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MEASUREMENT OF INDIVIDUAL RATE CONSTANTS OF IRREVERSIBLE INHIBITION OF A CYSTEINE PROTEINASE BY AN EPOXYSUCCINYL INHIBITOR

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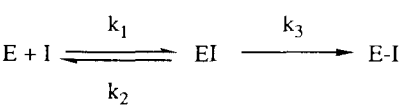
Abstract: The apparent binding constant (K_i) and the first order rate constant of inactivation (k_3) have been obtained for EP-475 inhibition of papain at partially saturating concentrations of inhibitor and various substrate concentrations by global analysis of the data. The value of k_3 ($k_3 = 0.71 \pm 0.25\ s^{-1}$) is larger whereas K_i ($2.4 \pm 0.91\ \mu M$) is comparable to or higher than those values for most nucleofuge methylketone inhibitors.

Introduction

Cysteine proteinases have become an increasingly important area of research as their physiological and pathological roles become better defined. For example cysteine proteinases are implicated in protein processing[1] and turnover,[2] apoptosis,[3] platelet aggregation,[4] tumor metastasis,[5] muscular dystrophy,[6] arthritis,[7] malaria,[8] and various viral infections.[9] One of the most important types of inhibitors of this class of proteinase is the epoxysuccinyl inhibitors. These compounds have been shown to irreversibly alkylate the active site cysteine thiol.[10,11] They have become useful diagnostic tools for identifying and elucidating the function of cysteine proteinases and have also been proposed as potential therapeutics.

Previous work to measure the inhibition of cysteine proteinases by epoxysuccinyl peptides has utilized the method of Kitz and Wilson.[12] In the absence of substrate, the kinetic mechanism assumed for irreversible inhibitors has been that of Scheme 1 where E is enzyme and I is inhibitor. In this mechanism the inhibitor



Scheme 1

forms a reversible complex with the enzyme before inactivating it. K_i is a kinetic constant defined as $k_1k_3/(k_2 + k_3)$ but is considered an equilibrium binding constant (k_2/k_1) when $k_2 >> k_3$; k_3 is the first order rate constant of inactivation. When $[I] >> [E_0]$ ([I] is inhibitor concentration; $[E_0]$ is initial free enzyme

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concentration) and [I] << K_i, then $k_{ap} \approx (k_3/K_i)([I])$ and the apparent second order rate constant of inactivation is $k_i' = k_3/K_i = k_{ap}/[I]$. Using this approximation, shown in Scheme 2, Barrett *et al.*[10] and other groups[13] have calculated k_i' for a wide variety of epoxysuccinyl inhibitors. Here we report the first determination

$$E + I \xrightarrow{k_i'} E\text{-}I$$

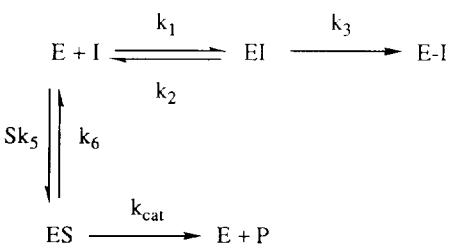
Scheme 2

of K_i and k_3 for any epoxysuccinyl inhibitor. The method used to obtain this information should be applicable to other fast irreversible proteinase inhibitors.

Results and Discussion

At the start of this work the inhibitor assay for papain developed by Barrett *et al.*[10] was employed with slight modification, and the data were analyzed using linear regression according to Kitz and Wilson.[11] However, many epoxysuccinyl inhibitors inactivated papain so quickly that they could only be tested at concentrations well below their K_i. Such concentrations allowed for reasonable estimates of k_i' but not of k_3 and K_i. Slight errors in the measurements made for large differences in k_3 and K_i, giving inconsistent results. Therefore a continuous assay[14] was designed based on the time-point assay of Barrett *et al.*[10] and progress curves were collected and analyzed using nonlinear regression as described.[15] Recently, others have also made use of continuous assays[16] to measure epoxysuccinyl inhibition of cysteine proteinases.

A continuous fluorometric assay utilizing Z-Phe-Arg-AMC as substrate was employed for all the inhibition kinetics reported herein. Scheme 3 shows the mechanism postulated under assay conditions. In a



Scheme 3

typical inhibition assay where [I] >> $[E_0]$ and $[S_0]$ >> $[E_0]$, ($[S_0]$ is substrate concentration at time zero) the resulting progress curve reflects the apparent first order loss of enzyme activity by asymptotically approaching a plateau level of fluorescence. The value of k_{ap} was obtained by fitting the progress curve to a first order exponential as given in the experimental. EP-475 was assayed at five or more concentrations and the resulting values of k_{ap} were fit to the integrated rate equation for competitive irreversible inhibition (Eqn. 2)[15] by non-linear regression to obtain K_i and k_3. The equation requires that the K_m of the substrate be known or that it is calculated as another parameter in the fit. The latter option is preferable, but is only possible when a sufficiently

large number of data points are collected as in this instance. The concentration of active enzyme was determined by titration with EP-475 or E-64 by use of a literature method.[10] Under the standard assay conditions (0.1 M phosphate, pH 6.8, 30 °C) the kinetic parameters determined for Z-Phe-Arg-AMC with papain are k_{cat} = 78.9 ± 1.6 s^{-1}, K_m = 44.9 ± 1.6 μM, and k_{cat}/K_m = 1.76 ± 0.03 x 10^6 $M^{-1}s^{-1}$. K_m and k_{cat}/K_m differ by an order of magnitude from commonly cited literature values;[17] the most likely cause of this discrepancy is that the literature values were obtained in an assay system containing 20% acetonitrile rather than the nearly pure aqueous solution used here.

For fast epoxysuccinyl inhibitors such as EP-475, reliable values of k_3 and K_i were difficult to obtain because of the limited inhibitor concentrations that were possible, and because substrate concentrations were limited to 100 μM or less by insolubility. Increasing the amount of organic solvent in the assay only increased K_m for the substrate and K_i for the inhibitor, and therefore did not help in the attempt to reach saturating levels of inhibitor. To mitigate these problems, inhibition data were collected at five different substrate concentrations, and the entire data set was fit at once to Eqn. 2 (ref. 15) in a global analysis. The kinetic parameters for EP-475 were calculated as k_3 = 0.71 ± 0.25 s^{-1}, K_i = 2.4 ± 0.91 μM, and k_i' = 2.95 ± 0.15 x 10^5 $M^{-1}s^{-1}$. The value for k_i' is in reasonable agreement with that obtained by Barrett *et al.*[10] (3.58 x 10^5 $M^{-1}s^{-1}$); the difference can be explained by both the higher assay temperature, 40 °C, and the different enzyme source[18] in that study. The first order rate constant, k_3, is relatively high compared to those observed for nucleofuge methylketone derived affinity labels (10^{-3} to 10^{-2} s^{-1}),[19] whereas the K_i is comparable or higher than many such irreversible inhibitors. For example peptidyl (acyloxy)methyl ketones with comparable k_i' have nanomolar values of K_i. This combination of

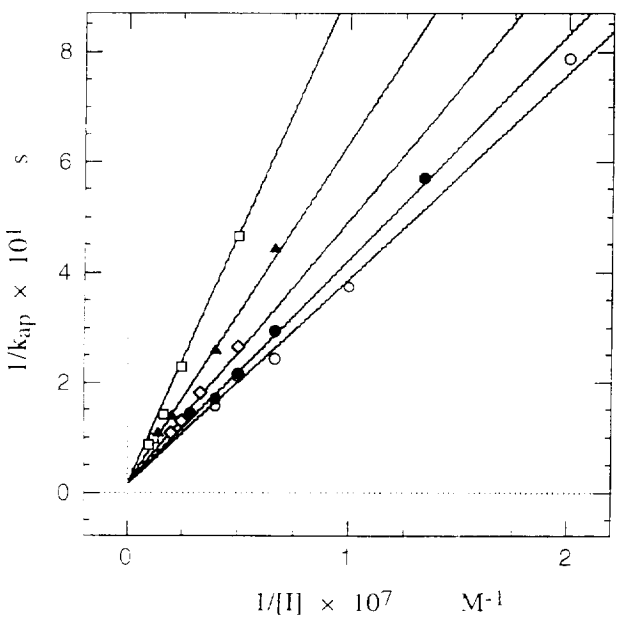


Figure Plot of $1/k_{ap}$ versus 1/[I] for the competitive irreversible inhibitor, EP-475, with papain. The pattern is identical to that for competitive reversible inhibition. Substrate concentrations are: o 5 μM, • 10 μM, ◊ 20 μM, Δ 40 μM. 80 μM. Assay conditions are descibed in footnote 15.

fast k_3 and micromolar values of K_i is the reason it is so difficult to reach saturating levels of epoxysuccinyl inhibitors.

The double reciprocal plot of these data is shown in the Figure. The pattern is identical to that for competitive reversible inhibition because Eqn. 2 has the same form as the equation for competitive reversible inhibition. Though expected from previous structural[20] and kinetic data,[10] this is the first time that an epoxysuccinyl peptide inhibitor has explicitly been shown to exhibit competitive irreversible kinetics. This analysis also shows that it is possible to extract good estimates of the individual rate constants at only partially saturating concentrations of inhibitor. Such an analysis should be applicable to other irreversible proteinase inhibitors with high values of k_3 and micromolar values of K_i.

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15. **Materials and Methods.**
Papain was purchased as a suspension in pH 4.0 acetate buffer from Boehringer Mannheim. Z-Phe-Arg-AMC was purchased from BaChem CA. E-64 was purchased from Sigma, and EP-475 was synthesized by standard methods (J.P. Meara, D.H. Rich, manuscript submitted). Water was distilled and deionized. The stock assay buffer for papain was 0.4 M phosphate (200 mM KH_2PO_4 and 200 mM Na_2HPO_4), 8 mM DTT (added the day of the assay), and 4 mM EDTA. Diluent was an aqueous solution of 0.1% Brij 35. The substrate was dissolved in DMSO to yield a stock solution (10 mM) which remained stable indefinitely when stored at 4° C. Water, buffer, and diluent were passed through 0.2 μm filters (Gellman) before use. Plastic ware was used for all enzyme solutions. Kinetic data were collected using a Perkin-Elmer MPF-4 fluorimeter interfaced with a Gateway 2000 computer running the OLIS data collection program.

Standard Assay Conditions.
Papain in 0.1% Brij 35 (30 μL, ~50-400 nM) was incubated with 30 μL of buffer at 30 °C for 5 min. A 20 μL aliquot of this enzyme solution was added to the assay solution (preequilibrated to 30 °C) to start the reaction. The assay solution consisted of 100 μL water (with or without inhibitor), 0.88 mL diluent, 0.5 mL substrate, 0.5 mL buffer (final concentrations: 0.1 M phosphate, pH 6.8 , 2 mM DTT, 1 mM EDTA, 0.25-2 nM papain). Enzyme assays were followed fluorimetrically at 370 nm excitation, 458 nm emission. The emission was calibrated using an assay solution containing 0.5 μM 7-amino-2-methyl-coumarin in place of substrate. The concentration of active papain was determined by titration with either E-64 or EP-475 by the method of Barrett *et al.*[10]

Substrate Kinetic Measurement.
The standard assay was performed as above with substrate concentrations of 5-100 μM. The first 2 min of the progress curves were collected (≤ 5% substrate depletion) for initial velocity determinations (see Data Analysis below).

Inhibitor Kinetic Measurements.
EP-475 was dissolved in 2% DMSO/water by dissolving in DMSO prior to adding water. Appropriate dilutions with water were then made, and the organic solvent present in an assay was less than 1%. The assay solution was as described above. Five different substrate concentrations from 5 to 80 μM were used. Progress curves were collected at five or more inhibitor concentrations at each substrate concentration.

Data Analysis.
Data were fit to integrated rate equations through least-squares non-linear regression (following the method of Duggleby, R. G. *Comput. Biol. Med.* **1984**, *14*, 447.) using KineTic, written by Petr Kuzmic (U. Wisconsin School of Pharmacy). For substrate kinetic constants, the initial velocities were determined by fitting the linear portion of the curves to a straight line using the OLIS program. The initial velocities were converted to enzyme activity (μmol substrate cleaved)/s and fit with [S] to the integrated form of the Michaelis-Menten equation to determine k_{cat} and k_{cat}/K_m. Time-dependent inhibitor progress curves were fit to a first order exponential (Eqn.1)[14] to yield an apparent inactivation rate (k_{ap}). P is product

$$P = (v_0/k_{ap})(1 - \exp(-k_{ap}t)) + d \qquad \text{(Eqn. 1)}$$

fluorescence; v_0 is the initial velocity; t is time; d is a displacement term to account for the fact that the emission is non-zero at the start of data collection. The values of k_{ap} at all substrate concentrations were then fit as a single set to Eqn. 2 for competitive irreversible inhibition to yield k_3, K_i,and K_m. [I] is

$$k_{ap} = (k_3[I]/([I] + K_i(1 + [S]/K_m)) \qquad \text{(Eqn. 2)}$$

inhibitor concentration, [S] is substrate concentration, K_m is the Michaelis-Menten constant for the substrate, k_3 is the first order inactivation rate, K_i is the apparent binding constant and is equal to $k_1k_3/(k_2 + k_3)$.

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