Mechanism of Inhibition of Human Leucocyte Elastase by β-Lactams. 3. Use of Electrospray Ionization Mass Spectrometry and Two-Dimensional NMR Techniques To Identify β-Lactam-Derived E−I Complexes†

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Received February 27, 1995; Revised Manuscript Received July 31, 1995⊗

ABSTRACT: A combination of NMR spectroscopy and electrospray ionization mass spectrometry (ESI-MS) was used to probe the identity of β-lactam-derived complexes with serine proteases. The carbon and proton NMR chemical shifts of the human leucocyte elastase (HLE)−inhibitor complex derived from [4-^{13}C]-L-680,833, [*S*-(*R**,*S**)]-4-[(1-(((1-(4-methylphenyl)butyl)amino)carbonyl)-3,3-diethyl-2-oxo-4-azetidinyl)oxy]benzeneacetic acid, were consistent with an sp^3 hybridized carbon. The ESI-MS spectrum of the L-680,833-derived HLE−I complex indicated an increase of 333 Da over the mass of the free enzyme. The data are consistent with acylation of the active site serine, loss of *p*-hydroxybenzeneacetic acid, and formation of a carbinolamine at the carbon deriving from C-4 of the lactam ring. The complexes produced from HLE and the diastereomers of L-680,833 display identical masses. Since the 4*R*-isomers produce more stable complexes [Green et al. (1995) *Biochemistry 34*, 14331−14343], these data suggest that these complexes differ in their stereochemistry or conformation. The structural model of the HLE−I complexes derived from the diastereomers predicts that the hydroxyl of the carbinolamine derives from a structurally observed water molecule yielding *S*-stereochemistry in all cases. In this model, the *4S*- and 4*R*-diastereomers produce complexes that differ by the location of the side chain of a phenylalanine residue. The mass of HLE was increased by that of L-684,481, (*R*)-1-(((1-(4-methylphenyl)butyl)amino)carbonyl)-3,3-diethyl-2-azetidinone, which lacks a leaving group at C-4 in the complex derived from this compound. L-691,886, [*S*-(*R**,*S**)]-4-[(1-(((1-(4-ethoxyphenyl)butyl)amino)carbonyl)-3,3-diethyl-4-oxo-2-azetidinyl)-oxy]benzeneacetic acid, produces two complexes of different mass that reactivate with different rates. The mass of the less stable complex is consistent with the acyl-enzyme of 2,2-ethyl-3-oxopropanoic acid while the mass of the more stable complex is analogous to the carbinolamine observed during L-680,833 inactivation. Porcine pancreatic elastase (PPE) produces a complex with a mass consistent with replacement of the C-4 leaving group by water to produce a carbinolamine from L-684,248, [*S*-(*R**,*S**)]-4-[(1-(((1-(4-methylphenyl)butyl)amino)carbonyl)-3,3-dimethyl-2-oxo-4-azetidinyl)oxy]benzoic acid. The C-4 diastereomer, L-684,249, produces two PPE−I complexes with different masses. One of these complexes has a mass identical to the mass of the complex derived from L-684,248 while the mass of the other complex indicates the presence of the entire inhibitor molecule. The mass of the latter complex indicates that the leaving group was not liberated during the reaction. These data demonstrate that the chemical mechanism of inhibition of elastases by β-lactams is dependent on the lactam structure. The molecular and conformational identity of the final complex controls its stability, and this is governed by the structure of the original inhibitor.

The common theme used to explain the stability of the acyl-enzymes produced from β-lactams and serine proteases has been the so-called "double hit" mechanism: acylation of the serine and alkylation of the histidine (Doherty et al., 1986; Navia et al., 1987). This is somewhat reminiscent of the proposed interaction of chloromethyl ketones with HLE (Navia et al., 1989) where a hemiketal forms between the serine and the ketone followed by alkylation of the histidine with displacement of chloride from the methylene group. Knight et al. (1993), using ESI-MS[1] technology, reported that a double hit is not required to form a stable complex between HLE[2] and a β-lactam. In the accompanying paper (Green et al., 1995), the stability of β-lactam-derived HLE−inhibitor complexes (HLE−I) and the products of the

† Preliminary results from portions of this work were presented by Knight et al. (1992b).

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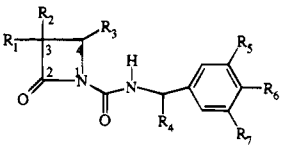
⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1995.

0006-2960/95/0434-14344$09.00/0

Table 1: Structures of the β-Lactams



	R_1	R_2	R_3	R_4	R_5	R_6	R_7
L-670,258	Et	Et	(*S*)-O-(C_6H_4)-4-CO_2H	H	−CH(CH)$_2$CH−		H
L-680,831	Et	Et	(*S*)-O-(C_6H_4)-4-CO_2H	(*R*)-propyl	H	CH_3	H
L-680,833	Et	Et	(*S*)-O-(C_6H_4)-4-CH_2CO_2H	(*R*)-propyl	H	CH_3	H
L-682,946	Et	Et	(*R*)-O-(C_6H_4)-4-CH_2CO_2H	(*R*)-propyl	H	CH_3	H
L-683,557	Et	Et	(*S*)-O-(C_6H_4)-4-CH_2CO_2H	(*S*)-propyl	H	CH_3	H
L-683,558	Et	Et	(*R*)-O-(C_6H_4)-4-CH_2CO_2H	(*S*)-propyl	H	CH_3	H
L-684,248	Me	Me	(*S*)-O-C_6H_4)-4-CO_2H	(*R*)-propyl	H	CH_3	H
L-684,249	Me	Me	(*R*)-O-(C_6H_4)-4-CO_2H	(*R*)-propyl	H	CH_3	H
L-684,481	Et	Et	H	(*R*)-propyl	H	CH_3	H
L-691,886	Et	Et	(*S*)-O-(C_6H_4)-4-CH_2CO_2H	(*R*)-propyl	H	$-OCH_2CH_3$	H

reactivation of these complexes were examined. In this work we report the results from ESI-MS and NMR spectroscopic studies that suggest that the identity of the stable HLE-I complexes derived from compounds such as L-680,833 is a carbinolamine. These results allow us to propose a mechanism consistent with the data collected to date that does not require a double hit mechanism to yield stable HLE−I complexes.

MATERIALS AND METHODS

The compounds listed in Table 1 were synthesized according to published procedures (for example, see European Patent Office Publication No. EPO O 337,549; Doherty et al., 1993; Shah et al., 1992). The synthesis of [4-^{13}C]-L680,833 will be published elsewhere (S. Shah, unpublished results). HLE (Elastin Products, St. Louis, MO) isozyme-4 (I-4) was purified according to Green et al. (1991). HLE was assayed, and the second-order rate constant for the inhibition of HLE by [4-^{13}C]-L680,833 was determined according to Knight et al. (1992a). The rate constants for reactivation of L-691,886-inactivated HLE were determined as in the accompanying work (Green et al., 1995). SLPI was purchased from R&D Systems (Minneapolis, MN). PPE (Serva) activity was determined versus 0.2 mM succ-AAPA-pNA in buffer A (45 mM TES at pH 7.5, 450 mM NaCl, and 10% DMSO). The pH of buffers was adjusted with either NaOH or NH_4OH. Inhibitor stock solutions were in DMSO except were noted. All other reagents were obtained or prepared as in the preceding paper in this series (Green et al., 1995).

HPLC−ESI-MS was conducted according to Knight et al. (1993) on either a Finnegan TSQ-700 or a Sciex API III spectrometer. For direct infusion[3] experiments, 20 μM PPE was dissolved in H_2O instead of buffer and inhibitor added in EtOH (final concentration ≤10%). After complete inhibition (95−98% in the case of the β-lactam inhibitors) was reached, 1−2 μL (19−38 pmol) was infused directly into the instrument flow of the Finnegan TSQ-700. For example, 1 μL of 2.36 mM L-684,248 was added to 9 μL of PPE. The amount of inhibition was assessed by diluting 2 μL of the sample to 40 μL with buffer A and assaying 10 μL (in 1 mL reactions) in duplicate. In the reaction of SLPI with PPE, 2 or 4 μL of 0.17 mM SLPI was added to 9 μL of 20 μM PPE. When diluted 1:20 and 10 μL assayed, 56% inhibition and 71% inhibition were observed, respectively. Aliquots (2−5 μL) of the undiluted solutions were analyzed by ESI-MS using direct infusion. Additional experiments were performed with SLPI and PPE in buffer as below. Most of the ESI-MS samples were dissolved in buffers and were introduced into the instrument stream by either microbore or capillary C-4, C-8, or C-18 HPLC. The buffers used in the ESI-MS experiments were prepared by titration with either NH_4OH or NaOH. In separate experiments it was determined that the presence of ammonium ion had no effect on the identity of the complexes produced. Inhibitors were dissolved in either DMSO or EtOH at concentrations such that the final solvent concentration was ≤10%. The solvent composition did not have an effect on the identity of the complexes produced. Specific conditions for the incubations are noted below when significantly different from standard reactions. For example, 1 μL of 2.36 mM L-684,248 in EtOH was added to 9 μL of 20 μM PPE in 50 mM NH_4-TES and 100 mM NH_4Cl (final concentrations). The reaction was incubated for 30−60 min at 25 °C and then 5 μL injected onto a 150 μm C-18 column eluted at 2 μL/min with a linear AcCN gradient. In experiments with I-4, typically 20−200 pmol was analyzed by ESI-MS. The amount was dependent upon the bore of the HPLC column used. In a typical reaction, 1 μL of 0.28 mM L-683,557 in DMSO was added to 9 μL of 0.22 mM I-4 in 50 mM NH_4-TES, pH 7.5, and

[1] Abbreviations: AcCN, acetonitrile; DMSO, dimethyl sulfoxide, ESI-MS, electrospray ionization mass spectrometry; HPLC, high-pressure liquid chromatography; HLE, human leucocyte elastase; MES, 2-(*N*-morpholino)ethanesulfonic acid; MWCO, molecular weight cutoff; NOE, nuclear overhauser effect; P_i, inorganic phosphate; PAGE, polyacrylamide gel electrophoresis; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PMN, polymorphonuclear neutrophils or leucocytes; PPE, porcine pancreatic elastase; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; TES, 2-[[tris(hydroxymethyl)-methyl]amino]ethanesulfonic acid; SLPI, secretory leucoprotease inhibitor.

[2] HLE or elastase refers to the mixture of isozymes as isolated from human sputum. I-4 is the most basic pooled fraction of HLE, based upon the order of elution from a Pharmacia Mono-S 5/5 column (Green et al., 1991). While this species displays a single band on PAGE gels, Knight et al. (1993) demonstrated by ES-MSI that this species is still somewhat heterogeneous. HLE−I refers to the HLE−inhibitor complex.

[3] The ESI-MS spectra of HLE isozyme-4 obtained by direct infusion were of poor quality, although the calculated mass of the major component present in I-4 was the same as that observed by HPLC−ESI-MS. The signal to noise ratio was insufficient to detect the minor components.

100 mM NH_4Cl. After incubation, 5 μL was injected onto a 150 μm C-18 column eluted at 2 μL/min with a linear AcCN gradient. The masses of the enzyme−inhibitor complexes derived from a number of β-lactams and HLE were determined by ESI-MS similarly at pH 7.5 (100 mM NH_4Cl and 10 mM NH_4-TES). Additional experiments with HLE were performed at pH 7.5 with 100 mM NaCl and 10 mM Na-TES and at pH 5.5 with 100 mM NaCl and 10 mM Na-MES and in separate experiments with 100 mM NH_4Cl and 10 mM NH_4-MES. In some experiments only 0.5 equiv of inhibitor were added to 1 equiv of enzyme.

The reaction of [4-^{13}C]-L-680,833 with HLE was examined by ^{1}H{^{13}C}HMQC NMR spectroscopy at 37°C. HLE (240 mg) was dissolved in 30 mL of 5 mM sodium acetate at pH 5.5 or NaP_i at pH 7.5 and dialyzed exhaustively versus H_2O, titrated to either pH 5.5 or pH 7.5, and then lyophilized. The enzyme was then dissolved in D_2O and lyophilized. This process was repeated three times. Prior to the last lyophilization step the enzyme was divided into 50 mg aliquots on the basis of activity. NMR enzyme stock solutions were prepared by dissolving the 50 mg aliquots in 1 mL of NMR buffer [(HLE) = 2 mM). The NMR buffers were prepared by titrating 150 mM NaCl and 50 mM NaP_i in H_2O to either pH 7.5 or pH 5.5. The NMR buffers were lyophilized and redissolved in D_2O for three cycles. The buffer was then redissolved in D_2O to the original volume. Stock [4-^{13}C]-L-680,833 was dissolved in DMSO-d_6 to a final concentration of 38 mM. Initial reactions were conducted at 5.0 mg/mL HLE (0.2 mM) and 0.6 mM [4-^{13}C]-L-608,833 in 150 mM NaCl and 50 mM NaP_i at pH 7.5 and 37 °C. The NMR spectra were collected at 37 °C. In a separate experiment, 2.2 mM [4-^{13}C]-L-680,833 and 10% DMSO-d_6 were added to 0.45 mL of the pH 5.5 NMR enzyme stock solution. The reaction was incubated at room temperature for 30 min and then added to 5 mm NMR tubes, and spectra were obtained at 15 °C. Control reactions contained either enzyme or inhibitor. Spectra were collected over 5 h periods alternating between the high-field (75 ± 55 ppm) and low-field regions (175 ± 55 ppm). Both of these reactions were filtered through Millipore filtration cups (10 kDa MWCO) to remove enzyme and spiked with [4-^{13}C]-L-680,833, and the spectra of the filtrate were collected.

The preceding paper (Green et al., 1995) reported that a number of β-lactams produced HLE−inhibitor complexes that displayed biphasic reactivation kinetics. The L-691,886-derived I-4 complex was chosen for additional studies. The enzymatic activity and ESI-MS spectra of the reaction of I-4 with this inhibitor was monitored over three cycles of addition of inhibitor, removal of excess inhibitor and metabolites by centrifugal gel filtration, partial reactivation, and addition of more inhibitor. A control solution of HLE was treated identically except inhibitor was omitted. For ESI-MS analysis 2 μL of the incubation solutions was diluted with 8 μL of H_2O, and 5−10 μL was injected onto a 300 μm (i.d.) C-4 column and eluted with an acetonitrile gradient into the instrument stream. The initial reaction containing 0.11 mM HLE in 1.7 mL of buffer A (450 mM NaCl, 10% DMSO, and 45 mM TES at pH 7.5) was incubated with 0.335 mM L-691,886 [approximately a 2-fold excess based upon the partition ratio (Chabin et al., 1993)] for 30 min at 25 °C. At this time an aliquot of the solution was diluted 1:30 and 10 μL assayed for activity. At this time the enzymatic activity was inhibited by 90%. A 2 μL aliquot was diluted for ESI-MS analysis (first inactivation). The original incubation solution (1.5 mL) was subjected to centrifugal gel filtration at 4 °C on 2.5 mL DEAE-Sephadex columns to remove excess inhibitor and any metabolites (0.5 mL aliquots and three separate columns equilibrated in buffer A). The resulting solution was pooled and labeled F1 for first filtrate. F1 was incubated at 37 °C until 50% reactivation had occurred. At that time, the ESI-MS spectrum of the enzyme solution was obtained (first reactivation). A 0.5 mL aliquot of F1 was saved and continually monitored for activity. After 2.5 h, 1 mL of F1 was made 0.17 mM (1.5 molar excess over total enzyme concentration) in L-691,886 and incubated for 30 min at room temperature. At that time the solution was assayed for enzymatic activity and by ESI-MS (second inactivation). The solution was then subjected to centrifugal gel filtration. This corresponds to F2, the second filtrate. The activity of F2 was monitored for 3 h, at which time 16% of the control activity had returned. An aliquot of the solution was subjected to ESI-MS (second reactivation). An aliquot of F2 was saved for continued monitoring for activity while the remainder was made 0.17 mM in L-691,886 and incubated at room temperature for 30 min. An aliquot was subjected to ESI-MS (third inactivation), and the remainder of the solution was filtered to yield filtrate 3 (F3). The activity of F3 was monitored over 118 h.

Data Analysis. The second-order rate constants (k_{inact}/K_i) for inactivation were determined by first fitting by nonlinear regression (using NLIN, a program developed at Merck by N. Thornberry) of progress curves (absorbance versus time) to eq 1 to determine k_o and then calculating k_{inact}/K_i from eq 2 on the basis of the inhibitor and substrate concentrations and the K_m (Knight et al., 1992a). The rate constants for reactivation of L-681,886-inactivated HLE were determined by nonlinear regression of the activity as a function of time to eq 3 or eq 4 using GraFit (Leatherbarrow, 1992).

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

$$k_{obs}/[I] = k_{inact}/(K_i(1 + [S]/K_m)) \quad (2)$$

$$y = ae^{-k_1 t} + be^{-k_2 t} + c \quad (3)$$

$$y = ae^{-kt} + c \quad (4)$$

RESULTS

The second-order rate constant for the inhibition of HLE by [4-^{13}C]-L-680,833 was 518 000 M^{-1} s^{-1}, which is within experimental error of the value obtained with unlabeled material (580 000 M^{-1} s^{-1}) in parallel experiments. The ^{1}H-detected ^{13}C 2D-NMR spectrum of [4-^{13}C]-L-680,833 at pH 7.5 displayed a contour at 5.9, 86.7 ppm (^{1}H, ^{13}C) (data not shown). In the presence of 5 mg/mL HLE at pH 7.5 the initial 2D-NMR spectrum of [4-^{13}C]-L-680,833 displayed only the parent compound, which disappeared over time producing four new species. The spectral region where the two cyclic products resonate (Green et al., 1995) is shown in Figure 1A. An additional species produced a contour at 9.8, 208.5 ppm (^{1}H, ^{13}C) and displayed a coupling constant (J_{C-H}) of 188 Hz (2,2-diethyl-3-oxopropanoic acid; data not shown). A contour at 5.9, 143 ppm (^{1}H, ^{13}C) was also observed (not shown). This species was observed in the base-catalyzed decomposition of L-680,833 (Knight et al.,

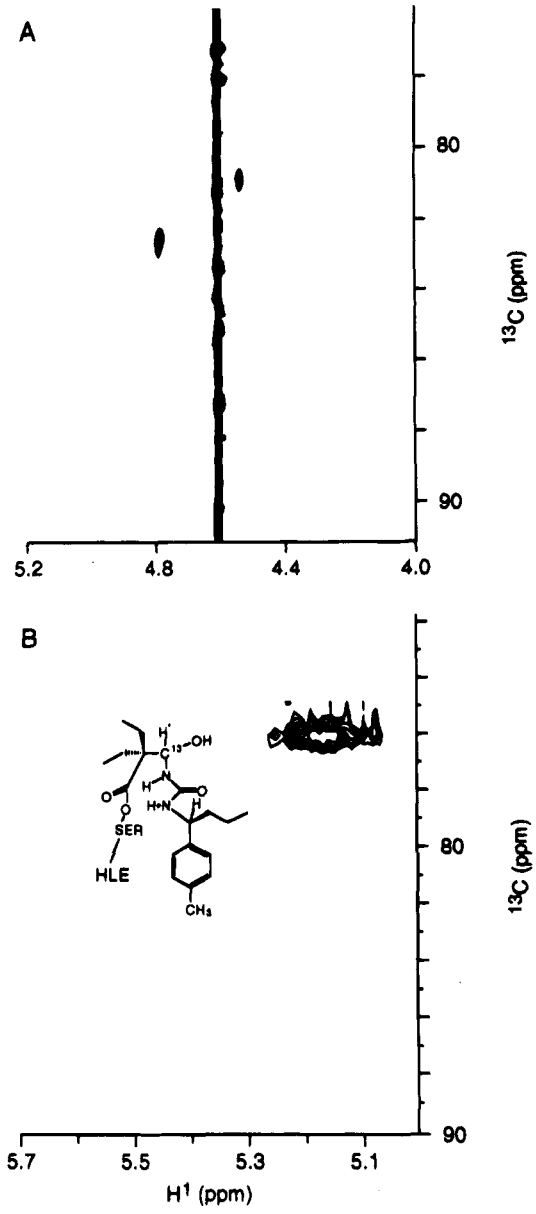
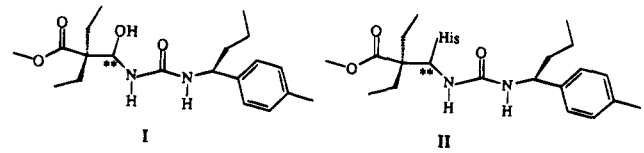


FIGURE 1: (A) ^{1}H-detected ^{13}C NMR spectrum of the two cyclic metabolites derived from the [4-^{13}C]-L-680,833-derived HLE−I complex at pH 7.5. (B) ^{1}H-detected ^{13}C NMR spectrum of the [4-^{13}C]-L-680,833-derived complex. The initial concentrations of inhibitor and enzyme were 2.2 and 2 mM, respectively, and the pH was 5.5.

1992a). There were no additional resonances observed in the region expected for sp^2 hybridized carbons. All four of the product resonances were evident in the NMR spectra after the enzyme was removed via filtration through 10 MWCO filter cups (Millipore). These resonances displayed narrow line widths, and there were no other resonances that could be attributed to the enzyme−inhibitor complex.

Due to the potential masking of protein-bound resonances by the water resonance and the line width expected for protein-bound inhibitor species, the NMR experiment was repeated at pH 5.5 and 50 mg/mL HLE.[4] The temperature was lowered to 15 °C to slow down reactivation of the HLE-I complex. The lower pH avoids potential complication due to the presence of multiple C-13-containing products since reactivation under these conditions generates essentially a single labeled product, 2,2-diethyl-3-oxopropanoic acid, whose ^{1}H and ^{13}C chemical shifts are known (Knight et al., 1992a; Green et al., 1995). A broad contour (5.2, 85 ppm; ^{1}H, ^{13}C) was observed in the initial spectrum (see Figure 1B) that was removed upon filtration to remove the enzyme (data not shown). The carbon and proton chemical shifts are consistent with an sp^3 hybridized carbon. A narrow low-field resonance (9.8, 208.5 ppm; ^{1}H, ^{13}C), consistent with an sp^2 hybridized carbon, was also observed before filtration of the sample. This resonance was observed in the NMR spectra of the filtrate and is consistent with the presence of 2,2-diethyl-3-oxopropanoic acid. The broad resonance is consistent with the carbinolamine predicted from the mass spectral results but does not rule out other sp^3 hybridized carbons. For example, the ^{13}C chemical shift of the labeled carbon predicted for the methyl ester **I** using STN Express (Chemical Abstracts Service, Columbus, OH) is 82 ± 5 ppm. A similar analysis for the histidine adduct **II** predicted a ^{13}C chemical shift of 84 ± 3 ppm for the labeled carbon.



I II

In Figure 2, the deconvoluted mass spectrum and the mass spectrum focusing only on the +11 to +14 ions of a mixture of I-4 and the L-680,833-derived I-4 complex (limiting L-680,833) are shown.[5] Focusing on the narrow mass range increases the digital signal to noise ratio for the mass determinations. In Table 2 the masses calculated from each of these ions are presented. This experiment was repeated six times to yield a shift in mass (mass of the I-4−inhibitor complex minus the mass of I-4) of 333 Da with a standard deviation of 1.2. This shift in mass corresponds to the mass of L-680,833 less the C-4 leaving group (*p*-hydroxyphenylacetic acid) plus the addition of 18 Da. This corresponds to the addition of one water molecule in the enzyme−inhibitor complex. The shift in mass relative to free I-4 of a number of β-lactam-derived I-4−inhibitor complexes is summarized in Table 3. The three diastereomers of L680,833, L-682,946, L-683,557, and L-683,558, also yielded complexes whose mass was indicative of loss of the C-4 leaving group and addition of one water molecule to the HLE−I complex. The mass spectrum of isozyme-4 after treatment with L-691,886 indicated the presence of two enzyme−inhibitor complexes and is discussed below. In Figure 3 the mass spectrum of the L-684,481-derived I-4 complex is shown. In this case the mass shift is equivalent to the mass of the inhibitor.

The return of enzymatic activity of the L-691,886-derived inhibitor complex at 25 °C after removal of excess inhibitor is presented in Figure 4. Biphasic reactivation curves were also observed at 37 °C. In Table 4 the half-lives obtained

[4] The partition ratio (k_{inact}/k_{H_2O}) was unaffected by either the ionic strength (Chabin et al., 1993) or pH. In separate experiments we confirmed that this value was not affected by P_i buffer. In addition, the rates of inactivation in P_i buffers were similar.

[5] Knight et al. (1993) reported that the deconvoluted mass spectrum of "purified" I-4 actually displays two to three species with different intensities depending upon the quality of the spectrum. All three components react with inhibitors of HLE. This work concluded that these species likely differ by carbohydrate content. The most predominant component yields a mass of 25 200 Da, while the next most predominant component is seen at approximately 25 050 Da. The least predominant component is not always clearly observed above background.

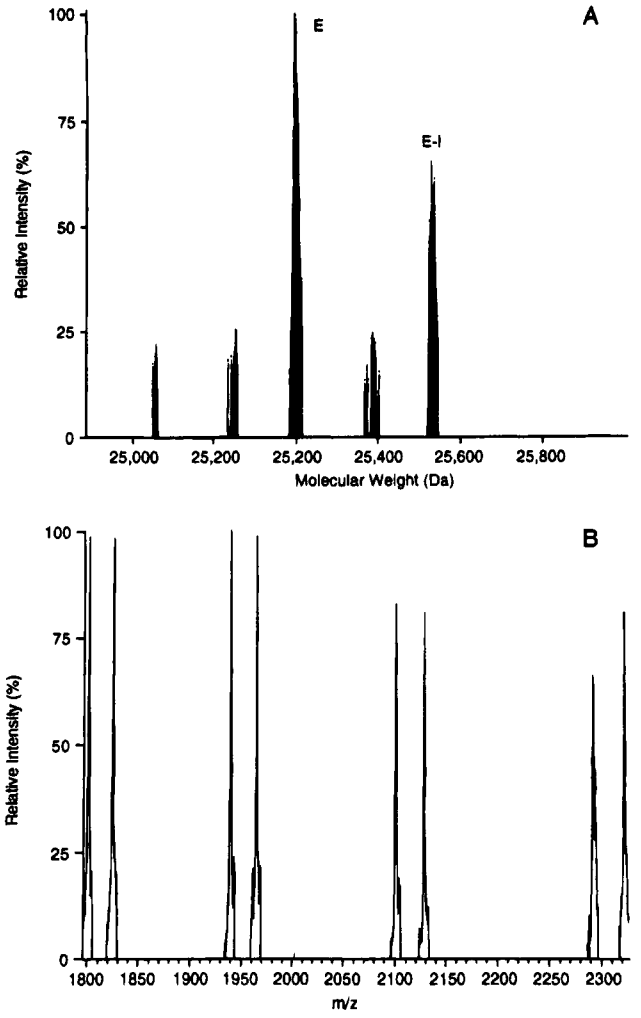


FIGURE 2: (A) Deconvoluted mass spectrum of the L-680,833-derived I-4 complex in the presence of excess HLE. The less intense peaks differ due to carbohydrate content (Knight et al., 1993). These data were obtained on a Sciex API-III instrument equipped with an Applied Biosystems 140 HPLC pump instrument using a 1 mm × 10 cm C-8 column eluted with 0–95% AcCN containing 0.1% TFA gradient over 8 min with a flow rate of 25 μL/min. The enzyme and inhibitor concentrations were 40 and 20 μM, respectively, at pH 7.5. A total of 200 pmol (5 μL) was injected. The ions with a *m/e* of +11 through +18 were used in the deconvolution. (B) Mass spectrum of the L-680,833-(26 μM) derived complex in the presence of excess I-4 (40 μM). The chromatography conditions were the same as in (A). The instrument was set to collect data for the eight ions due to the major I-4 and I-4–inhibitor complex species with a *m/e* of +11 to +14 to increase the digital signal to noise ratio.

from the reactivation of HLE–I complexes derived from multiple treatments of elastase with L-691,886 are shown. The mass spectrum of isozyme-4 after one inactivation cycle with L-691,886 is shown in Figure 5A. Two complexes were observed corresponding initially to mass shifts of 125 Da (886–complex 1) and 357 Da (886–complex 2). Loss of the C-4 leaving group, *p*-hydroxyphenylacetic acid, followed by addition of a water molecule could account for the mass of 886–complex 2. The mass of 886–complex 1 is indicative of further loss of the substituted urea portion of the inhibitor. After partial reactivation (≈50%) the 886–complex 1 has essentially disappeared with the concomitant production of free isozyme-4 (25 199.4 Da; see Figure 5B). This indicates that 886–complex-1 and 886–complex 2 are responsible for the fast and slow phases, respectively, of the

Table 2: Comparison of the Observed and Calculated Mass for the +11 to +14 Ions Produced from a Mixture of HLE and the L-680,833-Derived HLE–I Complex[a]

	actual *m/e*	predicted *m/e*	calculated mass
HLE–I	1825.0	1824.9	25 535.9
HLE–I	1965.2	1965.2	25 534.5
HLE–I	2128.9	2128.88	25 534.7
HLE–I	2322.10	2322.33	25 532.0
	calculated mass of HLE–I = 25 534.3 ± 1.6		
HLE	1801	1801.4	25 199.9
HLE	1939.5	1939.5	25 200.4
HLE	2101.2	2101.04	25 202.3
HLE	2291.9	2291.95	25 199.8
	calculated mass of HLE = 25 200.6 ± 1.16		

[a] These data were calculated using the Sciex Hypermass program from the data shown in Figure 2A.

Table 3: Mass Increase of β-Lactam-Derived HLE–Isozyme-4 Complexes[a] over Free Enxzyme

compd	condition	k_{inact}/K_I[b] (M^{-1} s^{-1})	$t_{1/2}$[b] (h)	mass inc. (Da)
L-680,833	pH 7.5	580 000	11.0	333 ± 1[c]
	pH 7.5			332
	after 70% react.			331[d]
	pH 5.5			332
L-682,946	pH 7.5	27 000	15.1	332
	50% inact.			330[d]
L-683,557	pH 7.5	18 000	1.6	333
L-683,558	pH 7.5	6 700	2.9	331
L-680,886	pH 7.5	1 100 000	0.16	124, 125[e] (886–complex 1)
			16.1	362, 357[e] (886–complex 2)
	after third cycle			362
L-684,481	pH 7.5	30 000	13.3	317[f]
	pH 5.5			317

[a] Except where noted the data were obtained on a Finnegan TSQ-700 spectrometer. In duplicate experiments with several compounds identical values were obtained on the Sciex Instruments. Abbreviations: inact, inactivation; inc., increase; react., reactivation. The molecular weight of L-680,833, L-682,946, L-683,557, and L-683,558 is 466. The molecular weight of these compounds less the C-4 leaving group, *p*-hydroxyphenylacetic acid, is 315. The molecular weights of L-684,481 and L-691,886 were 316 and 496, respectively. The mass of L-691,886 less the C-4 leaving group, *p*-hydroxybenzoic acid, is 345. [b] The values for k_{inact}/K_i and the reactivation half-times were from Chabin et al. (1993) or Doherty et al. (1994) and Green et al. (1995), respectively. [c] In these experiments (n = 6) the enzyme was inactivated by 50%. The spectral window was narrowed to collect data on a Sciex API III spectrometer for only the +11 to +14 ions due to the free enzyme and enzyme–inhibitor complex to increase the digital resolution (see Table 2 and Figure 2B). [d] Free enzyme was evident at 25 200 Da. [e] Two complexes were produced during the interaction of L-691,886 with isozyme-4. These data represent two separate determinations. The data in the second column were obtained from Table 5, first inactivation. [f] The same value was obtained on both the Sciex API III and Finnegan TSQ-700 spectrometers.

observed reactivation kinetics. In Figure 6, the mass spectrum of the L-691,886-derived complex after three filtrations and treatments with additional inhibitor is compared to that of untreated HLE. This treatment yields only the higher molecular weight HLE–inhibitor complex. The masses of isozyme-4 observed upon treatment cycles with L-691,886 are summarized in Table 5.

Porcine pancreatic elastase yielded the mass spectrum shown in Figure 7. The mass of the predominant species is 25 900 ± 1 Da (N = 3), but there is an additional species present at 25 788 ± 2 Da. These values are similar to those

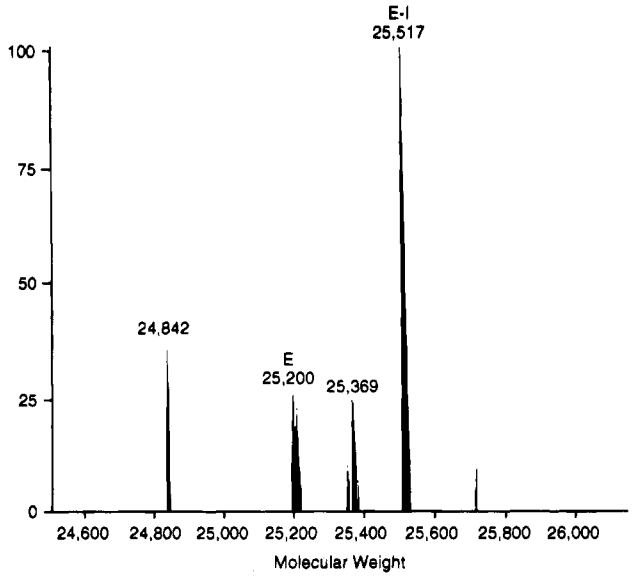


FIGURE 3: Deconvoluted mass spectrum of the L-684,481-derived I-4 complex in the presence of 69 μM inhibitor. The other conditions were the same as in Figure 2. A small amount of free enzyme is still evident at a mass of 25 200.

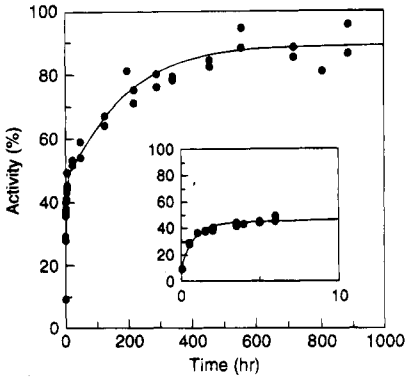


FIGURE 4: Return of HLE activity at 25 °C after inactivation with L-691,886 at 25 °C.

reported by Alpin et al. (1992) for this enzyme. The predicted molecular weight based upon the sequence and four disulfide bonds is 25 900 (Shotten & Hartley, 1970; Shirasu et al., 1986). Alpin et al. attributed the mass of the minor component observed in the mass spectra of PPE to loss of the C-terminal Asn residue (predicted 25 786 Da). Both of these species react with the inhibitors (*vide infra*).[6] The deconvoluted mass spectra of the L-684,248- and L-684,249-derived complexes of PPE are presented in Figure 8. The L-684,248-derived complex produced a species corresponding to loss of the C-4 leaving group and addition of one water molecule. Two distinct PPE−I complexes are observed in the presence of L-684,249. Direct infusion of the PPE−I complexes into the instrument flow without prior HPLC separation yielded similar mass spectra (see Table 6). The smaller mass likely corresponds to a complex that contains the inhibitor less the leaving group plus an additional water. The complex with the greater mass indicates the presence of the entire inhibitor molecule in this PPE−I complex. The mass shifts upon binding of the inhibitor are summarized in Table 6. The mass spectrum of PPE treated with the tight-binding noncovalent inhibitor, SLPI, displayed only free SLPI and PPE, and a complex was not observed.[7]

Table 4: Reactivation Studies with L-691,886[a]

temp (°C)					
inactivation	reactivation	k_1	$t_{1/2}$ (h^{-1})	k_2 (h)	$t_{1/2}$ (h^{-1})
25	25	1.3	0.52 ± 0.08	0.0058	119 ± 14
37	37	7.4	0.094 ± 0.03	0.044	16 ± 4
25	37	4.3	0.16 ± 0.02	0.043	16.1 ± 0.2
25	37 (F1)[c]	0.94	0.73 ± 0.2	0.041	16.9 ± 5.6
25	37 (F2)[d]			0.022	31 ± 5
25	37 (F2)[c]	0.25	2.8 ± 2.2	0.014	49 ± 24
25	37 (F3)[d]			0.0157	44 ± 8

[a] These data were obtained from a fit of the reactivation progress curves to eq 3 except where noted. HLE was inactivated in buffer A with L-691,886 at the temperature noted and then allowed to reactivate over time at the noted temperature. F1−F3 represent the reactivation kinetics of filtrates 1−3 which were generated by three cycles of addition of inhibitor followed with removal of excess inhibitor by centrigual gel filtration (see Materials and Methods for details). [b] These data are from the Green et al. (1995). [c] While these data were analyzed according to eq 3, the calculation of the first phase of these reactions was based upon only a few data points. Therefore, the rate constant for this phase in these experiments is not well determined. Each successive addition of inhibitor decreased the ability to determine the rate constant for the faster phase as less of the enzyme was in the state responsible for this phase. Therefore, the data were analyzed according to eq 4 as noted. [d] These data were obtained from a fit to eq 4.

DISCUSSION

The identity of the stable complexes derived from β-lactams and elastases was probed using a number of techniques. Chabin et al. (1993) demonstrated that the C-4 leaving group is liberated during the interaction of compounds such as L-680,833 with HLE. Therefore, three species were considered as likely candidates for the stable HLE−I complex derived from L-680,833; (1) the double hit complex proposed by Chabin et al. (1993), (2) the imine **2**, or (3) the carbinolamine **3** in Figure 9. The ^{13}C and ^{1}H NMR chemical shifts in the L-680,833-derived complex of the carbon and proton originating from C-4 of the lactam ring indicate that the carbon is not sp^2 hybridized, thus ruling out the imine. While the proton and carbon shifts are consistent with the original structure proposed by Chabin et al. (1993), i.e., alkylation of the active site histidine, they do not clearly distinguish between other sp^3 hybridized systems containing either oxygen or nitrogen substitutions. The mass spectrum of the L-680,833 complex clearly displays a shift that is 18 mass units higher than predicted on the basis of simple loss of the C-4 leaving group. This indicates the presence of an additional water molecule in the HLE−I complex. The mass of the complex derived from L-680,833 did not vary with pH, indicating that the same molecular species is responsible for the products generated at neutral and low pH (Green et al., 1995). The complex produced from a β-lactam (L-684,481) that does not contain a leaving group at C-4 yields a mass consistent with the presence of the entire inhibitor. These data demonstrate that a double hit is not required to produce stable acyl-enzymes since the interaction of L-684,481 with HLE would not be expected to yield a site for stable

[6] We have also observed that both of these species react with α_1PI, peptide chloromethyl ketones, and cephalosporin analogs to form covalent complexes (Knight et al., unpublished results). Alpin et al. (1992) have reported that both of these species also react with a peptide chloromethyl ketone specific for elastases.

[7] Kramps and Klasen (1985) reported that SLPI displayed a $K_i = 1 \times 10^{-9}$ M versus PPE.

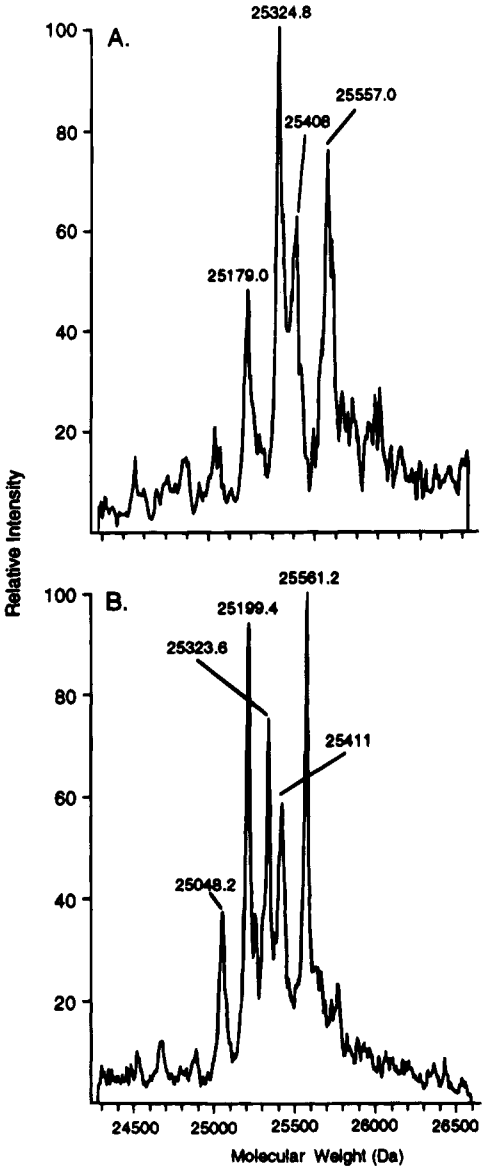


FIGURE 5: Mass spectrum of 220 pmol of the L-691,886-derived I-4 complex before (A) and after (B) approximately 50% reactivation. These data were obtained on a Finnegan TSQ-700 spectrometer. The sample was eluted at 2 μL/min from a 250 μm × 10 cm C-4 column with an acetonitrile gradient (10−90%) containing 0.1% TFA. Two of the isozymes which differ by approximately 150 Da of each enzyme form are clearly evident. For example, in panel B the two forms of the free enzyme are observed at 25 199.4 and 25 048 Da. These values are consistent with those reported in earlier work (Knight et al., 1995).

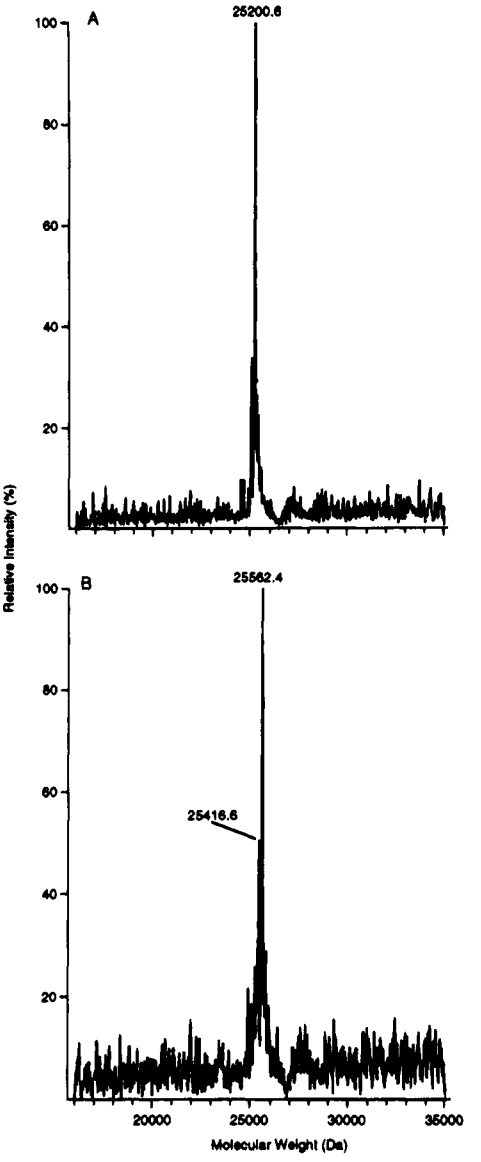


FIGURE 6: Deconvoluted mass spectrum of (A) I-4 and (B) the I-4−inhibitor complex derived from L-691,886 after three steps of complex isolation, reactivation, and treatment with additional compound. These data were obtained on a Finnegan TSQ-700 spectrometer with conditions as in Figure 5.

Table 5: Mass of Species Present during L-691,886 Inactivation and Reactivation Studies[a]

	mass (Da)		
	free isozyme-4	886−complex 1	886−complex 2
first inactivation	none detected	25 325 (25 179)	25 557 (25 408)
first reactivation	25 199.4 (25 048.2)	25 323.6 (nr)[b]	25 561 (25 411)
second inactivation	none detected	25 326 (25 190)	25 563 (25 410)
second reactivation	25 200	none detected	25 562 (25 409)
third inactivation	none detected	none detected	25 562 (25 417)

[a] The isozyme-4-derived species that is less one fucose (or hexose) residue is presented in parentheses when it was clearly resolved and above the signal to noise in the mass spectra. The data represent three cycles of inactivation followed by partial reactivation and addition of more L-691,886 (see Materials and Methods for details). [b] The minor species was not resolved from the free enzyme.

addition of active site nucleophiles. The three diastereomers of L-680,833 produce HLE−I complexes of essentially identical masses. One could argue that the water molecule is added to the imine during either the HPLC or ESI-MS analysis, but the NMR results argue against a sp^2 hybridized carbon. Given that the original C-4 carbon is neopentyl, it is also unlikely that hydrolysis of an alkylated histidine during the course of the experiments produces the +18 increase in mass, although we cannot rule this out entirely. The observation of an analogous shift in mass with β-lactam-derived PPE−I complexes when the sample is directly infused into the instrument flow suggests that the presence of an additional water is not an artifact of the HPLC chromatography (*vide infra*).

Structures **3** and **5** presented in Figure 9 are consistent with both the mass spectral data and the NMR chemical shifts of the L-680,833-derived HLE−I complex. In **3** water has added to the original C-4 of the lactam ring, while in **5** the active site histidine has added to this position while water

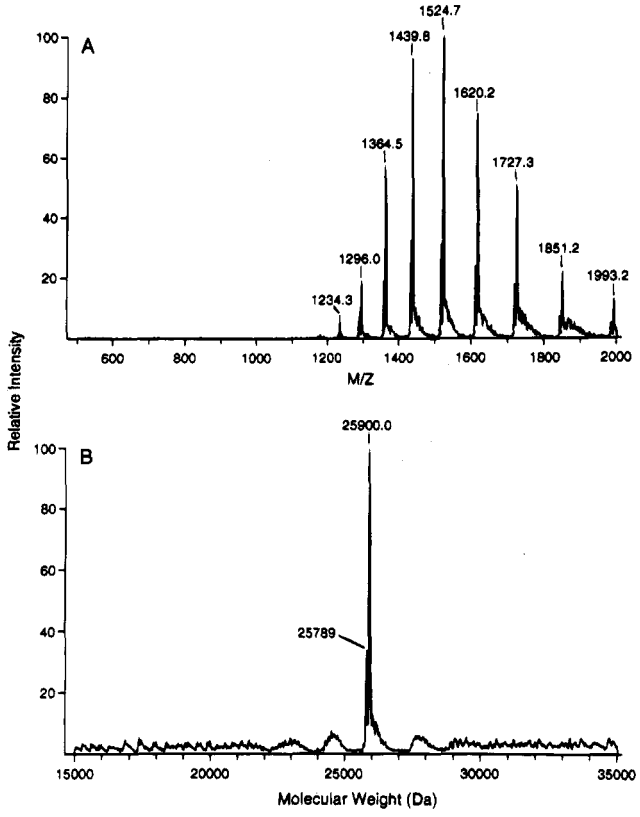


FIGURE 7: (A) ESI-MS (Finnegan TSQ-700 spectrometer) of 78 pmol of PPE at pH 7.5 after C-4 250 μm × 10 cm capillary HPLC eluted with a 10−90% AcCN−0.1% TFA gradient with a 2 μL/min flow rate. (B) Deconvoluted mass spectrum of the data presented in (A).

has hydrolyzed the acyl-enzyme. Both structures could derive from either direct S_N2-like displacement of the leaving group (route d with H_2O and route f with the active site His) or addition to the imine (routes b and g). While, sterically, a direct displacement at the neopentyl carbon is not favored, an enzyme-bound nucleophile (His or H_2O) could be favorably positioned to facilitate the displacement.[8] While we cannot rule out the production of structures analogous to **5** in some cases, the cyclic products generated during reactivation of both L-680,833- and L-670,258-derived complexes are more consistent with the carbinolamine structure **3** (Green et al., 1995). Direct displacement of the leaving group by histidine or addition to the imine requires hydrolysis of the acyl-enzyme to form the acid in the active site to be consistent with the determined mass of the complexes. While this would yield the same mass as the carbinolamine, the cyclic products observed would not likely be produced from this species as they require attack on an ester carbonyl (or otherwise activated carbonyl) for cyclization. Therefore, we conclude that the identity of the stable acyl-enzyme produced from HLE and L-680,833 is the carbinolamine **3**.

The observation of multiple complexes during the inactivation of HLE by β-lactams is not surprising given the biphasic reactivation kinetics observed with some of these HLE−I complexes (Green et al., 1995). In the case of

[8] Stepwise elimination of the leaving group to generate the imine followed by addition of the nucleophile would be more attractive on the basis of chemical precedence. Elimination could be facilitated by deprotonation of the nitrogen.

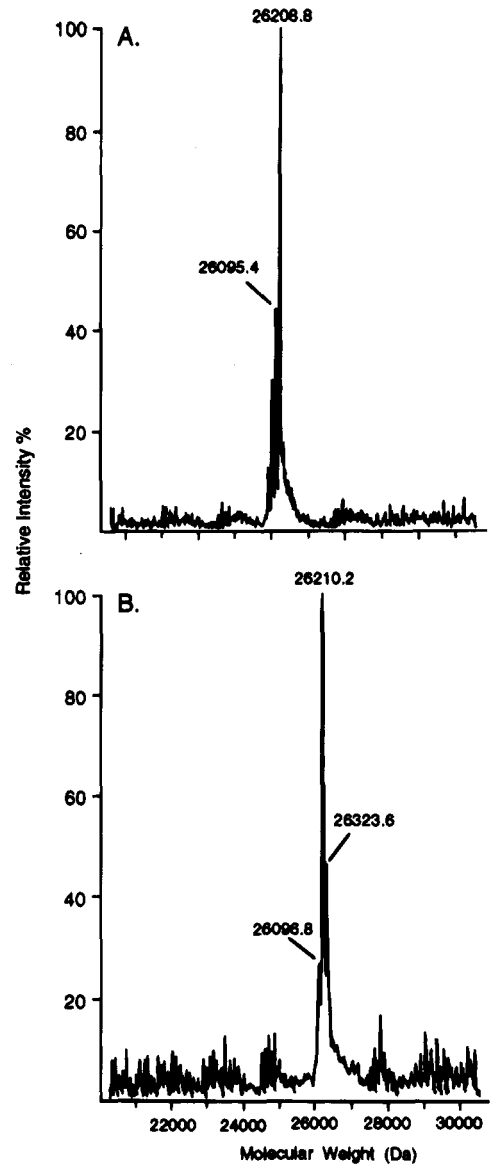


FIGURE 8: Deconvoluted mass spectra of PPE (18 μM) complexes produced from 236 μM L-684,248 (A) and L-684,249 (B). A total of 90 pmol of PPE was injected. The other conditions were the same as in Figure 7.

Table 6: Mass of Porcine Pancreatic Elastase and PPE−I Complexes

	major	minor	Δ^a	Δ^b
PPE	25 900 ± 1[c]	25 789 ± 2[c]		
PPE + L-684,248	26 209	26 095	309	306
PPE + L-684,249	26 210, 26 324	26 097	310, 424	308[d]
by direct infusion	26 212, 26 321	26 097	312, 421	308[c]
PPE + SLPI	25 899, 11 713	25 787		
SLPI	11 709			

[a] The difference is calculated from the major species. The molecular weight of L-684,248 and L-684,249 is 424. The molecular weight of the compound less the C-4 substituent (*p*-hydroxybenzoic acid) is 287. [b] The difference is calculated from the minor species. [c] From $n = 3$ determinations. [d] The other minor species derived from the PPE−I complex was not resolved.

L-691,886, there are initially two complexes produced. The complex with the higher mass is consistent with the carbinolamine resulting from loss of the C-4 leaving group followed by addition of water, in analogy with the reaction with L-680,833 (Δ mass = 357−361 Da, predicted 363 Da). The

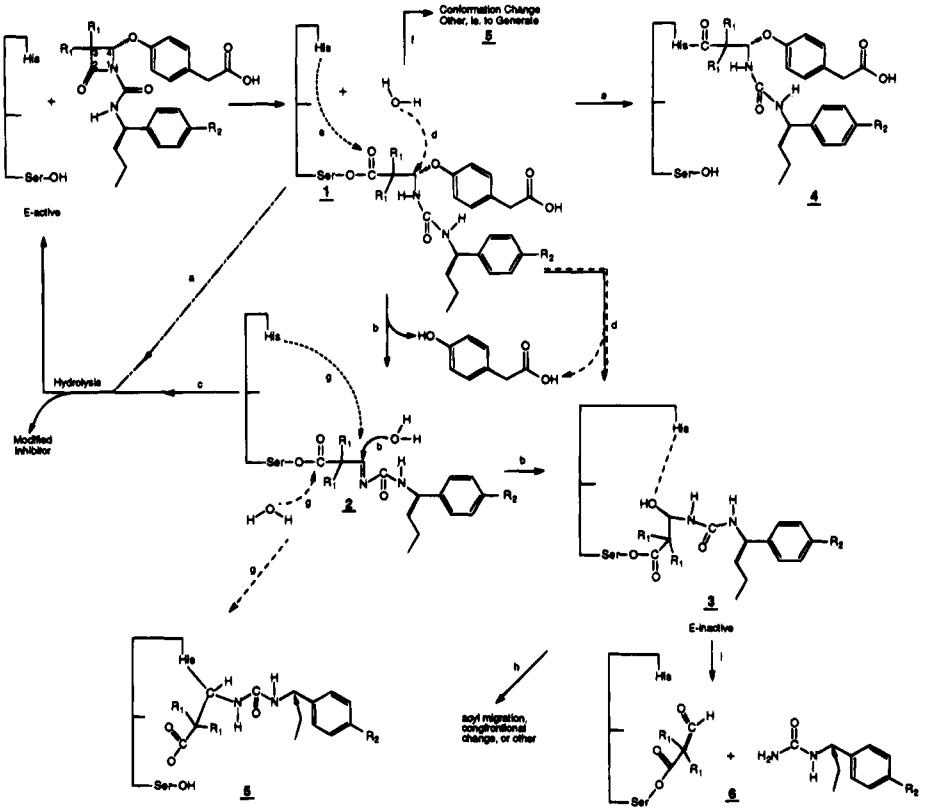


FIGURE 9: Proposed chemical mechanisms for the inhibition of elastases by β-lactams. For the purposes of the discussion, R_1 = ethyl except for L-684,248 and L-684,249 where it is methyl, and R_2 = methyl except for L-691,886 where it is ethoxy. In addition, the C-4 substitutions of L-684,248 and L-684,249 are *p*-hydroxybenzoic acid.

species with lower mass is consistent with the acyl-enzyme of 2,2-diethyl-3-oxopropanoic acid (Δ mass = 124−125 Da, predicted 122 Da; structure **6** in Figure 9). Given the biphasic reactivation kinetics observed with L-670,258 and the similarity of the rate constants for reactivation during the fast phase, **6** is likely formed during this reaction as well.[9] This complex would result from complete fragmentation of the inhibitor: loss of both the leaving group and the substituted urea. This complex reactivates faster than the species containing the carbinolamine and was suggested as an intermediate during one route for reactivation of the L-680,833 complex as well (Green et al., 1995). Multiple treatments with inhibitor drive all of the enzyme into the more stable complex whose mass is consistent with the carbinolamine. The final complex produced may also be different from the initial carbinolamine in some way other than mass as the final reactivation half-life increases from 17 to 49 h. One explanation for this increase in stability would be an additional change in conformation, possibly repositioning of F192,[10] as discussed by Green et al. (1995). Another possibility would be histidine attack on the acyl-enzyme to produce an acylimidazole. Nucleophilic participation of the active site histidine has been proposed for the "aging" of α-chymotrypsin complexes derived from phosphate esters but not carbonate esters (Bender & Wedler, 1972). Hubbard and Kirsch proposed that a transient acylimidazole intermediate was produced during the acylation of chymotrypsin by substituted *p*-nitrophenyl benzoates. Finally, Bachovchin et al. (1988) provided data consistent with nucleophilic participation of the histidine during formation of complexes of α-lytic protease with peptide boronic acids.

The discussion of the mechanism of inhibition of elastases by monocyclic β-lactams is complicated by the observation of multiple complexes and products which demonstrate the presence of multiple pathways for inactivation and reactivation. These are summarized in Figure 9. All of the possible routes initiate with formation of an acyl-enzyme **1** as a result of opening of the lactam ring by the active site serine. This likely occurs via a tetrahedral intermediate in analogy to the accepted mechanism for substrate hydrolysis (see **b** in Figure 10). Compounds that lack a leaving group must partition (if at all) from this species to produce a more stable complex by either migration of the acyl group to the histidine (route e to produce **4**, where the *p*-hydroxyphenylacetic acid would be replaced with a hydrogen) or a conformational change in the bound ligand and/or the enzyme (route f). The structure of the stable HLE−I complex derived from L-680,833 and its three diastereomers is likely the carbinolamine **3**. The four diastereomers of L-680,833 produce complexes of identical mass, but the compounds with *R*-stereochemistry at C-4, while inherently less active against the enzyme, produce more stable complexes (Doherty et al., 1994; Green et al., 1995; compare L-680,833 to L-682,946 and L-683,557 to L-683,558). The observation that the stereochemistry at

[9] The picture may not be quite this simple. All of the compounds that yield biphasic reactivation kinetics would produce **6** if they follow the mechanism suggested for L-691,886 inhibition of HLE, but the rates for the rapid return of activity vary 2−3-fold with these compounds (Green et al., 1995). This apparent discrepancy could either be due to the presence of different conformers (for example, different locations of F192) or simply be due to the increased error in measuring these rates, as they were defined by fewer time points than the slower reactivation phase.

[10] The sequence numbering system is derived from chymotrypsin.

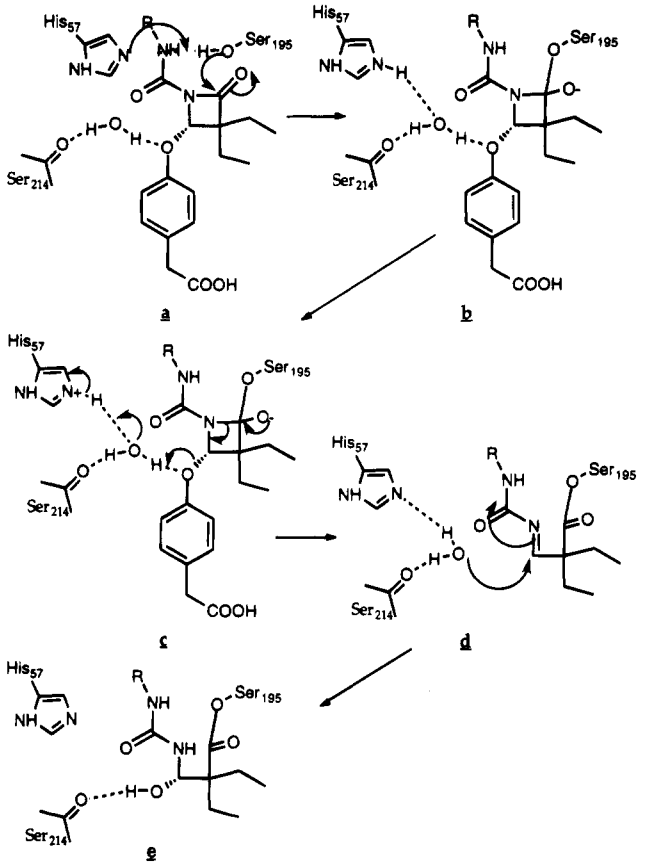


FIGURE 10: Molecular mechanism for the inhibition of elastases by compounds such as L-680,833 with *S*-stereochemistry at C-4 of the β-lactam ring. The residue numbering system is based upon chymotrypsin.

C-4 affects the stability of the final complex demonstrates that these complexes differ in some respect other than mass and argues against a common intermediate, for example, a sp^2 hybridized imine, during the reaction of the diastereomers of L-680,833 with HLE. On the other hand, partitioning of the L-680,833-derived complex liberates the same amount of leaving group as inhibitor required for inactivation, which argues that partitioning in this case occurs after leaving group departure (Chabin et al., 1993) and would be consistent with concerted departure of this group with opening of the lactam ring. Therefore, either one pair of diastereomers goes through the imine with stereospecific addition of water (route b) and the other pair occurs by direct displacement (route d) or both pairs generate the carbinolamine by direct displacement, hence stereospecific addition of water.[11] The (*R*)-hydroxy product generated by direct displacement from L-680,833 (4*S*) could be stabilized by sterically interfering with attack on the acyl-enzyme, while the (*S*)-hydroxy product generated from direct displacement on the 4*R*-diastereomers could be stabilized through hydrogen bonding to active site residues (*vide infra*). In this case, the carbinolamines would differ by their stereochemistry, but the structural model discussed below predicts that the carbinolamine derived from all of the diastereomers would have *S*-stereochemistry. Therefore, we favor mechanisms involving the intermediacy of the imine in the case of the 4*S*-stereoisomers and direct displacement of the leaving group or a modified elimination, hydrolytic mechanism for liberation of the leaving group[11] in the case of the 4*R*-stereoisomers.

Why is the carbinolamine produced? Production of this species relegates the partitioning of these reactions reported by Chabin et al. (1993) to an aborted hydrolytic reaction (see Figures 9 and 10). Either departure of the leaving group occurs after formation of the initial acyl-enzyme and opening of the lactam ring or in the case of the *S*-stereoisomers generation of the imine is concerted with these processes. The *R*-diastereomers would go through the stepwise opening of the lactam ring followed by displacement of the leaving group by water.[11] The latter is supported by the observation of a PPE−I complex that contains the leaving group when this enzyme is inactivated with L-684,249 (*vide infra*).[12] Once the acyl-enzyme is formed, the hydrolytic water can either attack the acyl-enzyme to regenerate active enzyme or attack at the original lactam C-4 to displace the leaving group (or add to the imine).

The mechanism of inhibition of HLE by L-691,886 proceeds by similar routes, but addition of water to the imine produces the carbinolamine **3** and the acyl-enzyme **6**. The simplest explanation for this result is that the imine is hydrolyzed with either a specific water (*vide infra*) to produce the (*S*)-carbinolamine or a bulk solvent water to produce the *R*-isomer. The structural model predicts that the former would be stabilized while the latter could eliminate urea to produce **6**. Alternatively, there could be an additional partitioning step to produce a more stable complex containing the carbinolamine and liberation of the urea to produce the less stable acyl-enzyme **6** (route i). Since the carbinolamine is a predicted intermediate along the course of imine hydrolysis, the additional partitioning step is required if one assumes that only the (*S*)-carbinolamine is produced. In this case the initial stable complex must derive from either an enzyme conformational change, acyl migration, or some as yet unknown mechanism that stabilizes the carbinolamine. In addition, prolonged treatment with this inhibitor appears to produce an even more stable complex, which would be consistent with the acyl migration mechanism to produce **5**.

The mechanism of inhibition of PPE by L-648,248 is likely analogous to that of L-680,833 inactivation of HLE. The mass of the L-684,248-derived PPE−I complex is consistent with loss of the *4S* leaving group and addition of water to produce a carbinolamine generating the *gem*-dimethyl analog of **3**. Generation of the carbinolamine could proceed concomitant with collapse of the tetrahedral intermediate and opening of the lactam ring (for example, see Figure 10). Inactivation of PPE with the *4R*-diastereomer, L-684,249, produces two complexes. In this case partitioning of the initial acyl-enzyme **1** produces a predominant species with the mass predicted for the carbinolamine, but a species that contains the entire inhibitor (analogous to **1** or **4**) is also evident. Either there are multiple conformations of **1** or

[11] If the leaving group were eliminated instead of displaced, the incoming water molecule must add either before complete formation of the imine or at the very least prior to dissociation of the leaving group from the active site to yield different stereochemical products.

[12] It is unlikely that the higher mass complex results from noncovalent binding of liberated *p*-hydroxybenzoic acid, since high-affinity noncovalent complexes of PPE or SLPI are not observed by HPLC−ESI-MS. A similar result was reported by Knight et al. (1993) for HLE and SLPI complexes.

Table 7: Crystallographic Waters in the Vicinity of S214 in Serine Protease X-ray Crystallographic Structures[a]

PDB name[b]	enzyme	H-bonding group	distance[c] S214 to C=O	distance[c] S to C=O
ALP2	native α-lytic protease with sulfate	H_2O	3.20	4.1 (SO_4^{2-})
CHG1	chymotrypsinogen	no H_2O's		
CHA6	chymotrypsin with α_1PI	H_2O	2.68	4.09
CSE1	subtilisin with eglin	Leu^{E45}-NH	3.76	2.90
EST1	PPE, tosylated	no close H_2O's		
EST2	PPE with (trifluoroacetyl)-LA-(trifluoromethyl)phenylanilide	no close H_2O's		
EST3	PPE with sulfate	H_2O	2.89	4.68 (SO_4^{2-})
GCH2	γ-chymotrypsin with sulfate	H_2O	3.30	2.51
PO21	α-lytic protease with AAP-boroAla[c]	boroAla-NH	2.93	2.79
TRM1	trypsin mutant (D102N)	H_2O	2.73	4.32
CHA2	chymotrypsin, tosylated	no close H_2O's		
CHA5	chymotrypsin dimer	Tyr^{B146}-OH	2.66	4.02
CHA4	chymotrypsin dimer	Tyr^{B146}-OH	2.59	4.04
PO11	α-lytic protease with Boc-AP-boroVal	boroVal-NH	3.25	2.82
PTB3	trypsin with benzamidine	H_2O	2.84	4.25
TPP1	trypsin with *p*-amidinophenylpyruvate	H_2O	2.76	4.31

[a] The structures were obtained from the Brookhaven Protein Data Bank (Bernstein et al., 1977). Some of the structures represent enzyme–inhibitor complexes while others represent native enzymes. [b] Protein database name. [c] Å, heavy atom to heavy atom. [d] BoroAla and boroVal represent substitution of the −COOH group of alanine and valine, respectively, with $-B(OH)_2$.

attack of the active site His on the acyl-enzyme generates **4**. Either way, these results demonstrate that departure of the leaving group is not concerted with opening of the lactam ring in this case and support an S_N2 or a modified elimination, hydrolytic mechanism for liberation of the *4R* leaving group.[11,12] These data could be taken to support a similar mechanism for the reaction of the *R*-diastereomers of L-680,833 with HLE. The data obtained with PPE further demonstrate that multiple complexes are possible between serine proteases and β-lactams and that expulsion of a C-4 leaving group is not a prerequisite for the production of relatively stable acyl-enzyme complexes from monocyclic β-lactams and elastases.

Proposed Mechanism Based upon the Structural Models of Serine Proteases. An examination of the structures of serine proteases suggests a reasonable molecular mechanism for the generation of the carbinolamine from L-680,833 and its diastereomers. A number of X-ray crystallographic structures of serine proteases from the Brookhaven Protein Data Bank (Bernstein et al., 1977) suggest the presence of a crystallographically defined water molecule which is well placed to hydrate the imine intermediate (see Table 7). This water molecule likely corresponds to the water molecule observed by Singer et al. (1993a) in Laue diffraction studies of trypsin (denoted in their studies as Wat1082). Their argument that this water molecule hydrolyzes the acyl-enzyme to generate the second product and regenerate free protease during peptide or protein hydrolysis is a matter of controversy (Perona et al., 1993; Singer et al., 1993). Nevertheless, this water molecule would provide a favorable component for stabilization of the HLE−I complexes derived from the L-680,833 class of compounds and a likely entity in the generation of the acylcarbinolamine. A suggested mechanism for this process is shown in Figure 10. A comparison of the native and the inhibited serine protease structures listed in Table 7 suggests that it is important to donate a hydrogen bond to the backbone carbonyl of S214. In the native structures and in those structures with bound inhibitors in which S_2[13] is unoccupied, water (Wat1082) fulfills this role. When the inhibitor occupies S_2, then the water is displaced and the S214 carbonyl is satisfied by donation of a hydrogen bond from the inhibitor [see, for example, the structure of HLE inhibited with peptide chloromethyl ketones (Navia et al., 1989)].

Although a structure of a HLE−I complex derived the L-680,833 class of β-lactams has not been forthcoming, the structure−activity relationships of these compounds and their structural models in the active site of HLE suggest that the hydrogen bond donor capability provided by a group such as Wat1082 bridges the acceptor properties of both the ether linkage of the leaving group and the amide carbonyl of S214. Wat1082 is postulated in Figure 10 to be pivotal in the creation of the imine and its stereospecific hydrolysis to produce the carbinolamine **e**. Donation of a H-bond via the intermediacy of Wat1082 to the ether linkage could facilitate departure of the leaving group concomitant with opening of the β-lactam ring. Concerted departure of *4S* leaving groups is supported by stopped-flow studies reported by Chabin et al. (1993). The hydroxyl group would then derive from the tightly bound water which remains essentially in the same position upon formation of the HLE−I complex **e** by addition to the imine.

Clearly the 4*R*-isomers of L-680,833 will interact with HLE in a very different manner, and this is borne out by the increased stability of complexes derived from these compounds. What is somewhat surprising is that the different isomers at C-4 yield ions of the same mass. In this case, the orientation of Wat1082 is such that it cannot H-bond to the *4R* oxygen. As discussed in Green et al. (1995), the orientation of the *4R* group in the initial HLE−I complex prior to acylation of the enzyme could force F192 into an alternate conformation away from the "lip" of the S_1 pocket where it would reside in a complex derived from a *4S*-isomer. Acylation of the enzyme by the *4R*-isomers would proceed through a tetrahedral intermediate, but collapse of this intermediate would not result in concomitant departure of the leaving group but rather proton transfer from the His residue to the lactam nitrogen resulting in ring opening. Direct displacement of the leaving group by Wat1082, catalyzed by hydrogen bonding to the histidine residue and/or S214 would yield the carbinolamine with *S*-stereochemistry. Alternatively, the *4R* leaving group could be elimi-

[13] The enzyme subsites are numbered according to the nomenclature of Schecter and Berger (1967).

nated, followed by addition of water. The model predicts that the carbinolamine generated from both (*S*)- and (*4R*)-β-lactams would have the same stereochemistry (*S*) and the difference in stability of each intermediate is the result of the induced fit of the enzyme to the ligand during the initial interactions, prior to the loss of the *4R*-substituent. The stereochemistry of the acylcarbinolamine produced from the *S*- and *R*-isomers cannot be ascertained from the cyclic products observed upon regeneration of the active enzyme since they likely are generated from an intermediate with the original lactam C-4 position sp^2 hybridized (Green et al., 1995); therefore, confirmation of this proposal awaits a structural determination.

The final question is how are the acyl-enzymes and carbinolamines stabilized? The rate of hydrolysis of the acyl-enzyme is determined by the accessibility of the acyl group to water (Green et al., 1995). The data and model suggest that the *4R*-ligand results in an acyl-enzyme which is protected from hydrolysis relative to the acyl-enzyme generated from *4S*-isomers. As discussed in the accompanying paper, this is likely do to slightly different orientations of the side chain of F192. If the hydroxyl that produces the carbinolamine derives from a tightly bound water that remains in essentially the same position, then the stability of the complexes derived from L-680,833 and the diastereomers can be explained by hydrogen bonding to either H57 and/or S214. In addition, if Wat1082 is the hydrolytic water as suggested by Singer et al. (1993a,b), then incorporation into the carbinolamine prevents its involvement in hydrolysis, although this would not explain the stability of complexes derived from lactams that lack a leaving group at C-4. Alternate explanations include leaving the histidine residue either in the wrong ionization state (protonated) after addition of water or simply out of position to act catalytically. The observation of a pH dependence of the products generated from L-680,833 (Green et al., 1995) argues against the former. In addition, the stability of the complexes lacking a leaving group suggests that this is not the case but would be consistent with displacement of the histidine residue such that it could not catalyze hydrolysis of the acyl-enzymes. Finally, it should be recognized that the carbonyl carbons of the acyl-enzymes produced from these lactams are sterically hindered due to the dialkyl substituents at C-3, and this may aid somewhat in complex stabilization, although this is not a sufficient determinant.

CONCLUSIONS

The identity of the stable complex formed between HLE and the four diastereomers of L-680,833 is a carbinolamine. The data are consistent with a mechanism in which departure of the 4*S* leaving group is concerted with opening of the β-lactam ring, while a 4*R* leaving group departs following ring opening. The structural model predicts that the complexes produced from the diastereomers are the result of addition of a specific bound water molecule to what was originally the 4 position of the lactam ring to produce (*S*)-carbinolamines, but the complexes differ in the conformation of active site residues. Multiple chemical complexes were observed between one β-lactam and HLE, which explains the biphasic reactivation kinetics observed with some of the β-lactam-derived HLE−I complexes. Multiple complexes were also observed during the interaction of PPE with a β-lactam. These observations indicate that multiple chemical reaction pathways are possible during the interaction of β-lactams with serine proteases.

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BI9504365