Electrospray Ionization Mass Spectrometry as a Mechanistic Tool: Mass of Human Leucocyte Elastase and a β-Lactam-Derived E–I Complex†

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ABSTRACT: We have utilized liquid chromatography electrospray ionization mass spectrometry (ESI-MS) to probe the nature of the covalent E–I complex of human leucocyte elastase (HLE) and a β-lactam. The mass spectrum of HLE isozyme 4 displayed one major and two minor components with masses of 25 202, 25 043, and 24 522 Da, respectively. Isozyme 3 displayed three components, with masses of 25 180, 24 030, and 24 523 Da. These data suggest that the isozymes differ in the type and not the content of carbohydrate. The minor components represent decreases in carbohydrate content. Inactivation of isozyme 4 with *trans*-4-(ethoxycarbonyl)-3-ethyl-1-[(4-nitrophenyl)sulfonyl]-azetidin-3-one increased the mass of the three components by that of the parent compound. Similar results were obtained with the mixture of HLE isozymes. These observations demonstrate that HLE does not catalyze the β-elimination of *p*-nitrophenylsulfinate as Firestone et al. [(1990) *Tetrahedron 46*, 2255) suggested. In addition, it suggests that a "double hit" of both the active-site serine and histidine is not required to form a stable acyl-enzyme. Noncovalent complexes between HLE and either the tight-binding secretory leucoprotease inhibitor (SLPI) or a slow tight-binding peptide difluoroketone inhibitor were not observed by ESI-MS. SLPI displayed a mass of 11 710 Da in the absence and presence of HLE. These data demonstrate the utility of ESI-MS to probe the mechanism of inhibition of enzymes by mechanism-based inhibitors.

Mechanism-based inhibitors of enzymes are chemically transformed during the reaction with the enzyme. The final enzyme–inhibitor complexes will contain the entirety or portion(s) of the original inhibitor. Knowledge of the components remaining in the E–I complex can lead to the proposal of a mechanism of inhibition consistent with both the chemical and kinetic data. The identities of these complexes are typically probed with radiolabeled material(s) or by analyzing the products produced. The recent application of electrospray ionization mass spectrometry (ESI-MS)[1] to accurately determine the mass of proteins adds a new technique to answer these questions (Fenn et al., 1989). This technique has recently been applied to enzyme–inhibitor complexes (Alpin et al., 1990; Menard et al., 1991) and to the observation of acyl-enzymes during the reaction between serine proteases and slow substrates (Ashton et al., 1991).

β-Lactams have recently been described as mechanism-based inhibitors of serine proteases (Knight et al., 1992a,b). The stability of the complexes formed between this class of inhibitors and human leucocyte elastase (HLE,[2] EC 3.4.21.37) was attributed to a "double hit" involving both acylation and alkylation of active-site serine and histidine residues, respectively. The X-ray crystallographic structure of porcine pancreatic elastase (PPE) and a cephalosporin derivative demonstrated both acylation of the serine and alkylation of the histidine by the 3′ exocyclic methylene group (Navia et al., 1987). In one report bicyclic β-lactams were shown to liberate the C-3′ group, a requirement for Michael addition to this position (Knight et al., 1992a). Firestone et al. (1990) proposed that compounds such as L-652117 inactivated HLE according to the mechanism shown in Figure 1. This requires that the enzyme catalyze a β-elimination of *p*-nitrophenylsulfinate to produce an electrophile for histidine alkylation. This was supported by the observation that the complex formed between this inhibitor and HLE displayed stability similar to that produced from the cephalosporin derivatives (Knight et al., 1992a,b). This proposal was tested in these studies with ESI-MS.

To determine if reversible HLE–I complexes survive LC/ESI-MS, we subjected the complexes produced from two reversible inhibitors of HLE, SLPI and L-708286, to the technique in this work. SLPI is a proteinaceous tight-binding reversible inhibitor of HLE. The reported dissociation constants range from 0.3 nM (Boudier & Bieth, 1989) to 0.01 nM (Gauthier et al., 1982). L-708286, a peptide-based

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[1] Abbreviations: ESI-MS, electrospray ionization mass spectrometry; LC, liquid chromatography; L-652117, *trans*-4-(ethoxycarbonyl)-3-ethyl-1-[(4-nitrophenyl)sulfonyl]azetidin-3-one; L-708286, (*S*)-(*N*-acetyl-L-alanyl)-*N*-[3,3-difluoro-1-(1-methylethyl)-2,4-dioxo-4-[(2-phenylethyl)-amino]butyl]-L-prolinamide; SLPI, secretory leucoprotease inhibitor; PPE, porcine pancreatic elastase, TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid.

[2] HLE refers to the mixture of elastase isozymes as isolated from human sputum. HNE is the mixture of elastase isozymes as isolated from human neutrophils. The nomenclature of the isozymes is based upon their order of elution from a cation-exchange column (Green et al., 1991).

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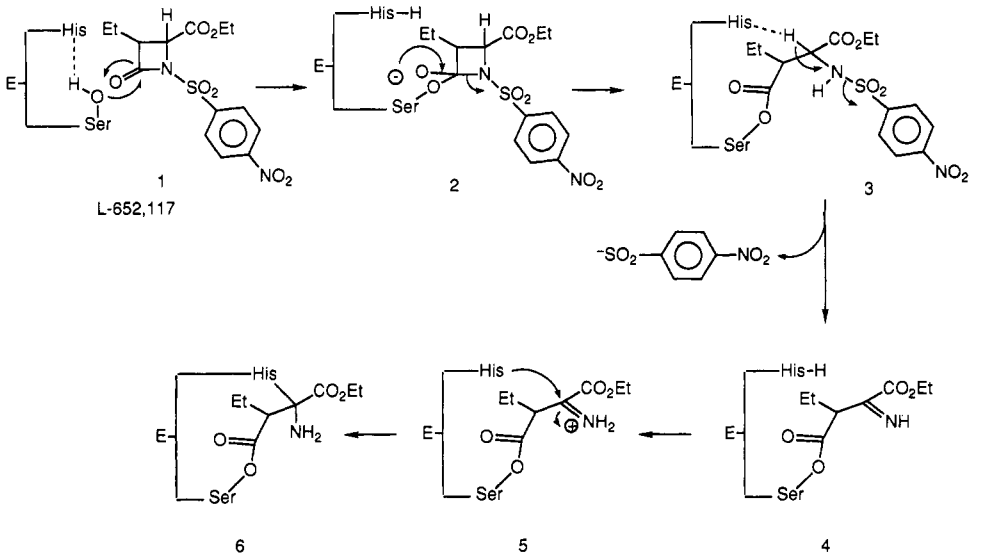
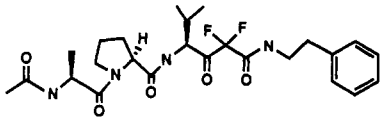


FIGURE 1: Mechanism proposed by Firestone et al. (1990) for the inhibition of HLE by L-652117. Formation of an acyl-enzyme followed by β-elimination of *p*-nitrophenylsulfinate would yield the electrophilic imine for the "second hit".

difluoromethyl ketone, was reported by Takahashi et al. (1988) to be a reversible inhibitor of PPE with a K_i of 0.1 μM. These



L-708,286

workers claimed that this component is a slow-binding inhibitor of HLE but did not present supporting data. The difluoroketones are thought to reversibly form hemiketals with the active-site serine of serine proteases. We have examined the mechanism of inhibition of HLE by this compound and report the kinetic constants.

HLE is known to contain a number of isozymes that are thought to differ only in carbohydrate content (Twumasi & Liener, 1977; Baugh & Travis, 1976). The carbohydrate content reported has ranged from 9–12% (Twumasi & Liener, 1977) to as high as 21% (Baugh & Travis, 1976). The most predominant isozymes present in HLE are isozymes 3 and 4 (Green et al., 1991). The isozymes are catalytically indistinguishable as they display identical kinetic constants versus a number of substrates and inhibitors of several classes (Green et al., 1991). The carbohydrate content of the HLE isozymes is examined in this work by ESI-MS.

EXPERIMENTAL PROCEDURES

Materials. HNE and HLE were purchased from Athens Research Products and Elastin Products, respectively. HLE isozymes 3 and 4 were purified from HLE by cation-exchange chromatography according to Green et al. (1991). The isozymes were exhaustively dialyzed versus deionized H_2O and lyophilized prior to use. The concentration of active HLE was based upon activity versus 1 mM methoxysuccinyl-AAPV-pNA[3] in 450 mM NaCl, 10% DMSO, and 45 mM Na-TES at pH 7.5 as determined spectrophotometrically (Green et al., 1991). SLPI, a gift from J. Stolk and J. Kramps (Department of Pulmonology, University of Leiden, The Netherlands) was dissolved in H_2O prior to use. L-652117 was synthesized according to Firestone et al. (1990) and was obtained from the Merck Chemical Collection. L-708286 was synthesized according to Takahashi et al. (1989). TES was purchased from Sigma Chemical Co. and titrated to the appropriated pH with either NaOH or NH_4OH.

Methods. Mass spectral analysis was performed on either a Finnegan TSQ-700 or Sciex API-III spectrometer. These were equipped with either ISCO-HPLC and Harvard syringe pumps or an Applied Biosystems 140 HPLC pump to create the gradients. In typical experiments, elastase was dissolved at 5–10 mg/mL and diluted 1/10 with either H_2O or buffer containing 10% EtOH. Aliquots (1–5 μL) were injected onto a 10-cm × 250-μm C-18 capillary column (or 25–50 μL onto 5-cm × 1-mm C-8 column) and eluted with a 0.1–0.07% TFA and 0–90% acetonitrile gradient at 2 μL/min (or 40 μL/min). Typically, 20–200 pmol of enzyme was analyzed. Reactions in buffer A contained 50 mM NH_4Cl, 10% EtOH, and 5 mM NH_4-TES at pH 7.5. Buffer B was 100 mM NaCl, 10% EtOH, and 10 mM Na-TES at pH 7.5. L-652117 was added in EtOH (final concentration 10%) at 3–50 times the enzyme concentration. In additional experiments, isozyme 4 was incubated with L-652117 for 30 min in 10% DMSO, 450 mM NaCl, and 45 mM TES, pH 7.5, followed by treatment with 4 M guanidine hydrochloride or 7 M urea for 1 h. The enzyme was then repurified on a 4.6-mm × 15-cm C-18 column eluted with an acetonitrile gradient. The protein was collected, lyophilized to dryness, and redissolved in 50% aqueous MeOH for analysis. SLPI (50 μM) was incubated with 30–50 μM HLE for 1 h in buffer A, and 1 μL was injected onto the column. L-708286 (10 equiv) was incubated with HLE for 1 h, and 1 μL was injected onto the column.

The second-order rate constant from the nonlinear progress curves for the inhibition of HLE by L-708286 was determined according to Green et al. (1991) in 450 mM NaCl, 10% DMSO, and 45 mM TES at pH 7.5. The K_i for this process was estimated from the final steady-state velocity (v_s) obtained from the first-order progress curves for the hydrolysis of 0.2 mM Suc-AAPA-pNA in the presence of 0.3 or 0.6 μM L-708286 according to eq 1 (Stein et al., 1987), where v_c is

$$K_i = \frac{[I]/(v_c/v_s - 1)}{(1 + [S]/K_m)} \quad (1)$$

[3] The standard one-letter abbreviations for the amino acids are used. pNA, *p*-nitroanilide, Suc, succinyl.

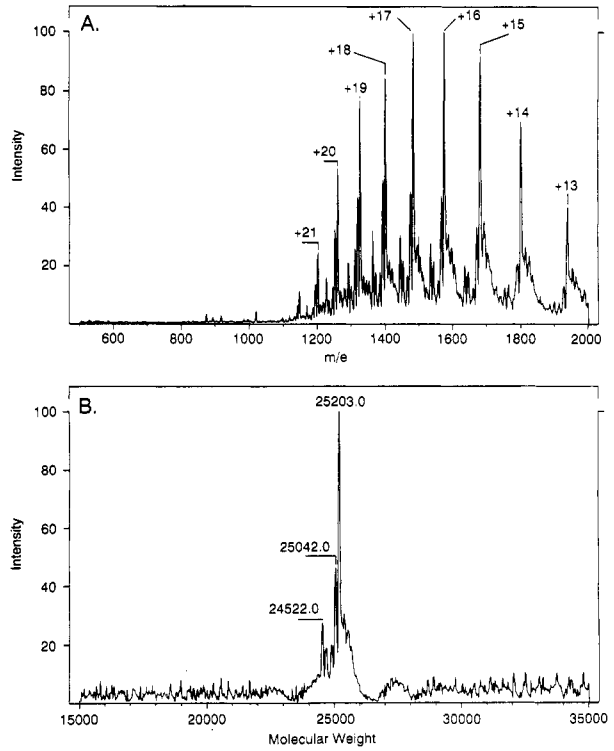


FIGURE 2: (A) Positive-ion ESI mass spectrum of 20 pmol of HLE isozyme 4 obtained using a Finnegan TSQ-700 quadrupole mass spectrometer. The multiple charge states observed are characteristic of ESI spectra of macromolecules. (B) Same spectrum after processing by Finnegan BioMass software to yield directly the average molecular weights of the sample components.

the control rate in the absence of inhibitor and $(1 + S/K_m) = 1.07$ under the assay conditions (Green et al., 1991). SLPI concentration was determined by titration of HLE activity (Stolk et al., 1992). In separate experiments, 40 nM HLE was preincubated for 0.5 h with 0.3 μM L-708286 and the reaction was initiated with substrate.

RESULTS AND DISCUSSION

Mass of HLE Isozymes. The electrospray mass spectrum of HLE isozyme 4 (Figure 2A) contains several series of ions resulting from different charge states for species in the sample mixture. With appropriate computer algorithms, the spectrum can be transformed to one which yields directly the molecular mass for all of the components observed (Figure 2B). A major component with a mass of 25 203 Da and minor components at 25 042 and 24 522 Da are evident in Figure 2B. The average of four determinations under identical conditions is reported in Table I. Similar results were obtained on both mass spectrometers. The mass calculated on the basis of the amino acid sequence is 23 295 Da.[4] These data suggest that the three forms present in isozyme 4 contain 7.6%, 7%, and 5% carbohydrate, respectively. This is consistent with the carbohydrate content reported by Twumasi and Liener (9–12%) in 1977 but considerably less than the maximum value of 21% reported by Baugh and Travis (1976). The species at −160 Da may represent one less hexose (−163 Da predicted) residue or, less likely, one less fucose (−148 Da predicted) residue. The low intensity of this species yielded significant errors in the determination of its mass. It is also possible this

[4] Calculated from the amino acid sequence reported by Sinha et al. (1987) using the program peptidesort of the Genetics Computer Group sequence analysis software package.

Table I: Mass of Human Leucocyte Elastases in the Presence and Absence of Inhibitors[a]

conditions	mass[a] (Da)	mass of (E–I – E)
HNE in H_2O	25 191 mj	
	25 050 mn	
HLE in H_2O[b]	25 186 mj	
	25 038 mn	
HLE + L-652117[b] in H_2O	25 540 mj	354
	25 398 mn	360
isozyme 4 in H_2O[b]	25 203 mj	
	25 053 mn	
	24 525 mn	
isozyme 3 in H_2O	25 180 mj	
	25 030 mn	
	24 523 mn	
isozyme 4 (n = 4) in buffer A	25 202 ± 1[a] mj	
	25 043 ± 8 mn	
	24 522 ± 6 mn	
isozyme 4 + L-652117 (n = 3) in buffer A	25 562 ± 3 mj	360 ± 3
	25 398 ± 3 mn	355 ± 9
	24 868 ± 5 mn	346 ± 8
isozyme 4 in buffer B	25 204 mj	
isozyme 4 + L-652117 in buffer B	25 566 ± 2 mj	362 ± 2
isozyme 4 prepurified[c]	25 200	
	25 044	
isozyme 4 + L-652117[c] + GuHCl	25 556	356
isozyme 4 + L-652117[c] + urea	25 556	356
isozyme 4 + SLPI in buffer A	25 200 mj	0
	25 045 mn	
	11 711 mj	
SLPI in buffer A	11 710 ± 1	
isozyme 4 + L-708286 in buffer A	25 200 mj	0
	25 048	

[a] The individual determinations typically have standard errors of ±1–2 amu from the deconvolution. The mass of the major form of isozyme 4 varied from 25 199 to 25 204 depending upon the instrument and experiment. When errors are quoted they are standard deviations from multiple determinations. The errors in the mass differences are propagated from the reported standard deviations. mj and mn refer to major and minor species, respectively, and refer to the observed intensity. In all cases, the three forms of isozyme 4 were observed even if not specified above. All three were shifted by similar mass upon reaction with L-652117. [b] These experiments were conducted on a Sciex API-III mass spectrometer. All other experiments were conducted on a Finnegan TSQ-700 mass spectrometer. [c] These experiments refer to isolation of isozyme 4 from a C-18 column prior to the LC–mass spectrometric analysis.

represents multiple species of very similar masses. We were unlikely to resolve clearly two species that differ by the absence of either a fucose or a hexose. Fucose and *N*-acetylglucosamine residues have been observed in an X-ray structure of HLE complexed to an inhibitor (Bode, 1988). The other minor component represents a decrease by multiple hexoses or *N*-acetylhexosamines. The mass spectrum of HLE isozyme 3 was similar to that displayed by isozyme 4 (data not shown), but the masses were slightly smaller (see Table I). These data suggest that the two isozymes may differ not by absolute carbohydrate content but possibly by subtle differences in the type of carbohydrate present. The mixture of isozymes of HLE isolated from human sputum and from neutrophils displayed masses intermediate between isozymes 3 and 4, suggesting that the spectra are averages of the two most predominant species (see Table I).

Mass of the L-652117-Derived HLE–I Complex. In Figure 3 the mass spectra of a sample containing both unreacted HLE isozyme 4 and the L-652117-derived HLE–I complex are shown. The compound increased the mass of the three components by approximately 360 Da. The average of three determinations is reported in Table I. In similar experiments with HLE, the shift in mass was 354 Da (see Table I). Treatment of HLE isozyme 4 with the inhibitor in the nonnucleophilic buffer Na-TES yielded similar results. The

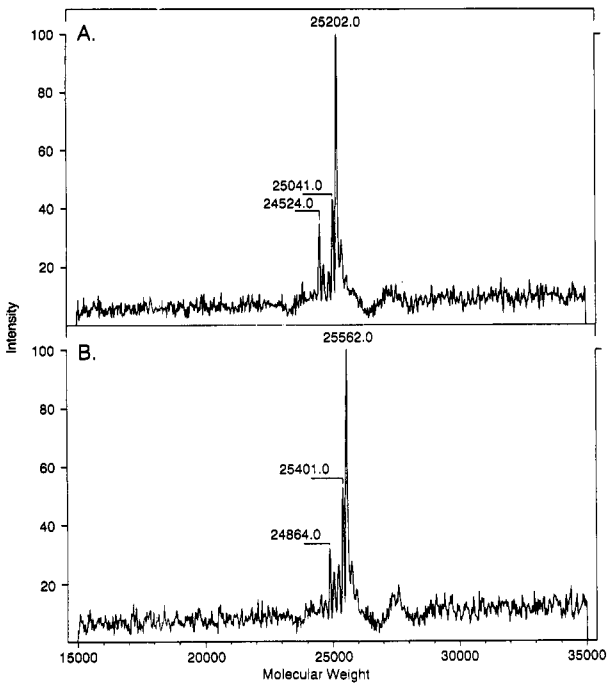


FIGURE 3: (A) Derived mass spectrum of excess HLE isozyme 4 (buffer A) in a sample containing L-652117. (B) Derived mass spectrum of the L-652117-derived HLE–I complex in the same sample. The free enzyme and HLE–I complex are resolved by chromatography.

mechanism presented in Figure 1 predicts an increase of only 168 amu between the stable E–I complex and free enzyme. These data demonstrate that the entire inhibitor molecule (MW = 356.4) is present in the enzyme–inhibitor complex; therefore, HLE does not catalyze the β-elimination of *p*-nitrophenylsulfinate as previously suggested unless this group is retained noncovalently. Since the liquid chromatography conditions are generally considered to be denaturing to proteins, the latter is unlikely.[5] In addition, treatment of the HLE–L-652117 complex with either 4 M guanidine hydrochloride or 7 M urea followed by isolation from a C-18 column resulted in the same mass spectrum.

In Figure 4, progress curves for the inhibition of HLE by L-708286 demonstrate slow-binding inhibition (Williams & Morrison, 1979). The final steady-state rates were equivalent whether the reaction was initiated with enzyme or after preincubation of enzyme inhibitors. The addition of a second aliquot of enzyme demonstrated that the steady-state rate did not result from inhibitor depletion. The K_i calculated from the final steady-state velocities was 64 ± 6 nM, while the second-order rate constant (k_{inact}/K_i) was 56 000 ± 5000 M^{-1} s^{-1}.

The enzyme–inhibitor complexes produced from the two tight-binding reversible inhibitors, SLPI and L-708286, did not survive the chromatography conditions (see Table I). Free HLE and not a complex was observed in both cases. SLPI eluted shortly after the injection front from a C-18 column eluted with a 30–90% acetonitrile gradient, while HLE elutes at the end of the gradient. The mass spectrum of SLPI originally in the presence of HLE is shown in Figure 5. The average experimentally determined mass of 11 710 is equivalent to that predicted from the amino acid sequence and eight disulfide bonds [see Müller et al. (1986)]. These data

[5] The chromatography conditions actually separated inhibitor, free HLE, and the HLE–inhibitor complex. The latter two eluted at greater than 80% acetonitrile.

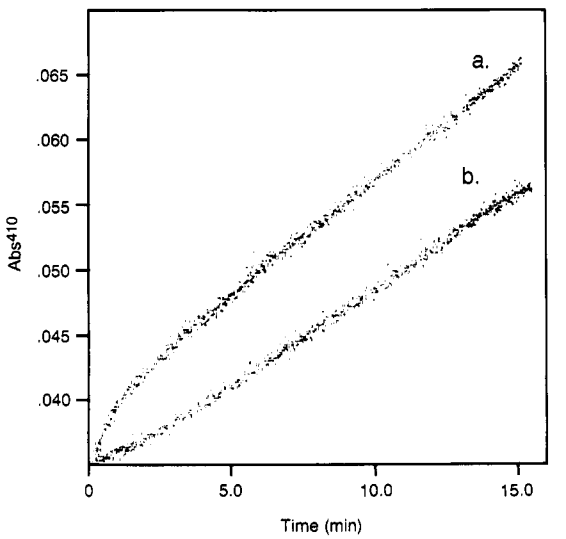


FIGURE 4: Progress curves of the hydrolysis of 0.2 mM Suc-AAPA-pNA by 40 nM HLE in the presence of 0.3 μM L-708286. (A) Reaction initiated with enzyme. (B) Reaction was initiated with substrate after preincubation of L-708286 for 1 h with HLE.

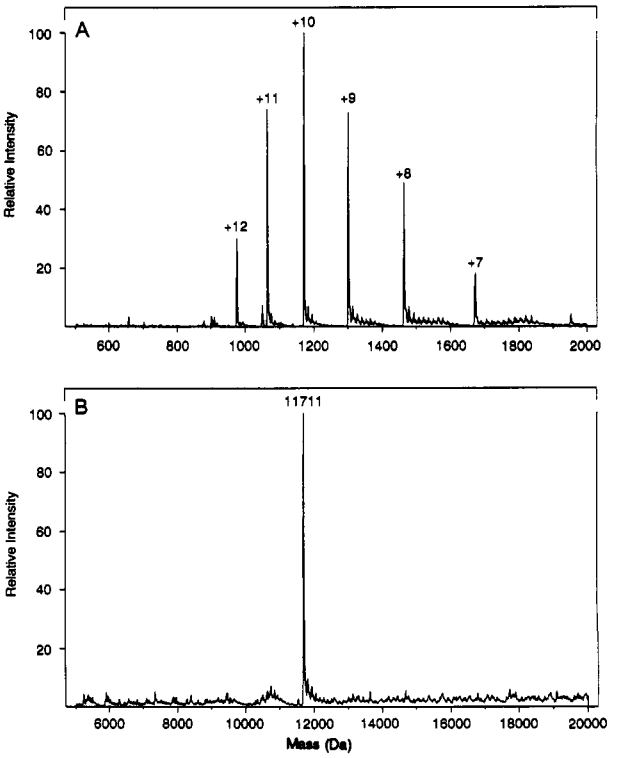


FIGURE 5: (A) Positive-ion ESI mass spectrum of 50 pmol of SLPI originally in the presence of 50 pmol of HLE. SLPI and HLE are separated by chromatography and were observed independently. (B) SLPI spectrum after processing by Finnegan BioMass software to yield directly the average molecular weights of the sample proteins.

suggest that noncovalent complexes are not observed with this system. Further evidence that these conditions are denaturing comes from the observation that the smaller peptide produced during the interaction of HLE with α_1PI is separated during C-18 chromatography of the α_1PI-HLE complex (Knight and Swiderek, unpublished results). Travis and Salvesen (1983) reported that this peptide could only be separated from the complex by "strong denaturing conditions such as sodium dodecyl sulfate".

These results suggest that a "double hit" of both the active-site serine and histidine is not required to form a stable acyl-enzyme between HLE and β-lactams. Since there is parti-

tioning of a L-652117-derived HLE–I complex between inhibitor turnover and inactivation (4:1) (Knight et al., 1992b), the initial acyl-enzyme **3** undergoes either hydrolysis or rearrangement to a more stable form without a decrease in mass. A conformational rearrangement of **3** that protects the acyl-enzyme from nucleophilic attack is consistent with these observations.

Conclusion. These data demonstrate the utility of electrospray mass spectrometry to probe the mechanism of inhibition of enzymes by mechanism-based inhibitors. A "double hit" of active-site residues is not required to produce a stable β-lactam-derived human leucocyte elastase–inhibitor complex.

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