{M}$, and the $k_r$ value was determined from the data derived from the slow decrease in fluorescence was $13.5 \pm 1 \times 10^{-6} \text{s}^{-1}$. These rate constants are in good agreement with that obtained from the kinetic analysis, therefore, the initial rapid fluorescence decrease can be correlated to the formation of the reversible complex $E_1$, whereas the slow, time-dependent decrease reflected the accumulation of the tight bound slow dissociating complex $E_1^*$.

**Effect of ATBI on the Isoindole Fluorescence of Xyl I by OPTA**—In our earlier report, we have investigated the role of essential histidine and lysine residues in the active site of the Xyl I and shown that binding of the chemoaffinity label OPTA to these residues of the active site resulted in the formation of an isoinodole derivative (36). The active site of Xyl I constitutes the catalytic carboxylic groups and the histidine residue, which play a crucial role in catalysis. To investigate the binding of ATBI to the active site and changes in the native intermolecular interactions, we have monitored the changes in the interaction of the lysine and histidine due to ATBI binding, and their influence on the isoinodole fluorescence of Xyl I (Fig. 9). The unbound enzyme did not show fluorescence when excited at $338 \text{ nm}$, however, incubation of OPTA with Xyl I resulted in an increase in the fluorescence with a $\lambda_{\text{max}}$ at $417 \text{ nm}$ due to the formation of the isoinodole derivative. The ATBI-preincubated Xyl I failed to react with OPTA as revealed by the total loss of isoinodole fluorescence, which not only confirmed the binding of ATBI to the active site of Xyl I but also further revealed that the binding of ATBI resulted in the formation of a new set of hydrogen bonding and other non-ionic interactions. These altered weak interactions cause disruption of the native hydrogen bonding network of the histidine and lysine residues, which are essential for the formation of isoinodole derivative.

**DISCUSSION**

Since the discovery of the potent inhibitory effects of deoxynojirimycin toward glycosidases in the early 1970s, this class of compound has been the subject of a vast amount of ongoing research interest concerning synthesis and the evaluation of biological properties (13). Although a plethora of synthetic inhibitors has been reported, there is a lacuna of peptidic inhibitors of glycosidases from extremophilic microorganisms. It is noteworthy to point out that several extremophiles are known to produce highly thermostable xylanases and cellulases, however, these organisms have not been studied extensively for their potential exploitation toward isolation of inhibitors of important enzymes. We present here the first report of a bifunctional peptidic inhibitor ATBI, from an extremophilic *Bacillus* sp., exhibiting slow-tight binding inhibition against xylanase. Previously we have reported the kinetic parameters involved in the inhibition of HIV-1 protease, pepsin, and the aspartic protease from the *Aspergillus saitoi* by ATBI. The inhibitor showed exceptionally high potency against Xyl I, the thermostable xylanase from a *Thermomonospora* sp., and its 1:1 molar ratio of interaction with the enzyme indicated its “tight binding” nature. The two-step inhibition mechanism was corroborated by the equilibrium binding studies of the enzyme and inhibitor and the correlation of the kinetic data with the conformational changes induced in the enzyme-inhibitor complexes.

In the presence of competitive inhibitors, a number of enzymatic reactions do not respond immediately but display a slow onset of inhibition, which is referred to as slow binding inhibition (37–40). The establishment of the equilibria between enzyme, inhibitor, and enzyme-inhibitor complexes, in slow binding inhibition occurs slowly on the steady-state time scale (41–45). 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 $K_r$ value and the concentration of $I$ varies in the region of $K_r$, both $k_rI$ and $k_r$ values would be low. Thus, a simple second-order interaction between enzyme and inhibitor, and low rates of association and dissociation would lead to slow binding inhibition. Alternatively, a two-step model depicts the rapid formation of an initial collisional complex $E_1$, which slowly isomerizes to form a tightly bound slow dissociating complex $E_1^*$.

**TABLE I**

**Inhibition constants of ATBI against Xyl I**

| Inhibition constants | Values   |
|----------------------|----------|
| $K_{IC_{50}}$        | $6.5 \pm 0.5 \times 10^{-6} \text{ M}$ |
| $K_r$                | $2.5 \pm 0.5 \times 10^{-6} \text{ M}$ |
| $K_{IC_{50}}^+$      | $7 \pm 1 \times 10^{-9} \text{ M}$ |
| $k_r$                | $13 \pm 1 \times 10^{-6} \text{ s}^{-1}$ |
| $k_{IC_{50}}$        | $5 \pm 0.5 \times 10^{-6} \text{ s}^{-1}$ |
| $k_r/k_r^+$          | $26 \pm 3 \times 10^2$ |
| $t$                  | $38.5 \pm 4 \times 10^2 \text{ h}$ |
inhibitor concentration is not equal to the added concentration of the inhibitor. The kinetic analysis of the xylanase inhibition in this report provides a unique opportunity for the quantitative determination of these rates and affinities, which can be extended to other slow-tight binding inhibition reactions. The formation of an EI complex between Xyl I and ATBI 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 or the ability of the inhibitor to stabilize the EI*. The $k_6$ values revealed very slow dissociation of the inhibitor from the EI* 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 biomedical applications.

The characteristic feature of slow binding inhibition is the

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**FIG. 6.** Steady-state fluorescence emission spectra of Xyl I as a function of ATBI. 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. Xyl I was dissolved in sodium-phosphate buffer, 0.05 M, pH 6.0, and the concentrations of ATBI used were 0 μM ( ), 0.31 μM ( ), 0.62 μM ( ), 1.24 μM ( ), 1.86 μM ( ), 2.48 μM ( ), 3.1 μM ( ), 3.72 μM ( ), 4.34 μM ( ), 4.96 μM ( ), 5.58 μM ( ), 6.2 μM ( ), 9.3 μM ( ), and 12.4 μM ( ). The curve in the inset represents the best fit of the fluorescence quenching data of Xyl I at 339 nm ($\lambda_{\text{max}}$) as a function of ATBI concentration.

**FIG. 7.** Time-dependent effect of ATBI on the fluorescence quenching of Xyl I. ATBI was added to Xyl I (2 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 339 nm, respectively. The data are the average of five scans with the correction for buffer and dilutions. The concentrations of ATBI used were 0 μM ( ), 0.62 μM ( ), and 1.24 μM ( ).
induction of conformational changes in the enzyme-inhibitor complex, resulting in the clamping down of the enzyme to the inhibitor, thus forming a stable enzyme-inhibitor complex. The two-step inhibition mechanism of Xyl I by ATBI was reflected in the quenching pattern of the fluorescence 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 $E_1$, whereas the subsequent slower decrease was correlated to the accumulation of the tightly bound complex $E_1^r$. Any major alteration in the three-dimensional structure of Xyl I due to the binding of ATBI can be ruled out, because there was no shift in the tryptophanyl fluorescence of the complexes. Any disturbance in the environment of tryptophan residues may be reflected in an alternation in emission wavelength, quantum yield, and susceptibility to quenching (46). Energy transfer to an acceptor molecule having an overlapping absorption spectrum can also contribute to fluorescence quenching (47). However, because the inhibitor has no absorption in the region of 290–450 nm, the fluorescence quenching due to the energy transfer between the inhibitor and the tryptophan residues of Xyl I have been neglected. The other possibility is the presence of multiple sites, where binding at one induced rapid fluorescence change and at a second site caused the slow fluorescence decrease. This was verified by titrating a fixed concentration of Xyl I with increasing concentrations of ATBI. The xylanolytic activity decreased linearly with increasing concentrations of ATBI yielding a stoichiometry close to 1:1 (also revealed by fluorescence) expected for the slow-tight binding inhibition, therefore inconsistent with the presence of multiple high affinity sites. From the physical explanation for the quenching process, it was apparent that the inhibitor induced fluorescence quenching followed the formation of both the complexes. The agreement of the rate constants concomitant with the fluorescence changes observed during the time-dependent inhibition led us to correlate the localized conformational changes in the enzyme-inhibitor complex to the isomerization of the $E_1^r$ to $E_1^*$. Conformational integrity of the active site of an enzyme is essential for its catalysis, and investigations on the molecular orientation of the functional groups of active site as well as their microenvironment are the areas of growing scientific interest. Chemoaffinity labeling is a powerful technique to assign the binding sites of ligand-macromolecule complexes, which combine some of the advantages of both the photoactivated and electrophilic affinity labeling (48). OPTA is a bifunctional, fluorescent chemoaffinity label, which until recently was known to have absolute specificity for amino and thiol groups (49) for the formation of an isoindole derivative. However, application of OPTA as a probe to ascertain the conformational flexibility and polarity of the active site of Xyl I by the formation of a fluorescent isoindole derivative with the lysine and histidine residue have been reported in our laboratory (36). OPTA contains two aldehyde groups; one of which reacts with the primary amine of lysine, whereas the second group reacts with the secondary amine of the imidazole ring of histidine, resulting in the formation of the isoindole derivative. A schematic model depicting the interaction of the aldehyde groups of OPTA with the secondary amine of His and the primary amine of Lys residue of the Xyl I has been proposed (Fig. 10A). Our foregoing results revealed that, when Xyl I was preincubated with ATBI, OPTA failed to form the isoindole derivative as reflected by the loss of fluorescence. The inability of OPTA to form the isoindole derivative with the ATBI-bound Xyl I could be attributed to the interaction of ATBI with either lysine or histidine or both the residues, thereby changing the native molecular interactions of these residues. The amino acid sequence of ATBI (Ala-Gly-Lys-Lys-Asp-Asp-Asp-Asp-Pro-Pro-Glu) revealed the presence of amino acid residues such as Asp, Lys, and Glu with charged side chains. We propose that the carboxyl group of the Asp residues of ATBI (Fig. 10B) can form hydrogen bonding with the amines of His and Lys residues of Xyl I, thereby preventing the binding of OPTA. This, however, does not exclude the possible role of steric hindrance exerted by the bound ATBI in preventing the binding of OPTA to the active site. The catalytic site of xylanases consists of two carboxyl groups and an essential lytic water molecule and follows a general acid-base catalytic mechanism (50). Based on the existing experimental evidences, we further propose that the charged side chains of the amino acids, the amide nitrogens, and the carbonyl oxygen groups of ATBI could form many intermolecular hydrogen bonds and other weak interactions (van der Waal’s, ionic, etc.) with the residues in or near the active site of Xyl I. We also visualize that the tight binding nature of ATBI in conjunction with the multiple non-bonded interactions may be sufficient to interfere in the native weak interactions between the carboxyl groups, the lytic water molecule, and the essential histidine residue of the active site, leading toward the inactivation of Xyl.
However, the crystal structure of the enzyme-inhibitor complex will aid in understanding the mechanism of inactivation of Xyl I in depth and will further shed light on the molecular interactions between the enzyme and inhibitor.

The kinetic analysis demonstrated that the inhibition of Xyl I by ATBI, followed slow-tight binding inhibition mechanism and the induced conformational changes, is conveniently monitored by fluorescence spectroscopy. Based on our observations, we conclude that, concomitant with the kinetic analysis, fluorescence spectroscopy plays a very important role in the determination of kinetic constants of enzyme inhibition, as discussed for the characterization of the mechanism of inhibition of Xyl I by ATBI and OPTA.
anism of inhibition of Xyl I by the slow-tight binding inhibitor ATBI.

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