Mechanism of Inhibition of Human Leucocyte Elastase by β-Lactams. 2. Stability, Reactivation Kinetics, and Products of β-Lactam-Derived E−I Complexes†

B. G. Green,‡ R. Chabin,‡ S. Mills,§ D. J. Underwood,|| S. K. Shah,§ D. Kuo,‡ P. Gale,‡ A. L. Maycock,‡,⊥ J. Liesch,# C. S. Burgey,§ J. B. Doherty,§ C. P. Dorn,§ P. E. Finke,§ W. K. Hagmann,§ J. J. Hale,§ M. MacCoss,§ W. M. Westler,○ and W. B. Knight*,‡,▽

Departments of Enzymology, Medicinal Chemistry, Molecular Sciences, and Natural Products Research, Merck Research Laboratories, Rahway, New Jersey 07065, and Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53711

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

ABSTRACT: The monocyclic β-lactams reported by Knight et al. [Knight, W. B., et al. (1992) *Biochemistry 31*, 8160; Chabin, R., et al. (1993) *Biochemistry 32*, 8970] as inhibitors of human leucocyte elastase (HLE) produce stable HLE−inhibitor complexes that slowly reactivate with half-lives ranging from less than 1 to 15 h at 37 °C. The complexes produced between PPE and two C-3 dimethyl-substituted β-lactams are less stable than those produced between HLE and analogous C-3 diethyl-substituted lactams. The stability of the HLE−I complexes is governed primarily by the structure of the substituted urea portion of the inhibitors and not by the identity or presence of a leaving group at C-4 of the lactam ring. In some cases substitutions on the urea portion of the inhibitors yielded complexes that displayed biphasic reactivation kinetics. This suggests the presence of at least two different complexes. The stereochemistry of the leaving group at C-4 has a small effect on the stability of the final complex (1.3−2-fold); therefore, the identity of the final complex is dependent upon the initial stereochemistry at that position. The stability of the complexes was relatively insensitive to hydroxylamine, which suggests that the acyl-enzymes are protected from nucleophilic "rescue". The rate of reactivation of the complex derived from L-680,833, [*S*-*R**,*S**)]-4-[(1-(((1-(4-methylphenyl)butyl)amino)carbonyl)-3,3-diethyl-2-oxo-4-azetidinyl)benzeneacetic acid, was pH independent, while the L-684,481, (*R*)-(1-(((1-(4-methylphenyl)butyl)amino)carbonyl)-3,3-diethyl-2-azetidinone generated complex displayed a pH-dependent reactivation rate. In the latter case, the increase in reactivation rate with pH displayed a pK_a of 7.2. This is consistent with the requirement for base catalysis by the active site histidine to regenerate enzymatic activity. Reactivation of the L-680,-833-derived complex produced different products as a function of pH, suggesting two different pH-dependent routes of reactivation. At low pH a route that produced primarily the substituted urea is favored, while at higher pH production of two six-membered ring diastereomers competes with urea generation. Thus, the apparent pH independence of the return of activity is the result of two offsetting pathways. Other compounds such as L-670,258, (*S*)-4-[((((2-naphthylmethyl)amino)carbonyl)-3,3-diethyl-4-oxo-2-azetidinyl)oxy]benzoic acid, reactivate by these two routes as well as by aminolysis by the other urea nitrogen to produce an additional regioisomer. The temperature dependence of the reactivation of the complexes derived from L-684,481 and L-680,833 suggests different mechanisms. The negative activation entropy in the former case is consistent with hydrolysis of an acyl-enzyme while the positive activation entropy in the latter case favors an intramolecular or dissociative mechanism.

Previous work demonstrated that monocyclic β-lactams could be developed as stable and specific mechanism-based inhibitors of serine proteases (Knight et al., 1992a; Chabin et al., 1993). The inherent specificity of these inhibitors for human leucocyte elastase (HLE,[1] EC 3.4.21.37) and their stability to nucleophilic attack in solution should minimize nonspecific acylation of endogenous nucleophiles, which will increase the potential therapeutic utility of this class of compounds. Moreover, when stable monocyclic β-lactams were designed as inhibitors of HLE, compounds that were active by oral administration in pharmacokinetic animal models, such as L-680,833, were developed (Doherty et al., 1993). Therefore, members of this class of compounds are under development as therapeutic agents for treatment of a number of disease states in which HLE has been implicated. These include emphysema (Kaplan et al., 1973; Powers, 1983) and other inflammatory diseases such as rheumatoid arthritis (Janoff et al., 1976).

The molecular mechanism of action of these compounds has been under intense study. The minimal kinetic mechanism is essentially the same as that reported by Knight et al. (1992b,c) for simple monobactams and the bicyclic cephalosporin derivatives (Chabin et al., 1993). The chemi-

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

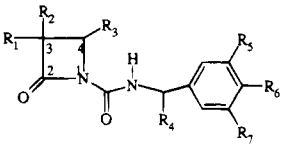
* Address correspondence to this author.
‡ Department of Enzymology.
§ Department of Medicinal Chemistry.
|| Department of Molecular Sciences.
⊥ Present address: Sterling Research Group, Malvern, PA 19355.
Department of Natural Products Research.
○ Department of Biochemistry, University of Wisconsin.
▽ Present address: Department of Enzymology, Glaxo Wellcome Inc., Research Triangle Park, NC 27709.

⊗ Abstract published in *Advance ACS Abstracts,* October 15, 1995.

[1] HLE or elastase refers to the mixture of isozymes as isolated from human sputum.

0006-2960/95/0434-14331$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[a]	Et	Et	(*R,S*)-O-(C_6H_4)-4-CO_2H	H		$-CH(CH)_2CH-$	H
L-679,723	Et	Et	(*S*)-O-(C_6H_4)-4-CH_2CO_2H	(*R*)-ethyl	H	CH_3	H
L-680,750	Et	Et	(*S*)-O-(C_6H_4)-4-CO_2H	(*R*)-propyl	H	H	CH_3
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-680,861	Et	Et	(*S*)-O-(C_6H_4)-4-CH_2CO_2H	(*R*)-propyl		$-OCH_2O-$	H
L-682,271	Et	Et	(*S*)-O-(C_6H_3)-3-NO_2-4-CO_2H	(*R*)-ethyl	H	H	H
L-682,272	Et	Et	(*R*)-O-(C_6H_3)-3-NO_2-4-CO_2H	(*R*)-ethyl	H	H	H
L-682,322	Et	Et	(*R*)-O-(C_6H_4)-4-CO_2H	(*R*)-propyl	H	CH_3	CH_3
L-682,946	Et	Et	(*R*)-O-(C_6H_4)-4-CH_2CO_2H	(*R*)-propyl	H	CH_3	H
L-683,537	Et	Et	(*S*)-O-(C_6H_4)-4-CO_2H	(*R*)-propyl	CH_3	H	CH_3
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-683,841	Et	Et	(*S*)-O-(C_6H_4)-4-CO_2H	(*R*)-propenyl		$-(CH_2)_4-$	H
L-683,845	Et	Et	(*S*)-O-(C_6H_4)-4-CH_2CO_2H	(*R*)-propyl		$-OCHCH-$	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_2	(*R*)-propyl	H	CH_3	H
L-684,481	Et	Et	H	(*R*)-propyl	H	CH_3	H
L-685,255	Et	Et	(*S*)-O-(C_6H_4)-4-CH_2CO_2H	(*R*)-ethyl	H	F	H
L-685,502	Et	Et	(*S*)-O-(2-C_5H_4N)	(*R*)-propyl	H	CH_3	H
L-686,402[b]	Et	Et	(*S,R*)-O-(C_6H_4)-4-CO_2H	(*R,S*)-propyl		$-CH_2CH_2CH_2O-$	H
L-686,403[b]	Et	Et	(*S,R*)-O-(C_6H_4)-4-CO_2H	(*R,S*)-propyl		$-CH_2CH_2CH_2S-$	H
L-687,666	Et	Et	(*S*)-O-(C_6H_4)-4-CH_2CO_2H	(*R*)-propyl	H	CF_3	H
L-687,676	Et	Et	(*S*)-O-(C_6H_4)-4-CH_2CO_2H	(*R*)-propyl	H	F	H
L-691,886	Et	Et	(*S*)-O-(C_6H_4)-4-CH_2CO_2H	(*R*)-propyl	H	$-OCH_2H_3$	H

[a] The *S*-enantiomer in the racemate preferentially inactivates HLE (Doherty et al., 1993; Chabin et al., 1993). [b] The 4(*S*), R_4(*R*) diastereomer preferentially inactivates HLE (Doherty et al., 1993; Chabin et al., 1993).

cal mechanism was examined by Chabin et al. (1993), but the identity of the final stable enzyme−inhibitor complexes remained elusive. The compounds initially form reversible Michaelis complexes with HLE which then react to form at least one acyl-enzyme species that partitions between turnover and inactivation of the enzyme. The partition ratio (k_{cat}/k_{inact}) of the best inhibitors such as L-680,833 approaches zero, thus rivaling the natural inhibitors such as α_1PI.[2] The initial hydrolysis product of partitioning is likely an acyclic imine that partitions in solution between hydrolysis to produce a urea and an aldehyde and decarboxylation to produce a vinyl-substituted urea and CO_2. Small amounts of other products were observed but not characterized. Prior to partitioning between enzyme reactivation and production of a stable enzyme−inhibitor complex, compounds that contain potential leaving groups, such as *p*-hydroxybenzoic acid, liberate these. Liberation of this group would yield an imine, which could potentially serve as an electrophile for an addition by an active site nucleophile such as the catalytic histidine. The addition of the histidine to an enzyme-generated electrophile was proposed to explain the stability of the complexes produced between PPE and cephalosporins (Navia et al., 1987) and by analogy HLE with this class of compounds (Knight et al., 1992b). The stability of the complexes and the products produced from HLE, PPE, and the compounds reported in the previous work (Knight et al., 1992b; Chabin et al., 1993) are examined in this work.

MATERIALS AND METHODS

The compounds listed in Table 1 and the substituted ureas were synthesized according to published procedures (for example, for Table 1 see European Patent Office Publication no. EPO O 337, 549; Doherty et al., 1993; Shah et al., 1992; for the substituted ureas see United States Patent US-05/276,139). The synthesis of [4-^{13}C]-L-680,833 will be published elsewhere (S. Shah, unpublished results). The β-lactams used in these studies are stable under the experimental conditions (Knight et al., 1992). HLE was purchased from Elastin Products (St. Louis, MO). HLE activity versus either 1 mM MeOsucc-AAPV-pNA[3] or 0.2 mM succ-AAPA-pNA was determined according to Knight et al. (1992b) in buffer A (450 mM NaCl, 10% DMSO, and 45 mM TES at pH 7.5). The active site concentration of HLE was determined according to Green et al. (1991). PPE was purchased from Serva Chemical Co. and assayed with succ-AAPA-pNA according to Knight et al. (1992a) in buffer A. Bovine nasal septum proteoglycan was a gift from Dr. M. Lark of Merck Research Laboratories (Rahway, NJ) and was prepared by $CeCl_2$ gradient according to Hascall and Kimura

[2] Abbreviations: AcCN, acetonitrile; 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; ESI-MS, electrospray ionization mass spectrometry; HLE, human leucocyte elastase; HPLC, high-pressure liquid chromatography; L-683,595, *N*-[(*R*)-(4-methylphenyl)butyl]urea; MES, 2-(*N*-morpholino)ethanesulfonic acid; MWCO, molecular weight cutoff; NOE, nuclear Overhauser effect; 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.

[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.

(1982). Buffers were titrated to the appropriate pH with NaOH. All experiments were conducted at 25 °C unless noted.

Electron impact mass spectra were recorded on Finnigan-MAT TSQ70B or MAT212 instruments at 70 eV. Trimethylsilyl derivatives were prepared with bis(trimethylsilyl)-trifluoroacetamide−pyridine (1:1) at 50 °C. Exact mass values were obtained using the peak matching method with perfluorokerosene as the internal standard. Two-dimensional $^1H\{^{13}C\}$ HMQC NMR spectra (Bax et al., 1983) were obtained according to Knight et al. (1992a) except that some experiments were conducted on a Bruker 600 MHz spectrometer operating at 600.13 and 150.924 MHz for the ^1H and ^{13}C frequencies, respectively. ^1H NMR spectra were obtained on 200 and 300 MHz Varian or Bruker 500 MHz spectrometers. UV−visible spectrophotometry was conducted on either Varian DMS-300 or Cary 210 spectrometers. Alternatively, the collection of kinetic data by UV−visible spectroscopy was automated according to Knight et al. (1992a). Reversed-phase HPLC was conducted with either Beckman 100 pumps equipped with a Waters 990 diode array spectrophotometer, software, and NEC computer for data collection and analysis or Waters 510 pumps with a Maxima-based data station for collection and analyzing data. The column eluants from the latter were monitored with a model 490E UV−visible detector. C-18 columns (4.6 × 150 cm) were eluted with a flow rate of 1 mL/min using one of two gradients. Gradient A was 2 min with 100% A (AcCN−H_2O−MeOH, 10:80:10, containing 0.1% acetic acid) followed by a 9 min linear gradient to 100% B (AcCN−H_2O−MeOH, 80:10:10, containing 0.1% acetic acid) and a 9 min wash with 100% B. The column was regenerated with a 3 min gradient to 100% A and a 4 min wash with 100% A. Gradient B was identical except acetonitrile−H_2O−MeOH, 45:45:10, containing 10% acetic acid was substituted for solvent A.

Reactivation of β-lactam-derived HLE−I complexes was determined according to Knight et al. (1992b). In typical experiments 36 μM HLE was incubated with excess inhibitor in 0.33 mL of buffer A at pH 7.5 and 25 °C until the reaction was complete. Excess inhibitor and any metabolites were removed by a modified centrifugal gel filtration technique (Penefsky, 1977; Knight et al., 1992b). A 2.5 mL DEAE-Sephadex A-25 column equilibrated in buffer at the pH of the reactivation study was centrifuged at 1000*g* for 5 min at 4 °C to remove excess buffer. The enzyme solution was then loaded onto the column and the column centrifuged again to elute the enzyme. A 0.3 mL aliquot of the eluant was diluted to 3 mL in the reactivation buffer, typically buffer A although in some experiments different buffers were used (*vide infra*). The resulting solution was then incubated at either 4, 15, 25, or 37 °C as noted, and 5−10 μL aliquots were assayed over time. Most experiments were conducted at 37 °C. In automated experiments 5 μL aliquots were assayed. In separate experiments with several β-lactams the reactivation process was monitored simultaneously for both the return of activity and the generation of metabolites (*vide infra*). Reactivation experiments in the presence of hydroxylamine were conducted by dilution of the column eluant into buffer A containing 0.125 M NH_2OH. The pH dependence of reactivation was examined with reactivation buffers containing 450 mM NaCl and 10% DMSO and 45 mM buffers as follows: MES, pH 5.5 and 6; PIPES, pH 6 and 7; TES and HEPES, pH 7.5; TAPS, pH 8 and 8.5. The dependence of the reactivation rate on ionic strength was examined in buffers containing either 150 or 450 mM NaCl, 10% DMSO, and 45 mM TES at pH 7.5. Similar experiments were conducted with buffers containing 150 mM NaCl in the presence and absence of 2 mg/mL proteoglycan.

The inhibitor-derived products generated during reactivation of HLE−I complexes were monitored and purified by reversed-phase HPLC on 15 × 4.6 cm C-18 columns with the two acetonitrile gradient systems. Sufficient quantities of the products were generated by adding additional inhibitor as it was consumed. In a typical reaction, 6 mL of buffer A containing 0.6 mM L-680,833 and 0.09 mM HLE was incubated at 37 °C. Over time, additional L-680,833 was added to a final concentration of 2 mM. The reactions were monitored by HPLC using gradient B. After 312 h, two metabolites, 833A and 833B, were purified by HPLC using gradient B. Aliquots were filtered through a 10 000 MWCO filtration cup under pressure (Millipore) to remove the enzyme and precipitated products and diluted 1:1 with aqueous 20% AcCN, 20% MeOH, and 0.2% acetic acid prior to injection. Fractions of 0.5 mL were collected (0.5 min). The content of each fraction was determined by analytical HPLC, and the fractions containing the unknowns were pooled. A total of 1.1 and 1.2 μmol of 833A and 833B were purified from 4.5 mL of the reaction, respectively.[4] In a similar fashion, 0.6 mM L-670,258 and 0.12 mM HLE were reacted in buffer A for 170 h at 37 °C. As time progressed, additional L-670,258 was added to a final concentration of 3 mM. The identity of the substituted ureas, L-683,595 (from L-680,833) and *N*-(2-naphthylmethyl)urea (from L-670,258), was confirmed both by mass spectrometry and by spiking enzyme-generated products with authentic materials followed by HPLC analysis. The previously identified products observed during base-catalyzed decarboxylation of L-670,-258 and L-680,833 were found in the precipitates produced in the respective reactions. The unidentified products were analyzed by mass spectrometry and ^1H NMR. ^{13}C-Labeled products were produced from the reactivation of the [4-^{13}C]-L-680,833-derived HLE−I complex similarly. ^{13}C-Labeled 833A and 833B were not separated but collected together. The material was lyophilized and exchanged four times with D_2O. The mixture of labeled 833A and 833B was dissolved in DMSO-d_6 (0.15 mL) and diluted with 1.35 mL of sodium phosphate at pH 7.5 in D_2O, and $^1H\{^{13}C\}$ HMQC NMR spectra were taken. In separate experiments with the same sample the pulse sequence was modified to observe carbon−proton coupling.

Synthetic 833A and 833B (*vide infra*) were added to enzyme-generated products from the reactivation of L-680,-833- and L-680,831-derived HLE−I complexes to confirm their identity and analyzed by HPLC using both gradients A and B. The products (833A and 833B) generated from L-680,833 enzymatically and the synthetic materials were examined in more detail. The ability of 833A and 833B to interconvert and their stability were examined by boiling for 2 h in buffer A. In a typical experiment 0.1 mg/mL 833A was boiled for 2 h in buffer A. Then 20 μL aliquots were analyzed by HPLC (gradient A) before and after boiling. The ability of 40 μM HLE at pH 7.5 and at 37 °C to catalyze the interconversion of 0.1 mg/mL 833A to 833B (and vice versa) was also examined by HPLC.

[4] This reaction was not complete as there was still L-680,833 present in the reaction solution.

The products produced from the L-680,833-derived HLE−I complex at pH 5.5 were also examined. HLE (0.18 mM) was inactivated with 0.36 mM L-680,833 at pH 7.5 in buffer A. The buffer was exchanged; excess inhibitor and any metabolites produced from inactivation were removed on a Sephadex A-25 column equilibrated in 450 mM NaCl, 10% DMSO, and 45 mM MES at pH 5.5. The sample was then incubated for 96 h at 37 °C. After 24 h an additional 0.11 mM L-680,833 was added. The reaction was monitored by HPLC using gradient A.

Chemical Synthesis of the Products Observed during the Reactivation of the L-680,833-Derived HLE−I Complex. The two diastereomers proposed as the enzymic products generated from L-680,833 were synthesized chemically. Intermediates and products were identified by ^{1}H NMR spectra of $CDCl_3$ solutions and FAB mass spectrometry. Key NMR assignments of the final products were made on the basis of NOE difference experiments.

(A) (R)-1-[(Aminocarbonyl)amino]-1-(4-methylphenyl)-3-butene. A solution of 0.5 g (3.1 mmol) of (*R*)-1-amino-1-(4-methylphenyl)-3-butene (see European Patent 0 337 549) in 5 mL of dry methylene chloride was transferred by cannula into a suspension of 0.503 g (3.1 mmol) of carbonyldiimidazole in 5 mL of methylene chloride over 10 min. After 2 h, the mixture was cooled to 0 °C, and a stream of gaseous ammonia was bubbled into it for 5 min. The mixture was allowed to warm to room temperature and stirred for 42 h. The reaction mixture was then concentrated *in vacuo* and the residue dissolved in 10 mL of ethyl acetate. The pH was adjusted to 3 with 2 N aqueous HCl and aqueous $NaHCO_3$. The mixture was extracted with 5 × 10 mL of ethyl acetate, and the combined organic layers were dried over sodium sulfate, filtered, and concentrated *in vacuo*. The residue was purified by flash chromatography on 40 g of silica gel eluting with 1 L of 4:100 $CH_3OH-CH_2Cl_2$ to give 0.56 g (88%) of a white solid [^{1}H NMR (ppm) δ 2.3 (s, 3H), 2.43 (t, 2H), 4.5−4.7 (m, 3H), 5−5.15 (m, 2H), 5.55−5.8 (m, 2H), 7.05−7.2 (m, 4H)].

(B) (R)-1-[1-(4-Methylphenyl)-3-butenyl]-5,5-diethyl-2,4,6-trioxohexahydropyrimidine. To 1 mL of dry ethanol under N_2 was added 0.034 g (1.47 mmol) of Na metal. When the Na had dissolved, the solution was charged with 0.318 g (1.47 mmol) of diethyl 2,2-diethylmalonate and then 0.3 g (1.47 mmol) of (*R*)-1-[(aminocarbonyl)amino]-1-(4-methylphenyl)-3-butene, and the mixture was heated at reflux under N_2 for 46 h. An additional 2 mL of dry ethanol was added, and the mixture was heated for an additional 72 h. The mixture was then cooled to room temperature and treated with ethyl acetate and water. The resulting mixture was acidified to pH 4−5 with 2 N aqueous HCl and extracted with 6 × 5 mL of ethyl acetate. The combined organic layers were dried over sodium sulfate, filtered, and concentrated *in vacuo*. The residue was purified by flash chromatography on 40 g of silica gel eluting with 9:91 ethyl acetate−hexane to give 240 mg (50%) of an oil [FAB mass spectrum, *m/z* 481 (M + H + matrix, 6%), 329 (M + H, 1%), 299 (M − ethyl, 24%), 145 (butyl − tolyl side chain, 100%), 105 (46%); ^{1}H NMR ($CDCl_3$, ppm) δ 0.75 (t, 3H), 0.87 (t, 3H), 1.9−2.2 (m, 4H), 2.37 (s, 3H), 2.9−3.1 (m, 1H), 3.3−3.5 (m, 1H), 5.1−5.35 (m, 2H), 5.75−5.76 (m, 1H), 6.1−6.25 (m, 1H), 7.2 (d, 2H), 7.42 (d, 2H), 8.88 (br s, 1H)].

(C) (R)-1-[1-(4-Methylphenyl)butyl]-5,5-diethyl-2,4,6-trioxohexahydropyrimidine. A solution of 0.24 g (0.73 mmol) of (*R*)-1-[1-(4-methylphenyl)-3-butenyl]-5,5-diethyl-2,4,6-trioxohexahydropyrimidine in 3.5 mL of ethyl acetate was treated with 13 mg of 5% platinum on carbon, and the mixture was stirred under 1 atm of H_2 for 60 min. The mixture was filtered through Celite and the filtrate concentrated *in vacuo*, yielding 228 mg (95%) of an oil (FAB mass spectrum, *m/z* 481 (M + H + matrix, 8%), 331 (M + H, 20%), 185 (M − butyl − tolyl side chain, 29%), 147 (butyl − tolyl side chain, 36%), 105 (100%); ^{1}H NMR (ppm) δ 0.73 (t, 3H), 0.82 (t, 3H), 1 (t, 3H), 1.2−1.5 (m, 3H), 1.9−2.1 (m, 5H), 2.1−2.6 (m, 3H), 2.32 (s, 3H), 5.95 (app t, 1H), 7.1 (d, 2H), 7.35 (d, 2H), 7.98 (br s, 1H)].

(D) (R)-1-[1-(4-Methylphenyl)butyl]-5,5-diethyl-2,4-dioxo-6(R)- or 6(S)-hydroxyhexahydropyrimidine (833A). A solution of 0.05 g (0.15 mmol) of (*R*)-1-[1-(4-methylphenyl)-butyl]-5,5-diethyl-2,4,6-trioxohexahydropyrimdine in 0.6 mL of dry toluene was cooled to −60 °C under N_2 and treated with 0.4 mL (0.61 mmol) of a 1.5 M solution of diisobutylaluminum hydride in toluene. After 25 min, 2 drops of acetone was added, and the solution was stirred for an additional 15 min at 65 °C. The reaction mixture was then poured into an ice-cold mixture of 4 mL of 1 M aqueous tartaric acid and 3 mL of hexanes and the mixture allowed to warm to room temperature. After being stirred for 30 min, the layers were separated, the aqueous phase was extracted with 3 × 4 mL of hexanes, and the combined organic layers were dried over sodium sulfate, filtered, and concentrated *in vacuo*. The residue was purified by flash chromatography on 9 g of silica gel, eluting with 78:22 hexanes−ethyl acetate to give 35 mg (70%) of a light yellow oil [FAB mass spectrum, *m/z* 335 (30%), 333 (M + H, 24%), 315 (M + H − H_2O, 34%), 147 (butyl − tolyl side chain, 70%), 105 (100%); ^{1}H NMR (ppm) δ 0.56 (t, 3H), 0.73 (t, 3H, CH_2CH_3, syn to ring methine), 0.97 (t, 3H, $CHCH_2CH_2CH_3$), 1.35−1.45 (m, 2H, $CHCH_2CH_2CH_3$), 1.63 (hextet, 2H, CH_2CH_3, syn to ring methine H), 1.84 (hextet, 2H), 2.10 (q, 2H, $CHCH_2CH_2CH_3$), 2.36 (s, 3H), 3.32 (d, J_{H-H} = 7.4 Hz, 1H, CHO*H*), 4.47 (d, 1H, J_{H-H} = 6.9 Hz, C*H*OH), 5.56 (t, 1H, J_{H-H} = 8.0 Hz, $CHCH_2CH_2CH_3$), 7.16 (d, 2H), 7.25 (d, 2H), 8.08 (s, 1H)].

(E) (R)-1-[1-(4-Methylphenyl)butyl)]-5,5-diethyl-2,4-dioxo-6(S)- or 6(R)-hydroxyhexahydropyrimidine (833B). A solution of 0.01 g (0.03 mmol) of (*R*)-1-[1-(4-methylphenyl)-butyl)]-5,5-diethyl-2,4-dioxo-6(*R*)- or 6(*S*)-hydroxyhexahydropyrimidine in 1 mL of dry 1,2-dichloroethane was treated with 7 mg of *p*-toluenesulfonic acid hydrate at room temperature for 2.5 h. TLC on silica gel in 75:25 hexanes−ethyl acetate showed that a new species (R_f = 0.2) was produced in an amount comparable to that of the remaining starting material (R_f = 0.12). The mixture was diluted with aqueous $NaHCO_3$ and extracted with ethyl acetate. The organic phase was dried over sodium sulfate, filtered, and concentrated *in vacuo*. The residue was purified by flash chromatography on 4 g of silica gel, eluting with 78:22 hexanes−ethyl acetate to give 4 mg (40%) of the title compound as an oil [FAB mass spectrum, *m/z* 335 (30%), 333 (M + H, 72%), 315 (M + H − H_2O, 36%), 147 (butyl − tolyl side chain, 78%), 105 (100%); ^{1}H NMR (ppm) δ 0.8 (t, 3H), 0.88 (t, 3H), 0.97 (t, 3H), 1.15−2.1 (m, 9H), 2.35 (s, 3H), 4.68 (d, J_{H-H} = 2.2 Hz, 1H), 5.7 (t, 1H, J_{H-H} = 7.7 Hz), 7.22 (d, 2H, J_{H-H} = 8.2 Hz), 7.36 (d, 2H, J_{H-H} = 8.2 Hz), 8.08 (s, 1H)].

Data Analysis. The data obtained from monitoring the return of enzymatic activity versus time after inactivation by β-lactams were fit by nonlinear regression to eq 1 to

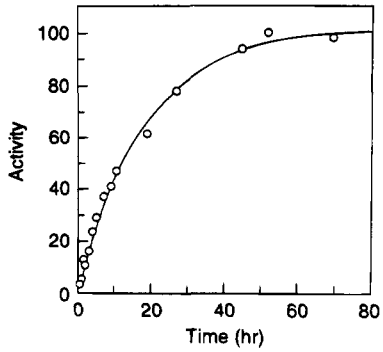


FIGURE 1: Recovery of HLE activity after inhibition by L-680,-833 at 37 °C in 450 mM NaCl and 45 mM TES at pH 7.5. The curve was generated from the theoretical fit to eq 1.

obtain the first-order rate constant and thus the half-life for the complex. Either GraFit (Leatherbarrow, 1992) or NLIN (a program developed at Merck by N. Thornberry) was used. Both software packages yielded the same values. In some cases biphasic reactivation kinetics were evident and eq 2 was used. In cases where insufficient data points were obtained to allow determination of the rate constant for the more rapid phase of obviously biphasic reactions, k_2 for the slower phase was calculated using eq 1. The errors reported are standard errors from the fit of the data except where noted. The thermodynamic parameters for reactivation were determined by fitting the reactivation rate constants (in units of s^{-1}) as a function of temperature (K) to eq 3 to determine the activation energy (E_a) and the preexponential factor (A). The former was used to calculate $\Delta H^{\ddagger}$ and the latter to calculate $\Delta S^{\ddagger}$ according to eq 4 and 5, respectively. The free energy of activation was then determined from these values according to eq 6.

$$y = ae^{-kt} + c \quad (1)$$

$$y = ae^{-k_1 t} + be^{-k_2 t} + c \quad (2)$$

$$k_r = Ae^{-E_a/RT} \quad (3)$$

$$\Delta H^{\ddagger} = E_a - RT \quad (4)$$

$$\Delta S^{\ddagger} = R \ln(ANh/RT) - R \quad (5)$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} = T\Delta S^{\ddagger} \quad (6)$$

RESULTS

Reactivation of β-Lactam-Inhibited HLE. HLE inactivated by monobactams slowly regains activity. In Figure 1, a plot of a typical monophasic return of HLE activity after inhibition by L-680,833 is presented. This compound yields a relatively stable HLE−I complex. For comparison, inhibition of HLE by the simple 4-acetoxy-β-lactam produced a complex with a half-life of only 1.5 h at 25 °C.[5] The half-life and rate constants for reactivation of HLE−I complexes generated from a number of β-lactams are reported in Table 2. Neither ionic strength nor the presence of a macromolecule (proteoglycan) affected the stability of the E−I complexes (see Table 3). The nucleophile NH_2OH increased the rate of reactivation by only 30−35% (see Table 3). The reactivation of L-680,833- and L-684,481-inhibited HLE was also examined as a function of pH (see Table 4 and Figure 2). While the reactivation of L-680,833-inhibited HLE was pH independent, the complex produced from L-684,481 reactivated in a pH-dependent process that displayed a single ionization with a pK_a of 7.2 ± 0.1.[6] The temperature dependence for the first-order rate constants for the return of HLE activity after inhibition by L-680,833 and L-684,-481 is presented in Table 5 and Figure 3. In Table 6 the activation parameters calculated from the temperature dependence data are reported.[7] A number of compounds produced complexes that displayed biphasic reactivation kinetics. In Figure 4, a typical biphasic return of HLE activity after inactivation by L-683,537 is presented. These data yield individual rate constants for each phase of the reaction, and these are summarized for the complexes

Table 2: Kinetic Constants[a] for the Reactivation of β-Lactam-Derived Elastase Complexes at 37 °C

inhibitor	k_{obs} (h^{-1})	$t_{1/2}$ (h)	app Log P^b
L-679,723	0.122	5.68 ± 0.12	1.93
L-680,831	0.0724	9.6 ± 0.1	3.11
L-680,833	0.063	11.0 ± 1[c]	3.11
L-680,861	0.0469	14.8 ± 0.4	1.82
L-682,271	0.17	4.1 ± 0.3	1.93
L-672,272	0.115	6.03 ± 0.5	1.93
L-682,946	0.0459	15.1 ± 0.5	3.11
L-683,557	0.438	1.58 ± 0.08	3.11
L-683,558	0.238	2.9 ± 0.1	3.11
L-683,845	0.116	6.0 ± 0.3	3.05
L-684,481	0.0521	13.3 ± 1.0	3.11
L-685,255	0.228	3.04 ± 0.08	2.08
L-685,502	0.0622	11.1 ± 1.6	
L-686,402	0.101	6.8 ± 4	
L-687,676	0.115	6.01 ± 0.03	2.6

[a] All rate constants were obtained from buffer A. The errors in the half-lives are the standard errors determined from the fit of the data to eq 1 and calculated from the percent error in the rate constants except where noted (see footnote *c*). [b] The apparent Log *P* was calculated using only atoms from the substitution on the exocyclic urea nitrogen. [c] The error in this value is a standard deviation from $n = 3$ determinations.

Table 3: Effect of Ionic Strength, Macromolecules, and Nucleophiles on the Stability of β-Lactam-Derived Elastase Complexes

inhibitor	condition[a]	k_{obs} (h^{-1})	$t_{1/2}$ (h)
L-680,831		0.0724	9.6 ± 0.1
	500 mM NaCl	0.058	12.0 ± 0.7
	150 mM NaCl	0.053	13.0 ± 2
	+proteoglycan[b]	0.08	8.7 ± 1
	−proteoglycan	0.076	9.1 ± 0.9
L-680,833		0.063	11.0 ± 1[c]
	NH_2OH	0.0971	7.1 ± 0.7
L-684,481		0.0521	13.3 ± 1.0
	NH_2OH	0.0747	9.3 ± 0.4

[a] All conditions except as noted were the same as those quoted in Table 2. [b] 2 mg/mL. [c] From Table 2.

[5] This value was estimated from the activity that returned in 1.5 h. The initial activity after removal of inhibitor was 40% of the control. After 1.5 h the activity was 64%, and after 2.5 h 92% activity was attained.

[6] While we have chosen to fit the data in Figure 3 to a simple model for a single ionization, close examination of the data suggests that the rate of reactivation may be leveling off at lower pH. This is reminiscent of the pH dependence of both substrate hydrolysis and the rate of inactivation of HLE by β-lactams reported by Chabin et al. (1993) and suggests that there may be a pH-independent component to the reactions at low pH.

[7] The authors are indebted to the diligence of the reviewers, who pointed out the correct way to propagate the error in $\Delta S^{\ddagger}$. This was confirmed by Bevington (1969).

Table 4: Dependence of β-Lactam-Derived Complexes on pH[a]

	L-680,833		L-684,481	
pH	k_0 (h^{-1})	$t_{1/2}$ (h)	k_0 (h^{-1})	$t_{1/2}$ (h)
5.5	0.037	18.7 ± 1.5	0.00264	262 ± 12
	0.0576	12.0 ± 1		
6.0	0.0587	12.0 ± 1.2	0.00375	185 ± 11
			0.00342	203 ± 10
6.5	0.062	11.2 ± 0.9	0.0143	48.5 ± 3.5
			0.0152	45.6 ± 2.7
7.0	0.0728	9.5 ● 0.8	0.0215	32.2 ± 3.2
			0.0237	29.2 ± 1.7
7.5	0.0571	12.1 ± 0.6[b]	*d*	*d*
	0.0714	9.7 ± 0.4[c]		
8.0			0.0695	10.0 ± 0.8
			0.0513	13.5 ± 1.3
8.5	0.0462	15 ± 3.4	0.0834	8.3 ± 0.6
			0.09	7.7 ± 0.5
9.0			0.10	6.9 ± 0.8
			0.090	7.7 ± 0.4

[a] These studies were conducted at 37 °C. The experimental conditions were the same as those quoted in Table 2 except the reactivation buffer was varied as described in Materials and Methods. [b] HEPES (45 mM) buffer was used in this experiment. [c] TES (45 mM) buffer was used in this experiment. [d] See Table 2.

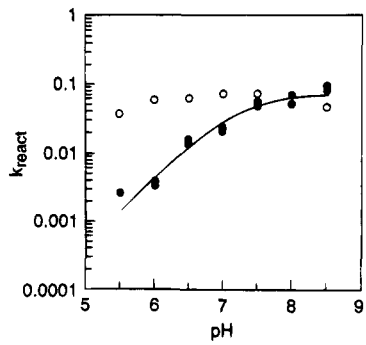


FIGURE 2: Dependence of the reactivation rate constants of the L-680,833- (○) and L-683,841- (●) derived HLE−I complexes on pH.

Table 5: Dependence of β-Lactam-Derived Complexes on Temperature[a]

temp	L-680,833		L-684,481	
(°C)	k_0 (h^{-1})	$t_{1/2}$ (h)	k_0 (h^{-1})	$t_{1/2}$ (h)
47			0.21	3.3 ± 0.6
			0.27	2.6 ± 0.44
37		11 ± 1[b]	0.0521	13.3 ± 1.0[b]
25	0.0104	67 ± 5	0.0136	51 ± 3.5
			0.0142	48.8 ± 1.6
15	0.00182	380 ± 10	0.00439	158 ± 12
			0.00418	166 ± 7
4	0.000216	3210 ± 130		

[a] The temperature dependence was examined at pH 7.5. The other conditions were the same as those quoted in Table 2. [b] From Table 2.

produced from a number of β-lactams and HLE in Table 7. Inactivation of HLE with L-670,258 also produced biphasic reactivation kinetics at pH 5.5. There was approximately a 6-fold decrease in both the rate constants when the reactivation was monitored at the lower pH.

In previous work we reported that the two monocyclic β-lactams, L-684,248 and L-684,249, were relatively good inhibitors of PPE due to the decreased steric constraints of the C-3 dimethyl substitution (Knight et al., 1992). When PPE was inactivated with these β-lactams, activity returned over time (see Table 8). The reactivation kinetics were monophasic. The PPE−I complexes derived from L-684,-

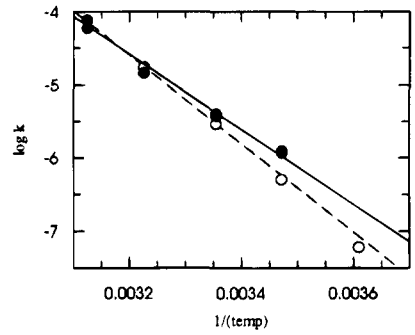


FIGURE 3: Temperature dependence of the first-order rate constant for reactivation of the L-680,833- (○) and L-684,481- (●) derived HLE-I complexes. The units of temperature are in kelvin. The curves were calculated from the parameters (E_a and A_0) determined from the fit of the data to the Arrhenius equation (eq 3). The dashed and solid lines correspond to the fits of L-680,833 and L-684,481 data, respectively.

Table 6: Activation Parameters for the Reactivation of L-680,833- and L-684,481-Derived Complexes[a]

	L-680,833	L-684,481
E_a (kcal/mol)	27.6 ● 1.0	23.5 ± 0.3
$\Delta G^{\ddagger}$ (kcal/mol)	24.9	24.2
$\Delta H^{\ddagger}$ (kcal/mol)	25.0	22.3
$\Delta S^{\ddagger}$ (eu)	6.6 ± 0.4	−6.3 ± 0.9

[a] The thermodynamic activation parameters are calculated at 25 °C from the data presented in Table 5. The errors in E_a are standard errors from the fit of the data to eq 3. The absolute errors in $\Delta S^{\ddagger}$ were propagated from the standard error in the determination of A (the preexponential factor) from eq 3 according $R(\sigma_A/A)$, where σ_A is the standard error in A and R is the gas constant in cal K^{-1}.[7]

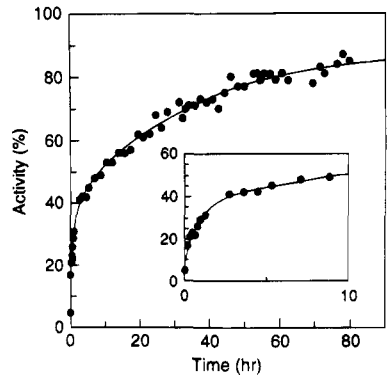


FIGURE 4: Recovery of HLE activity after inhibition by L-683,-537 at 37 °C. The curve was generated from the theoretical fit to eq 2. The inset represents the first 10 h of the main graph.

248 and L-684,249 were 6- and 4-fold less stable at 25 °C than the L-680,833-generated HLE−I complex.

HPLC of the Reaction of a Monobactam with HLE. An HPLC chromatogram of the reaction of L-670,258 over extended time with HLE is presented in Figure 5. In addition to *p*-hydroxybenzoic acid and *N*-(2-naphthylmethyl)urea, two new species were observed. Small amounts of two species with HPLC retention times identical to these were previously observed as a result of partitioning of an acyl-enzyme intermediate prior to the inactivation event (Knight et al., 1992a). The vinyl-substituted *N*-(2-naphthylmethyl)urea, which was observed during both base-catalyzed decomposition and partitioning during inactivation of HLE by L-670,-258, was found in the precipitate formed during the reaction and is therefore not clearly evident in Figure 5. The UV−visible spectra of these metabolites are compared in Figure 5C. From 3 mM L-670,258, 2.99 mM *p*-hydroxybenzoic

Table 7: Kinetic Constants[a] for the Reactivation of β-Lactam-Derived Elastase Complexes That Displayed Biphasic Reactivation Kinetics

inhibitor	k_1 (h^{-1})	$t_{1/2}$ (h)	k_2 (h^{-1})	$t_{1/2}$ (h)
L-670,258	6.34	0.11 ± 0.03	0.33	2.1 ± 0.2
L-670,258, pH 5.5	1.02	0.7 ± 0.2	0.054	12.8 ± 4.2
L-680,750	1.2	0.58 ± 0.09	0.027	25.7 ± 1.4
L-682,322	2.86	0.24 ± 0.05	0.0395	17.5 ± 1.7
L-683,537	1.15	0.60 ± 0.09	0.032	21.7 ± 1.8
L-683,841[b]	5.9	0.12 ± 0.05	1.31	0.53 ± 0.16
			1.43	0.48 ± 0.04
L-686,403	c	c	0.158	4.4 ± 0.6
L-687,666	1.71	0.41 ± 0.2	0.022	31.5 ± 10.0
L-691,886	4.3	0.16 ± 0.02	0.043	16.1 ± 0.2

[a] These data were obtained under the same conditions as quoted in Table 2. The rate constants were obtained by fitting the data to eq 2. The errors in the half-lives are the standard errors determined from the fit of the data to eq 2 and calculated from the percent in the rate constants. In some cases there were insufficient data to assess the more rapid rate constant of obviously biphasic reactivation profiles. In these cases, the value reported for k_2 is the fit of the return of the last 50% of the activity to eq 1. [b] In a second experiment the data were of insufficient quality to clearly distinguish between first-order and biphasic reactivation kinetics. [c] Insufficient data.

Table 8: Kinetic Constants[a] for the Reactivation of β-Lactam-Derived Porcine Pancreatic Elastase Complexes at 25 °C

inhibitor	k_{obs} (h^{-1})	$t_{1/2}$ (h)
L-684,248[b]	0.091	7.6 ± 1.0
L-684,249[b]	0.052	13.3 ± 7.7

[a] The conditions were the same as those used to generate the data presented in Table 2. [b] The activity of L-684,249 returned to 69% of control after 28 h, which was the last time point taken. Over the same time course the L-684,248-inhibited enzyme had returned to 90% of control. It was clear from the raw data that the L-684,248-derived PPE-I complex reactivated faster.

acid, 0.92 mM urea, 0.72 mM metabolite 258A, and 0.83 mM metabolite 258B were produced. The latter concentrations were estimated by assuming that 258A and 258B have extinction coefficients similar to that of the urea. When the L-670,258-derived HLE−I complex was isolated in a separate experiment and the products generated were monitored by HPLC, only the urea, 258A and 258B were observed (data not shown).[8] This further demonstrates that the original C-4 moiety is not present in the final HLE−I complex derived from the β-lactam. Neither hydroxybenzoic acid nor the vinyl-substituted naphthyl-2-methyleneurea were produced during the reactivation process.

Identity of 258A and 258B. The structures shown below are consistent with the spectral data for the identity of the two HLE-generated metabolites. The molecular weight, as determined by mass spectrometry for both 258A and 258B, was 326 Da ($C_{19}H_{22}N_2O_3$). Both products formed di-TMS derivatives, but the fragmentation patterns of both 258A and 258B and their TMS derivatives were substantially different (data not shown). Figure 6 shows the low-field region of the proton NMR spectrum of 258A and 258B.[9] Of particular

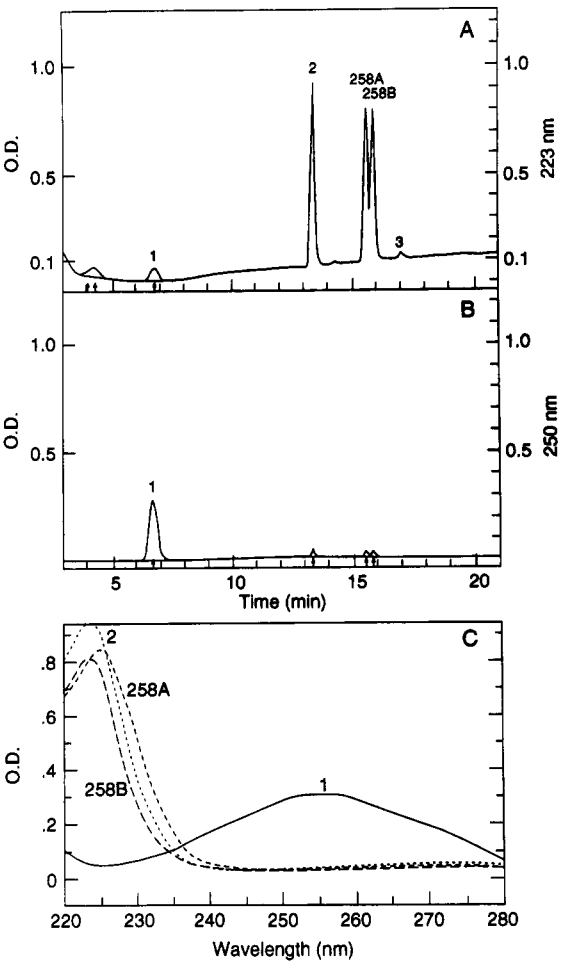


FIGURE 5: HPLC chromatogram of the soluble metabolites produced from L-670,258 during reaction with HLE at 37 °C. This experiment represents continued turnover of the compound and was monitored at 223 (A) and 250 (B) nm. The 4.6 × 15.0 cm C-18 column was eluted with a flow rate of 1 mL/min using gradient A (see Materials and Methods). (C) UV−visible spectra of (1) *p*-hydroxybenzoic acid, (2) β-methylenenaphthyl)urea, and two new products observed in the chromatogram. Unreacted L-670,258 elutes at 17 min (3). A small amount of the vinyl-substituted urea is evident at approximately 14 min (see panel A) although the bulk of this material was present in the precipitate (data not shown).

importance are the chemical shifts of the amide protons and the methine and methylene protons. In 258A, the broad amide proton due to the quadrupolar nitrogen appears at 8.07 ppm while the methine proton appears as a singlet at 4.57 ppm. The addition of CD_3OD causes the resonance at 8.07 ppm to disappear (data not shown). This downfield shift is consistent with the amide α to two carbonyls and is almost identical to that observed for the amide proton in spectra of synthetic 833A and 833B. The two doublets at 5.17 and 4.35 ppm with a 15-Hz coupling constant are consistent with two diastereotopic methylene protons close to a stereochemical center (the carbinolamine carbon). The NMR spectrum of 258B displays a broad amide proton at 6.77 ppm which is coupled to the methine proton at 4.61 ppm (J_{H-H} = 4.8 Hz). The methine carbon collapses to a sharp singlet, and the amide proton disappears upon the addition of CD_3OD (data not shown). The diastereotopic methylene protons of 258B yield the singlet at 5.01 ppm since they are further removed from the methine stereochemical site than in 258A.

After isolation, the L-680,833-derived HLE−I complex produced approximately 0.33 equiv each of the urea and two unknowns (833A and 833B) but no *p*-hydroxyphenylacetic

[8] Mass balance requires that the remainder of the inhibitor molecule, the aldehyde acid 2,2-diethyl-3-oxopropanoic acid, while not observed spectrophotometrically, must also be present when the urea is generated. It should be noted that some of the *N*-(2-naphthylmethyl)urea evident in Figure 5 was likely generated during the inactivation process.

[9] It must be noted that H_2O was present in the NMR samples, and therefore hydroxyl protons are not easily observed. Close examination of Figure 6 suggests the presence of a broad doublet in the baseline around 4.2 ppm that may be due to the hydroxyl group.

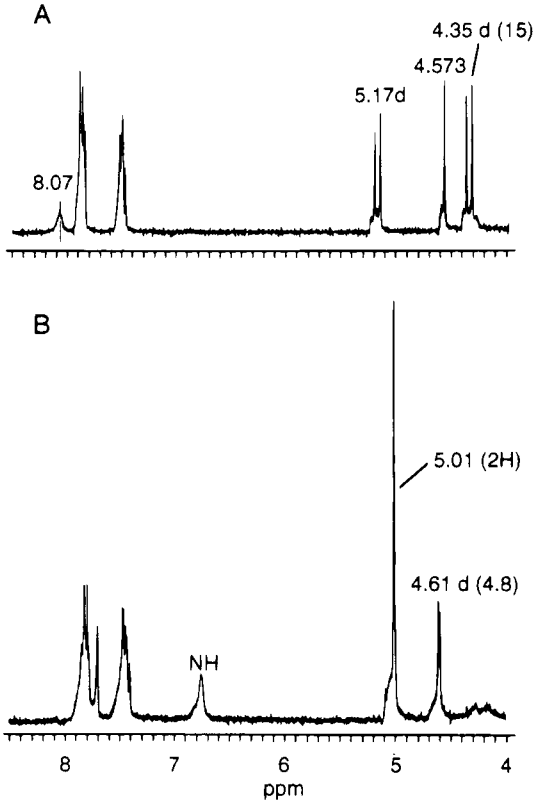
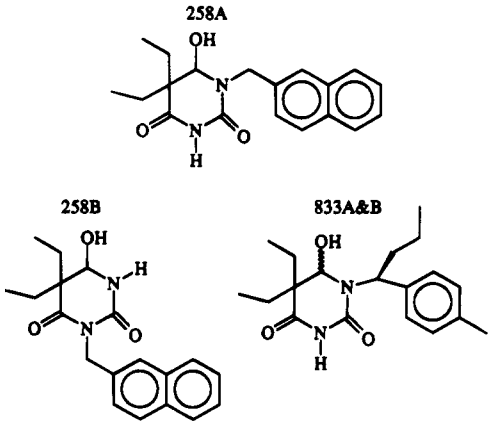


FIGURE 6: Proton NMR spectra of enzyme-generated 258A (0.32 mg) and 258B (0.47 mg) obtained in CD_3CN on a Varian 300 spectrometer. There were resonances due to water (2.18 ppm) evident in both spectra (not shown).



acid at pH 7.5 upon reactivation. Products displaying HPLC retention times identical to those obtained from L-680,833 were also obtained from the L-680,831-derived HLE–inhibitor complex. The molecular mass of both unknowns was 332 Da ($C_{19}H_{28}N_2O_3$). Unlike the L-670,258-derived HLE products, the mass spectral fragmentation patterns for 833A and 833B were identical (data not shown). The mass of both the unknowns produced from [4-^{13}C]-L-680,833 was 333 Da. The ^{1}H-decoupled 2-D NMR spectrum of a mixture of the two ^{13}C-labeled enzyme-generated unknowns, 833A and 833B, yielded two resonances. The chemical shifts of the proton and carbon originating from the C-4 position of the lactam ring were 4.55, 80.8 and 4.8, 82.6 (^{1}H, ^{13}C). Four resonances were observed in the coupled spectrum with J_{C-H} coupling constants of 155 Hz. When 833A was boiled in buffer A for 2 h, 6% of the starting material was converted to a new species with the same retention time as 833B (see Figure 7). This material coeluted with authentic 833B (both

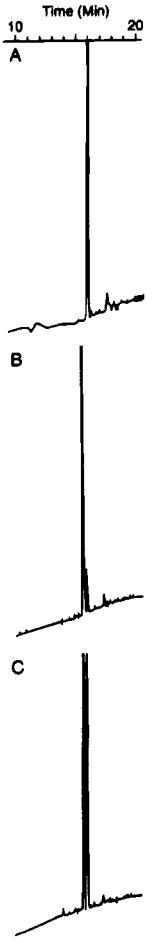


FIGURE 7: Reverse-phase HPLC chromatogram of (A) synthetic 833A and (B) after boiling and (C) after addition of synthetic 833B. Gradient A (see Materials and Methods) was used in these experiments.

synthetic and enzymatically generated). In a similar experiment 7% of a species that coeluted with 833A was produced from 833B by boiling. In neither case was the urea L-683,-595 liberated in these experiments. The two metabolites were not interconverted in the presence of HLE at 37 °C over the same time frame. Reactivation at pH 5.5 of the L-680,833-derived HLE–I complex produced a significantly different product profile (data not shown). Under these conditions very little 833A and 833B (less than 5% of an equivalent each) were produced, and the substituted urea (>90% of an equivalent) was observed.

DISCUSSION

The β-lactams of the L-680,833 class produce relatively stable complexes with HLE. For example, the complex formed between HLE and L-680,833 reactivates with a half-life of 11 h at 37 °C at pH 7.5. The long half-lives of these complexes, even in the presence of proteinaceous components of connective tissue such as proteoglycan, suggest that, in the context of drug development, agents such as L-680,-833 can be considered functionally irreversible inhibitors.

The stability of the final complex formed between the β-lactams and HLE is dependent on the structure of the original compound. The identity of the leaving group at C-4 of the β-lactam ring has no effect on the final stability of the complex (compare L-680,831, L-680,833, and L-685,-502). In fact, L-684,481, which lacks a C-4 leaving group, produces a complex at least as stable as that derived from

L-680,833 and even more stable at lower pH (*vide infra*). The stereochemistry of the leaving group did have a slight effect on the stability of the final HLE−I complex. For example, the complex derived from L-680,833 (4*S*) was 37% less stable than that derived from L-682,946 (4*R*). While one could argue that these values are not significantly greater than the experimental error, a similar trend is seen when the stabilities of the complexes derived from L-683,557 (4*S*) and L-683,558 (4*R*) are compared. In this case the difference is almost 2-fold. Furthermore, the same trend is observed when the stabilities of the L-682,271- and L-682,272-generated HLE−I complexes are compared. Given that the C-4 leaving group is not present in the E−I complexes derived from the four diastereomers of L-680,833 since the masses of the final complexes are identical (*vide infra*; Underwood et al., 1995), the difference in stability is somewhat surprising. Therefore, the stereochemistry at C-4 must affect a step with a stereochemical preference along the reaction pathway prior to departure of the leaving group.

The stability of the β-lactam-derived HLE−I complexes was governed primarily by the identity of the substituted urea. Propyl versus ethyl substitution at the benzylic carbon (α to the urea nitrogen) produced a 2-fold increase (L-679,723 versus L-680,833) in stability. The stereochemistry of the benzylic carbon also affected the stability of the final complex. For example, L-680,833 [(*R*)-benzylic carbon] produces a complex approximately 7-fold more stable than the complex produced from L-683,557 [(*S*)-benzylic carbon]. A similar trend is observed when L-682,946 and L-683,558 are compared (5-fold difference in stability). Substitutions on the phenyl ring of the urea also had dramatic effects on the stability of the complexes. Substitution of fluorine for the *p*-methyl of L-680,833 (compound L-687,676) resulted in a 2-fold decrease in stability. The effects were additive as a combination of ethyl versus propyl on the benzylic carbon and *p*-fluoro versus *p*-methyl on the phenyl ring decreased the stability by 4-fold (compare the complex derived from L-685,255 to that derived from L-680,833). Addition of a fused ring system decreased the stability in many cases (for example, the tetralin, L-683,841, and benzofuran, L-683,845), but in the case of the benzodioxolane, L-680,861, slightly enhanced stability was observed.[10]

In many cases the substitution on the phenyl ring of the urea resulted in biphasic reactivation kinetics. In particular, meta substitution of the ring in general yields biphasic reaction kinetics (see the reactivation rates for the HLE−I complexes derived from L-680,750, L-682,322, and 683,-537 in Table 7). For example, the reactivation of the L-683,-537-derived complex exhibited a rapid return of approximately 40% of the control activity followed by a 20-fold slower return of the remaining activity. This general rule does not completely hold for fused ring systems as they can produce complexes that yield either biphasic or first-order reactivation kinetics. For example, both the benzofuran (L-683,845) and the benzodihydropyran (L-686,402) yield first-order reactivation kinetics while the tetralin (L-683,841), naphthalene (L-670,258), and benzodihydrothiopyran (L-686,403) yield biphasic reactivation kinetics. The simplest explanation for these results is that some of the inhibitors produce two different complexes that reactivate with different rates while others produce a single complex. Assuming this is the case, the stability of the slower reactivating complex is often greater than the complex produced from L-680,833. In addition, some para substitutions on the phenyl ring, such as trifluoromethyl (L-687,666) and ethoxy (L-691,886), also yield biphasic reactivation kinetics. Data presented in the accompanying paper demonstrate that the interaction of L-691,886 with HLE does indeed produce two complexes: one that corresponds to a 2,2-diethyloxopropanoyl−HLE complex and the other corresponding to a carbinolamine−HLE complex (Underwood et al., 1995). The latter is analogous to the single complex produced from the interaction of L-680,833 and HLE.

The stability of the final HLE−I complex derived from L-680,833 is independent of pH and ionic strength and is relatively insensitive to nucleophiles such as NH_2OH. The latter contrasts with the sensitivity to the presence of NH_2-OH, when the nucleophile is included in the inactivation buffer. Chabin et al. (1993) reported that NH_2OH dramatically decreased the susceptibility of HLE to inactivation by L-680,833, indicating the presence of an acyl-enzyme along the reaction pathway that was sensitive to nucleophiles. The final complex produced from L-684,481 was also relatively insensitive to the NH_2OH. The data reported in this work demonstrate that the acyl-enzymes in the final complexes derived from L-680,833 and L-684,481 are protected from "rescue" by nucleophiles. The failure to observe an ionizable group with a pK_a of 7 during reactivation of the L-680,833-derived complex would appear to suggest that the active site histidine is not involved in the reactivation process. Initially, this was interpreted as kinetic evidence for "blocking" of this group possibly by alkylation to produce a "double hit"; however, the pH independence of the stability is somewhat misleading since different products are observed at low and neutral pH (*vide infra*).

In contrast to the case with L-680,833, the stability of the L-684,481- and L-670,258-derived HLE−I complexes increases at lower pH. The data obtained with L-684,481 indicated a single ionizable group whose protonation decreases the rate of reactivation. This observation parallels the pH dependence of the second-order rate constants for the inactivation of HLE by L-680,831 and for substrate hydrolysis (Chabin et al., 1993). This ionization has been attributed to the active site histidine residue. Therefore, reactivation of the L-684,481-derived complex requires deprotonation by the catalytic base. There is, however, one difference in that in the ES complex the pK_a of this group as measured by the dependence of V_m on pH is perturbed to a lower pH (5.7) while the pK_a of this group in the L-684,-481-derived E−I complex is essentially the same as that of free enzyme as measured by the pH dependence of *V*/*K* for substrate hydrolysis. Both phases of the reactivation kinetics observed with L-670,258 were decreased by 6-fold upon going from pH 7.5 to pH 5.5. These data suggest that if there are two complexes derived from this inhibitor as the biphasic reactivation kinetics suggest, they both require base catalysis by the active site histidine for reactivation.

The temperature dependence of the reactivation of the L-680,833- and L-684,481-derived complexes yielded linear Arrhenius plots. This suggests that if there are two routes of reactivation, as suggested from the product analysis discussed below for the L-680,833-generated complex, then

[10] While one can argue that the 34% difference reported in this work between the stability of L-680,861 and L-680,833 is not significant, the same trend has been observed in another series of compounds containing a benzodioxolane-substituted urea that possesses different C-4 substituents.

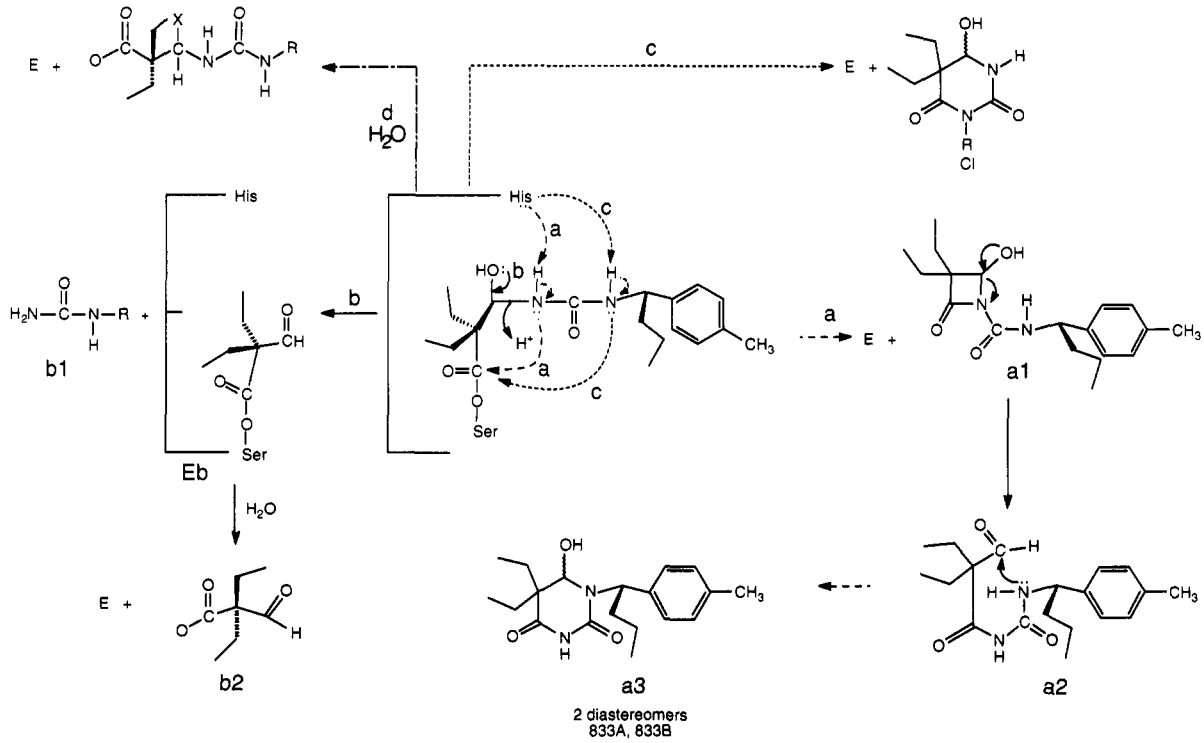


FIGURE 8: Mechanisms for reactivation of β-lactam-derived HLE−I complexes. X is either hydrogen or hydroxyl. In the Discussion R is either methylenenaphthyl (**C1**) for L-670,258 or (4-methylphenyl)butyl for compounds such as L-680,833 (**b1** and route d). In the product of route d, X = OH for carbinolamine products or H for the product generated from L-684,481.

they have indistinguishable activation energies at neutral pH. The free energy of activation for the reactivation of the L-680,833-derived complex is dominated by a large positive enthalpy of activation. As pointed out by Knight et al. (1992b), this should not be interpreted as evidence for a double hit mechanism as similar activation enthalpies are observed during the hydrolysis of acylchymotrypsins (Kunigi et al., 1979; Wang et al., 1981, Adams & Stewart, 1977). Reactivation of the L-680,833-derived HLE−I complex yielded a positive $\Delta S^{\ddagger}$ which contrasts with the negative values obtained for $\Delta S^{\ddagger}$ during the reactivation of the cephalosporin-derived HLE−I complexes reported in earlier work (Knight et al., 1992b) and the hydrolysis of acylchymotrypsins. The mechanism for reactivation of the cephalosporin derivatives involves hydrolytic steps while at least one of the proposed routes of reactivation of the L-680,833 complex can be considered truly an intramolecular event (see Figure 8, route b). According to Jencks (1969), unimolecular dissociative mechanisms should yield positive entropies of activation. The free energy of activation for the reactivation of the L-684,481-derived complex is similar to that observed with L-680,833, although relative to the L-680,833-derived complex, a slight decrease in $\Delta H^{\ddagger}$ is offset by a negative $\Delta S^{\ddagger}$, which is common for the hydrolysis of acyl-enzymes. The obvious differences between the activation parameters for the complexes derived from L-680,833 and L-684,481 are the sign of $\Delta S^{\ddagger}$ and a 3 kcal/mol difference in $\Delta H^{\ddagger}$ and suggest at the least significant differences between the mechanisms of reactivation.

A complete understanding of the mechanism of inhibition by β-lactams required analysis of the products derived from the interaction of HLE with the monobactams. The products released from the HLE−I complexes generated from monobactams containing a C-4 leaving group during reactivation were examined in this work and can be compared to those observed during the inactivation process (Chabin et al., 1993). Reactivation of the L-680,833- and L-680,831-derived HLE−I complexes produced roughly equal amounts of a substituted urea and two new species, 833A and 833B, which contain the elements of the urea. Small amounts of three species that coelute during HPLC with all three of these products were observed during the inactivation process as well. Mass balance requires that production of the urea should also yield 2,2-diethyl-3-oxopropanoic acid, which was indeed observed when [4-^{13}C]-L-680,833 was used to inactivate HLE (Underwood et al., 1995). We did not observe the production of any *p*-hydroxyphenylacetic acid during the reactivation process whereas 1.3 equiv of this group was liberated during inactivation. The 833A and 833B generated from L-680,833 and L-680,831 appear to be diastereomers due to the stereochemistry of the methine carbon. The cyclic products interconvert when heated but are not interconverted by HLE. These data suggest that 833A and 833B derive from a common intermediate in solution and are not directly released by the enzyme. Interaction of L-670,258 with HLE produces similar products except in this case the NMR results suggest that the two new products formed are cyclic regioisomers. Once again, small amounts of species with identical HPLC retention times were observed during the inactivation process. Interestingly, at pH 5.5 only the substituted urea was observed as a result of reactivation of the L-680,833-derived HLE−I complex. The analysis of the reactivation products demonstrates two different pH-dependent pathways, both an acid- and base-catalyzed route, for reactivation of the L-680,833-derived complex. These results explain the lack of pH dependence observed during reactivation of the L-680,833-derived complex.

The observation that compounds, such as L-684,481, which do not contain a potential leaving group at C-4 of the β-lactam ring, while 30-fold less potent than L-680,833, produce final E−I complexes of similar stability is not consistent with the mechanism originally proposed by Chabin

et al. (1993). In that work, the mechanism proposed involved acylation of the enzyme, followed by loss of the C-4 leaving group which produces an electrophile for addition of the active site histidine. This mechanism is analogous to the double hit mechanism originally demonstrated for the inactivation of HLE by cephalosporin derivatives (Doherty et al., 1986; Navia et al., 1987). Opening of the β-lactam ring of L-684,481 could not generate an electrophilic carbon for histidine alkylation. In addition, the final complex formed from L-684,481 displayed similar sensitivity to hydroxylamine as the complex-derived L-680,833. This indicates that the acyl-enzyme formed must be protected from access by small nucleophiles. These observations suggest that a double hit is not required to produce a stable acyl-enzyme. Furthermore, Knight et al. (1993) provided mass spectral evidence that a double hit mechanism was not required to produce stable HLE−I complexes from a simple monocyclic β-lactam. To explain these results, the mechanism must be modified to accommodate the monobactam inhibitors such that alkylation of the histidine residue is not required. In the final step of this mechanism either a conformational change or possibly acyl migration to the histidine must result in protection of the acyl-enzyme from hydrolysis. Inhibitors that have leaving groups could ultimately alkylate the histidine, but this is not required to form a stable HLE−inhibitor complex. This suggests that a great deal of the inhibitory potency of the monobactams results from the ability of the substituted urea and the diethyl substitution on the β-lactam ring to drive the appropriate conformational change to stabilize the enzyme−inhibitor complex.

The spectroscopic results in the accompanying work suggest a possible mechanism that could unify the results obtained with compounds such as L-684,481 and L-680,-833. The mass of the L-680,833-derived HLE−I complex (and others) is consistent with addition of water to the imine to produce a carbinolamine (Underwood et al., 1995). Furthermore, the products derived from the L-680,833-derived HLE−I complex observed in this work are consistent with an HLE−I complex containing a hydroxyl group at the original C-4 position of the β-lactam ring. Therefore, inactivation of HLE by these compounds could be considered an abortive deacylation with addition of water to the original C-4 carbon rather than the carbonyl. A hydrogen bond with the His-57 could aid in stabilizing the complex. In the case of L-684,481 inactivation, a water molecule could be trapped as a result of a conformational change in a similar position and hydrogen bonded to His-57 in the HLE−I complex.

In Figure 8 possible mechanisms for reactivation of the monocyclic β-lactam-derived HLE−I complexes are summarized. The carbinolamine HLE−I complex identified in the accompanying work (Underwood et al., 1995) can reactivate in an intramolecular fashion by two routes (a and b) at neutral pH. In route a, reclosure of the β-lactam ring, an intramolecular deacylation and aminolysis event, catalyzed by proton transfer to the active site histidine results in release of the hydroxy-β-lactam (**a1**). Kamata et al. (1979) have demonstrated that 4-hydroxyazetidin-2-ones are unstable ($t_{1/2}$ at −15 °C is 30−60 min); therefore, the β-lactam ring of **a1** would be expected to open to produce an acyclic product. In the case of the substituted ureas, the initial acyclic aldehyde product **a2** can reclose to form a stable six-membered cyclic species forming an intramolecular hemiaminal. Bimolecular hydration of this aldehyde is not favorable due to the fact that the carbonyl is a neopentyl carbon,[11] but intramolecular ring closure is entropically favored. The generation of the two diastereomers suggests that this process occurs in solution and is not enzyme catalyzed, as the latter would be expected to show stereospecificity. Furthermore, the fact that 833A and 833B interconvert in the absence of HLE supports the acyclic product **a2** as a solution intermediate. Reclosure of the lactam ring is obviously energetically unfavorable, but the reaction could be driven by the solution equilibria. Alternatively, **a2** could be generated directly in the enzyme active site via a modification of route b. Liberation of the free urea in the active site could be followed by attack of the urea nitrogen on the acyl-enzyme. The latter could be facilitated by proton transfer back to the active site His. If the rate of urea release from the active site was similar to the rate of attack on the acyl-enzyme, **a2** would be generated in the active site. At neutral pH, 33% of the time, reactivation follows the acid-catalyzed route b which liberates the substituted urea and the acyl-enzyme **Eb**. A similar species was observed by mass spectrometry during the interaction of HLE with β-lactams that produce less stable E−I complexes such as L-691,886 (Underwood et al., 1995). Hydrolysis of **Eb** with a half-life of approximately 0.2−0.5 h produces 2,2-diethyl-3-oxopropanoic acid.[12] This would preclude the observation of **Eb** during the course of reactivation of the L-680,833-inactivated enzyme since the slow step would be expulsion of the urea to generate **Eb**. At low pH, route a is decelerated while route b is enhanced; thus the rate of reactivation of the L-680,833-derived HLE−I complex appears to be independent of pH. Complexes derived from compounds such as L-670,258 reactivate by route b to produce the urea and route a or the modified route b. In this case the latter two possibilities generate two enantiomers of 258A rather than diastereomers since there is only a single stereochemical site in this structure. The other product observed is likely due to intramolecular deacylation (route c) by attack of the other urea nitrogen to produce the six-membered ring regioisomer **C1** (258B) directly. Stereospecific addition of water to the original C-4 position of the lactam ring would yield a single enantiomer for 258B, although this has not been confirmed. This alternate route for reactivation can be explained on the basis of the model (*vide infra*). Compounds that do not contain a leaving group at C-4 could reactivate by route c or by slow hydrolysis of the acyl-enzyme (route d, X = H). The latter is most consistent with the negative activation energy. Route d is also more likely since L-684,481 will produce a complex that has the *p*-tolyl group in the same orientation in the active site as the complex derived from L-680,833, and the other regioisomer is not observed during reactivation of this complex. Finally, it is also possible that the complexes derived from compounds that originally contain a C-4 leaving group reactivate by route d. Simple hydrolysis of the acyl-enzyme would release the carbinolamine (route d, X = OH) in solution, and this species would be expected to readily eliminate the substituted urea (**b1**) and produce an aldehyde (**b2**).

Interpretation of the Data from the Model of β-Lactam Binding to HLE. An active site structural model of the

[11] In fact, 2,2-diethyl-3-oxopropanoic acid exists as the free aldehyde (Underwood et al., 1995).

[12] The rapid phase of reactivation of the L-691,886-generated HLE−I complex(es) predicts a half-life for **Eb** of 0.2 h.

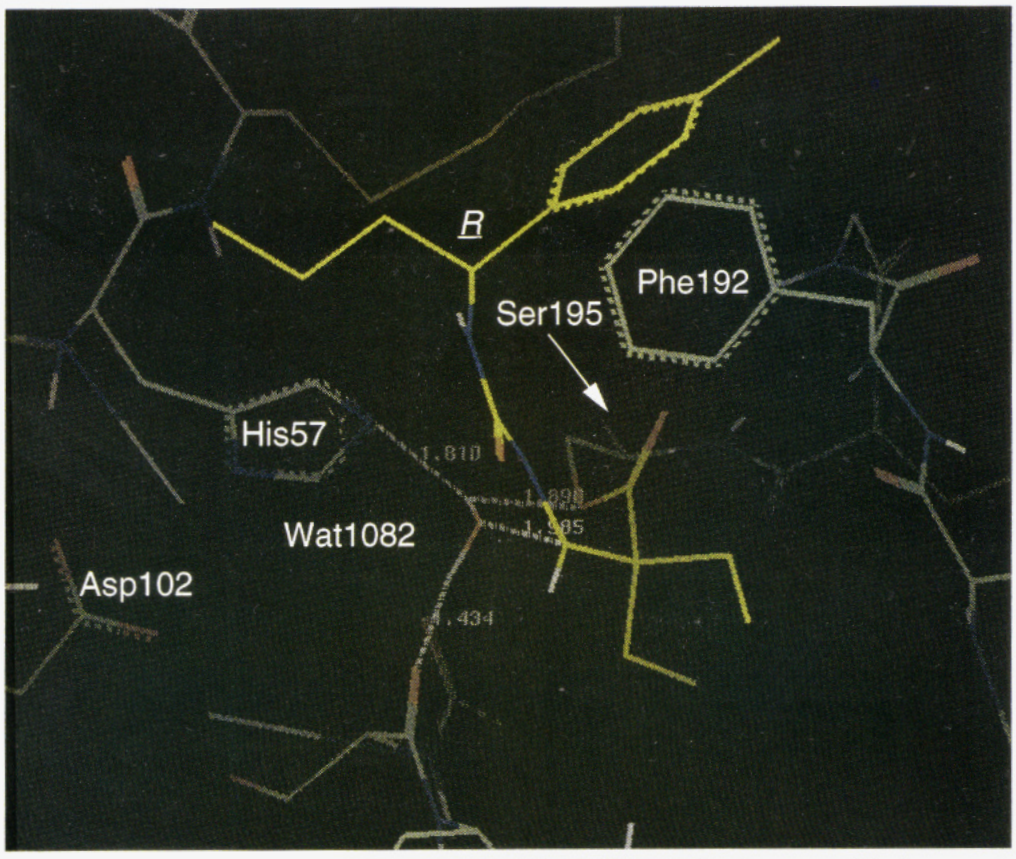


FIGURE 9: Model of the acylimine derived from L-682,946 and HLE placing F192 in the alternate position juxtaposed to the substituted urea portion of these inhibitors. The sequence numbering system is derived from chymotrypsin.

Michaelis complex formed between HLE and monocyclic β-lactams has been reported (Knight et al., 1992; Shah et al., 1992). This model is based upon the structure of the HLE−I complex derived from a chloromethyl ketone (Navia et al., 1990). The C-2 dialkyl, C-4, and N-1 urea substituents bind into the S_1, S_2, and hydrophobic prime subsites,[13] respectively. The model predicted that F192[14] was positioned at the mouth of the S_1 pocket forming a hydrophobic "lip" which closes over the group occupying this subsite when substrates and inhibitors bind. Examination of the X-ray structures of serine proteases indicates that the side chain of this residue packs closely around the P_1 group in stable intermediates, but in the absence of ligands this group would shield the S_1 pocket. This implies that the dynamics of ligand binding and the induced fit of a ligand in the active site most likely involve movement of the F192 side chain. There is an alternate position for the F192 side chain observed in the X-ray structure of HLE with chloromethyl ketones. This position would pack the side chain against the substituted urea portion of the β-lactams used in this study. The positioning of this side chain in the various intermediates along the reaction pathway will be determined by the character of the ligand. In Figure 9 the model of the L-680,-833 (or L-682,946) derived acylimine is shown with F192 in the alternate position. In this position the side chain would be expected to protect the acylated serine from attack by either water or intramolecular reclosure of the lactam. When the C-4 stereochemistry of the original β-lactam is *R*, as in L-682,946, steric avoidance of this group could force the F192 side chain to adopt the alternate configuration. Furthermore, this would be consistent with a stepwise displacement of the leaving group by attack of the bound water molecule possibly catalyzed by H57.[15] Compounds with 4*S* stereochemistry would produce complexes with F192 in the position originally reported by Navia et al. (1990). In these cases the C-4 leaving group extends toward the S_2 site and would not block the F192 from the S_1 pocket. Furthermore, the stereoelectronics are such that a C-4 leaving group with an *S*-stereochemistry group could depart in a concerted fashion to produce the imine followed by addition of the bound water to the imine. Examination of the model also suggests that the (*R*)-propyl group on the methine carbon of L-680,833 would yield an optimal fit into a hydrophobic pocket in both orientations of F192. This could explain the increased stability of compounds that possess *R*-stereochemistry at this position. *S*-Stereochemistry at the benzylic carbon would also preclude location of F192 in the alternate position further destabilizing the complex. For example, the complex derived from L-680,833 is 7-fold more stable than that derived from L-683,557. In the case of L-670,258, the lack of a methine substitution and an imperfect fit in the hydrophobic prime sites increase the accessibility of the β-urea nitrogen and lead to the alternate route (c) of reactivation.

The location of the benzyl in the hydrophobic prime sites suggests that there might be a correlation between stability and hydrophobicity (Log *P*; see Table 2). The apparent Log *P* was estimated for the subset of compounds in Table 2

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

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

[15] As discussed in the following paper, direct displacement at the C-4 carbon is not sterically favorable, but a water molecule may be strategically placed to effect this process (Underwood et al., 1995). Alternatively, stepwise elimination of the leaving group followed by addition of water is also consistent with the results.

that yield monophasic reactivation kinetics and have 4*S*-stereochemistry and (*R*)-benzylic stereochemistry. In general, groups with higher Log *P* produce a greater $t_{1/2}$. The correlation is not perfect as L-680,861, the benzodioxolane, does not conform to this rule. The steric shape of the ligand in the urea region is also likely a key factor in both the placement of F192 and the stability of the complex. This is evident with L-683,557 and L-683,558 which, while possessing a high Log *P*, produce relatively unstable complexes due to the *S*-stereochemistry of the benzylic carbon.

The model also predicts that substitutions at the meta and para positions of the phenyl ring either can fit in the hydrophobic pocket or can extend into the solvent shell by rotation around a single bond (Knight et al., 1992; Shah et al., 1993). In particular, the two different orientations of a meta substitution could lead to the ultimate formation of two different complexes that may result in biphasic reactivation kinetics. Once again, the analogy is not perfect as fused ring systems yield both mono- and biphasic reactivation kinetics and there is not an apparent correlation with ring size.

CONCLUSIONS

The stability of monocyclic β-lactam-derived HLE−I complexes is governed primarily by the substituted urea portion of the molecule. This substitution also governs the mechanism of reactivation and dictates the products generated. Both acid- and base-catalyzed routes of reactivation can occur. The model for the acyl-enzymes generated from these compounds predicts that the differences in the stability of these complexes can be explained both by interactions with two hydrophobic pockets and by different orientations of a Phe residue. The latter is influenced in part by the stereochemistry of the C-4 substitution.

REFERENCES

Adams, P. A., & Swart, E. R. (1977) *Biochem. J. 161*, 83−92.

Bax, A., Griffey, R. H., & Hawkins, B. L. (1983) *J. Am. Chem. Soc. 105*, 7188.

Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, p 64, McGraw-Hill, New York.

Chabin, R., Green, B. G., Gale, P., Maycock, A. L., Weston, H., Dorn, C. P., Finke, P. E., Hagmann, W. K., Hale, J. J., MacCoss, M., Shah, S. K., Doherty, J. B., & Knight, W. B. (1993) *Biochemistry 32*, 8970−8980.

Doherty, J. B., Ashe, B. M., Argenbright, L. W., Barker, P. L., 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. 90*, 8727−8731.

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.

Hascall, V. C., & Kimura, J. H. (1982) *Methods Enzymol. 82*, 769−800.

Janoff, A., Feinstein, G., Malemud, J., & Elias, M. (1976) *J. Clin. Invest. 57*, 615−624.

Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, pp 608−609, McGraw-Hill, New York.

Kamata, S., Yamamoto, S., Haga, N., & Nagata, W. (1979) *J. Chem. Soc., Chem. Commun.*, 1106−1107.

Kaplan, P. O., Kuhn, C., & Pierce, J. A. (1973) *J. Lab. Clin. Med. 82*, 349.

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. (1992a) *Biochemistry 31*, 8160−8170.

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. (1992b) *Biochemistry 31*, 4980−4986.

Knight, W. B., Chabin, R., & Green, B. G. (1992c) *Arch. Biochem. Biophys. 296*, 704−708.

Knight, W. B., Swiderek, K., 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. R. (1993) *Biochemistry 32*, 2031−2035.

Kunigi, S., Hirohara, H., & Ise, N. (1979) *J. Am. Chem. Soc. 101*, 3640−3646.

Leatherbarrow, R. J. (1992) *GraFit Version 3.0*, Erithacus Software Ltd., Staines, U.K.

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.

Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891−2899.

Powers, J. C. (1983) *Am. Rev. Respir. Dis. 127*, 554−558.

Schecter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157−162.

Shah, S. K., Dorn, C. P., Finke, P. E., Hale, J. J., Hagmann, 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.

Underwood, D. J., Green, B. G., Chabin, R., Mills, S., Doherty, J. B., Finke, P. E., MacCoss, M., Shah, S. K., Burgey, C. S., Dickinson, T. A., Griffin, P. R., Lee, T. E., Swiderek, K. M., Covey, T., Westler, W. M., & Knight, W. B. (1995) *Biochemistry 34*, 14344−14355.

Wang, C.-L. A., Calvo, K. C., & Klapper, M. H. (1981) *Biochemistry 20*, 1401−1408.

BI950437X