Mechanism of Inhibition of Human Leucocyte Elastase by Monocyclic β-Lactams†

R. Chabin,‡ B. G. Green,‡ P. Gale,‡ A. L. Maycock,‡,§ H. Weston,‡ C. P. Dorn,∥ P. E. Finke,∥ W. K. Hagmann,∥ J. J. Hale,∥ M. MacCoss,∥ S. K. Shah,∥ D. Underwood,⊥ J. B. Doherty,∥ and W. B. Knight*,‡,●

Departments of Enzymology, Medicinal Chemistry Research, and Molecular Systems, Merck Research Laboratories, Rahway, New Jersey 07065

Received February 17, 1993; Revised Manuscript Received May 13, 1993

ABSTRACT: The kinetic and catalytic mechanisms of time-dependent inhibition of human polymorphonuclear leukocyte elastase (HLE) by the monocyclic β-lactams described by Knight et al. [Knight, W. B., et al. (1992) *Biochemistry 31*, 8160] are investigated in this work. The dependence of the pseudo-first-order rate constant (k_{obs}) on inhibitor concentration was saturable. The individual kinetic constants for the inhibition by L-680,833, [*S*-(*R**,*S**)]-4-[(1-(((1-(4-methylphenyl)butyl)amino)carbonyl)-3,3-diethyl-4-oxo-2-azetidinyl)oxy]benzeneacetic acid, and L-683,845, [*S*-(*R**,*S**)]-4-[(1-(((1-(5-benzofuranyl)butyl)amino)carbonyl)-3,3-diethyl-4-oxo-2-azetidinyl)oxy]benzeneacetic acid, at pH 7.5 were k_{inact} = 0.08 and 0.06 s^{-1} and K_i = 0.14 and 0.06 μM, respectively. The relative potency of this class of compounds as measured by k_{inact}/K_i is primarily controlled by the K_i, term which ranged from 6 nM to 8 mM, while k_{inact} was relatively insensitive to structural changes and varied by only an order of magnitude. Inactivation by the β-lactams was efficient, requiring only 1.3 and 1.7 equiv of L-680,833 and L-683,845 to inactivate HLE. These values are indicative of some partitioning between turnover of inhibitor and inactivation. The partition ratio ranged as high as 3.5:1 depending upon the structure of the inhibitors, but this ratio was essentially independent of the availability and identity of a leaving group at C-4 of the lactam ring. Inactivation and partitioning liberate the leaving group when present at C-4. *p*-Hydroxy-*m*-nitrophenylacetic acid is liberated from this position at a rate similar to that for enzyme inactivation, suggesting kinetic competence of this process. Other products observed during the interaction of L-680,833 with HLE include a substituted urea, a species previously observed during the base-catalyzed decomposition of this class of compounds, and small amounts of products observed during reactivation of β-lactam-derived HLE–I complexes. Both the pH dependence of k_{inact}/K_i for the inactivation of HLE by [*S*-(*R**,*S**)]-4-[(1-(((1-(4-methylphenyl)butyl)amino)carbonyl)-3,3-diethyl-4-oxo-2-azetidinyl)oxy]benzoic acid and V/K for HLE-catalyzed substrate hydrolysis indicate that a single ionizable group with a pK of ~7 must be deprotonated for both processes. This group is likely the active site histidine. The data are consistent with initial formation of a Michaelis complex, acylation of the catalytic serine, and loss of the leaving group at C-4 of the original β-lactam ring followed by partitioning between regeneration of active enzyme and production of a stable enzyme–inhibitor complex. The latter could involve alkylation of the histidine, although this does not appear to be an absolute requirement. These data suggest that these compounds are mechanism-based inhibitors of the HLE.

Inhibitors from diverse structural classes for human leukocyte elastase (HLE, EC 3.4.21.37), a potential therapeutic target in a number of disease states, have been described. These range from macromolecular inhibitors such as α_1-proteinase inhibitor and antileukoprotease inhibitor (ALP) to low molecular weight inhibitors [for a review of the latter, see Stein et al. (1985)]. The structural diversity of HLE inhibitors has produced a medley of inhibition mechanisms even within the same general inhibitor class. For example, α_1 PI[1] is an irreversible inhibitor of HLE, while ALP is a tight-binding reversible inhibitor of the enzyme. Fatty acids such as oleic acid and their derivatives have been described as both competitive and noncompetitive inhibitors of HLE (Tyagi & Simon, 1990; Ashe & Zimmerman, 1977; Hornebeck et al., 1985). Peptide chloromethyl ketones are irreversible inhibitors of HLE, while trifluoromethyl ketones are slow, tight-binding, reversible inhibitors of the enzyme (Williams et al., 1991). A number of potential acylating or alkylating reagents have been reported as HLE inhibitors. Recently, cephalosporin derivatives were described that may produce a "double hit", both acylating the serine and alkylating the active site histidine (Doherty et al., 1986; Knight et al., 1992a). A similar mechanism was originally proposed by Firestone et al. (1990) for the inhibition of HLE by simple monocyclic β-lactams. Both of these classes of β-lactams inhibited HLE according to the minimal kinetic mechanism shown in Scheme I (Knight et al., 1992a,b) consisting of reversible formation of a Michaelis complex followed by formation of an acyl–enzyme intermediate(s) and partitioning between turnover of the inhibitor and further reaction to produce a relatively stable acyl–enzyme. The latter complex slowly hydrolyzes to regenerate active enzyme. The stability of the acyl–enzymes produced from these compounds is consistent with the double

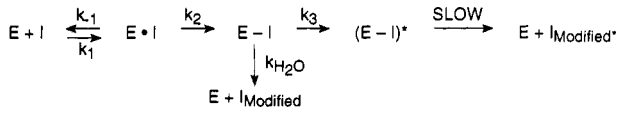
† Portions of this work were presented by Green et al. (1992).

* Address correspondence to this author.
‡ Department of Enzymology.
§ Present address: Sterling Research Group, Malvern, PA 19355.
∥ Department of Medicinal Chemical Research.
⊥ Department of Molecular Systems.
● Present address: Department of Biochemistry, Glaxo Research Inst., Research Triangle Park, NC 27709.

[1] Abbreviations: α_1PI, α_1-proteinase inhibitor; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; Cat-G, cathepsin G; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; DFP, diisopropyl fluorophosphate; DMSO, dimethyl sulfoxide; HLE, human leukocyte elastase; MES, 2-(*N*-morpholino)ethanesulfonic acid; MWCO, molecular weight cut-off; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PMN, polymorphonuclear neutrophils or leukocytes; PPE, porcine pancreatic elastase; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

0006-2960/93/0432-8970$04.00/0

Scheme I



hit hypothesis (Knight et al., 1992b). Evidence that a double hit is not required to produce a stable β-lactam-derived acyl–enzyme was recently provided by Knight et al. (1993). Both of these classes of β-lactams were considered mechanism-based inhibitors of HLE. A series of highly substituted β-lactams were described as potent, specific, and time-dependent inhibitors of HLE (Knight et al., 1992c). In this work, we explore the mechanism of inhibition of HLE by these inhibitors.

EXPERIMENTAL PROCEDURES

Materials

[^{14}C]DFP (sp act. = 125 mCi/mmol) was purchased from New England Nuclear. The synthesis of the other compounds listed in Table I, the substituted ureas, [1,2-^{3}H]ethyl-L-680,833 (sp act. = 40.8 Ci/mmol), and [2,3-^{3}H]propyl-L-669,844 (sp act. = 61.7 Ci/mmol) have been published elsewhere (Shah et al., 1992).[2] Labeled L-680,833 and L-669,844 were diluted with cold material prior to use. The final specific activities of radiolabeled stock solutions were determined by counting under the same conditions. For example, a typical stock solution of [^{3}H]L-680,833 yielded 1.11×10^{8} dpm/μmol. *p*-Hydroxybenzoic acid, *p*-hydroxy-*m*-nitrophenylacetic acid, and *p*-hydroxyphenylacetic acid were purchased from Aldrich Chemical Co. All other reagents and buffers were purchased as in Knight et al. (1992c). Buffers were titrated to the appropriate pH with either HCl or NaOH prior to use. HLE was purchased from Elastin Products. In some cases HLE was passed over a 3-mL DEAE Sephadex A-25 column prior to use. Succ-AAPA-pNA[3] and MeOsucc-AAPV-pNA were purchased from Chemical Dynamics and Calbiochem Co., respectively.

Methods

UV-visible spectroscopy was performed on either Varian DMS-300 or Cary 2200 spectrophotometers. Alternatively, spectrophotometric assays were automated by equipping an Aviv-14DS spectrophotometer with a Zymark robot for sample preparation and a Hewlett-Packard 900/320 series computer for data analysis and storage. HLE activity was determined in buffer A (450 mM NaCl, 10% DMSO, and 45 mM TES at pH 7.5) versus MeOsucc-AAPV-pNA according to Knight et al. (1992c). The active site concentration of HLE was determined by activity according to Green et al. (1991). All kinetic experiments were conducted in buffer A except where noted. The dependence of the kinetics of the HLE reactions with substrate and inhibitors on pH was determined in 450 mM NaCl, 10% DMSO, and 45 mM of the following buffers: succinate, pH 4.75; MES, pH 5.64; MES, pH 6.03; PIPES, pH 6.5 and 7.0; TES, pH 7.5; TAPS, pH 7.97 and 8.66; and CHES, pH 9.1 and 9.58. The enzyme concentrations ranged from 0.016 to 0.0054 μM, and the data were normalized to 0.0054 μM HLE. In control experiments in mixed buffer compositions with overlapping pK_a's, there were no artifacts due to the buffers. The second-order rate constants for the inhibition of HLE by the β-lactams were determined as in Knight et al. (1992c) at pH 7.5. The second-order rate constants for the inhibition of HLE by L-680,831 as a function of pH were determined similarly except versus 2 mM MeOsucc-AAPVpNA with the buffers and enzyme concentrations discussed above. The potency of L-680,833 versus HLE was also determined at pH 5.5 and 8.5 in 450 mM NaCl and 10% DMSO with 45 mM MES and TAPS, respectively. The dependence of the rate of inactivation by L-680,833 and L-684,481 in the presence of 0.125 M NH_2OH was also examined. In control experiments, NH_2OH did not affect the rate of substrate hydrolysis. The liberation of *p*-hydroxy-*m*-nitrophenylacetic acid during the reaction of L-682,271 was monitored by UV-visible spectroscopy at 428 nm (ϵ = 3500 M^{-1} cm^{-1}). This reaction was also monitored as a function of time by stopped-flow spectrophotometry on an Applied Photophysics SX.17MV stopped-flow spectrometer operating in the single-mixing mode over 50 s. The final reaction solutions contained 303 μM L-682,271 and 8.5 μM HLE in buffer A. The substituted ureas were tested as inhibitors versus HLE by varying MeOsucc-AAPV-pNA from 0.04 to 0.6 mM. *N*-[1-(*R*)-(4-methylphenyl)butyl]urea, (L-683,595) was varied from 0-0.12 mM, while *N*-β-naphthylmethylurea was varied from 0 to 0.4 mM. *p*-Hydroxyphenylacetic acid (0.130 mM) was tested as an inhibitor versus the HLE-catalyzed (40 nM) hydrolysis of 0.2 mM succ-AAPA-pNA.

The number of equivalents of the inhibitors necessary to completely inactivate HLE was determined by incubating 4 μM HLE at 25 °C with varying amounts of inhibitor in buffer A. Ten-microliter aliquots were assayed for activity over time to determine the extent of inactivation. The inactivation was over in minutes for the better inhibitors, but was monitored for 1 h. The remaining activity was defined as that present at 1 h. In separate experiments it was determined that removal of enzyme-generated metabolites utilizing centrifugal gel filtration (Penefsky, 1977; Green et al., 1991) columns did not affect the results (vide infra). Similar titrations were conducted with L-680,833, L-682,946, and L-684,481 in the presence of 0.125 M hydroxylamine in buffer A and with L-680,833 at pH 5.5 (45 mM MES, 450 mM NaCl, and 10% DMSO) and in 50 mM NaCl, 10% DMSO, and 50 mM $NaPO_4$ at pH 7.5.

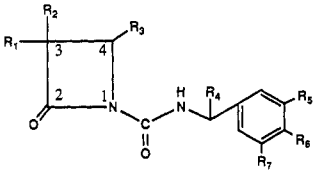
In experiments with radiolabeled β-lactams and DFP, excess inhibitor and any metabolites were removed by centrifugal gel filtration. Either 1- or 3-mL DEAE Sephadex A-25 column equilibrated in buffer A were centrifuged at 1000*g* for 5 min at 4 °C to remove excess buffer. The enzyme solution was loaded onto the column, and the column was centrifuged again to elute the protein. The results were corrected for recovery from the column upon the basis of either the A^{280} or the HLE activity of a control reaction treated similarly. The protein recovery was typically 80%. In a typical experiment, 29 μM HLE in 0.6 mL was incubated with 180 μM [^{3}H]L-680,833 for 1 h. A 0.5-mL aliquot was passed over a 3-mL DEAE Sephadex column, and 100-μL aliquots were added to 10 mL of scintillation cocktail and counted on a Packard Tricarb 2000A counter.

HPLC analysis of the interaction of HLE with the β-lactams was conducted on 150 × 4.6-mm C-18 columns with two acetonitrile gradient elution systems (Knight et al., 1992c).

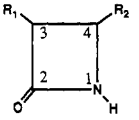
[2] For other examples, see European Patent Office Publication EPO O 337 549, Doherty et al. (1993), and Hanlon et al., European Patent Application 92202533.3.

[3] Peptide-based substrates and inhibitors are abbreviated using the standard one-letter representation of the amino acids. Additional functionalities present were abbreviated as follows; MeOsucc; methoxysuccinyl; pNA, *p*-nitroanilide; succ, succinyl.

Table I: Structures of β-Lactams



L-number	R_1	R_2	R_3	R_4	R_5	R_6	R_7
669,844	ethyl	(*S*)propyl	(*S*)-*p*-hydroxybenzoic acid	H	H	H	H
670,258	ethyl	ethyl	(*S*)-*p*-hydroxybenzoic acid	H	–CH(CH)$_2$CH–		H
680,831	ethyl	ethyl	(*S*)-*p*-hydroxybenzoic acid	(*R*)-propyl	H	CH_3	H
680,833	ethyl	ethyl	(*S*)-*p*-hydroxyphenylacetic acid	(*R*)-propyl	H	CH_3	H
682,271	ethyl	ethyl	(*S*)-*p*-hydroxy-*m*-nitrophenylacetic acid	(*R*)-ethyl	H	H	H
682,946	ethyl	ethyl	(*R*)-*p*-hydroxyphenylacetic acid	(*R*)-propyl	H	CH_3	H
683,537	ethyl	ethyl	(*S*)-*p*-hydroxybenzoic acid	(*R*)-propyl	CH_3	H	CH_3
683,557	ethyl	ethyl	(*S*)-*p*-hydroxybenzoic acid	(*S*)-propyl	H	CH_3	H
683,558	ethyl	ethyl	(*R*)-*p*-hydroxyphenylacetic acid	(*S*)-propyl	H	CH_3	H
683,841	ethyl	ethyl	(*S*)-*p*-hydroxybenzoic acid	(*R*)-propyl		$-(CH_2)_4-$	H
683,845	ethyl	ethyl	(*S*)-*p*-hydroxyphenylacetic acid	(*R*)-propyl		–CHCHO–	H
684,202	ethyl	ethyl	H	(R)-CH_2CHCH_2	H	CH_3	H
684,481	ethyl	ethyl	H	(*R*)-propyl	H	CH_3	H
691,886	ethyl	ethyl	(*S*)-*p*-hydroxyphenylacetic acid	(*R*)-propyl	H	OCH_2CH_3	H
692,357	ethyl	ethyl	(*S*)-*p*-hydroxyphenyl-$(CH_2)OC(O)-CH_2-N(H)C(O)CH_2$-phenyl	(*R*)-propyl	H	CH_3	H



L-number or compound	R_1	R_2
C-4-acetoxy-β-lactam	H	$OC(O)CH_3$
651,173	$CHCH_2$	$C(O)OCH_2CH_3$

The eluants were monitored at 223 and 250 nm. The reaction mixtures were filtered through a 10 000 MWCO filter to remove protein prior to injection. The products observed during the complete inactivation of 1 equiv of HLE by L-680,831, L-680,833, L-683,845, L-669,844, and L-670,258 were examined by incubating HLE with 1-10 equiv of inhibitor for 1 h and subjecting the solutions to HPLC analysis. In a typical experiment, 0.094 mM HLE was treated with 0.89 mM L-669,844, and 10-150-μL aliquots were assayed by HPLC. Separate experiments with L-680,833 were conducted in the presence of 0.125 M NH_2OH. The concentrations of resulting products and inhibitor consumed were estimated upon the basis of the extinction coefficients calculated from standards of the inhibitors, the leaving groups (*p*-hydroxybenzoic acid or *p*-hydroxyphenylacetic acid), or the substituted ureas. The suspected identity of enzyme-generated products was confirmed by spiking authentic standards and conducting the HPLC analysis with both acetonitrile gradient systems. The amount of unidentified products was estimated by assuming the extinction coefficient of the corresponding urea since the UV spectra indicated that the group originally present at C-4 of these inhibitors was absent from these species.

Data Analysis. Initial rates were determined by least linear squares regression of the change in absorbance as a function of time to eq 1. The kinetic parameters for substrate hydrolysis as a function of pH were determined by fitting the initial velocities as a function of substrate concentration (0.2-3 or 4 × K_m) to eq 2 by nonlinear least squares regression. The pseudo-first-order rate constants, k_{obs}, for the inhibition of HLE as a function of time by β-lactams were determined according to eq 3. The second-order rate constants (k_{inact}/K_i) were estimated according to eq 4 in at least duplicates. The individual kinetic constants (k_{inact} and K_i) for the inhibition of HLE by the β-lactams were obtained by determining k_{obs} (in duplicate or triplicate) as a function of the inhibitor concentration according to eq 5 (Kitz & Wilson, 1962). The apparent K_i and k_{inact}/K_i values were corrected for the substrate concentration according to eq 6. The pH dependence of the kinetic parameters (Y) for substrate hydrolysis and β-lactam inhibition were analyzed according to eq 7. The data obtained from the inhibition of HLE by the ureas were fit to eqs 8 (competitive) and 9 (mixed noncompetitive).

$$Y = v_i X + B \quad (1)$$

$$Y = V_m[S]/(K_m + [S]) \quad (2)$$

$$Y = v_s t + ((v_o - v_s)(1 - e^{-k_{obs}t})/k_{obs}) + A_o \quad (3)$$

$$k_{obs}/[I] = k_{inact}/(K_i(1 + [S]/K_m)) \quad (4)$$

$$k_{obs} = K_{app}[I]/(K_{iapp} + [I]) \quad (5)$$

$$K_i = K_{iapp}/(1 + [S]/K_m) \quad (6)$$

$$\log Y = (\log Y_{lim})/(1 + [H]/K_a) \quad (7)$$

$$Y = V_m[S]/(K_m(1 + [I]/K_i) + [S]) \quad (8)$$

$$Y = V_m[S]/(K_m(1 + [I]/K_{is}) + [S](1 + [I]/K_{ii})) \quad (9)$$

RESULTS

Progress curves of the HLE-catalyzed hydrolysis of MeO-succ-AAPV-pNA in the presence or absence of L-680,833 and hydroxylamine are shown in Figure 1. In the absence of the nucleophile the reaction follows a first-order loss of activity in agreement with Doherty et al. (1993), while the presence of the hydroxylamine afforded protection of the enzyme from inactivation. The inhibition of HLE by L-684,481 followed first-order kinetics in the presence and absence of hydroxylamine (see Figure 2). The second-order rate constants for inactivation of HLE by 18 β-lactams are reported in Table II.

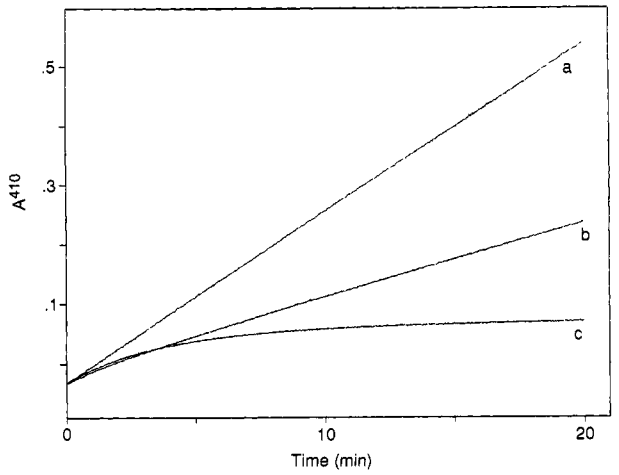


FIGURE 1: Progress curves for (a) the HLE-catalyzed (7 nM) hydrolysis of 1 mM MeOsucc-AAPV-pNA; (b) the reaction in the presence of 0.11 μM L-680,833, and 0.125 M NH_2OH; and (c) the reaction in the presence of 0.11 μM L-680,833.

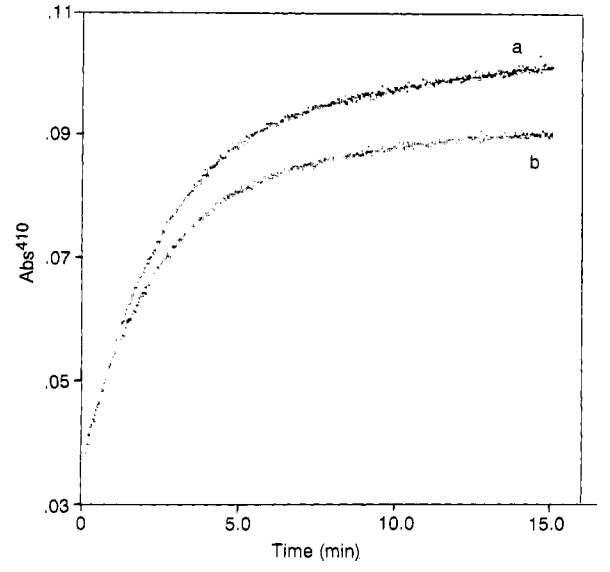


FIGURE 2: Progress curves for the hydrolysis of 0.2 mM succ-AAPA-pNA by 30 nM HLE (a) in the presence of 0.356 μM L-684,481 and (b) in the presence of 0.356 μM L-684,481 and 0.125 M NH_2OH. The theoretical fits of the individual data sets to eq 3 are shown as dotted curves.

pH Studies. The kinetic parameters for the hydrolysis of MeOsucc-AAPV-pNA by HLE and the second-order rate constant for the inactivation of HLE by L-680,831 and L-680,833 were determined as a function of pH. There were single ionizations evident in both the V/K (see Figure 3A) and the V_m (data not shown) pH profiles for the substrate hydrolysis reaction. The pK_a's, assuming a simple model for a single ionization, were 7.13 ± 0.05 and 5.73 ± 0.09, respectively. The limiting values for k_c/K_m and k_c were 1.1 ± −0.07 × 10^5 M^{-1} s^{-1} and 8.7 ± 0.6 s^{-1}, respectively. The pH dependence of k_{inact}/K_i for the inhibition of HLE by L-680,831 exhibited a pK_a of 6.91 ± 0.04 (Figure 3B). The limiting value of k_{inact}/K_i was 4.6 ± 0.2 × 10^5 M^{-1} s^{-1}. In Table II the pH dependence of the inhibition of HLE by L-680,833 is presented. The compound is less potent versus HLE at lower pH by about 30-fold, indicating the same trend observed with L-680,831.

In Figure 4 the dependence of the first-order rate constant (k_{obs}) calculated from primary data on the concentration of L-680,831 is presented. Similar curves were observed with L-680,833, L-683,845, L-684,481, L-692,357, and *C*-4-acetoxy-β-lactam. The observation of saturation kinetics in these cases allowed the calculation of the individual kinetic constants (K_i and k_{inact}), which are summarized in Table II. L-651,173 did not clearly display saturation kinetics versus HLE up to the limit of compound solubility; therefore, we could only place lower limits on the individual kinetic parameters (see Table II).

L-683,595 displayed competitive inhibition versus HLE with a K_i of 43 ± 8 μM. *N*-β-naphthylmethylurea displayed noncompetitive inhibition of HLE with a K_i of 140 ± 20 μM (K_{is} was equal to K_{ii}). There was no inhibition of HLE-catalyzed hydrolysis of 0.2 mM succ-AAPA-pNA (~0.1K_m) by 130 mM *p*-hydroxyphenylacetic acid.

A typical titration of HLE activity with a monocyclic β-lactam is presented in Figure 5. It required approximately 1.3 equiv of L-680,833 to inactivate the enzyme to approximately 99%. Similar values were obtained at pH 5.5, at 37°C and at lower ionic strength. In the presence of 0.125 M hydroxylamine, 18 equiv was required to inactivate the enzyme (see Figure 5). The presence of hydroxylamine had virtually no effect on the amount of L-684,481 and L-682,946 required to inactivate HLE. The results from similar titrations of HLE activity with additional compounds are reported in Table II.

HLE (0.0023 μmol) inactivated with [^{3}H]ethyl-L-680,833 retained 231 000 ± 2000 dpm after removal of excess inhibitor and metabolites. This corresponds to 0.89 ± 0.01 equiv of label per active site. Similar experiments conducted with [2,3-^{3}H]propyl-L-669,844 indicated the incorporation of 0.86 ± 0.04 equiv of label per enzyme. HLE inactivated with [^{14}C]-DFP retained 1.13 equiv of label on the basis of the reported specific activity of the inhibitor. Enzyme pretreated with L-680,831 until approximately 99% inhibition was observed only retained 0.0017 equiv of DFP.

In Figure 6 a typical HPLC chromatogram of the reaction of L-669,844 with HLE is shown. During the inactivation of 1 equiv of HLE in the presence of 10 equiv of L-669,844, which resulted in the consumption of 2.5 ± 0.3 equivalents of inhibitor, 2.6 ± 0.1 equiv of *p*-hydroxybenzoate were produced. Additional metabolites observed included species with identical retention times to the *N*-benzylurea (0.65 equiv) and the vinyl product (1.0 equiv) observed during the base-catalyzed decarboxylation of this compound (Knight et al., 1992c). The identity of these species was further confirmed by spiking with authentic materials and repeating the HPLC analysis with two different acetonitrile gradients. By monitoring the production of *p*-hydroxybenzoic acid and the disappearance of parent by HPLC during the inactivation of HLE by L-670,258, we confirmed the titration data obtained kinetically (see Figure 7). In the HPLC chromatogram of Figure 7 we observed *p*-hydroxybenzoic acid, L-670,258, *N*-β-naphthylmethyleneurea, the vinyl adduct produced during the base-catalyzed decomposition, and two additional metabolites (labeled as c). The UV-visible spectra of the unknown products indicate that they do not contain *p*-hydroxybenzoic acid (data not shown).[4] The products observed by HPLC resulting from the inactivation of HLE by L-680,833 are summarized in

[4] In previous work we demonstrated that the decarboxylated product tended to precipitate from aqueous buffer at higher concentrations (Knight et al., 1992c). In subsequent work we have demonstrated that species with similar retention times to the other two unknowns reported in this work are produced during reactivation of the corresponding β-lactam-derived HLE–I complexes. These species also tended to precipitate from buffer at higher concentrations. Mass spectral analysis of these metabolites demonstrated that they contain the ureas. (B. G. Green and W. B. Knight, unpublished data).

Table II: Kinetic Constants[a] for the Inhibition of HLE by β-Lactams

inhibitor	K_i, μM	k_{inact}, s^{-1}	k_{inact}/K_i, M^{-1} s^{-1}	no. of equiv[b] consumed: based upon activity	no. of equiv[b] consumed: based upon HPLC
C-4-acetoxy-β-lactam	7770 ± 1400	0.00872 ± 0.0003	1.12	nd	nd
α_1P_I	nd	nd	2.2×10^6 [c]	1.0	nd
651,173	>3000	>0.021	107	nd	nd
669,844	nd	nd	5000	2.7 ± 0.03	2.5
670,258	nd	nd	21 000	3.2 ± 0.2[d]	3.0
680,831	0.27	0.07	260 000, 300 000[e]	1.1	1.0
680,833	0.14 ± 0.04	0.083 ± 022	570 000	1.3 ± 0.1	1.2
+NH_2OH	nd	nd	nd	18	12
25 °C			510 000	1.4	nd
37 °C			800 000	1.3	nd
pH = 8.5	nd	nd	720 000	nd	nd
pH = 7.5			565 000	1.3	nd
pH = 5.5	nd	nd	23 200	1.3	nd
pH = 7.2, 50 mM P_i, 50 mM NaCl			305 000	1.5	nd
682,271	nd	nd	18 500	1.2	nd
682,946	nd	nd	27 000[e]	1.2	nd
+NH_2OH	nd	nd	nd	1.3	nd
683,537	nd	nd	675 000	1.5	nd
683,557	nd	nd	18 000[e]	1.5	nd
683,558	nd	nd	6700[e]	4.5	nd
683,841	nd	nd	2.93×10^6	1.3	1.3
683,845	0.06 ± 0.015	0.06 ± 0.009	980 000	1.7 ± 0.3	nd
			848 000[c]		
684,202	nd	nd	25 500	1.5	nd
684,481	4.3 ± 1.1	0.13 ± 0.03	30 000	1.1	nd
			22 000		
+NH_2OH	nd	nd	21 800	1.3	nd
691,886	nd	nd	1.1E6	1.4	nd
692,357	0.0065 ± (7 × 10^{-4})	0.012 ± (6 × 10^{-4})	(1.8 ± 0.17) × 10^6	nd	nd

[a] All constants were obtained at pH 7.5 in buffer A at 25 °C except where noted. Experiments were conducted with L-680,833 under a variety of conditions for direct comparison. At pH 8.5 and 5.5, 45 mM TAPS and MES were used in 450 mM NaCl and 10% DMSO. The reaction in the presence of 0.125 M NH_2OH was conducted in buffer A. The temperature dependence was examined in buffer A at 25 and 37 °C. The ionic strength dependence was examined in the presence of 50 mM P_i, 50 mM NaCl, and 10% DMSO. The standard deviations in the second-order rate constants from at least two determinations were less than or equal to 15% of the reported values. The second-order rate constants were estimated as in Knight et al. (1992c) unless individual kinetic constants are reported; then the values are the results of fitting the data to eqs 5 and 6 in the text. Standard errors in the individual kinetic constants obtained from the fit of these data are reported. [b] The standard errors in the determination of the number of equivalents required to inactivate HLE were less than 10% of the reported values. Where errors are quoted, they are deviations from the mean of two determinations. [c] These values were reported by Knight et al. (1992c). [d] These plots displayed curvature at the higher I/E ratios (>80% inhibition). The value reported is from $n = 2$, extrapolated from the linear portion of the curve. [e] These values were reported by Doherty et al. (1993).

Scheme II (data not shown). *p*-Hydroxyphenylacetic acid, 1.2 equiv, was produced from the complete inactivation of HLE in the presence of a 5-fold molar excess of L-680,833 with the consumption of 1.3–1.5 equiv of compound. In addition, small amounts of L-683,595 (urea) and the decarboxylated species (I, the vinylogous urea in Scheme II), previously observed during the base-catalyzed decomposition (Knight et al., 1992c), were produced from L-680,833 (data not shown). Two new species analogous to those observed during the L-670,258 reaction were also observed during the interaction of L-680,833 with HLE. Assuming an extinction coefficient at 223 nm equivalent to the urea, the amount of the unknown products, the urea, and I produced was approximately 0.2-0.3 equiv. Mass balance requires that the production of the urea and I be accompanied by production of 2,2-diethyl-3-oxopropanoic acid and CO_2, respectively, although these experiments do not address these issues. Identical products were observed as a result of the inactivation of HLE by L-680,831 except *p*-hydroxybenzoate replaced *p*-hydroxyphenylacetic acid. In similar experiments the titration equivalents were confirmed during the reaction of L-683,845 with HLE. Products with similar retention times relative to the parent compound were produced from L-683,845 as well. In the presence of hydroxylamine 12 equiv of L-680,833 was consumed, producing 10 equiv of *p*-hydroxyphenylacetic acid and 1 equiv (based upon the extinction coefficient displayed by the urea) of a species displaying the same retention time as the urea, L-683,595. In addition, three other less polar species (longer retention times on the C-18 column) were observed. These products have not been isolated and characterized.

Experiments with L-682,271. It required 1.3 equiv of L-682,271 to completely inactivate 1 equiv of HLE. The reaction of HLE (11.2-36 μM) with L-682,271 (0.28 mM) resulted in the liberation of 1.15 ± 0.05 equiv of *p*-hydroxy-*m*-nitrobenzoic acid. In stopped-flow experiments monitored over 50 s, *p*-hydroxy-*m*-nitrophenylacetic acid was produced from L-682,271 in a first-order process with k_{obs} equal to 0.14 ± 0.01 s^{-1}. The second-order rate constant can be estimated from k_{obs} and the enzyme concentration (8.5 μM) to be 16 500 ± 1200 M^{-1} s^{-1} [5]. This value is similar within experimental error to the second-order rate constant for the inactivation of HLE by this compound (see Table II).

DISCUSSION

In agreement with Stein (1983), the pH dependence of both V_m and V/K for the reaction of HLE with substrate indicates that a single group must be ionized for maximal

[5] Preliminary experiments demonstrated that the concentration of inhibitor used in this study was saturating.

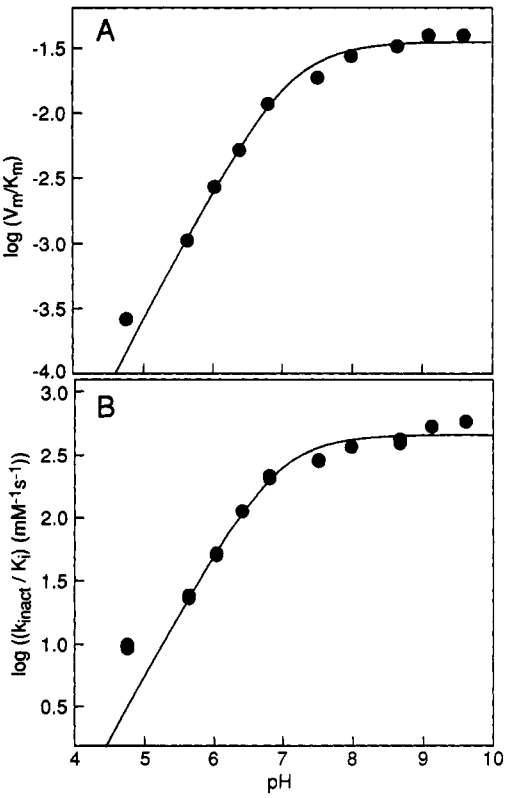


FIGURE 3: (A) pH dependence of the relative V/K for the hydrolysis of MeOsucc-AAPV-pNA by 5.4 nM HLE. (B) pH dependence of the second-order rate constant for the inhibition of HLE by L-680,831. The curves in panels A and B are the theoretical fits of the data to eq 7.

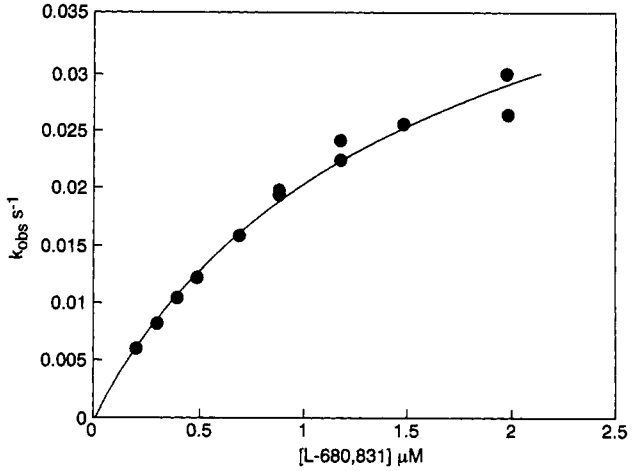


FIGURE 4: The dependence of the first-order rate constant for inhibition on the concentration of L-680,831. The curve is the theoretical fit of the data to eq 5.

activity. The identity of the group is likely the active site histidine. From the dependence of V_m on pH, it is clear that the pK_a for this group is perturbed to lower pH in the ES complex. This is not unusual in enzymatic reactions. Another interpretation of this perturbation is a change in the rate-limiting step at lower pH, as suggested by Stein (1983). This is also a common occurrence in enzymatic reactions [for example, see Schmerlik and Cleland (1977) or Grimshaw et al. (1981)]. The dependence of the second-order rate constant for inhibition of HLE by L-680,831 was similar to that observed in the pH dependence of V/K for substrate hydrolysis. The limiting second-order rate constant for inactivation by L-680,831 was 4.62 ± 0.24 × 10^5 M^{-1} s^{-1}. The activity of L-

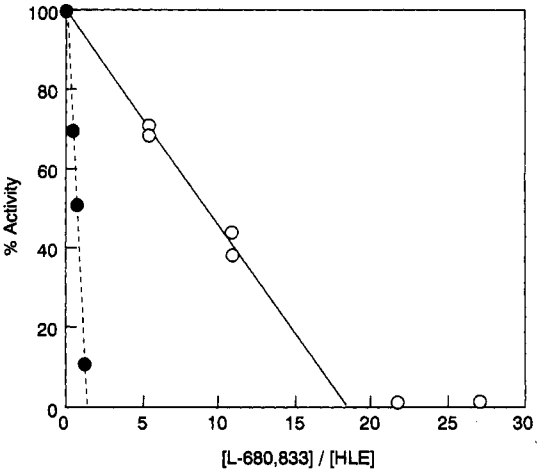


FIGURE 5: Titration of HLE activity with L-680,833 in the absence (●) and the presence (○) of 0.125 M NH_2OH. The lines are linear least squares fits to the individual data sets.

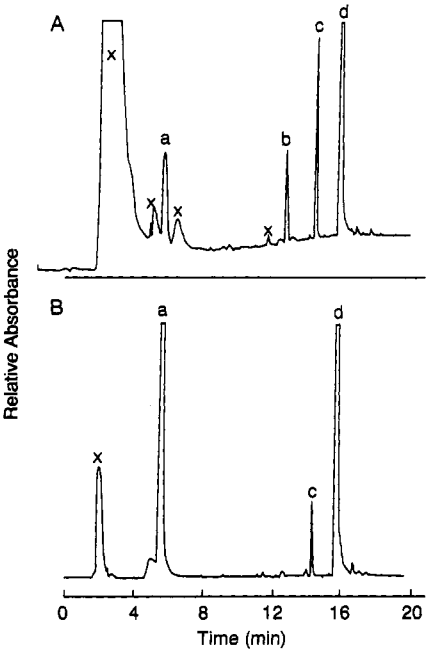


FIGURE 6: HPLC chromatograms of the reaction of 0.63 mM L-669,844 with 0.063 mM HLE at 25°C and pH 7.5. Panels A and B show chromatograms that were monitored at 223 and 250 nm, respectively. One hundred and fifty microliters of the reaction solution was injected. These data were collected on a Varian CDS 504 data station for analysis. The species present are (a) *p*-hydroxybenzoic acid, (b) *N*-benzylurea, (c) the vinyl decarboxylated product also observed during base-catalyzed decomposition of L-669,844 [see text and Knight et al. (1992c)], and (d) L-669,844. The areas labeled X are artifacts due to either buffers or the gradients, as they are present when 150 mL of control solutions is injected.

680,833 versus HLE also decreased with pH. These data demonstrate that the active site histidine must be deprotonated for maximal rate of inactivation. These results indicate that the inactivation process utilizes the same ionization state of HLE as substrate during hydrolysis and adds credence to the classification of the β-lactams as mechanism-based inhibitors.[6]

The data presented in this work demonstrate that the monocyclic β-lactams follow the minimal kinetic mechanism during their interaction with HLE shown in Scheme I until formation of the stable E–I complex. The last step, slow reactivation of the HLE–I complexes, will be discussed in subsequent work. This is the same kinetic mechanism reported for the inhibition of HLE by cephalosporin derivatives (Knight

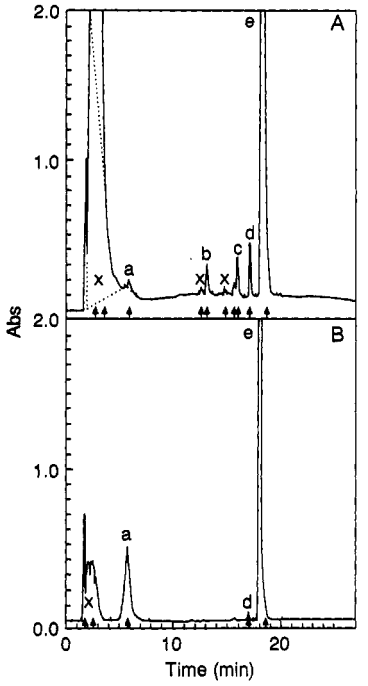


FIGURE 7: HPLC chromatograms of the reaction of excess L-670,258 with HLE after 1 h. Panels A and B show spectra that were monitored at 223 and 250 nm, respectively. These data were collected on a Waters Maxima system for analysis. The areas labeled X are artifacts due to either buffers or the gradient, as they are present in injections of equal volumes of control solutions. The species present and (a) *p*-hydroxybenzoic acid, (b) *N*-β-naphthylmethylurea, (c) unknown(s), (d) the vinyl decarboxylated product observed during the base-catalyzed decomposition of L-670,258 [see text and Knight et al. (1992c)], and (e) L-670,258.

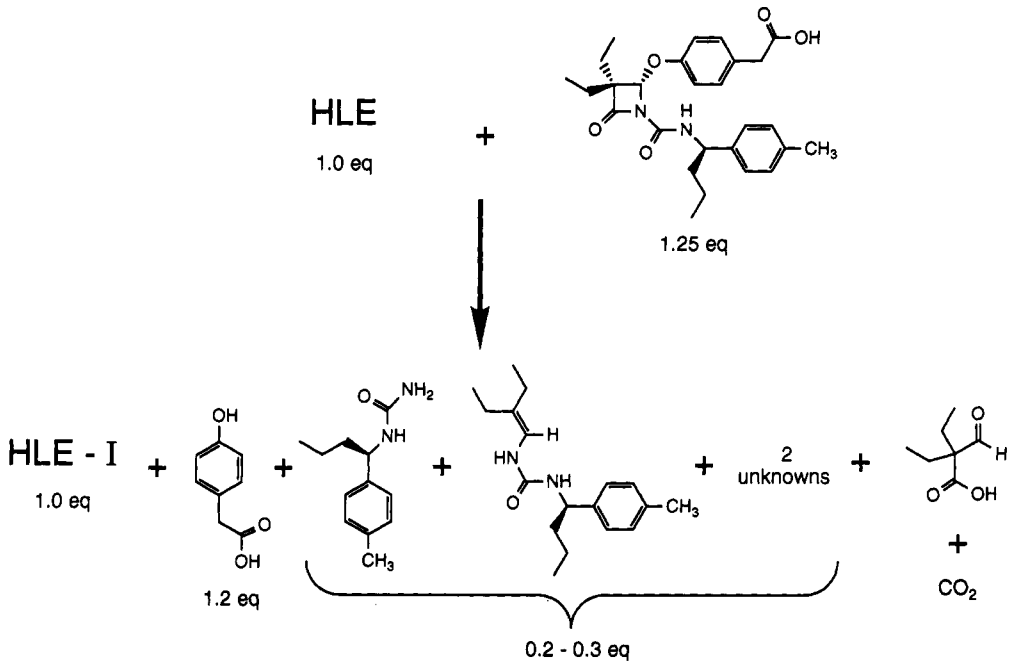
et al., 1992). The observation of saturation kinetics with these compounds indicates that they initially form reversible complexes with the enzyme. The next step(s) involves formation of an acyl–enzyme(s). Pretreatment of the enzyme with L-680,831 prevents the uptake of radiolabeled DFP. Conversely, pretreatment with DFP prevents the uptake of labeled L-680,833 (data not shown). This is consistent with acylation of the serine by the monolactam. This conclusion is supported by the additional observation that the initial complex formed between HLE and L-680,833 is sensitive to nucleophiles such as hydroxylamine. L-680,833 was consumed in 10-14-fold greater amounts in the presence of hydroxylamine, demonstrating that the nucleophile can "rescue" the acyl–enzyme from further inactivation. This indicates that k_{nuc} times the hydroxylamine concentration is 10-14-fold greater than $k_{H_2O} + k_3$ in Scheme I. The observation that the amount of *p*-hydroxyphenylacetic acid produced in the presence of NH_2OH was essentially equivalent to the amount of inhibitor consumed suggests that the nucleophile acts on an acyl–enzyme after departure of this group. Therefore, the acyl–enzyme partitions between turnover to produce modified inhibitor and formation of a stable inactive HLE–inhibitor complex. Titration of HLE activity with most of the monobactams in Table I indicates that the partition ratio is almost 0, indicating an optimally efficient inhibitor.[7] The most efficient monocyclic β-lactams were severalfold more efficient than the cephalosporin derivatives (Knight et al., 1992c) and rival the efficiency of α_1PI versus HLF, whose partition ratio is essentially 0. The partition ratio was not affected by pH, ionic strength, or temperature. The partition ratio was not affected by the stereochemistry of the leaving group at C-4 (compare L-680,833 to L-682,946). When HLE was titrated with L-684,202 and L-684,481, it required only 1.1-1.5 equiv to inactivate HLE. These values are similar to that observed for L-680,833 and L-680,831 (1.3 equiv) but less than that observed for L-670,258 (3.0 equiv). This indicates that the nature of the substituted urea has a greater effect on the partition ratio between turnover and inactivation than does the presence of a potential leaving group on the ring. Comparison of L-670,258 to L683,841 suggests that the propyl substitution on the benzylic carbon has a greater effect on the partition ratio than the size of the substitution on the phenyl ring, although this is not a perfectly matched set, as the former is a naphthyl derivative and the latter is a tetralin. Other substitutions on the phenyl ring have essentially no effect on the partition ratio. L-683,558, the enantiomer of L-680,833, has a partition ratio of 3.5:1, indicating that hydrolysis of the intermediate E–I complex is faster than production of the final complex. Comparison of these data to those displayed by the other three diastereomers, L-683,-557, and L-680,833, and L-682,946, indicates that a combination of incorrect stereochemistry at both C-4 and the benzyl carbon α to the urea nitrogen adversely affects the partition ratio, while these individually have no effect on this parameter.

The data obtained with L-684,481, which lacks a potential leaving group, suggests an inconsistency to the kinetic mechanism of Scheme I. The reaction of L-684,481 with HLE was independent of hydroxylamine. This suggests that the rate-limiting step in the reaction is either formation of the tetrahedral intermediate or its collapse to open the lactam ring, producing the stable acyl–enzyme directly without partitioning. The amount of this compound required to completely inactivate HLE was 1.1 equiv, which is too close to unity to definitively establish a partitioning step. This value increased somewhat in the presence of hydroxylamine, but once again the increase is not clearly definitive. If there is an additional step after formation of the initial acyl–enzyme from L-684,481, it must be a conformational change leading to the stable acyl–enzyme. The relative insensitivity to hydroxylamine would require either that this step be fast relative to formation of the initial acyl–enzyme or that both acyl–enzymes be inaccessible to bulk solvent. In the latter case, the water required for partitioning must be trapped in the active site. Additional data in support of this proposal

[6] The purpose of the pH studies was to demonstrate that the inactivation by inhibitors such as L-680,831 utilizes the same ionization state as substrate hydrolysis. The data in Figure 3 adequately fulfill this purpose, but close examination of these data suggests that the pH dependence of the second-order rate constants for both substrate hydrolysis and inactivation by L-680,831 may deviate from that predicted by the simple model. In particular there may be a pH-independent component to the inhibition data at low pH, as the rate appears to level off rather than continuing to decrease. It would be tempting to attribute this to a greater rate of inactivation by the acid over the carboxylate of L-680,831 offsetting protonation of the histidine, but to a lesser extent V/K for substrate hydrolysis also appears to level off at low pH. Matta et al. (1976) reported a similar observation during the hydrolysis of *N*-acetyl-L-tyrosine-4-acetylanilide by subtilisin. The simplest explanation for this phenomenon is that there is an alternate pathway at low pH. Either general base catalysis by the active site histidine is not absolutely required for catalysis, but does increase the rate by 2 orders of magnitude, or protonation of another active site residue offsets the decrease in rate upon functional loss of the histidine. For example, a protonated acidic residue could function as an acid catalyst for both the inactivation and the substrate hydrolysis processes. More detailed studies at low pH are required to firmly establish (a) if there is deviation from the simple model and (b) the explanation for deviation.

[7] The partition ratio equals the number of equivalents required for complete inactivation minus 1.

Scheme II



will be presented in subsequent work concerning the molecular identity of the stable acyl–enzymes derived from β-lactams.

The increase in potency of the highly substituted monocyclic β-lactams such as L-680,833 versus the cephalosporin derivatives results from both a lower K_i, the concentration of inhibitor that produces half the maximal rate of inactivation, and an increase in k_{inact}, the maximal rate constant for the inactivation of the enzyme, by an order of magnitude. For example, Green et al. (1991) reported that k_{inact} and K_i equal to 0.007 s^{-1} and 1.7 μM for the inhibition of HLE by the cephalosporin derivative L-658,758. The improvement over the chloromethyl ketones is primarily due to a dramatically lower K_i (0.14 μM for L-680,833 versus 30 μM for MeOsucc-AAPV-CMK), while the chloromethyl ketones inactivate HLE with similar maximal rates to those displayed by the monobactams (Green et al., 1991). The K_i reported in this work should not be equated to a true dissociation constant ($K_d = k_{-1}/k_1$) for the initial E–I complex since the kinetic mechanism in Scheme I will yield complex terms for both K_i and k_{inact} (Waley, 1980, 1985).[8] The individual structural components of these inhibitors, the ureas and the C-4 substituents, display only very weak inhibitory activity (high micromolar K_i's, if any at all) versus HLE. In addition, simple peptides bind to HLE with K_i's in the millimolar range even when they take advantage of binding subsites on both sides of the scissile bond including the S_1 specificity pocket (McRae et al., 1980). These two sets of observations further suggest that it is highly unlikely that the low K_i determined in these experiments can be equated to the K_d for the initial Michaelis constant formed from the β-lactams and HLE. In the future, the determination of the rate constants for the individual steps in Scheme I, in particular k_2, should allow the calculation of K_d.

Within the monocyclic β-lactam series the K_i has the greatest effect on the observed potency, varying over 7 orders of magnitude, while k_{inact} varies only 10-fold (compare *C*-4-acetoxy-β-lactam to L-680,833). In fact, dramatic decreases in K_i offset decreases in k_{inact} in the case of L-692,357. Both the substituted urea portion (compare L-683,845 and L-680,833) and the leaving group portion of the molecule (or the lack thereof; compare L-680,831, L-680,833, L-684,481, and L-692,357) affect K_i. Comparison of the maximal rate of inactivation of HLE by L-680,833 and L-692,357 within the phenoxy-substituted series suggests that the presence of a group at C-4 with decreased potential as a leaving group will decrease the maximal rate, but the data obtained with L-684,481, which lacks a leaving group, yield a similar maximal rate to that displayed by L-680,833. Care must be taken in making these comparisons, as the kinetic terms that define k_{inact} will likely differ in the latter and be similar in the former cases. For example, k_{inact} will contain rate constants for step(s) that liberate the leaving group and its release from the enzyme in the case of L-680,833 and L-692,357 unless these steps occur after the rate-determining step of the reaction. It should be noted that HPLC experiments were not conducted with L-692,367 to demonstrate that the C-4 substituent was indeed liberated during the reaction with HLE, but experiments with compounds of similar structure (B. G. Green and W. B. Knight, unpublished results) suggest that this should be the case. Given the considerations discussed above, it would be premature to draw conclusions concerning the structure–activity relationships of the individual kinetic constants in these reactions. These must await a detailed analysis of the microscopic kinetic constants.

The interaction of the β-lactams with *S*-stereochemistry at C-4 of the ring with HLE yielded several products in addition to the inactive HLE–I complexes. The *p*-hydroxybenzoate and *p*-hydroxyphenylacetate groups present at C-4 of these β-lactams were liberated in amounts similar to the amount of the particular inhibitor required to completely inactivate the enzyme. In addition, *p*-hydroxy-*m*-nitrophenylacetate is liberated from C-4 of L-682,271 similar to the rate of enzyme inactivation with this inhibitor. This demonstrates kinetic competence for this step with this inhibitor. These two sets of observations are consistent with the branch point of the reaction occurring after the step that liberates the C-4 substituents. The amount of *p*-hydroxyphenylacetic acid produced from L-680,833 in the presence of hydroxylamine

[8] For example, the kinetic mechanism described in Scheme I will yield $k_{inact} = (k_2k_3)/(k_2 + k_{H_2O} + k_3)$ and $K_i = ((k_{-1} + k_2)/k_1)((k_{H_2O} + k_3)/(k_2 + k_{H_2O} + k_3))$.

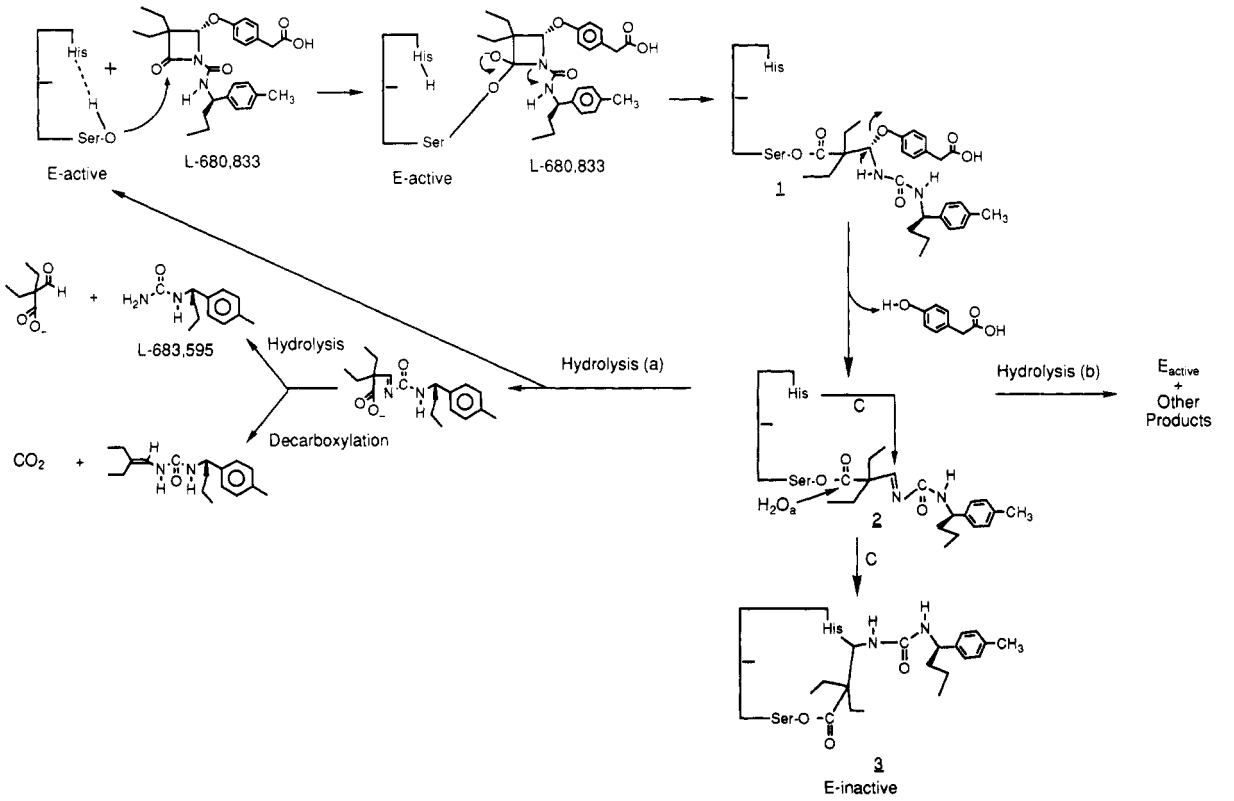


FIGURE 8: Catalytic mechanism proposed for the inhibition of HLE by compounds such as L-680,833 (see text for discussion). The ultimate products resulting from partitioning of imine **2** by route a have been identified. The products produced from partitioning by route b are similar to those observed during reactivation of the L-680,833-derived HLE–I complex (B. G. Green and W. B. Knight, unpublished data). Most of **2** partitions to produce an inactive HLE–I complex such as **3**. The absolute identity of this stable complex will be explored in subsequent work.

is also consistent with this proposal (*vide infra*). One could argue that the leaving group could be liberated nonenzymatically from an initial product released from the enzyme. The most likely candidate for this species would be the ring-opened β-lactam. Analogous species were suggested as products during the reaction of β-lactams with β-lactamases [for example, see Faraci and Pratt (1984) and Pratt et al. (1983)]. In the case of the L-680,833 series of lactams, the acyclic lactam hydrolysis product would have to undergo either (a) an S_N2 hydrolysis at the neopentyl carbon, (b) a β-elimination (or an S_N1 elimination of the group), on (c) a decarboxylation to liberate the group originally at C-4. While proposal a is highly unlikely, we cannot rule out the latter two possibilities a priori. In fact, small amounts of the decarboxylated product as well as the substituted urea were produced, but they can also be produced as a result of decarboxylation of the imine, as shown in Figure 8. The production of the urea also requires that equivalent amounts of 2,2-diethyl-3-oxopropanoic acid be produced. In unpublished NMR experiments W. M. Westler and W. B. Knight have observed a species displaying both proton and carbon chemical shifts consistent with this species during the interaction of HLE with ^{13}C-labeled L-680,833. Knight et al. (1992c) argue that the products produced from the base-catalyzed decarboxylation of L-680,833 and similar compounds support the imine as the intermediate that partitions in this case. The ratio of urea to decarboxylated products (1:2) in the enzymatic reactions is essentially identical to that reported for the nonenzymatic reactions. The simplest interpretation of the data is that the product released from the enzyme is the imine, which then partitions as in the nonenzymatic reaction to produce the urea and the decarboxylated adduct. In addition, small amounts of two other products are produced that do not contain the chromophore at C-4. These products are also observed during reactivation of β-lactam-derived HLE–I complexes and will be discussed in subsequent work (B. G. Green et al., unpublished data). The products produced from the reaction of L-680,833 with HLE in the presence of hydroxylamine have not been thoroughly characterized. The amount of *p*-hydroxyphenylacetic acid produced is consistent with partitioning from acyl–enzyme **2** in Figure 8. A species that displayed an HPLC retention time identical to that displayed by the urea L-683,595 was observed. The hydroxamate containing the imine produced from **2** might be expected to hydrolyze in solution to produce the urea. Isolation and characterization of any additional products in the presence of hydroxylamine should shed further light on this reaction.

In Figure 8 a catalytic mechanism for the inhibition of HLE by L-680,833 and other 4-(*S*)-β-lactams consistent with the data is presented. Initial formation of the acyl–enzyme utilizes the catalytic machinery of the enzyme. The active site histidine facilitated by the aspartate residue (not shown) increases the nucleophilicity of the serine by removing a proton as the serine attacks the carbonyl of the β-lactam ring. In the proposed model for the binding of L-680,833 in the active site of HLE, this attack would occur from the bottom face of the lactam ring (Doherty et al., 1993; Knight et al., 1992c). This leads to an initial tetrahedral intermediate, which opens the β-lactam ring as it collapses to form **1**. We have represented **1** with the leaving group still present, but we cannot rule out departure of the leaving group as the ring opens. The stereoelectronics required for concerted departure of the group at C-4 would preclude both C-4 stereoisomers from reacting by this route. In the case of porcine pancreatic elastase, the two C-4 diastereomers of C-3-dimethyl-substituted β-lactams inactivate the enzyme with similar rates, which again argues

against the absolute requirement for concerted liberation of the leaving group during the reaction with this enzyme (Knight et al., 1992c). However, it is possible that the mechanism of inhibition by L-680,833 and compounds with *S*-stereochemistry with HLE results in concerted departure of the leaving group with ring opening, while that of the C-4 diastereomer, L-682,946, operates by stepwise ring opening followed by departure of the leaving group. In the model of L-680,833 binding in the HLE active site, both the protonated histidine and the *p*-hydroxyphenylacetate leaving group are on the bottom face of the lactam ring. While this likely explains the difference in potency reported for the two diastereomers by Doherty et al. (1992), this orientation does not necessarily distinguish between a concerted departure of the leaving group and a stereochemical preference in the rate of liberation of the group in a separate step. The observation that the stereochemistry or identity of the leaving group does not effect the partition ratio for the inactivation of HLE by the β-lactams suggests partitioning from a common intermediate after departure of the leaving group. Inconsistent with this proposal is the observation that the partition ratio for the reaction of L-682,946 is unaffected by hydroxylamine, which argues against partitioning of the L-680,833- and L-682,946-derived complexes from a common intermediate such as the imine (*vide infra*). It could be simply fortuitous that the partition ratios are similar for the two compounds in the absence of hydroxylamine.[9]

Partitioning of E–I complexes derived from inhibitors with *S*-stereochemistry likely occurs from the imine as shown in Figure 8. This proposal is consistent with the lack of dependence of the partition ratio on the identity of the leaving group, the liberation of these leaving groups in amounts equivalent to the amount of inhibitor required for inactivation, and the kinetic competence of this step when *p*-hydroxy-*m*-nitrophenylacetic acid is the leaving group. Addition of the active site histidine to the imine as shown would lead to a stable enzyme–inhibitor complex containing a double hit. This would be analogous to the complex observed between PPE and a cephalosporin derivative by X-ray crystallography (Navia et al., 1987). The observation that the stability of the final complex formed between the enzyme and these complexes is pH independent (B. G. Green and W. B. Knight, unpublished data) suggests that the histidine is unable to participate during reactivation and supports this proposal. But Knight et al. (1993) have recently provided evidence that a double hit is not required to form a stable β-lactam-derived HLE–I complex. Two other possible identities for the final complex are the imine in Figure 8 or a carbinolamine that results from the addition of the water molecule normally destined to hydrolyze the acyl–enzyme to the imine. There is support for the latter from preliminary NMR and mass spectrometry studies as well as the observation that L-682,946, the C-4 diastereomer of L-680,833, yields an HLE–I complex slightly more stable than L-680,833 (B. G. Green and W. B. Knight, unpublished data). The latter observation suggests some difference between these two complexes and would further argue against the imine as a common product or intermediate for that matter. This suggests that the original stereochemistry at C-4 controls the identity of the final E–I complex. This could result from addition of water as the leaving group departs or stereospecific addition of water to the imine possibly prior to dissociation of the leaving group into solution. The presence of the leaving group could then control the stereospecificity. It is also possible that histidine could add to the imine prior to departure of the leaving group from the active site, thereby controlling the stereochemistry of addition. Establishing the identity of the stable enzyme–inhibitor complexes should resolve some of these questions.

The mechanism in Figure 8 should be considered only a working model to describe the interaction of β-lactams with HLE. While the steps involving attack of the active site serine on the lactam ring to form a tetrahedral intermediate are likely shared by all β-lactams, opening of the ring and any subsequent steps may depend upon the inhibitor structure. In particular, only the data for the 4-*S* isomers of the β-lactams are completely consistent with the mechanism proposed in Figure 8. For example, on the basis of the data obtained with L-682,946 and hydroxylamine, the identity of the complex that partitions is different from that produced from L-680,833. In addition, compounds such as L-684,481 that lack a leaving group at C-4 will not produce an electrophile upon opening of the lactam ring that can undergo stable addition by either the active site histidine of other nucleophiles such as water. In this case the stable acyl–enzyme must be that of the serine and the acyclic derivative of L-684,481. Any partitioning in this case (if this occurs) must be due to either different conformational states or possibly partitioning of an active site water between hydrolysis of the acyl–enzyme and solution. As discussed above, the stereochemistry of the leaving group may affect not only the potency of these compounds but also the catalytic mechanism of inactivation, including the identity of both the complex that partitions and the final E–I complex. These issues will be explored in subsequent work.

[9] It should be noted that we have recently demonstrated by electrospray ionization mass spectroscopy that *p*-hydroxyphenylacetic acid is not present in the stable complex derived from HLE and L-682,946 (P. Griffin, B. G. Green, and W. B. Knight, unpublished data).

ACKNOWLEDGMENT

We wish to acknowledge W. W. Cleland for critical reading of this work.

REFERENCES

Ashe, B. M., & Zimmerman, M. (1977) *Biochem. Biophys. Res. Commun. 75*, 194–199.

Cleland, W. W. (1986) *Techniques of Chemistry. Vol. 6: Investigation of rates and mechanisms of reactions, Part 1* (Bernasconi, C. F., Ed.) 4th ed., pp 791–870, Wiley, New York.

Doherty, J. B., Ashe, B. M., Argenbright, L. W., Barker, P. I., Bonney, R. J., Chandler, G. O., Dahlgren, M. E., Dorn, C. P., Finke, P. E., Firestone, R. A., Fletcher, D., Hagmann, W. K., Mumford, R., O'Grady, L., Maycock, A. L., Pisano, J. M., Shah, S. K., Thompson, K. R., & Zimmerman, M. (1986) *Nature 322*, 192–194.

Doherty, J. B., Shah, S. K., Finke, P. E., Dorn, C. P., Hagmann, W. K., Hale, J. J., Kissinger, A. L., Thompson, K. R., Brause, K., Chandler, G. O., Knight, W. B., Maycock, A. L., Ashe, B. M., Weston, H., Gale, P., Mumford, R. A., Anderson, O. F., Williams, H. R., Underwood, D., Vyas, K. P., Kari, P. H., Dahlgren, M. E., Mao, J., Fletcher, D. S., Dellea, P. S., Hand, K. M., Osing, D., Peterson, L. P., Williams, D. T., Metzger, J. M., Bonney, R. J., Humes, J. L., Pacholok, S. P., Hanlon, W. A., Opas, E., Stolk, J., & Davies, P. (1993) *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Faraci, W. S., & Pratt, R. F. (1984) *J. Am. Chem. Soc. 106*, 1489–1490.

Firestone, R. A., Barker, P. L., Pisano, J. M., Ashe, B. M., & Dahlgren, M. E. (1990) *Tetrahedron 46*, 2255–2262.

Green, B. G., Weston, H., Ashe, B. M., Doherty, J. B., Finke, P. E., Hagmann, W. K., Lark, M., Mao, J., Maycock, A. L.,

Mumford, R., Walakovits, L., & Knight, W. B. (1991) *Arch. Biochem. Biophys. 286*, 284–292.
Green, B. G., Chabin, R., Kuo, D., Weston, H., Doherty, J. B., Dorn, C. P., Finke, P. E., Hagmann, W. K., Hale, J. J., MacCoss, M., Shah, S. K., & Knight, W. B. (1992) *Biochemistry 31*, 2202.
Grimshaw, C. E., Cook, P. F., & Cleland, W. W. (1981) *Biochemistry 20*, 5655–5661.
Hornebeck, W., Moczar, E., Szecsi, J., & Robert, L. (1985) *Biochem. Pharmacol. 34*, 3315–3321.
Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245–3249.
Knight, W. B., Maycock, A. L., Ashe, B. M., Gale, P., Weston, H., Finke, P. E., Hagmann, W. K., Shah, S. K., & Doherty, J. B. (1992a) *Biochemistry 31*, 4980–4986.
Knight, W. B., Chabin, R., & Green, B. G. (1992b) *Arch. Biochem. Biophys. 296*, 704–708.
Knight, W. B., Green, B. G., Chabin, R., Gale, P., Maycock, A. L., Weston, H., Kuo, D., Westler, W. M., Dorn, C. P., Finke, P. E., Hagmann, W. K., Hale, J. J., Liesch, J., MacCoss, M., Navia, M., Shah, S. K., Underwood, D., & Doherty, J. B. (1992c) *Biochemistry 31*, 8160–8170.
Knight, W. B., Swiederek, K. M., Sakuma, T., Calacay, J., Shively, J. E., Lee, T. D., Covey, T. R., Shushan, B., Green, B. G., Chabin, R., Shah, S., Mumford, R., Dickinson, T. A., & Griffin, P. (1993) *Biochemistry 32*, 2031–2035.
Matta, M. S., Green, C. M., Stein, R. L., & Henderson, P. A. (1976) *J. Biol. Chem. 251*, 1006–1008.
McRae, B., Nakajima, K., Travis, J., & Powers, J. C. (1980) *Biochemistry 19*, 3973–3978.
Navia, M. A., Springer, J. P., Lin, T. Y., Williams, H., Firestone, R. A., Pisano, J. M., Doherty, J. B., Finke, P. E., & Hoogsteen, K. (1987) *Nature 327*, 79–82.
Navia, M. A., McKeever, B. M., Springer, J. P., Lin, T.-Y., Williams, H., Fluder, E. M., Dorn, C. P., & Hoogsteen, K. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7–11.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.
Pratt, R. F., Surh, Y. S., & Shaskus, J. J. (1983) *J. Am. Chem. Soc. 105*, 1006–1014.
Renard, M., & Ferscht, A. R. (1973) *Biochemistry 12*, 4713–4718.
Shah, S. K., Dorn, C. P., Finke, P. E., Hale, J. E., Hagman, W. K., Brause, K. A., Chandler, G. O., Kissinger, A. L., Ashe, B. M., Weston, H., Knight, W. B., Maycock, A. L., Dellea, P. S., Fletcher, D. S., Hand, K. M., Mumford, R. A., Underwood, D. J., & Doherty, J. B. (1992) *J. Med. Chem. 35*, 3745–3754.
Shimerlik, M. I., & Cleland, W. W. (1977) *Biochemistry 16*, 576–583.
Stein, R. L. (1983) *J. Am. Chem. Soc. 105*, 5111–5116.
Stein, R. L., Trainor, D. A., & Wildonger, R. A. (1985) in *Annual Reports in Medicinal Chemistry* (Bailey, D. M., Ed.) Vol. 20, pp 237–246, Academic Press, New York.
Tyagi, S. C., & Simon, S. R. (1990) *Biochemistry 29*, 9970–9977.
Waley, S. G. (1980) *Biochem. J. 185*, 771–773.
Waley, S. G. (1985) *Biochem. J. 227*, 843–849.
Williams, J. C., Falcone, R. C., Knee, C., Stein, R. L., Strimpler, A. M., Reaves, B., Giles, R. E., & Krell, R. D. (1991) *Am. Rev. Respir. Dis. 144*, 975–883.

CORRECTIONS

Kinetic Studies on the Peroxidase Activity of Selenosubtilisin, by Ian M. Bell, Megan L. Fisher, Zhen-Ping Wu, and Donald Hilvert*, Volume 32, Number 14, April 13, 1993, pages 3754–3762.

Page 3759. Equation 4 was inadvertently altered during proofreading and should read as follows:

$$\frac{v_o}{[E]_o} = \frac{k_{max}[ArSH][t\text{-BuOOH}]}{[ArSH][t\text{-BuOOH}] + K_{t\text{-BuOOH}}[ArSH] + K_{ArSH}[t\text{-BuOOH}]} \quad (4)$$