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Kinetics and Mechanism of Human Leukocyte Elastase Inactivation by Ynenol Lactones†

Leslie J. Copp, Allen Krantz, and Robin W. Spencer*
Syntex Research, Mississauga, Ontario, Canada L5N 3X4
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ABSTRACT: Human leukocyte elastase (HLE), a serine protease involved in inflammation and tissue degradation, can be irreversibly inactivated in a time- and concentration-dependent manner by ynenol lactones (**1–4**). Ynenol lactones that are α-unsubstituted do not inactivate but are alternate substrate inhibitors that are hydrolyzed by the enzyme. Ynenol lactones that are both substituted α to the lactone carbonyl and unsubstituted at the acetylene terminus are rapid inactivators of HLE and inactivate pancreatic elastase and trypsin more slowly. 3-Benzyl-5(*E*)-(prop-2-ynylidene)tetrahydro-2-furanone inactivates HLE with biphasic kinetics and an apparent second-order rate of up to 22 000 M^{-1} s^{-1} (pH 7.8, 25 °C). The rate of inactivation is pH-dependent and is slowed by a competitive inhibitor. The partition ratio is 1.6 ± 0.1. Rapid removal of ynenol lactone during the course of inactivation yields a mixture of acyl and inactivated enzyme species, which then shows a partial recovery of activity that is time- and pH-dependent. Inactivation is not reversible with hydroxylamine. The enzyme is not inactivated if the untethered allenone is added exogenously. All of these results are consistent with a mechanism involving enzyme acylation at serine-195 by the ynenol lactone, isomerization of the acyl enzyme to give a tethered allenone, and capture of a nucleophile (probably histidine-57) to inactivate the enzyme. Substitution at the acetylene terminus of ynenol lactones severely reduces their ability to inactivate HLE, because allenone formation is slowed and/or nucleophile capture is hindered. Chemical competence of each of these steps has been demonstrated [Spencer, R. W., Tam, T. F., Thomas, E. M., Robinson, V. J., & Krantz, A. (1986) *J. Am. Chem. Soc. 108*, 5589–5597].

The design and characterization of serine protease inhibitors, especially inhibitors of human leukocyte elastase (HLE),[1] is an active area of research since such compounds may have therapeutic utility in a number of degradative diseases (Starkey, 1977; Barrett, 1980). Inhibition by a variety of small molecules (i.e., excluding natural or recombinant polypeptide inhibitors) is known and may be subdivided into three mechanistic classes: (1) inhibition that is reversible with respect to both the inhibitor and the enzyme, (2) inhibition that is reversible with respect to the enzyme but in which the inhibitor is permanently altered (i.e., the inhibitor is actually a substrate), and (3) inhibition that is irreversible with respect to both the enzyme and the inhibitor. Table I expands on this classification with specific examples. Fully reversible inhibitors (class 1 above) include noncovalent and carbonyl agents. Inhibitors that form acyl enzymes belong in class 2,[2] though in some cases deacylation is so slow that the inhibition is, for practical purposes, irreversible. Alkylating agents are generally fully irreversible (class 3) in their action.

Known alkylating inhibitors may be further classified as those that are intrinsically electrophilic and those in which the electrophilic functionality is initially masked and exposed only

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[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HLE, human leukocyte elastase; HSE, human sputum elastase; MES, 2-(*N*-morpholino)ethanesulfonic acid; Me_2SO, dimethyl sulfoxide; PPE, porcine pancreatic elastase; SAAPVC, 7-(methoxysuccinylalanylalanylprolylvalinamido)-4-methylcoumarin; SAAPVFC, 7-(methoxysuccinylalanylalanylprolylvalinamido)-4-(trifluoromethyl)coumarin; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]-propanesulfonic acid; TMAC, 7-[(trimethylacetyl)oxy]-4-methylcoumarin (see Materials and Methods).

[2] The lactones of Tobias et al. (1969) and Izbicka and Bolen (1981) are an interesting exception. Though these compounds acylate chymotrypsin, they can deacylate intramolecularly to regenerate the initial inhibitor.

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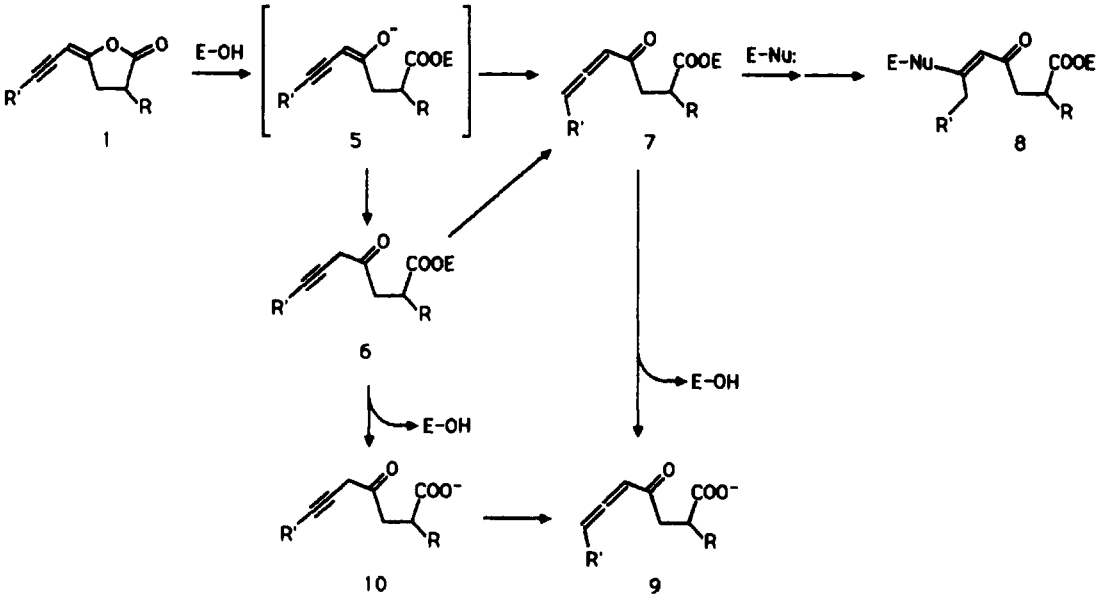
Table I: Classes of Serine Protease Inhibitors

type	example	references
noncovalent	fatty acids	Ashe & Zimmerman, 1977
	elesnin & analogs	Omura et al., 1978; Ohno et al., 1978; Groutas et al., 1984; Spencer et al., 1985
carbonyl agents	peptidyl aldehydes	Hassall et al., 1985
	peptidyl trifluoromethyl ketones	Gelb et al., 1985
boron analogs	peptidyl boronic acids	Kettner & Shenvi, 1984; Kinder & Ketzenellenbogen, 1985
acylating agents		
acyclic	azapeptide carbamates	Gupton et al., 1984; Powers et al., 1984
	peptidyl carbamates & related acylating agents	Tsuji et al., 1984; Groutas et al., 1985a,b
cyclic	lactones	Tobias et al., 1969; Izbicka & Bolen, 1981
	saccharins	Zimmerman et al., 1980; Ashe et al., 1981
	chloropyrones	Westkaemper & Abeles, 1983
	isatoic anhydrides, etc.	Moorman & Abeles, 1982; Weidmann & Abeles, 1984
	benzoxazinones	Teshima et al., 1982; Hedstrom et al., 1984
	chloroisocoumarins, phthalides, etc.	Harper et al., 1985; Hemmi et al., 1985
heteroatomic	aryl sulfonyl fluorides	Yoshimura et al., 1982
	peptidyl phosphonyl fluorides	Lamden & Bartlett, 1983
alkylating agents		
direct	peptidyl chloromethyl ketones	Shaw et al., 1981
masked	2-bromomethylbenzoxazinone	Alazard et al., 1973
	6-bromomethyl-3,4-dihydrocoumarins	Béchet et al., 1973
	nitrosoamides	White et al., 1977
	7-amino-4-chloroisocoumarins	Harper & Powers, 1984, 1985
	halo enol lactones	Chakravarty et al., 1982; Daniels et al., 1983; Daniels & Ketzenellenbogen, 1986
	ynenol lactones	Tam et al., 1984; this work

Scheme I



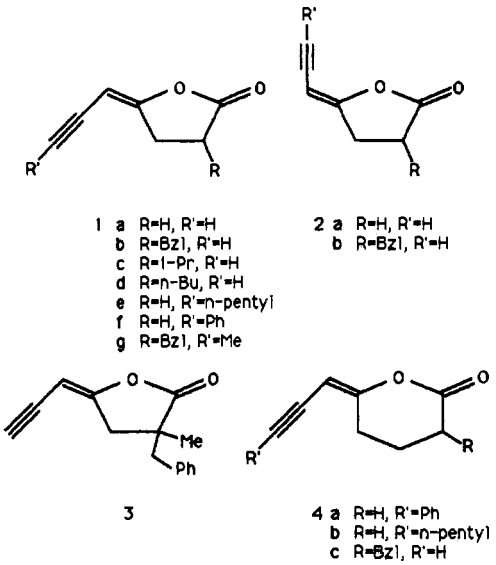
at the enzyme active site. Halomethyl ketones are the best known examples of intrinsically electrophilic alkylating agents. They are very potent inactivators and with appropriate peptidyl substitution can be very enzyme specific (Shaw et al., 1981). Nonetheless, their consideration as drugs has been limited by their chemical reactivity since nonspecific alkylation in vivo raises the specters of toxicity and mutagenicity.

Masked alkylating agents are designed to overcome this potential problem by rearranging during the course of enzyme catalysis to produce electrophiles only at enzyme active sites. They are therefore "suicide substrates" in the most stringent sense[3] (Walsh, 1982; Rando, 1984). In addition, this strategy for serine protease inactivators has involved molecules which produce an electrophile that is covalently tethered to serine-195 as an acyl enzyme, in the hope that the long lifetime of the acyl enzyme will maximize the opportunity for attack on the exposed electrophile.

In this paper we address the mechanism of inactivation of human leukocyte elastase by a class of such suicide substrates.

[3] Many types of compounds have been called suicide substrates. Our criteria are those of the original example of Bloch (1969), which involved (1) normal catalysis by the target enzyme, (2) resulting production of a reactive intermediate, and (3) irreversible reaction between the intermediate and the enzyme that is not part of normal catalysis.

HL elastase is a serine protease that may be involved in tissue destruction in several diseases, including emphysema and arthritis (Janoff, 1983; Powers, 1983; Schnebli, 1985). The suicide substrates are ynenol lactones **1–4**, which are designed



to inactivate by the mechanism of Scheme I. The key steps in this scheme are enzyme acylation (**1** → **5**), formation of a tethered allenone (the electrophile) (**5** → **7** or **5** → **6** → **7**), and finally alkylation (**7** → **8**). Inactivation would be thwarted by deacylation of the intermediates (**6** → **10** and **7** → **9**); such deacylation is also undesirable because it would release potentially reactive molecules from the active site. The chemical competence of ynenol lactones in model reactions of these steps has been demonstrated (Spencer et al., 1986), and some of the biochemical results have been previously communicated (Tam et al., 1984).

Specifically, we wish to establish whether ynenol lactones are substrates for HL elastase, and, if so, what products are released. If ynenol lactones inactivate the enzyme, the concentration dependence of the inactivation rate and the partition ratio should be determined. Inactivation according to Scheme I should also be shown to proceed from an acyl intermediate, be active site directed, and be irreversible. We also address substituent effects on HL elastase inactivation and the specificity of inactivation of other serine proteases by ynenol lactones.

Materials and Methods

Human sputum elastase (HSE) was obtained from Elastin Products Co., Inc. (Pacific, MO). Porcine pancreatic elastase (type I), bovine pancreatic α-chymotrypsin (type II), bovine pancreatic trypsin (type III), Brij 35 [poly(oxyethylene 23) lauryl ether], N^{α}-benzoyl-L-arginine-*p*-nitroanilide hydrochloride, 7-(glutaryl-L-phenylalaninamido)-4-methylcoumarin, 7-(*N*-benzoyl-L-argininamido)-4-methylcoumarin, 4-methylumbelliferyl *p*-(trimethylammonium)cinnamate chloride, and 4-methylumbelliferone were all purchased from Sigma Chemical Co. 7-(*N*-Succinyl-L-alanyl-L-prolyl-L-alaninamido)-4-methylcoumarin was purchased from Chemical Dynamics Corp. (South Plainfield, NJ). 7-(Methoxysuccinylalanylalanylprolylvalinamido)-4-methylcoumarin and elastatinal were purchased from Peninsula Laboratories, Inc. (Belmont, CA). 7-(Methoxysuccinylalanylprolylvalinamido)-4-(trifluoromethyl)coumarin was purchased from Enzyme Systems Products (Livermore, CA). Gel filtration media and PD-10 columns were purchased from Pharmacia (Dorval, Quebec).

Syntheses. Ynenol lactones **1a–g**, **2a,b**, **3**, and **4a–c** were synthesized as described in Spencer et al. (1986).

7-[(Trimethylacetyl)oxy]-4-methylcoumarin (2,2-Dimethylpropanoic Acid 4-Methyl-2-oxo-2H-1-benzopyran-7-yl Ester; TMAC). To 8.8 g of 4-methyl-7-hydroxycoumarin (Sigma) in 50 mL of pyridine was added 11.2 mL of trimethylacetic anhydride (Aldrich). The mixture was fitted with a drying tube, stirred overnight at 60 °C, and then poured into 100 mL of 1 M HCl at 0 °C and extracted with EtOAc. The organic phase was washed with 1 M HCl, water, 5% (w/v) $NaHCO_3$, and twice with water and then dried with Na_2SO_4 and evaporated to a pale yellow oil. The oil was dissolved in EtOAc, and addition of approximately 2 volumes of petroleum ether (30–60 °C mixture of hexanes) gave fine white needles, which were washed with petroleum ether. The crystals were dissolved in ≈150 mL of EtOH with heating and treated with water to give 8.40 g (68%) of the product as fine, white, nonfluorescent needles: mp 95–95.5 °C; IR (Perkin-Elmer 298 spectrometer; Nujol) 2926, 2856, 1731, 1615, 1462 cm^{-1}; ^1H NMR (Bruker WP-80 SY spectrometer, $CDCl_3$, ppm from tetramethylsilane) δ 1.38 [s, 9 H, $C(CH_3)_3$], 2.43 (d, 3 H, J = 1.2 Hz, 4-CH_3), 6.26 (q, 1 H, J = 1.2 Hz, H3), 6.9–7.8 (m, 2 H, H5, H6), 7.08 (s, 1 H, H8).

Preparation of Human Leukocyte Elastase. Fresh human leukocytes, obtained by leukapheresis from a healthy donor, were frozen and kept at −75 °C until use. Enzyme preparation followed published methods (Englebrecht et al., 1982; Barrett, 1981) with slight modifications: cells were washed in 0.14 M NaCl and homogenized in the presence of 1 M NaCl and 0.1% (w/v) Brij 35. After centrifugation and concentration by dialysis against dry poly(ethylene glycol) (M_r 20 000), the material was chromatographed on Sephacryl S-300. Active fractions were combined, concentrated as before, and chromatographed on an affinity gel of bovine lung trypsin inhibitor attached to Sepharose CL-6B. Active fractions were combined, concentrated to approximately 0.3 μM in active elastase, and frozen in 1-mL aliquots (in 50 mM sodium phosphate, 1 M NaCl, 0.1% Brij 35, pH 6.0) at −75 °C until use.

Quantitation of HL and HS Elastase with TMAC. Burst titration of elastase using a *p*-nitrophenyl ester has been described by Bender et al. (1966). We have used a related fluorogenic reagent to titrate elastase with routine 20-pmol sensitivity. The reagent (TMAC) has been previously described (Akada et al., 1978) but has not been used as a burst titrant. Upon enzymatic hydrolysis of TMAC, the product 7-hydroxy-4-methylcoumarin is released, fluorescing at $\lambda_{max,excitation}$ = 365 nm and $\lambda_{max,emission}$ = 450 nm.

In a typical assay, the background hydrolysis of TMAC is followed for several minutes, using 2.0 mL of assay buffer (described below) with 10 μM TMAC (from a 10 mM stock solution in Me_2SO). An aliquot of HSE (in its storage buffer of 0.1 M KOAc, 0.5 M NaCl, and 0.05% Brij 35, pH 5.0) or HLE is added to the mixture. A rapid burst of fluorescence is observed, followed by a slow steady-state rate of TMAC hydrolysis. Analysis follows that of Bender et al. (1966) with interpolation from a standard curve of 7-hydroxy-4-methylcoumarin fluorescence.

Inhibition Assays: Progress Curve Method. The HLE assay buffer was 25 mM K^+-HEPES, 1 M NaCl, and 0.1% Brij 35, pH 7.8 (hereafter referred to as "assay buffer"). To 2.0 mL of assay buffer were added substrate to 2.5 μM (as 5 μL of 1 mM of SAAPVC in Me_2SO) and 20 μL of HLE (≈0.3 μM). Fluorescence increase was monitored by excitation at 370 nm and emission at 460 nm on a Perkin-Elmer 650-40 fluorescence spectrometer at 25 °C. An alternate substrate

used was SAAPVFC according to the same procedure with excitation at 400 nm and emission at 505 nm. The ynenol lactone inhibitor was added in aliquots of a stock solution (2–20 mM in Me_2SO). Where high inhibitor concentrations were required to measure K_i, the assays were done in 5% Me_2SO.

PPE and α-chymotrypsin were assayed as above in 25 mM K^+-HEPES and 0.1 M KCl at pH 7.8. To 2.0 mL of buffer were added 5 μL of enzyme (3 μM PPE in 1 mM HOAc or 0.7 mM α-chymotrypsin in 1 mM HCl) and 5 μL of substrate [5 mM 7-(*N*-succinylalanylprolylalaninamido)-4-methylcoumarin or 10 mM 7-(glutaryl-L-phenylalaninamido)-4-methylcoumarin, respectively].

Bovine trypsin was assayed with either a chromogenic or fluorogenic substrate. In the first case 1.0 mL of buffer (25 mM K^+-HEPES, 0.1 M KCl, 50 mM $CaCl_2$, pH 7.75), 10 μL of substrate (5 mM N^{α}-benzoyl-L-arginine-*p*-nitroanilide in Me_2SO), and 5 μL of enzyme ($\approx$1 mM in 1 mM HCl) were mixed, and increase of absorbance at 400 nm was monitored on a Perkin-Elmer 559A spectrophotometer. The fluorescence assay was monitored as described for HLE by using 2.0 mL of buffer with 5 μL of substrate [2 mM 7-(*N*-benzoyl-L-argininamido)-4-methylcoumarin in Me_2SO] and 5 μL of enzyme ($\approx$70 μM in 1 mM HCl).

Inhibition Assays: Incubation Method. HL elastase (100 μL of 0.3 μM) was incubated with 5 μL of ynenol lactone inhibitor (2–20 mM in Me_2SO) in 0.9 mL of assay buffer at 25 °C for up to 1 h. At various times 50-μL aliquots were removed and added to 1.94 mL of assay buffer containing 10 μL of SAAPVC (1 mM in Me_2SO). A control in the absence of inhibitor was assayed over the same time course.

Irreversibility Test by Gel Filtration. A 1.0-mL aliquot of HLE was loaded onto a 1-mL Sephadex G-50 column equilibrated with assay buffer. The column was spun for 10–15 s in a clinical centrifuge, and the eluant (now containing HLE at pH 7.55) was collected. An incubation mixture consisting of 0.8 mL of this HLE and an excess of **1b** (4 μL of 2 mM in Me_2SO) at 25 °C was monitored for activity at various times. Since there is a 100-fold dilution of the inhibitor in these assays, the activities reflect inactivation rather than inhibition. After 17 min (with less than 20% activity remaining) the incubation mixture was loaded onto the G-50 column and spun again to remove the excess **1b**. The resulting eluant was assayed for activity at various times over the course of an hour in the presence or absence of 10 mM β-mercaptoethanol.

Incubation of HL Elastase with Exogenous Allenone. To produce allenone **9b**, 10 μL of **1b** (20 mM in Me_2SO) and 20 μL of 0.1 M NaOH were added to 1.8 mL of 1 M NaCl and 0.1% Brij 35. The production of **9b** was followed at 240 nm (Spencer et al., 1986). When the reaction was complete, 0.2 mL of 0.25 M K^+-HEPES, pH 7.8, and 2 μL of 1 M HCl were added to give the usual assay buffer. To 1.5 mL of this were added 15 μL of 0.3 μM HLE and 4.5 μL of 1 mM SAAPVC, and the enzyme activity was measured for 5 min. As a control, addition of **1b** (1 μL of 20 mM) resulted in immediate, complete inhibition. The presence of allenone **9b** was shown by the addition of *n*-butylamine (20 μL of 1 M in H_2O) to 1 mL of the assay mixture. Formation of the cis amino–enone adduct was indicated by the rapid appearance of its ultraviolet spectrum (λ_{max} = 312 nm, ϵ = 23 000 M^{-1} cm^{-1}; Spencer et al., 1986).

Titration of HSE Inactivation by **1b**. A mixture of 0.95 mL of assay buffer, 50 μL of HSE (11.7 μM), and 0.5 μL of **1b** (0.5 mM in Me_2SO) was incubated at 25 °C. The enzyme activity of 10-μL aliquots was determined in 1.0 mL of assay buffer with 10 mM β-mercaptoethanol and 2.5 μM SAAPVC. When no further change in activity was observed, another aliquot of **1b** was added to the incubation. The procedure was repeated until negligible enzyme activity remained. A control incubation in the absence of **1b** showed no loss of activity over the time course of the experiment.

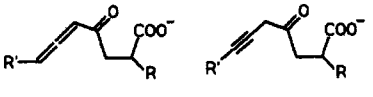
Elastatinal Protection of HLE from Inactivation by **1b**. The apparent rate of inactivation of HLE by **1b** (progress curve method) in the presence of elastatinal (0–12 μM) was calculated by single-exponential analysis.

Data Analysis and Simulations. Data were digitized from chart recordings with a Summagraphics MM1200 tablet attached to an NEC PC-8001A microcomputer. Rate constants were estimated by nonlinear least-squares regression (Bevington, 1969) of the real (i.e., not logarithmic) data to exponential ($Ae^{-kt} + B$), exponential/linear ($Ae^{-kt} + Bt + C$), or double-exponential ($Ae^{-k_1 t} + Be^{-k_2 t}$) equations as indicated. Reported errors are the standard errors of estimates obtained from the covariance matrices (Cleland, 1979). Initial rates were estimated as the derivatives of the above equations evaluated at mixing time. Inhibition constants were obtained with program VKKI, a special case of Cleland's program COMP (Cleland, 1979), which fits data to $v = (V_{max}/K_m)[A]/(1 + [I]/K_i)$, where V_{max}/K_m is a single parameter. The use of this equation is justified by the low substrate concentrations used: $K_{m,SAAPVC}$ = 150 ± 4 μM, so that $[A]/K_m$ = 0.017 or 0.033 throughout. In addition, these low concentrations assure that the fluorogenic substrate will not significantly perturb the kinetics or extents of inhibition or inactivation by the suicide substrates. Reported pK_a values were estimated with the program ABELL (Cleland, 1979).

Simulations were performed by integration (modified Euler's method) of the differential equations implied by Scheme II, except that the equilibrium constant (k_{-1}/k_1) was assumed to be rapid and therefore evaluated analytically at each cycle. For the simulation of Figure 6, the numeric integration agreed with the analytical solution within 0.6% at t = 8 min. Of the four independent constants in the simulation (k_{-1}/k_1, k_2, k_3, and k_4), only k_4 was obtained from rescue experiment data. The other constants were obtained from total inhibition experiments (k_{-1}/k_1, k_2, Figure 2) and from the deacylation of isolated enzyme (k_3, Figure 5).

RESULTS

Ynenol Lactones as Enzyme Substrates. Ynenol lactone **1a** inhibits HL elastase with a K_i of 5.3 ± 0.1 μM (based on initial rate assays) but does not show significant time-dependent inactivation. Whether this inhibition represents fortuitous noncovalent inhibition or alternate substrate inhibition (Segel, 1975) due to acylation and deacylation without inactivation (i.e., deacylation of **6** or **7** in Scheme I) can be determined by the ultraviolet spectra of elastase and **1a**. Figure 1a shows ultraviolet difference spectra in the course of the nonenzymatic alkaline hydrolysis of **1a**. The difference λ_{max} at 234 nm is characteristic of the conversion of **1a** to its corresponding allenone **9a** (Spencer et al., 1986). Figure 1b



9 a R = H, R' = H
b R = Bzl, R' = H
f R = H, R' = Ph

10

shows difference spectra taken during the reaction of HS elastase with excess **1a** at pH 6.8 (difference spectra were

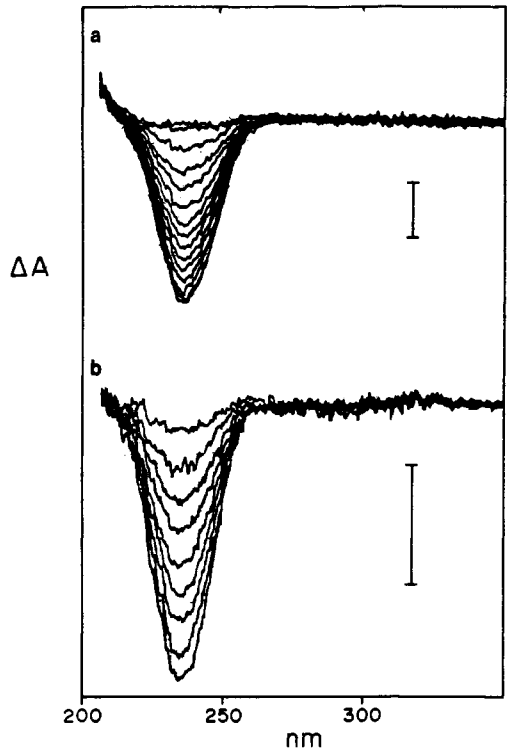


FIGURE 1: Difference spectra of alkaline and enzymatic hydrolysis of **1a**. Vertical scale bars represent 0.01 absorbance unit in each case. (a) Reference and sample were in split cuvettes, each containing 50 μL of **1a** (0.26 mM in CH_3CN) in 0.95 mL of water on one side and 1.0 mL of 1 mM potassium borate, pH 9.0, on the other, all at 25 °C. The sample cuvette was mixed, and scans were taken every 2 min. Succeeding spectra decrease at 234 nm. (b) Reference and sample cuvettes each contained 1.0 mL of 50 mM KP_i, 0.5 M NaCl, and 0.05% Brij 35, pH 6.8, 25 °C, and 5 μL of **1a** (4.7 mM in CH_3CN). To start the reaction, 20 μL of HSE (11.7 μM) was added to the sample. The base line was immediately rezeroed, and spectra were recorded at 2-min intervals. As in (a), succeeding spectra decrease at 234 nm.

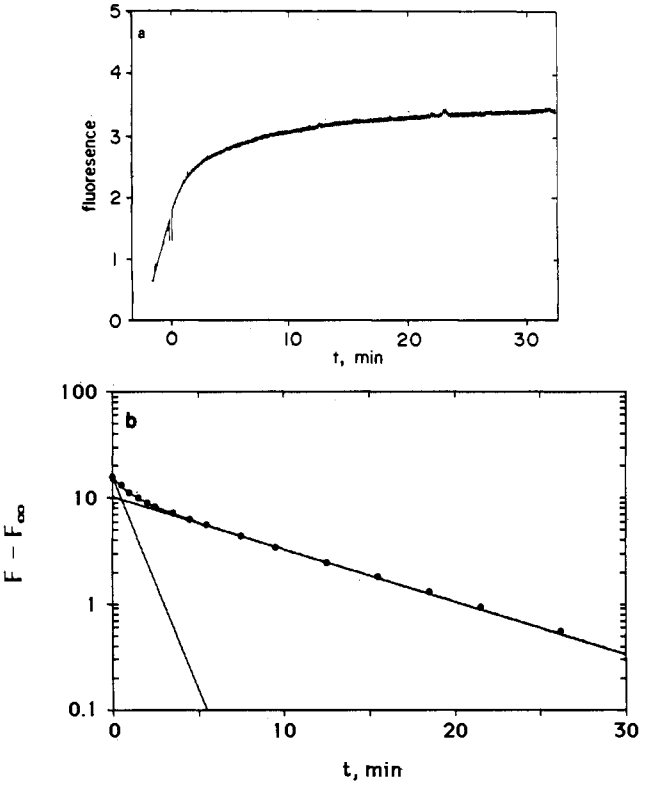


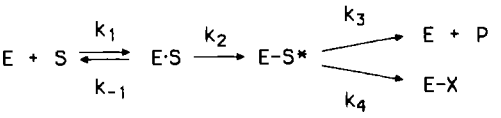
FIGURE 2: (a) HLE inhibition by ynenol lactone **1b**: progress curve assay. The HLE-catalyzed hydrolysis of SAAPVC was monitored by product fluorescence, and at $t = 0$ ynenol lactone **1b** was added (1 μL of 2 mM in Me_2SO; final concentration = 1 μM). (b) The points are the data of (a), and the curve is the best fit to $F - F_\infty = Ae^{-k_1 t} + Be^{-k_2 t}$: $A = 5.31$, $B = 10.36$, $k_1 = 0.0154$ s^{-1}, and $k_2 = 0.0019$ s^{-1}. The straight lines are the asymptotes $F - F_\infty = (A + B)e^{-k_1 t}$ and $F - F_\infty = Be^{-k_2 t}$.

taken to cancel any nonenzymatic reactions of **1a**, and in the absence of enzyme the base line was stable over this time scale). The similarity of these figures shows that HS elastase catalyzes the hydrolysis of ynenol lactone **1a** to allenone **9a**.

The experiment of Figure 1b also allows the calculation of a limit to the partition ratio for HS elastase and **1a**. In this experiment the final observed change at 234 nm was 0.05 absorbance unit, and with $\Delta\epsilon_{234} = 9200$ M^{-1} cm^{-1} from the model chemistry and [HS elastase] = 0.23 μM, the partition ratio must be greater than 24. The limit to the partition ratio for HL elastase and **1a** was also determined by assay of enzyme activity: 0.03 μM HL elastase was incubated with 10 μM **1a** (pH 7.8, 25 °C) and retained 90% of activity vs. control (without **1a**) after 1 h, which implies that the partition ratio is ≫50. The turnover number (k_{cat}) of HS elastase with **1a** can also be estimated from Figure 1b. In this and similar experiments the rate of the reaction ($\Delta A_{234}/\Delta t$) was first order in enzyme, with a rate of 0.013 ± 0.001 s^{-1} (pH 6.8, 25 °C). This rate is a good estimate of k_{cat} since the concentration of **1a** was 23.5 μM, or 4.4K_m (note that $K_m = K_i$ for alternate substrate inhibitors; Segel, 1975).

The same behavior is also seen with ω-phenyl ynenol lactone **1f**. In initial rate assays with a fluorogenic substrate, it is an inhibitor ($K_i = 110 \pm 10$ μM) that shows no time-dependent inactivation. As in Figure 1, difference spectra during incubation of **1f** with HSE are identical with those for the alkaline hydrolysis of **1f**, showing that the accumulating product is the ω-phenyl allenone **9f**. The partition ratio is ≫16, and k_{cat} is approximately 0.009 s^{-1} (pH 6.8, 25 °C).

Scheme II



These experiments show that HS elastase catalyzes the production of allenones **9** (R = H, R′ = H or Ph) from the corresponding ynenol lactones **1**, most likely via an acyl enzyme as expected for any ester or amide hydrolysis by a serine protease. Thus in Scheme I, either **7** deacylates to give active enzyme and **9** directly or **6** deacylates to give enzyme and propargyl ketone **10**, which then nonenzymatically rearranges to the allenone. Such a propargylic rearrangement would be facile and would be catalyzed by buffer salts (Spencer et al., 1986), and we would not expect to see **10** even if it were the immediate enzyme product. Additional evidence for acyl enzyme involvement is given by the pH dependence of inactivation and recovery from partial inactivation (vide infra).

Enzyme Inactivation by 3-Benzyl Ynenol Butyrolactone: Concentration Dependence and Irreversibility. In contrast to ynenol lactones **1a** and **1f**, the 3-benzyl-substituted **1b** is a rapid and complete inactivator of HL elastase. The time course of this inactivation by 1 μM **1b** is given in Figure 2a, as monitored continuously by a fluorogenic substrate in the incubation. The kinetics will be discussed with reference to Scheme II, in which E is free enzyme, S is the suicide substrate, E·S and E–S* are intermediates (perhaps a Michaelis complex and acyl enzyme, respectively), and E–X is inactivated enzyme.

Close inspection of Figure 2a shows that the inactivation is not strictly first order in remaining enzyme; a fit of the data

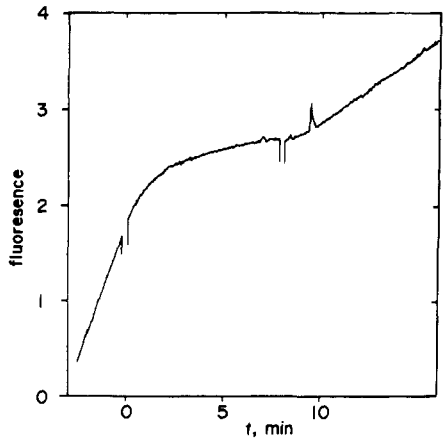


FIGURE 3: Nucleophilic "rescue" of HLE inactivation. Conditions were as in Figure 2a, except that β-mercaptoethanol was added to a concentration of 10 mM at 8 min after **1b** addition.

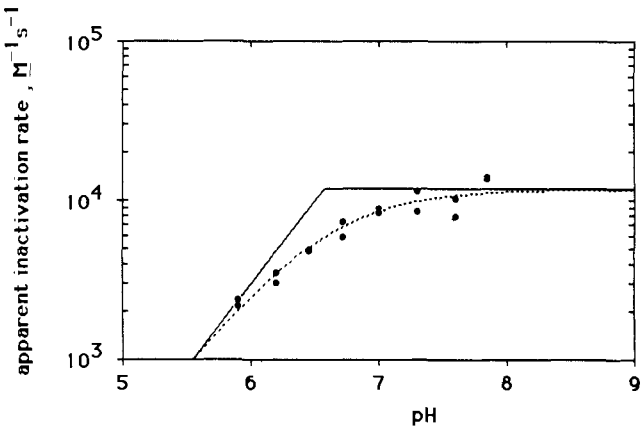


FIGURE 4: pH dependence of HLE inactivation by **1b**. Conditions were as described for progress curve assays, with buffers of 10 mM MES/HEPES, 0.5 M NaCl, and 0.1% Brij 35. In each assay [**1b**] was less than 1 μM. Each point represents a separate determination of $k_{app}/[\mathbf{1b}]$, where k_{app} is the apparent inactivation rate as determined by exponential/linear fitting.

to a single exponential ($y = Ae^{-kt} + B$) is poor with correlated residuals. There are several reasons why enzyme inactivation could be more complex than first order. First, **1b** is a mixture of enantiomers that could acylate and inactivate at different rates. Second, the enzyme is a mixture of isozymes (Barrett, 1981; also, HS elastase, which shows the same biphasic inactivation, has three bands on gel electrophoresis). Third, the concentration of **1b** can change during lengthy inactivation assays due to its lability at pH 7.8 ($k_{OH^-} \approx 260$ M^{-1} s^{-1}). The first two possibilities suggest that double-exponential analysis ($y = Ae^{-k_1t} + Be^{-k_2t} + C$) is appropriate, and the third suggests that exponential/linear ($y = Ae^{-kt} + Bt + C$) is appropriate (Purdie & Heggie, 1969). We find that any given set of data (e.g., Figure 2a) is equally well fit by either equation, but exponential/linear fitting typically gives an apparent rate of inactivation that is up to 3-fold lower than the first phase of double-exponential fitting. Which method of analysis is used will be noted, and for comparisons between different inactivators only exponential/linear analysis is used.

A double-exponential fit to the data of Figure 2a is shown in Figure 2b. For double-exponential fitting we limit our analysis to the fast phase of inactivation since it is predominant, accounting for 60–100% of the inactivation depending on [S]. The first phase of inactivation is saturable in [**1b**], and analysis of the rates (Kitz & Wilson, 1962; Walsh et al., 1978) gives $k_{max} = 0.090 \pm 0.007$ s^{-1}, $K = 4.1 \pm 0.7$ μM, and $k_{max}/K = 22\,000 \pm 2000$ M^{-1} s^{-1}. In addition to inactivation, **1b** inhibits HL elastase in initial rate assays (i.e., in the same sense that **1a** and **1f** inhibit). This initial inhibition has $K_i = 4.3 \pm 0.7$ μM. The identity of the inhibition constants for rapid inhibition and time-dependent inactivation suggests that these processes share a common intermediate, namely, E·S and/or E–S* in Scheme II. Analysis of the same data by exponential/linear fitting gives a second-order rate of inactivation (k_{max}/K) of 7600 ± 900 M^{-1} s^{-1}.

Continuous assays, such as that of Figure 2, measure only free enzyme (E in Scheme II) and cannot distinguish between enzyme that is reversibly inhibited (in the form of E·S or E–S*) from that which is inactivated (E–X). Thus the values determined above represent an apparent inactivation that is a combination of inhibition and inactivation. Determination of the rate of inactivation alone requires that all active enzyme species (E + E·S + E–S*) be measured. This can be done by separating enzyme from excess free suicide substrate and measuring total enzyme activity after intermediates E·S and E–S* have completely partitioned to E and E–X. We achieve the removal of S in situ by the addition of a nucleophile (β-mercaptoethanol or hydroxylamine) that reacts rapidly with the ynenol lactone.[4]

Figure 3 demonstrates the in situ technique. The experiment is identical with that of Figure 2a, except at time Δt (8 min here) after addition of **1b**, β-mercaptoethanol was added to a final concentration of 10 mM. In separate experiments in the same buffer the rate of reaction of **1b** with β-mercaptoethanol was found to be 4.5 ± 0.3 M^{-1} s^{-1}. Thus in Figure 3 the half-time for disappearance of **1b** after thiol addition is 15 s, which in addition to the rate of enzyme deacylation (k_3 in Scheme II) accounts for the brief lag between thiol addition and the new steady state due to "rescued" enzyme. A full Kitz and Wilson treatment for real inactivation is available by varying [**1b**] and Δt, with the result that $K_{inact} = 0.63 \pm 0.08$ μM, $k_{inact} = (3.7 \pm 0.1) \times 10^{-3}$ s^{-1}, and $k_{inact}/K_{inact} = 6000 \pm 600$ M^{-1} s^{-1}. A separate series of "rescue" experiments with [**1b**] = 5 μM and hydroxylamine added to 10 mM at Δt were in excellent agreement, with $k_{inact} = (3.8 \pm 0.5) \times 10^{-3}$ s^{-1}.

The irreversibility of HL elastase inactivation by **1b** is also demonstrated by rescue experiments. At long Δt (>30 min) the addition of hydroxylamine resulted in less than 1% recovery of activity. In a separate experiment, enzyme was incubated with **1b** and then separated by gel filtration. The remaining enzyme activity did not increase over a period of 1 h in pH 7.8 buffer, with or without 10 mM β-mercaptoethanol present. The stability of E–X to gel filtration and nucleophiles suggests that the inactive enzyme has been alkylated and is not simply a stable acyl enzyme species.

Dependence on pH: Evidence for Acyl Intermediates. The effect of pH on the apparent second-order rate of inactivation (i.e., as in Figure 2a, with exponential/linear analysis) is presented in Figure 4. The rate decrease below pH 7 is consistent with the protease hydrolysis of esters (Bender et al., 1962; Fersht, 1977) and supports the mechanisms of Schemes I and II in which one or more acyl enzyme intermediates precede inactivation. If k_2 is irreversible, then Figure 4 represents the effect of pH on acylation and/or rearrangement, but not on inactivation (k_4) (Fersht, 1977; Stein, 1983). The data are fit by $v = V/(1 + [H^+]/K_a)$ surprisingly well (apparent $pK_a = 6.58 \pm 0.03$, $V = 11\,700 \pm 300$ M^{-1} s^{-1}), con-

[4] Because the rate constants for general-base hydrolysis of ynenol lactones are small (Spencer et al., 1986), this reaction probably represents attack at the carbonyl followed by ring opening and additional attack at the β carbon of the allenone.

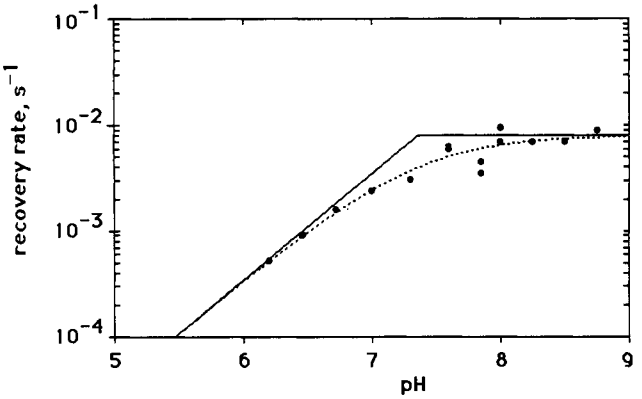


FIGURE 5: pH dependence of HLE recovery. HLE (0.4 mL of 0.3 μM) was partially inactivated by mixing with 0.6 mL of assay buffer and 3 μL of **1b** (10 mM in Me_2SO) and immediately removing excess inhibitor by gel filtration on a PD-10 column at pH 5–6. A 50-μL aliquot of the protein fraction was added to 2.0 mL of buffer (10 mM MES/HEPES/TAPS, 0.5 M NaCl, 0.1% Brij 35) containing 2.5 μM SAAPVFC. Rates of recovery to a final steady-state rate were determined by exponential/linear analysis.

sidering that the ynenol lactone is racemic and that the kinetics become noticeably biphasic at concentrations ≥1 μM.

A simple dependence on pH is expected of enzyme rescue. Gel filtration of partially inactivated enzyme will isolate all enzyme species from excess suicide substrate, and if the resulting enzyme is kept cold at low pH (ca. 5), its partial recovery can be followed in time as it is diluted into higher pH buffer containing fluorogenic substrate. The effect of pH on these recovery rates is shown in Figure 5, where $pK_a = 7.36 \pm 0.04$ and $k_{max} = (8.1 \pm 0.4) \times 10^{-3}$ s^{-1}. Since the recovery rate is k_3 in Scheme II, this pK_a is excellent evidence that E–S* represents an acyl enzyme (Bender et al., 1962). In addition, by knowing k_3 at pH 7.8 (=0.0056 s^{-1}) and assuming that $k_4 = k_{inact}$ (=0.0038 s^{-1}, vide supra), the partition ratio (Walsh, 1982) can be estimated as $k_3/k_4 = 1.5$. This is in good agreement with the following determination by direct titration.

Titration of HS Elastase with **1b**. The partition ratio (r) for suicide substrate inactivation can generally be determined in two ways: first, as the ratio of the separately measured rate constants for inactivation and catalysis (as above), and second, by direct stoichiometric titration of the enzyme activity. HS elastase [used since it was available in much larger quantities than the leukocyte enzyme; it is apparently identical with HLE (Twumasi & Liener, 1977)] was titrated with **1b**. Linear regression of $[E_{active}]/[E_{initial}]$ vs. $[\mathbf{1b}_{initial}]/[E_{initial}]$ gives a horizontal intercept of 2.7 ± 0.5. This intercept equals $r + 1$ (Nagahisa et al., 1983) so that $r = 1.7 \pm 0.5$. The low partition ratio implies that **1b** is a very efficient suicide substrate, and it is tempting to speculate that one enantiomer of **1b** might inactivate 100% of the time while the other enantiomer is turned over at 1.7 times the rate.

Inactivation Is Active Site Directed. Two experiments establish that elastase inactivation by **1b** is active site directed. First, the rate of inactivation is inhibited by a reversible inhibitor that is known to bind at the active site. Second, the enzyme is not inactivated when allenone **9b** is added exogenously rather than being produced at the active site.

Elastatinal is a peptidyl aldehyde (Okura et al., 1975), a class of compound that inhibits by forming a hemiacetal at serine-195 (Delbaere & Brayer, 1985). Inhibition is achieved rapidly, and we find $K_i = 5.2 \pm 0.5$ μM vs. our fluorogenic substrates.[5] When elastatinal is included in experiments such as that of Figure 2a, inactivation is slowed. The effect of elastatinal on the apparent rates of inactivation by **1b** gives a K_i for elastatinal of 4.3 ± 0.5 μM [determined by COMP (Cleland, 1979) with [A] = [**1b**] and [I] = [elastatinal]], in good agreement with its inhibition of turnover.

[5] Feinstein et al. (1976) report $K_i \approx 50$ μM in assays where [E] ~ [I].

Table II: HL Elastase Inhibition and Inactivation by Ynenol Lactones

compd	inhibition[a] K_i (μM)	inactivation rate[b] (M^{-1} s^{-1})	inactivation partition ratio
1a	5.3 ± 0.1	*c*	>50
1b		7600 ± 900	1.6 ± 0.1
1c	≈3	≈600	
1d		12000 ± 2000	
1e	120 ± 5	*c*	*d*
1f	110 ± 8	*c*	>16
1g	90 ± 10	2.4 ± 0.4	
2a	72 ± 4	*c*	*d*
2b	55 ± 10	3200 ± 90	
3	120 ± 20	63 ± 4	
4a	22 ± 1	*e*	*d*
4b	210 ± 20	*c*	*d*
4c		28000 ± 2000	5.3 ± 1.2

[a] Inhibition in initial rate assays. [b] Apparent second-order inactivation rates, as measured by exponential/linear fits to progress curves. [c] No time-dependent behavior observed in continuous assays. [d] Not determined, but probably ≫10. [e] No time-dependent behavior was observed in continuous assays, but in an extended preincubation experiment the rate was ≈3 M^{-1} s^{-1}.

Table III: Serine Protease Inactivation by Ynenol Lactones[a]

compd	enzyme: HL elastase	PP elastase	trypsin	chymotrypsin
1b	7600 ± 900	730 ± 70	17 ± 3	*b*
1d	12000 ± 2000	260 ± 30	27 ± 2	
3	63 ± 4	6.7 ± 0.5		≈400
4c	28000 ± 2000	5500 ± 700		*b*

[a] Data are apparent inactivation rates in M^{-1} s^{-1}. [b] Complex behavior: incomplete inactivation.

The putative alkylating agent in Scheme I is **7**, which represents an allenone (**9**) tethered at serine-195 as an acyl intermediate. In any suicide inactivation scheme it is important to know whether the reactive species inactivates the enzyme while it is within the active site (**7** → **8**) or whether it is released (**7** → **9** and/or **6** → **10** → **9**) and inactivates by nonspecific alkylation. This can be tested if the reactive species can be generated separately and added to active enzyme. Allenones **9** are reactive and difficult to purify, but they can be generated in situ and have characteristic UV spectra with known extinction coefficients (Spencer et al., 1986). Allenone **9b** was generated from **1b** by mild alkaline hydrolysis and then added to excess assay buffer to give a solution 100 μM in **9b**. Enzyme and fluorogenic substrate were added, and neither inhibition nor inactivation was observed. Since 1 μM **1b** inhibits dramatically (Figure 2a), this is good evidence that inactivation cannot proceed by release of **9b** and subsequent alkylation.

Inhibition and Inactivation by Other Ynenol Lactones: Substituent Effects and Enzyme Specificity. We have demonstrated that ynenol lactones **1a** and **1f** are alternate substrate inhibitors of HLE and that **1b** is an efficient suicide inactivator. The characterization of HLE inhibition by other ynenol lactones is given in Table II. Enzyme inactivation is very sensitive to substitution, since simple modifications of structure result in up to 10 000-fold variations in the apparent rates of inactivation.

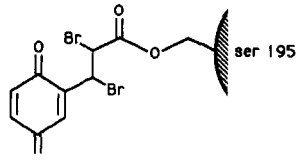
Table III presents the apparent rates of inactivation of three other serine proteases. The ynenol lactones shown are among

the better inactivators of HLE. In general, HL elastase is inactivated more rapidly than either PP elastase or bovine trypsin by factors of approximately 10 and 400, respectively. The reaction of chymotrypsin with ynenol lactones is complex; acyl intermediate(s) is (are) clearly formed, but subsequent inactivation is incomplete. These results will be presented separately.

Discussion

The alkylating inactivators of Table I involve two principal types of electrophilic functionality: activated haloalkanes and Michael acceptors. On the basis of chemical precedent, haloalkanes probably react with displacement of the halide by a nucleophile Nu: to produce the C–Nu bond, and Michael acceptors react by conjugate addition of Nu: to an electron-deficient olefin. The chemistry of these reactions is very different, especially with respect to geometric constraints, and it is interesting that both strategies have led to effective alkylating inactivators of serine proteases.

The Michael acceptor strategies of Bêchet et al. (1973) and Harper and Powers (1984, 1985) are similar in the quinone methide structures proposed for the tethered electrophiles (**11** and **12**, respectively). Bêchet et al. (1973) have shown that



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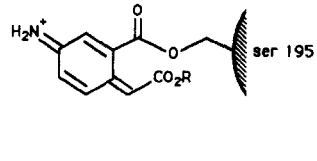

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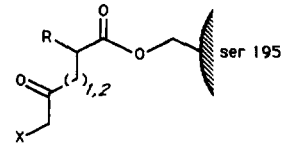
histidine is the nucleophile that adds to **11**. Harper and Powers (1985) give evidence to suggest that histidine-57 is the nucleophile in their case as well, including a high-resolution crystal structure of PP elastase and an acetate adduct of **12** in which histidine-57 is hydrogen-bonded to the ester (Meyer et al., 1985). In addition, this crystal structure and that of the adduct of 5-benzyl-6-chloro-2-pyrone with chymotrypsin (Ringe et al., 1985) establish that O^{γ} of serine-195 is the point of attachment for these small heterocyclic inhibitors.

The ynenol lactones presented here are more similar in their initial chemistry and steric constraints to the haloenol lactones of Katzenellenbogen and colleagues (Chakravarty et al., 1982; Daniels et al., 1983; Naruto et al., 1985; Daniels & Katzenellenbogen, 1986), which are proposed to inactivate via the tethered halomethyl ketone **13**. There is good evidence that



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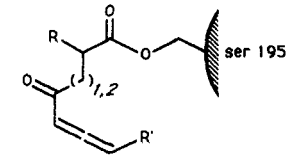

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serine-195 is also the point of initial acylation for the haloenol lactones and that histidine-57 is also the nucleophile that is alkylated by the unmasked halomethyl ketone (Naruto et al., 1985). The calculations of Naruto et al. (1985) and the variety of tethered electrophiles represented by **11**–**13** underscore the nucleophilicity of histidine and the flexibility of the active sites. In light of the evidence presented here that ynenol lactones are substrates for HLE and inactivate via an acyl intermediate, we suggest that the electrophilic intermediate is **7** and that the nucleophile is most likely to be histidine-57.

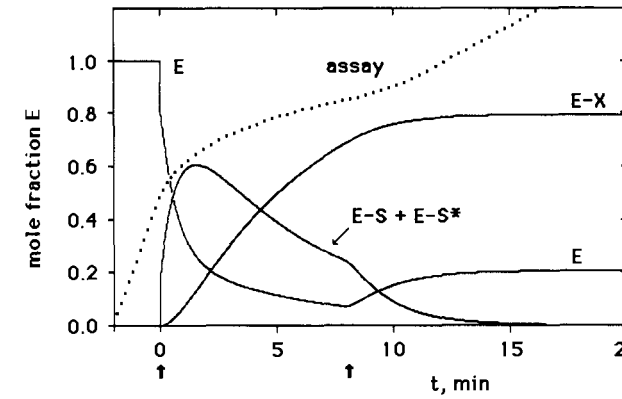

FIGURE 6: Computer simulation of the rescue experiment of Figure 3. Scheme II was used as the basis for a simulation with k_{-1}/k_1 = 4.1 μM, k_2 = 0.090 s^{-1}, k_3 = 0.0056 s^{-1}, and k_4 = 0.0037 s^{-1}. The initial conditions were [E] = 0.003 μM and [S] = [E·S] = [E–S*] = [E–X] = 0. At t = 0, [S] was changed from 0 to 1.0 μM, and at t = 8 min a first-order decrease in [S] of 0.045 s^{-1} was begun to simulate the addition of β-mercaptoethanol.

The kinetics of HL elastase inactivation by ynenol lactone **1b** are complex. Since **1b** is a racemic mixture, the observed rates of acylation and deacylation probably represent a combination of rate constants; for example, the deacylation rates of the chymotrypsin esters of D- and L-amino acids vary by large factors (Blow, 1971). Scheme II is therefore a very simplified model for the kinetics of inactivation. Nonetheless, calculations based on Scheme II give reasonable simulations of rescue experiments as shown in Figure 6, which is a simulation of the experiment of Figure 3. The dotted line marked "assay" in Figure 6 is proportional to $\int$[E] dt and should therefore parallel the accumulating fluorescence of the product in Figure 3. The approximate agreement in the extent of irreversible enzyme inactivation also provides support for the simple kinetic model: in Figure 3 the ratio of final rate to initial rate ($\equiv[E_\infty]/[E_0]$) is 0.32, and in the simulation it is 0.21.[6] Note in the simulation that the disappearance of [E] (from t = 0 to 8 min) is clearly not first order and that E·S and E–S* are never constant enough to warrant a steady-state treatment (Tatsunami et al., 1981).

The range of apparent inactivation rates in Table II (from <2 to 28 000 M^{-1} s^{-1}) illustrates the importance of substitution in the design of suicide substrates. The most striking pattern in the data of Table II is that substitution α to the lactone carbonyl is essential for inactivation of HLE. Those compounds that are α-unsubstituted (**1a**, **1e**, **1f**, **2a**, **4a**, **4b**) exhibit initial inhibition in the 20–200 μM range but little or no subsequent time-dependent inhibition. They are probably all alternate substrate inhibitors, as demonstrated for **1a** and **1f**, and so their negligible inactivation implies that deacylation (Scheme I, **6** → **10** or **7** → **9**) is much more rapid than alkylation (**7** → **8**). Westkaemper and Abeles (1983) have similarly shown that substitution on 6-chloro-2-pyrones is essential for enzyme inactivation and indeed directs the regiochemistry of serine-195 attack. As has been demonstrated for the chloropyrones (Ringe et al., 1985), we suggest that the α-substituent of ynenol lactones occupies the P_1 site of HLE and thereby provides an orientation essential for inactivation.

Lactone ring size is also an important factor. Daniels et al. (1983) find that with chymotrypsin the haloenol valerolactones are more rapid inactivators than the corresponding butyrolactones by an average factor of 6000[7] and also have

[6] If β-mercaptoethanol acts as a catalyst in the deacylation of E·S or E–S* (Gelb & Abeles, 1984) as well as in the decomposition of S, then the calculated $[E_\infty]/[E_0]$ can increase from 0.21 to 0.31.

lower partition ratios (Daniels & Katzenellenbogen, 1986). For ynenol lactones with HLE the rate factor is much smaller; the fastest inactivator is the 3-benzyl valerolactone **4c**, which is 3.7-fold more rapid than the 3-benzyl butyrolactone **1b**. Note that for haloenol lactones the electrophilic carbon is generated α to the carbonyl (**13**) and for ynenol lactones the electrophilic carbon is β (**7**), suggesting that rapid inactivation requires a minimum chain length of six carbon atoms from serine O^{γ} to the electrophilic carbon.

Only those ynenol lactones that are unsubstituted at the acetylene terminus (**1**, R′ = H) are rapid inactivators. Comparison of **1b** and **1g** shows that methyl substitution slows apparent inactivation 3000-fold, and ynenol lactones with other acetylene substituents (**1e**, **1f**, **4a**, **4b**) show little or no inactivation. We have shown that allenone formation is slowed 300-fold by such substitution in a model system (1-hexyn-4-one → 1,2-hexadien-4-one vs. 2-hexyn-5-one → 2,3-hexadien-5-one; Spencer et al., 1986). In addition, steric constraints imposed by allenone γ-substitution (**7**, R′ ≠ H) could hinder conjugate addition of the nucleophile Nu:, thereby preventing inactivation. In model systems the effects of allenone γ-substitution on hydroxide or *n*-butylamine addition are small (Spencer et al., 1986), but the effect could be greater in the more restricted environment of an active site. One possibility is that γ-substituted allenones are formed in the active site with a chirality that is unfavorable for capture of an enzyme nucleophile.

Registry No. **1a**, 93040-44-9; **1b**, 103437-63-4; **1c**, 103437-65-6; **1d**, 103437-67-8; **1e**, 103437-69-0; **1f**, 103437-70-3; **1g**, 103437-71-4; **2a**, 103437-72-5; **2b**, 103437-73-6; **3**, 103437-74-7; **4a**, 103437-75-8; **4b**, 103437-76-9; **4c**, 103437-77-0; HLE, 9004-06-2; SAAPVC, 72252-90-5; 7-[(trimethylacetyl)oxy]-4-methylcoumarin, 66185-69-1; 4-methyl-7-hydroxycoumarin, 90-33-5; trimethylacetic anhydride, 1538-75-6.

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Thermodynamic Study of Yeast Phosphoglycerate Kinase

Cui Qing Hu and Julian M. Sturtevant*

Departments of Chemistry and of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511

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ABSTRACT: Enthalpies of binding of MgADP, MgATP, and 3-phosphoglycerate to yeast phosphoglycerate kinase have been determined by flow calorimetry at 9.95–32.00 °C. Combination of these data with published dissociation constants [Scopes, R. K. (1978) *Eur. J. Biochem. 91*, 119–129] yielded the following thermodynamic parameters for the binding of 3-phosphoglycerate at 25 °C: $\Delta G^\circ = -6.76 \pm 0.11$ kcal mol^{-1}, $\Delta H = 3.74 \pm 0.08$ kcal mol^{-1}, $\Delta S^\circ = 35.2 \pm 0.6$ cal K^{-1} mol^{-1}, and $\Delta C_p = 0.12 \pm 0.32$ kcal K^{-1} mol^{-1}. The thermal unfolding of phosphoglycerate kinase in the absence and presence of the ligands listed above was studied by differential scanning calorimetry. The temperature of half-completion, $t_{1/2}$, of the denaturation and the denaturational enthalpy are increased by the binding of the ligands, the increase in $t_{1/2}$ being a manifestation of Le Chatelier's principle and that in enthalpy reflecting the enthalpy of dissociation of the ligand. Only one denaturational peak was observed under all conditions, and in contrast with the case of yeast hexokinase [Takahashi, K., Casey, J. L., & Sturtevant, J. M. (1981) *Biochemistry 20*, 4693–4697], no definitive evidence for the unfolding of more than one domain was obtained.

Yeast phosphoglycerate kinase (EC 2.7.2.3) (PGK)[1] consists of a single polypeptide chain of some 420 amino acid residues and has a molecular mass of 45 000–50 000 daltons (Scopes et al., 1973). High-resolution X-ray studies (Banks et al., 1979) show that in the crystalline state the molecule is composed of two globular lobes of approximately equal size separated by a cleft. As with other kinases (Anderson et al., 1979), substrate binding induces a hinge-bending conformational change which closes the cleft.

In previous work from this laboratory (Takahashi et al., 1981) the thermodynamics of the binding of glucose to yeast hexokinase and of the thermal unfolding of the enzyme in the absence and presence of glucose were investigated. In this paper we report similar work on yeast phosphoglycerate kinase in the absence and presence of the ligands 3-phosphoglycerate, MgATP, and MgADP.

[1] Abbreviations: PGK, yeast phosphoglycerate kinase; 3-PG, 3-phosphoglycerate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); DTE, dithioerythritol; DSC, differential scanning calorimetry.

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