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Mechanism of Inhibition of Human Leukocyte Elastase by Two Cephalosporin Derivatives

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ABSTRACT: The cephalosporin derivatives L 658758 [1-[[3-(acetoxymethyl)-7α-methoxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-2-yl]carbonyl]proline *S,S*-dioxide] and L 659286 [1-[[7α-methoxy-8-oxo-3-[[(1,2,5,6-tetrahydro-2-methyl-5,6-dioxo-1,2,4-triazin-3-yl)thio]methyl]-5-thia-1-aza-(6*R*)-bicyclo[4.2.0]-oct-2-en-2-yl]carbonyl]pyrrolidine *S,S*-dioxide] are mechanism based inhibitors of human leukocyte elastase (HLE). The mechanism involves initial formation of a Michaelis complex followed by acylation of the active site serine. The group on the 3′-methylene is liberated during the course of these reactions, followed by partitioning of an intermediate between hydrolysis to regenerate active enzyme and further modification to produce a stable HLE–inhibitor complex. The partition ratio of 2.0 obtained for the reaction with L 658758 approaches that of an optimal inhibitor. These compounds are functionally irreversible inhibitors as the recovery of activity after inactivation is slow. The half-lives at 37 °C of the L 658758 and L 659286 derived HLE–I complexes were 9 and 6.5 h, respectively. The complexes produced by both inhibitors are similar chemically since the thermodynamic parameters for activation to regenerate active enzyme are essentially identical. The free energy of activation for this process is dominated primarily by the enthalpy term. The stability of the final complexes likely arises from Michael addition on the active site histidine to the 3′-methylene.

Elastases are potent serine proteases with the ability to degrade a number of proteinaceous components of connective tissues. Since the degradation of connective tissues is evident in the pathogenesis of a number of chronic inflammatory diseases, human leukocyte elastase (HLE, EC 3.4.21.37) has been implicated in disorders such as emphysema (Kaplan et al., 1973), atherosclerosis (Travis et al., 1980), and rheumatoid arthritis (Janoff et al. 1976).[1] The evidence[2] for the involvement of HLE in disease has led to the development of a number of synthetic inhibitors [for a review, see Stein et al. (1985)]. The observation that the benzylic esters of clavulanic

Scheme I

$$E + I \rightleftharpoons E \cdot I \longrightarrow \underbrace{(E - I)}_{\text{Acyl-Enzyme}} \longrightarrow \text{Further Modification}$$

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[1] This list is far from complete as a role for HLE has been suggested in a number of other inflammatory disease states [for example, see Davies et al. (1991)].
[2] For a concise discussion of the evidence, see Davies et al. (1991).

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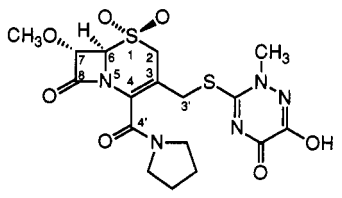
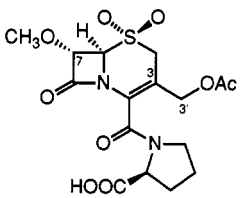
acid and penicillin sulfone inhibited HLE led to the development of a series of cephalosporin derivatives as inhibitors of HLE (Doherty et al., 1986). These workers proposed the kinetic mechanism presented in Scheme I to explain the kinetics of the interaction of HLE with these inhibitors. Green et al. (1991) reported that the apparent first-order rate constant for the inhibition of HLE by two members of this class of inhibitors (L 658758 and L 659286)[3] was saturable. This provided evidence for the formation of a reversible Michaelis complex, the first step in Scheme I. These workers also reported that there was partitioning of an L 659286 derived enzyme–inhibitor complex between turnover of inhibitor and inactivation of the enzyme. This suggests that Scheme I does not fully explain the kinetics of inhibition of HLE by these inhibitors.



L-659,286



L-658,758

β-Lactamases are mechanistically similar to serine proteases in that they contain an active site serine residue and utilize covalent catalysis. Knowles (1981) proposed a chemical mechanism for the inhibition of β-lactamases by penicillin sulfones involving (1) initial acylation of the active site serine by the β-lactam carbonyl, thus opening the ring, and generating an electrophilic site and (2) partitioning of the acyl-enzyme between hydrolysis and attack of an active site nucleophile on the electrophile to produce the final enzyme inhibitor complex. A similar "double hit" chemical mechanism was proposed for the interaction of porcine pancreatic elastase (PPE) and a cephalosporin derivative (L 647957)[3] on the basis of the structure of the PPE–I complex determined by Navia et al. (1987). The inhibition of HLE by this class of compounds may display mechanistic similarities to PPE.

We have examined the kinetic and chemical mechanism of inhibition of HLE by the two cephalosporin derivatives L 658758 and L 659286. The results presented below suggest a plausible chemical mechanism and a minimal kinetic mechanism.

Experimental Procedures

Materials. [^{3}H]DFP[3] (specific activity, 3.5 Ci/mmol) was purchased from Amersham. MeOsucc-AAPV-pNA[4] was purchased from Calbiochem Co. Substrate and inhibitor stock solutions were prepared in DMSO. Peptide *p*-nitroanilide stock concentrations were determined either from the absorbance at 315 nm (ϵ = 14000 M^{-1} cm^{-1}) or by complete hydrolysis to free *p*-nitroanilide (ϵ = 9350 at 410 nm in buffer A (45 mM TES at pH 7.5, 450 mM NaCl in 10% DMSO)). Buffers were purchased from Sigma Chemical Co. and titrated to the appropriate pH with either NaOH or HCl prior to use. HLE was purchased from Elastin Products. Pyruvate kinase, acetate kinase, and lactate dehydrogenase were purchased from Sigma Chemical Co. and used without further purification. NADH, ATP, and PEP were purchased from Sigma Chemical Co.

L 658758 was synthesized according to the method of Doherty et al. (1990). [^{14}C]methoxy-labeled L 658758 was synthesized similarly with a specific activity of 5 μCi/mg. The preparation of L 659286 followed similar methodology. 7α-Methoxycephalosporanic acid was converted to the pyrrolidine amide by DCC coupling with pyrrolidine (Finke et al., 1990). Subsequent peracid oxidation yielded the sulfone. The C-3′-acetoxy group was converted to a chloride (Shah et al., 1990) which was displaced with 1,2,5,6-tetrahydro-5,6-dioxo-3-thio-2-methyl-1,2,4-triazine (Rhone Poulanc Industries, 1981) in the presence of aqueous sodium bicarbonate. The compound was isolated as a light beige powder (monohydrate, mp 150 °C dec).

Methods. UV–visible spectroscopy was conducted on either Varian DMS-300 or Cary 2200 spectrophotometers. The hydrolysis of peptide *p*-nitroanilides was monitored at 410 nm. Prolonged reactivation studies were monitored on an AVIV-14DS spectrophotometer equipped with a Zymark robot for sample preparation and a Hewlett-Packard 900/320 series computer for data collection, analysis, and storage. The UV–visible spectra of compounds were obtained with a Waters-990 photodiode array detector. Scintillation counting was conducted on a Packard Tri-Carb 2000CA liquid scintillation analyzer.

Elastase activity was determined in buffer A versus 1 mM MeOsucc-AAPV-pNA. The data were fit to eq 1 to determine the initial velocity. The active site concentration of HLE stock solutions was determined on the basis of activity according to Green et al. (1991).

Experiments with radiolabeled L 658758 were carried out as follows: 10 mg of HLE was inactivated to less than 0.5% of the control in the presence of a 5-fold molar excess of [^{14}C]methoxy-labeled inhibitor. The reaction mixture was dialyzed against 1 mM acetic acid at 4 °C for 48 h, and the specific activity and the enzyme activity were determined.

The binding of [^{3}H]DFP to HLE in the presence and absence of L 659286 was determined as follows: 2.8 nmol of HLE was incubated in buffer B (1 mL of 150 mM NaCl, 50 mM TES at pH 7.5) at 25 °C with either 8 nmol of [^{3}H]DFP (added in 5 μL of propylene glycol) or 53 nmol of L 659286 (added in 5 μL of DMSO). The control contained 2.8 nmol of HLE in 1 mL of buffer B. Aliquots of the three solutions were removed after 30 min and assayed for activity. Then, 53 nmol of L 659286 was added to the solution pretreated with [^{3}H]DFP and 8 nmol of [^{3}H]DFP was added to the solution pretreated with L 659286. The two inhibitor treated reactions and the control were exhaustively dialyzed at 4 °C versus three changes of 100 mL of buffer B. Aliquots were assayed for activity, and 20 μL of each dialysate was counted. The results are expressed in disintegrations per minute of the basis of addition of an internal standard of [^{3}H]toluene to each sample.

The Michaelis constant of control HLE (20 μL of a 1:10 dilution) and L 659286 treated enzyme (50 μL) was deter-

[3] Abbreviations; ATP, adenosine triphosphate; DFP, diisopropyl fluorophosphate; HLE, human PMN elastase; DMSO, dimethyl sulfoxide; L 647957, 3-(acetoxymethyl)-7α-chloro-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate *S,S*-dioxide *t*-butyl ester; L 658758, 1-[[3-(acetoxymethyl)-7α-methoxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-2-yl]carbonyl]proline *S,S*-dioxide); L 659286, 1-[[7α-methoxy-8-oxo-3-[[(1,2,5,6-tetrahydro-2-methyl-5,6-dioxo-1,2,4-triazin-3-yl)thio]-methyl]-5-thia-1-aza-(6*R*)-bicyclo[4.2.0]oct-2-en-2-yl]carbonyl]-pyrrolidine *S,S*-dioxide); NADH, reduced nicotinamide adenine dinucleotide; PEP, phosphoenolpyruvate; PMN, polymorphonuclear neutrophils or leucocytes; PPE, porcine pancreatic elastase; TES, *N*-[tris-(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; thiotriazine, 1,2,5,6-tetrahydro-5,6-dioxo-3-thio-2-methyl-1,2,4-triazine.

[4] Peptide based substrates and inhibitors are abbreviated using the standard 1-letter representation of the amino acids. Additional functionalities present were abbreviated as follows: MeOsucc-, methoxysuccinyl; pNA, *p*-nitroanilide.

Table I: HLE Incorporation of [^{3}H]DFP

treatment (first/second)	% inhibition	dpm in 20 μL of dialysate
[^{3}H]DFP/-	99.6	235500
[^{3}H]DFP/L 659286	99.4	237900
L 659286/[^{3}H]DFP	99.5	7500

mined by varying MeOsucc-AAPV-pNA in 1 mL of buffer C (10% DMSO, 135 mM NaCl, and 45 mM TES at pH 7.5). The initial velocities as a function of substrate concentration were fit to eq 2 to determine the Michaelis constants. An additional 2.4 nmol of DFP was added to the L 659286 treated enzyme, and an aliquot was assayed and the solution was exhaustively dialyzed.

The minimum amount of L 658758 required to yield complete inactivation was determined by two methods under several conditions. Method A was an adaptation of the method used by Green et al. (1991) to determine a similar constant for L 659286. In method A, either 0.4 μM or 40 μM HLE was preincubated with L 658758 at concentrations ranging from either 0 to 1.6 μM or 16 to 128 μM at 25 °C in 50 mM TES at pH 7.5 and 150 mM NaCl. The residual enzyme activity was determined by adding an aliquot of each solution to 1 mL of 1 mM MeOsucc-AAPV-pNA in buffer C. The percentage of inhibition was calculated by comparing the observed residual rates to those of control samples incubated in the absence of inhibitor. In preliminary experiments, we determined that after a 2-h preincubation period there was no further loss of activity at all levels of inhibitor. The maximum percentage of inhibition was plotted as a function of the molar ratio of inhibitor to enzyme. The error is expressed as the standard error from the linear least-squares fit to the curve. In method B, the preincubation mixtures were subjected to centrifugal gel filtration at 700*g* for 5 min at 4 °C on 3-mL Sephadex A-25 columns equilibrated in buffer A prior to the activity assay. This procedure removes any excess inhibitor or metabolites produced from the inhibitor during the inactivation process. The residual enzyme activity was determined as above. Method A was also used to determine the amount of L 659286 required to completely inactivate HLE. In addition, a similar experiment was conducted but with the addition of 0.125 mM hydroxylamine to the enzyme assay solutions.

The return of catalytic activity of L 658758 and L 659286 derived HLE–inhibitor complexes was monitored at 25 and 37 °C utilizing two methods. In a typical experiment utilizing method 1, 2-mL solutions of 100 μg/mL HLE in buffer B were treated with either 20 μL of DMSO or 20 μL of a 1 mg/mL solution of L 658758 in DMSO at 25 °C. After 1 h, the enzyme was >98% inhibited. The solution of inactive enzyme and the control were dialyzed at 4 °C for 21 h against three changes of 100 mL of buffer A; 20-μL aliquots were assayed for activity, and the percentage of activity was calculated as a percent of the control. One milliliter of each solution was incubated at 37 °C while the remainder was incubated at 25 °C. Enzyme activity was monitored over 2 days and 4 days, respectively. The data were fit to eq 3 to determine the first-order rate constant for the return of activity. In a typical experiment utilizing method 2, 0.05 mM HLE in 0.6 mL of buffer A was incubated with 0.25 mM L 659286 for 20 min at 25 °C (activity was 96% inhibited). Excess inhibitor and any metabolites were removed from a 0.5-mL aliquot of the incubation mixture by centrifugal gel filtration (vide supra). The filtrate was diluted to 5 mL and incubated at either 25 or 37 °C, and 5- or 10-μL aliquots were removed over time and assayed for activity. A control was treated similarly and the percentage of inhibition was calculated as above. The activation energy for reactivation was estimated by fitting the first-order rate constants (with units of s^{-1}) to eq 4. The enthalpy and entropy of activation were estimated from eqs 5 and 6, respectively, at 298 °K.

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

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

$$y = ae^{(-kt)} + c \quad (3)$$

$$\log k_r = (-E_a/2.3R)(1/T) + \log A \quad (4)$$

$$\Delta H^\ddagger = E_a - RT \quad (5)$$

$$\Delta S^\ddagger = R \ln (ANh/RT) - R \quad (6)$$

Analysis of HLE Derived Products from L 658758 and L 659286. The liberation of acetate from the exocyclic 3′-methylene of L 658758 was determined by incubating a 10-fold excess of L 658758 with 0.06 mM HLE for 3 h at 25 °C. Enzyme was removed from the reaction mixture by filtration through a 10000-MW-cutoff membrane, and the phosphorylation of acetate by acetate kinase was coupled to the oxidation of NADH (ϵ = 6220 M^{-1} cm^{-1} at 340 nm). The 1-mL coupled assay solution contained 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 1 mM PEP, 40 units of lactate dehydrogenase, 40 units of pyruvate kinase, 0.3 mM NADH, 30 units of acetate kinase, 35 mM TES, pH 7.5, and 350 mM NaCl. Acetate kinase was added to the coupling system prior to the addition of an aliquot of the filtrate to consume contaminating acetate. The concentration of acetate produced in the enzymatic reaction mixture was corrected for the nonenzymatic decomposition of L 658758 (0.132 mM over 3 h).

The products produced during the interaction of HLE with L 659286 were monitored by HPLC. In a typical experiment, 0.096 μM HLE was incubated for 3 h at 25 °C with 1.0 mM L 659286 in buffer A. The reaction and control solutions were filtered through a 10000-MW-cutoff membrane, and an aliquot of each was applied to a 4.6 mm × 150 mm Beckman Ultrasphere (C-18) column. The column was washed with methanol/water (2:98) containing 0.1% acetic acid for 8 min to elute the thiotriazine liberated from the 3′ methylene of L 659286. The column was then eluted with a linear methanol/acetonitrile gradient for 11 min to 100% of 10:90:0 methanol/acetonitrile/water, followed by a 5-min wash with this last gradient solution to elute L 659286 and other metabolites. The concentrations of L 659286 and the thiotriazine were determined from chromatographing standard concentrations of both compounds. The final concentrations of thiotriazine liberated and L 659286 were corrected for the nonenzymatic decomposition of the L 659286 at pH 7.5 (11% of the amount of the L 659286 over 3 h).

Results

When HLE was inactivated to greater than 98% with [^{14}C]-L 658758 and then dialyzed, 2.36 × 10^6 cpm ^{14}C was associated with the protein. This represents 1.2 mol of label/mol of enzyme. In Table I the results of treating HLE in the presence and absence of L 659286 are presented. Prior to treatment with the second inhibitor, the [^{3}H]DFP and L 659286 treated HLE had 99.1% and 96.1% of the control activity, respectively. The Michaelis constant for the residual HLE activity after treatment with L 659286 was determined after dialysis to be 0.19 ± 0.2 mM toward MeOsucc-AAPV-pNA in buffer C. This was virtually identical to that displayed by the control HLE sample (0.20 ± 0.2 mM) under the same conditions. Addition of DFP to the L 659286 treated enzyme

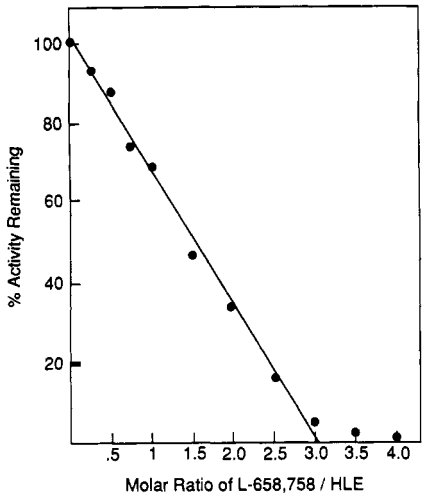


FIGURE 1: Titration of HLE activity with L 658758. Experimental conditions are reported in the text.

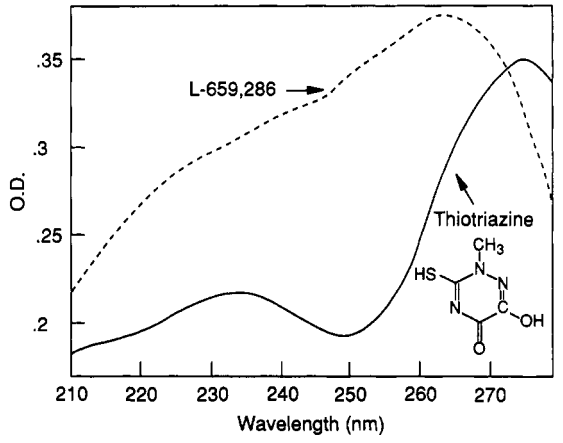


FIGURE 2: UV–visible spectra of L 659286 and the thiotriazine.

resulted in 99.5% inhibition before and after dialysis.

The data presented in Figure 1 indicate that 3.0 ± 0.1 equivalents of L 658758 are required to inactivate 1 equivalent of HLE. This value did not change when the reaction solutions were passed over a DEAE-Sephadex A-25 column prior to assay. The interaction of HLE with L 658758 liberated 0.191 mM acetate. This corresponded to 3.2 equivalents. The interaction required 4.8 ± 0.2 equivalents of L 659286 to inactivate HLE (data not shown). This agrees with the value reported by Green et al. (1991). When the titration solutions were assayed in the presence of hydroxylamine, the interaction required 5.0 equivalents L 659286 to inactivate HLE.

In Figure 2, the UV–visible spectra of L 659286 and the thiotriazine obtained from standard chromatograms indicate absorbance maxima of 264 nm and 274 nm, respectively. In Figure 3, a typical chromatogram monitored at 274 nm of L 659286 before and after reaction with HLE is shown. The thiotriazine elutes at 7 min, and the L 659286 elutes at 20.8 min. Contaminants present initially in the L 659286 appear at 16.7 min and 20 min. The UV–visible spectra of these two species are shown in Figure 4. The latter eluting impurity was consumed during the reaction with HLE. It is interesting to note that this species increases in concentration in the control reaction (not shown). There is initially a 4% contamination of the leaving group in the preparation of L 659286. There were 4.2 ± 0.6 (n = 6) equivalents of thiotriazine liberated and 5.0 ± 1.1 (n = 6) equivalents of L 659286 consumed per mole equivalent of HLE in the reaction. The latter value determined by HPLC was in excellent agreement with the value determined by titration of enzymatic activity (Green et

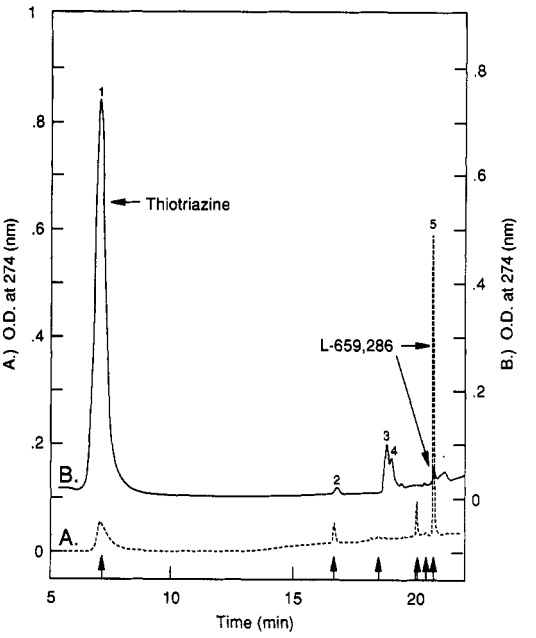


FIGURE 3: HPLC chromatograms of L 659286 before and after reaction with HLE.

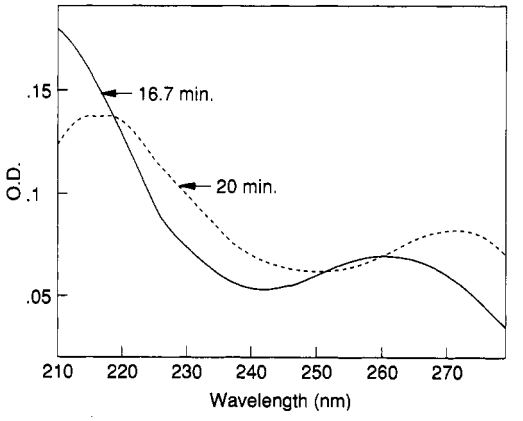


FIGURE 4: UV–visible spectra of the two contaminants present in L 659286.

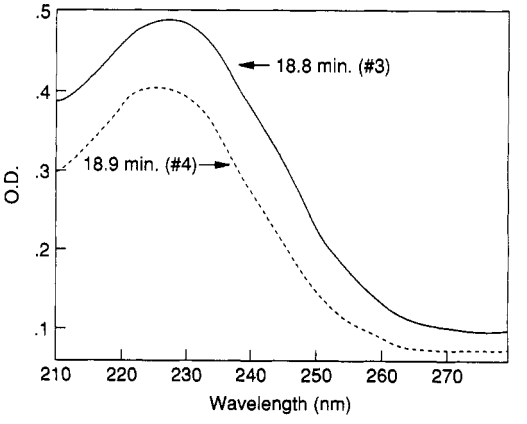
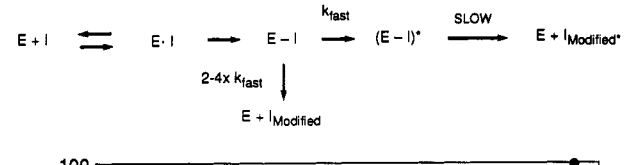


FIGURE 5: UV–visible spectra of two of the enzyme generated products.

al., 1991) and repeated in this work (vide supra). In addition, two products that elute at 18.8 and 19 min from the HPLC column were evident. The UV–visible spectra of these metabolites are presented in Figure 5.

Upon incubation, enzyme inactivated with either L 658578 or L 659286 slowly reactivated. In Figure 6, the return of HLE activity at 37 °C after inactivation with L 659758 is shown. Similar data were obtained with L 659286. In Table

Scheme II



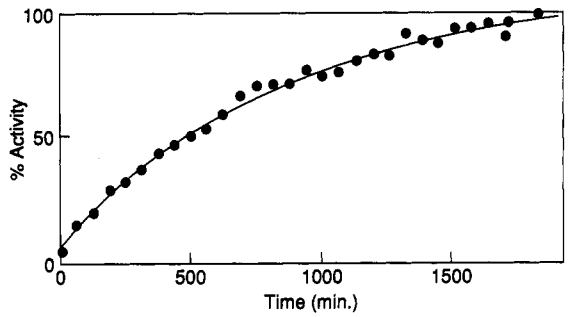


FIGURE 6: Spontaneous reactivation of L 658758 derived HLE–inhibitor complex at 37 °C.

II, the first-order rate constants, half-lives, and thermodynamic activation parameters for these processes are summarized.

Discussion

A minimal kinetic mechanism for the inhibition of HLE by L 658758 and L 659286 consistent with the data presented in this work and by Green et al. (1991) is shown in Scheme II. The observation by Green et al. that the first-order rate constants for the inhibition of HLE by these compounds were saturable establishes the first step, reversible formation of a noncovalent Michaelis complex. The binding of L 659286 prevents the incorporation of labeled DFP, suggesting that the cephalosporins also react with the active site serine to form an acylenzyme.

As suggested by Green et al. (1991), an intermediate along the reaction pathway partitions between inhibitor turnover and inactivation of the enzyme as it requires more than a single equivalent to inactivate the enzyme. The partition ratio represents the ratio of the rate constant for hydrolysis of the E–I complex to the rate constant for the production of the (E–I)* complex. This ratio for the reaction of HLE with L 658758 is 2, which is 2-fold lower than that reported by Green et al. (1991) for the reaction of L 659286 with HLE. The efficiency of the interaction of these compounds approaches that of an optimal inhibitor which would not partition. When these values are compared to the partition ratios obtained during the interaction of β-lactamases with β-lactams, this is evident. For example, it required 115 and 7000 molar equiv of clavulanic acid (Fisher et al., 1978) and penicillinic acid sulfone (Brenner & Knowles, 1981) respectively to inactivate the RTEM β-lactamase.

Table II: Kinetics Parameters[a] for the Reactivation of L 658758 and L 659286 Derived HLE–Inhibitor Complexes

		compound	
	temp (°C)	L 658758	L 659286
k_{react} ($min^{-1} \times 10^4$)	25	2.9 ± 0.3	4.4 ± 0.9
	37	13.0 ± 2	18.0 ± 6
	37	11.6 ± 0.7	
$t_{1/2}$ (h)	25	39.0 ± 4.0	27.0 ± 6.0
	37	9.0 ± 1.1	6.5 ± 2.2
	37	10.0 ± 0.6	
E_a (kcal/mol)		22.3	21.4
$\Delta G^{\ddagger}$ (kcal/mol)	25	24.7	24.4
$\Delta H^{\ddagger}$ (kcal/mol)	25	21.4	21.1
$\Delta S^{\ddagger}$ (eu)	25	–9.9	–12.0

[a] Standard errors are reported.

A 1:1 covalent complex is formed between the cephalosporins and HLE as 1 equivalent of [^{14}C]methoxy-L 658758 is associated with the protein after dialysis. This complex, (E–I)*, is relatively stable but slowly releases active enzyme and modified inhibitor. The complexes produced from L 658758 and L 659286 are chemically similar since the thermodynamic activation parameters associated with regeneration of active enzyme from both complexes are essentially identical. The free energy of activation for this process is dominated primarily by a large enthalpy of activation in both cases.

In Figure 7, a plausible chemical mechanism for the inhibition of HLE by the cephalosporins is presented. This

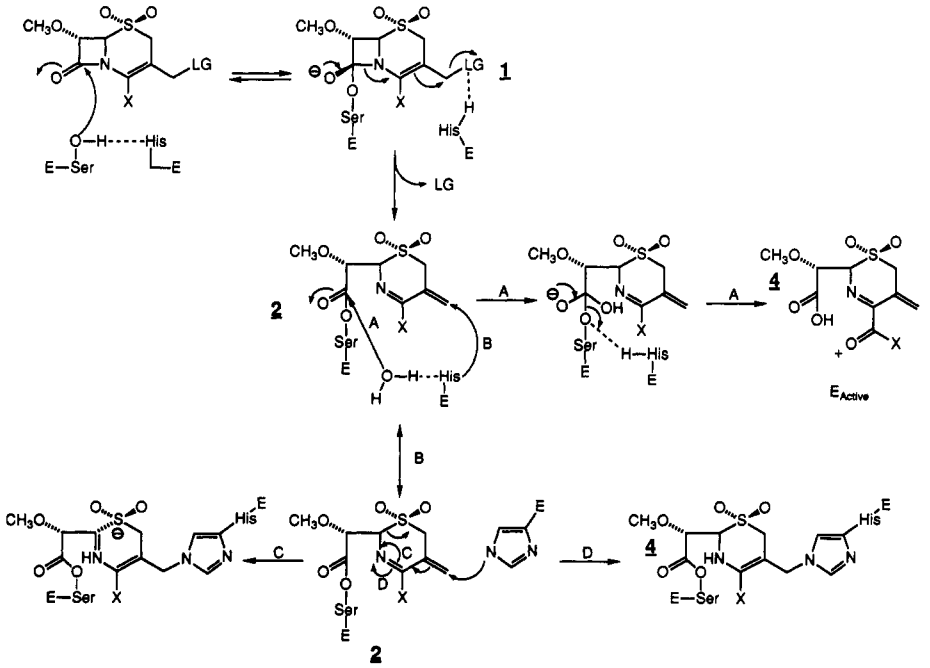


FIGURE 7: Proposed chemical mechanism for the inhibition of HLE by the cephalosporin derivatives. The ratio of A:B represents the partition ratio. Pathway A regenerates active enzyme. Pathway B produces the stable E–I complex, which results from Michael addition of the histidine with (C) or without (D) opening of the dihydrothiazine ring.

mechanism assumes that the reaction takes advantage of the catalytic triad present in serine proteases. Attack of the active site serine facilitated by the catalytic histidine and aspartate (not shown) on the β-lactam ring would produce a tetrahedral intermediate (**1**) that collapses, opening the β-lactam ring. This could be facilitated by concerted expulsion of the group from the 3′-methylene as shown in Figure 7, or this group could depart in a separate step to produce **2**. During the interaction of HLE with the two cephalosporin derivatives, the groups at this position are liberated. The amount of acetate generated from the interaction of HLE with L 658758 equaled the amount of inhibitor consumed. The amount of thiotriazine liberated from the 3′-methylene of L 659286 during reaction with HLE was equivalent to the amount of inhibitor consumed. These observations suggest that partitioning occurs after departure of the group at C-3′.

Attack of water on the acylenzyme **2** (pathway A) results in formation of a tetrahedral intermediate that would collapse to release active enzyme and the carboxylic acid **4**. On the basis of the results from HPLC, there appear to be two additional metabolites released as a result of the partitioning event. These presumably contain the remaining portion of the inhibitor (minus the thiotriazine). They displayed UV–visible spectra similar to each other but distinct from either parent or the thiotriazine leaving group. Either these two metabolites are chemically very similar or quite possibly there is a single entity produced and the apparent doublet was an artifact of the chromatography. In fact, we have observed that carboxylic acids often behave in this fashion during reverse-phase chromatography. The other possibility is that the second metabolite observed is produced from the contaminant initially present. In the control reaction, this contaminant was produced over the course of the incubation, but it was consumed during the course of the enzyme reaction.

In pathway B, Michael addition of the active site histidine to the 3′-methylene produces a stable complex that is insensitive to hydroxylamine as the partition ratio was unaffected by quenching the reaction in this reagent. In fact, the addition of histidine to C-3′ was observed in the crystallographic structure of the PPE–I complex derived from L 647957 (Navia et al., 1987). This was proposed as the explanation for the stability of this complex to nucleophiles such as hydroxylamine. While it is tempting to use the high activation enthalpy for deacylation of these HLE–I complexes in support of a histidine Michael addition, similar activation enthalpies were reported for the hydrolysis of acylchymotrypsins derived from nonspecific esters (Kunugi et al., 1979; Wang et al., 1981; Adams & Swart, 1977).

There are two structures proposed for the final HLE–I complex in Figure 7. In pathway C the attack results in opening of the dihydrothiazine ring while in pathway D the integrity of the second ring is maintained. In the structure of the complex of PPE and L 647957 reported by Navia et al. (1987), the dihydrothiazine ring appeared intact. On the basis of this observation we suggest that **4** is the stable HLE–I complex. However, we advise that some caution be exercised in the application of mechanistic conclusionns drawn from PPE–I complexes to those produced by HLE. First, there are substantial differences in the substrate (Zimmerman & Ashe, 1977) and inhibitor (B. G. Green, P. Gale, R. Chabin, A. Maycock, H. Weston, J. Doherty, C. Dorn, W. Hagmann, J. Hale, J. Liesch, M. Navia, S. Shah, and W. B. Knight, unpublished data, 1991) specificities between HLE and PPE. In addition, in the L 647957 derived PPE–I complex, the chloride present at C-7 of the original β-lactam ring was absent. This was the result of the elimination of HCl from C-7 and C-6. In the case of the [^{14}C]methoxy-L 658758 derived HLE–I complex, the methoxy group at C-7 is still present in the final HLE–I complex. While this difference may be solely the result of the leaving group potential of chloride ion versus the methoxy group and not differences between the two enzymes, it should be considered. Pathway C should result in increased fragmentation of the inhibitor as a result of hydrolysis of the substituted imine. We are currently examining this possibility.

The observation that the inhibition of HLE by L 659286 did not appear to go to completion is not readily explained by the mechanism discussed above. The residual activity was inhibitable and labeled by [^{3}H]DFP, indicating that it resulted from a serine protease. The Michaelis constant toward the HLE substrate MeOsucc-AAPV-pNA displayed by the residual activity was identical to that of the native enzyme, suggesting that it was HLE. One possible explanation for this apparent discrepancy was the presence of an HLE isozyme that was not susceptible to the inhibitor. But, Green et al. (1991) provided evidence that the HLE isozymes are catalytically indistinguishable toward substrates and inhibitors. It is possible that a small percentage of the L 659286 derived HLE–I complex exists in a form that is sensitive to the presence of substrate. Rapid reactivation of this complex in the presence of substrate could explain this apparent discrepancy. We have some evidence for this possibility in the case of other β-lactam inhibitors (unpublished data), and are investigating this possibility.

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Oxidation of Phenolic Arylglycerol β-Aryl Ether Lignin Model Compounds by Manganese Peroxidase from *Phanerochaete chrysosporium*: Oxidative Cleavage of an α-Carbonyl Model Compound†

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ABSTRACT: Manganese peroxidase (MnP) oxidized 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(4-(hydroxymethyl)-2-methoxyphenoxy)-1,3-dihydroxypropane (I) in the presence of Mn^{II} and H_2O_2 to yield 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(4-(hydroxymethyl)-2-methoxyphenoxy)-1-oxo-3-hydroxypropane (II), 2,6-dimethoxy-1,4-benzoquinone (III), 2,6-dimethoxy-1,4-dihydroxybenzene (IV), 2-(4-(hydroxymethyl)-2-methoxyphenoxy)-3-hydroxypropanal (V), syringaldehyde (VI), vanillyl alcohol (VII), and vanillin (VIII). MnP oxidized II to yield 2,6-dimethoxy-1,4-benzoquinone (III), 2,6-dimethoxy-1,4-dihydroxybenzene (IV), vanillyl alcohol (VII), vanillin (VIII), syringic acid (IX), and 2-(4-(hydroxymethyl)-2-methoxyphenoxy)-3-hydroxypropanoic acid (X). A chemically prepared Mn^{III}–malonate complex catalyzed the same reactions. Oxidation of I and II in $H_2{}^{18}O$ under argon resulted in incorporation of one atom of ^{18}O into the quinone III and into the hydroquinone IV. Incorporation of one atom of oxygen from $H_2{}^{18}O$ into syringic acid (IX) and the phenoxypropanoic acid X was also observed in the oxidation of II. These results are explained by mechanisms involving the initial one-electron oxidation of I or II by enzyme-generated Mn^{III} to produce a phenoxy radical. This intermediate is further oxidized by Mn^{III} to a cyclohexadienyl cation. Loss of a proton, followed by rearrangement of the quinone methide intermediate, yields the C_α-oxo dimer II as the major product from substrate I. Alternatively, cyclohexadienyl cations are attacked by water. Subsequent alkyl–phenyl cleavage yields the hydroquinone IV and the phenoxypropanal V from I, and IV and the phenoxypropanoic acid X from II, respectively. The initial phenoxy radical also can undergo C_α–C_β bond cleavage, yielding syringaldehyde (VI) and a C_6–C_2-ether radical from I and syringic acid (IX) and the same C_6–C_2-ether radical from II. The C_6–C_2-ether radical is scavenged by O_2 or further oxidized by Mn^{III}, subsequently leading to release of vanillyl alcohol (VII). VII and IV are oxidized to vanillin (VIII) and the quinone III, respectively.

Lignin is a random, heterogeneous phenylpropanoid polymer that constitutes 15-30% of woody plant cell walls (Sarkanen & Ludwig, 1971). Since the biodegradation of the wood polysaccharides cellulose and hemicellulose is retarded by the presence of lignin (Crawford, 1981), lignin-degrading microorganisms play a key role in the Earth's carbon cycle (Crawford, 1981; Kirk & Farrell, 1987; Buswell & Odier, 1987; Gold et al., 1989). White-rot fungi are primarily responsible for the initial decomposition of lignin in wood (Buswell & Odier, 1987; Kirk & Farrell, 1987; Gold et al., 1989), which occurs via an oxidative and relatively nonspecific process (Kirk & Farrell, 1987; Gold et al., 1989; Higuchi, 1990; Schoemaker, 1990).

When cultured under ligninolytic conditions, the white-rot basidiomycete *Phanerochaete chrysosporium* produces two heme peroxidases, lignin peroxidase (LiP)[1] and manganese

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