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Inhibitors Directed to Binding Domains in Neutrophil Elastase†

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ABSTRACT: Human neutrophil elastase (HNE) can be inhibited by unsaturated fatty acids, including oleic acid [Ashe, B. M., & Zimmerman, M. (1977) *Biochem. Biophys. Res. Commun. 75*, 194-199; Cook, L., & Ternai, B. (1988) *Biol. Chem. Hoppe-Seyler 369*, 627-631], but is not affected by saturated fatty acids. We have shown that the interaction of oleic acid with HNE can be characterized by two apparent inhibitory modes: a high-affinity mode (K_i = 48 ± 3 nM), resulting in partial noncompetitive inhibition (87% residual activity), and a competitive inhibitory mode of lower affinity (K_i = 16 ± 1 μM). Binding of oleate in the high-affinity mode induces a blue shift in the endogenous fluorescence arising from the tryptophan residues in HNE. This shift is maximal in the presence of 1 μM oleate; higher concentrations of fatty acid have no further effect on the fluorescence spectrum. The negatively charged fluorescent ester of oleic acid and hydroxypyrenetrisulfonate (HPTSoleate) interacts with HNE at an apparent single site (K_i = 44 ± 3 nM), resulting in competitive inhibition. A blue shift in the emission maximum of the pyrene fluorescence at 410 nm and a decrease in the ratio of the intensities of the maximum at 388 and 410 nm indicate that upon binding to HNE the environment of the pyrene ring in HPTSoleate becomes more hydrophobic. In order to probe further the roles of nonpolar and electrostatic interactions in binding of negatively charged hydrophobic inhibitors to HNE, the enzyme was modified with the arginine-specific reagent pyreneglyoxal (PYG). Under conditions in which only two arginines are modified by PYG, the catalytic activity of HNE is eliminated. Upon reaction with HNE, the two maxima in the emission spectrum of PYG are both blue-shifted, and the ratio of the intensities around 378 and 395 nm is decreased, indicating increased hydrophobicity of the environment surrounding the pyrene ring. An additional blue shift of both maxima and a further change in intensity ratio are seen in the presence of oleic acid, but only at high concentrations (200 μM), suggesting that the apparent high-affinity mode of binding for oleate may no longer be accessible after reaction of HNE with PYG. These results suggest a role for at least one arginine residue in a hydrophobic environment in regulating substrate binding and catalysis by HNE. Inhibitors which interact with both this positively charged center and the neighboring hydrophobic environment should be especially potent and selective for HNE.

Human neutrophil elastase (HNE)[1] is believed to be responsible for much of the damage to connective tissues associated with inflammatory processes (Travis & Salvesen, 1983; Janoff, 1985; Bieth, 1986). Interactions between this protease and the proteins of the extracellular matrix are dominated by hydrophobic interactions (Lonky & Wohl, 1983; Lestienne & Bieth, 1980). The extended substrate binding pocket of HNE contains an abundance of hydrophobic amino acid side chains, including a number of phenylalanine rings (Bode et al., 1986).

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[1] Abbreviations: HNE, human neutrophil elastase; DMF, dimethylformamide; PYG, pyreneglyoxal; HPTSoleate, ester of oleic acid and 8-hydroxypyrene-1,3,6-trisulfonate; MeOSucAAPVpNa, methoxysuccinylalanylalanylprolylvalyl-*p*-nitroanilide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; PPE, porcine pancreatic elastase; IEF, isoelectric focusing; PBS, phosphate-buffered saline.

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Hydrophobic inhibitors of HNE may interact with these nonpolar domains in the vicinity of the substrate binding site of the enzyme. In addition to the nonpolar side chains in the extended substrate binding cavity, a center of positive charge near the substrate binding site has been inferred from the increased potency and specifity associated with negatively charged inhibitors of HNE (Ashe & Zimmerman, 1977; Harper et al., 1983; Bode et al., 1986; Cook & Ternai, 1988, 1989). A number of studies have appeared which identify the unsaturated fatty acid oleic acid as an inhibitor of HNE (Ashe & Zimmerman 1977; Cook & Terani, 1988, 1989). Other serine proteases, including pancreatic elastase, trypsin, chymotrypsin, and neutrophil cathepsin G, are not affected by oleic acid. Saturated fatty acids, such as stearic acid do not inhibit HNE.

The positively charged site on HNE which is assumed to participate in electrostatic interactions with anionic inhibitors most likely involves at least 1 of the 19 arginine residues in this enzyme (Bode et al., 1989), since there are no lysines in the protein (Sinha et al., 1987). A recent report on inhibition of HNE by EDTA suggests that the polyanionic chelator may associate with at least one arginine side chain (Burnett et al., 1988). Davril et al. (1984) have reported on inactivation of porcine pancreatic elastase by a number of vicinal diones, but comparable studies on arginines in HNE have not been described. Bode et al. (1986) have noted that all but 2 of the 19 arginines in HNE are located in patches on the surface of the enzyme, whereas the side chains are Arg-177 and Arg-217 are located in proximity to the hydrophobic extended substrate binding domain.

The present work is aimed at characterizing further the nature of the inhibition of HNE by oleic acid and the relationship of this inhibition to interactions involving hydrophobic domains and positive charged guanidino side chains in the enzyme. In order to probe these two types of interactions, we have employed two pyrene derivatives: a polyanionic ester of oleic acid and 8-hydroxypyrene-1,3,6-trisulfonate (HPTSoleate), and the vicinal dione 1-pyreneglyoxal (PYG). The fluorescence of the pyrene ring on these derivatives is typically shifted to the blue as the hydrophobicity of the surrounding environment is increased. In addition, we have studied the effect of oleic acid on the endogenous fluorescence of the three tryptophan residues in HNE to see if this inhibitor induces any perturbation in the structure of the protein.

Materials and Methods

Materials. HNE was obtained from Elastin Products Co. (St. Louis, MO); its purity was confirmed by SDS–PAGE. PYG and HPTSoleate were from Molecular Probes (Eugene, OR). Methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (MeOSucAAPVpNa), L-arginine, and oleic and stearic acids were from Sigma (St. Louis, MO). Ampholytes and disposable minicolumns packed with Sephadex G-25 were from Isolab, Inc. (Akron, OH).

Assays of HNE Activity. The amidolytic activity and HNE was assayed with MeOSucAAPVpNa as a substrate (Nakajima et al., 1979). The release of *p*-nitroaniline was monitored by recording the absorbance at 405 nm with a V_{max} multiwell microplate reader (Molecular Devices, Menlo Park, CA) operating in the kinetic mode. The substrate concentrations were varied from 0.02 to 0.60 mM. The final HNE concentration was set at 0.03 μM for determinations of K_i for HPTSoleate, at 0.1 μM for determinations of K_i for oleic acid, and at 0.50 μM for determination of stoichiometry of binding. The buffer used throughout contained 3.3% dimethylformamide and 0.01% Triton X-100. Kinetic data were collected for 5 min, except for studies involving simultaneous addition of substrate and PYG. During this 5-min period, reaction rates remained linear. Activities in the presence of inhibitor (v) were all normalized to controls determined in the absence of inhibitor (v_0).

Inactivation of HNE by PYG. (*a*) *Stoichiometry and Rate of Inactivation.* The reaction of 0.04 μM HNE with different concentrations of PYG in a total reaction volume of 2 mL was allowed to proceed in 100 mM borate buffer, pH 8.3, containing 3.3% DMF and 0.01% Triton X-100 at 25 °C. At various times, 112.5-μL aliquots were removed and added to 37.5 μL of a 1.2 mM MeOSucAAPVpNa solution (final substrate concentration = 0.3 mM). The kinetics of the increase of absorbance at 405 nm were monitored on the V_{max} microplate reader as described above.

(*b*) *Protection by Substrate.* An aliquot of HNE was added to a mixture of PYG and MeOSucAAPVpNA to measure the rate of inactivation in the presence of substrate. The final concentrations were PYG = 15 μM, MeOSucAAPVpNa = 0.3 mM, and HNE = 0.033 μM. The reaction was allowed to proceed at 25 °C with continuous recording by the V_{max} microplate reader for 40 min.

Preparative Isolation of a PYG–HNE Complex. A reaction mixture containing 70 μM HNE and 0.2 mM PYG in 0.5 mL of PBS, pH 7.4, containing 3.3% DMF and 0.01% Triton X-100, was incubated at 25 °C for 1 h. Measurements of residual enzyme activity indicated that no further activity could be detected after 45 min. The reaction mixture was separated from unreacted PYG by two cycles of centrifugation through a Sephadex G-25 column (Isolab) according to the method of Penefsky (1979). The isolated PYG–HNE complex was stable for 6 months at −20 °C and for 2 weeks at 4 °C as judged by the invariance of the fluorescence spectrum of the bound PYG.

Isoelectric Focusing Gels. Isoelectric focusing in 12 × 10 cm polyacryamide slab gels was carried out at 22 °C by the methods of Righetti and Drysdale (1971) and Vesterberg (1972) with minor modifications. The final concentrations of reagents in the gels were 4% acrylamide, 0.16% *N,N'*-methylenebis(acrylamide), 0.02% *N,N,N',N'*-tetramethylenediamine, 0.02% ammonium persulfate, 6 M urea, and 2% (w/v) ampholyte buffer (3–10 pH range). The gel solutions were deaerated and left for 30–60 min to set.

The anode and cathode reservoirs were filled with solutions of 0.01 M H_3PO_4 and 0.02 M NaOH, respectively, with the anode at the top of the gel. After prerunning the gels at 10 mA/slab for 10 min to remove excess ammonium persulfate, samples (10–20 μL) containing 10–20 μg to protein in 2% ampholyte and 6 M urea were applied. Focusing was performed at constant current (10 mA) until the voltage had risen to 400 V. The voltage was then maintained at this value for 6 h. Gels were stained with Coomassie brilliant blue.

SDS–PAGE Gels. SDS–PAGE was performed on vertical slab gels according to a modification of the procedure of Laemmli (1970), using a 4.5% separating gel (pH 6.8). All solutions contained 0.1% SDS.

Inhibition of HNE by HPTSoleate. (*a*) *Stoichiometry.* Various concentrations of HPTSoleate were incubated with 1.5 μM HNE in PBS, pH 7.4, containing 0.01% Triton X-100. An aliquot of 50 μL was removed from each incubation mixture and added to 62.5 μL of the same buffer along with 37.5 μL of a 1.2 mM MeOSucAAPVpNa solution, and the absorbance at 405 nm was followed immediately thereafter with the V_{max} microplate reader as above. The final enzyme concentration during the assay was 0.5 μM.

(*b*) *Determination of* K_i. Various concentrations of HPTSoleate (final concentration = 15–600 nM) were preincubated with HNE (final concentration = 0.03 μM), and the residual activity was assayed in the V_{max} microplate reader with six different concentrations of MeOSucAAPvpNa (final concentration = 0.02–0.60 mM). All reactions were carried out in PBS, pH 7.4, containing 0.01% Triton X-100. Data were analyzed according to the methods discussed by Green and Work (1953), Henderson (1972), Beith (1974), and Empie and Laskowski (1982) for inhibition under conditions in which a substantial fraction of the inhibitor is bound to the enzyme.

Inhibition of HNE by Oleic Acid. Inhibition of HNE by oleic acid was performed as described for HPTSoleate except that the final concentration of HNE was increased to 0.10 μM, and the oleic acid final concentration range was extended from 50 nM to 80 μM. The data were analyzed by the methods referred to above.

Spectroscopic Methods. Absorption spectra were recorded with a Hewlett Packard 8452A diode array spectrophometer. PYG and HPTSoleate were kept as 0.1 M stock solutions in dry DMF and were diluted into buffer containing 3.3% DMF before use. The concentrations of PYG and HPTSoleate in these final working solutions were confirmed by using an extinction coefficient for PYG of 46 000 M^{-1} cm^{-1} at 346 nm, and for HPTSoleate of 37 000 M^{-1} cm^{-1} at 370 nm, which are both assigned to the 0,0–1,1 transition in the pyrene ring (Jaffe & Orchin, 1967; Knopp & Weber, 1967). Oleic acid, stearic acid, and hydroxylamine concentrations were based on weight measurements. The concentrations of a set of HNE standards were calculated by using a value of $\epsilon_{280}^{1\%} = 9.85$ (Baugh & Travis, 1976), and these samples were then used to generate a standard curve employing the protein assay procedure of Bradford (1976). L-Arginine concentration was determined by weight and confirmed by the extinction coefficient of 1360 M^{-1} cm^{-1} at 205 nm (Fasman, 1976).

All fluorescence measurements were recorded on a computer-controlled Spex Datamate spectrofluorometer. The band-pass width was set at 1.25 nm for excitation and 2.5 nm for emission. The spectra were recorded at 1-nm intervals and corrected for base line and instrument response. Micro cells (0.3 × 0.3 cm, 100-μL capacity) were used for all the experiments. Samples were prepared and incubated for the appropriate time before their spectra were recorded. All spectroscopic measurements were carried out at 25 °C.

Results

Inhibition of HNE by Reaction with PYG. The reaction of HNE with PYG results in a time-dependent loss of catalytic activity (Figure 1A). In borate buffer at pH 8.3, 100 nM PYG inactivates 33 nM HNE with a $t_{1/2}$ of approximately 8 min at 25 °C. By 40 min, less than 2% residual activity remains. This loss of catalytic activity is slowly reversed by incubating the inhibited enzyme with hydroxylamine; about 30% of the activity is restored after 30 min of incubation with 1 μM hydroxylamine at 25 °C. The rate of inactivation is a function of the PYG concentration in the reaction mixture; at 15 μM PYG, the $t_{1/2}$ for inactivation of 33 nM HNE is less than 0.5 min (data not shown). The extent of inhibition at completion is a function of the molar ratio of PYG to HNE in the reaction mixture (Figure 1B). There is a roughly linear dependence of loss of activity on this molar ratio, with complete inactivation achieved at a ratio of PYG to HNE of about 2:1. The rate of inactivation is slowed if substrate is present; in the presence of 0.3 mM MeOSucAAPVpNa, the $t_{1/2}$ for inactivation of 33 nM HNE by 15 μM PYG is increased at least 10-fold, to 4–5 min (Figure 1C). These data suggest that

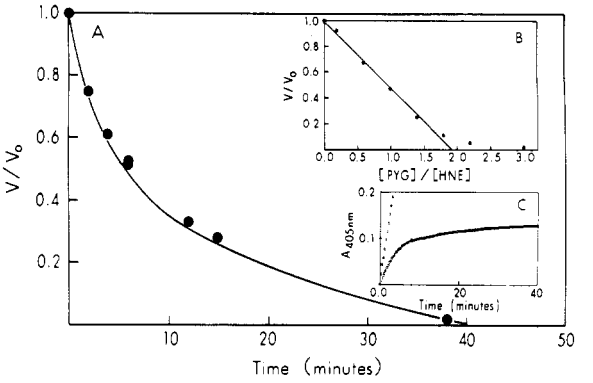

FIGURE 1: (A) Time-dependent inactivation of HNE by PYG. HNE (33 nM) was incubated with PYG (0.1 μM) at 25 °C in 100 mM borate (pH 8.3) containing 3.3% DMF and 0.01% Triton X-100. Amidolytic assays were started by addition of 0.3 mM MeOSucAAPVpNa. The rates of release of *p*-nitroaniline over the first 5 min of assay (v) were all normalized to the amidolytic rate in the absence of PYG (v_0). The line is a visually smoothed fit to the data points. (B) Stoichiometry of inhibition of HNE amidolytic activity by PYG. HNE (33 nM) was incubated for 45 min at 25 °C with different aliquots of a PYG stock solution (concentration determined spectrophotometrically). Residual HNE amidolytic activity was detected by addition of 0.3 mM MeOSucAAPVpNa in 100 mM borate (pH 8.3) containing 3.3% DMF and 0.01% Triton X-100. The x-intercept value of 1.9 ± 0.1 calculated from a least-squares fit of the data is consistent with complete inactivation of HNE by two molecules of PYG. (C) Effect of the presence of substrate on the rate of inhibition of HNE by PYG. HNE (33 mM) was added to a mixture of PYG (15 μM) and MeOSucAAPPVpNa (0.3 mM) in borate (pH 8.3) containing 3.3% DMF and 0.01% Triton X-100 at 25 °C. The release of *p*-nitroaniline (absorbance at 405 nm) was recorded as a function of time. The trace with the steep linear slope in the panel is the record of amidolytic activity in the absence of PYG.

interaction of the oligopeptide analogue chromogenic substrate, MeOSucAAPPVpNa, with some part of the extended substrate binding site protects HNE from reaction with PYG.

Reaction of vicinal diones with the guanidino side chain of arginine leads to a measurable spectral change. We have observed an increase in the extinction at 430 nm upon reaction of PYG with arginine, analogous to that reported for the reaction of arginine with (*p*-nitrophenyl)glyoxal (Yamasaki et al., 1981). This extinction difference permits determination of the apparent reaction stoichiometry by spectrophotometric titration. We have measured the increase in absorbance at 430 nm as a function of the concentration of PYG added to fixed concentrations of arginine or HNE and have estimated the apparent stoichiometries from the breaks in the curves (Figure 2). In a separate experiment, we determined that the increase in absorance at 430 nm is time-dependent, consistent with the rate of inactivation HNE by PYG. Reaction of arginine with all concentrations of PYG was complete within 30 min, and reaction of HNE was complete within 45 min under the conditions we have employed (data not shown). We therefore allowed all reaction mixtures to incubate for 1 h prior to recording their absorbance in the spectrophotometric titration experiments. No further increases in molar extinction of the reaction mixtures occur beyond a 1:1 molar ratio of PYG to arginine, or beyond a 2:1 ratio of PYG to HNE, suggesting that one PYG combines with each guanidino side chain and that two arginines are modified by PYG in the HNE molecule. We have separated the PYG–HNE complex from excess reagent by gel filtration prior to submitting the modified enzyme to isoelectric focusing. The p*I* of the protein is decreased after modification, consistent with reaction of PYG with arginine side chains.

Inhibition of HNE by Oleic Acid. In order to conform to the criteria outlined by Silverman (1988) for analysis of in-

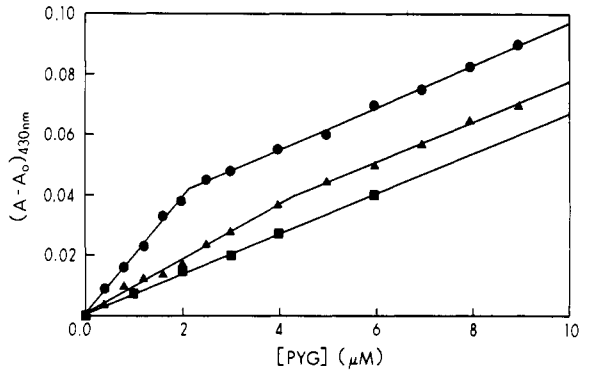

FIGURE 2: Stoichiometry of modification of HNE (1 μM) and of arginine (4.5 μM) by PYG. The increase in absorbance at 430 nm is shown as a function of PYG added. (■) PYG alone; (▲) PYG + arginine; (●) PYG + HNE. Samples were incubated in 100 mM borate (pH 8.3) containing 3.3% DMF and 0.01% Triton X-100 at 25 °C for 1 h to ensure all reactions had gone to completion before the absorbance was recorded.

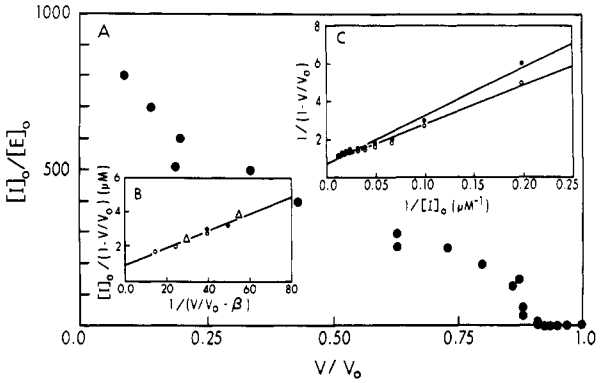

FIGURE 3: (A) Inhibition of HNE (0.1 μM) by various concentrations of oleic acid. After oleic acid was mixed with the enzyme, the residual amidolytic activity was assayed by addition of various concentrations of MeOSucAAPVpNa as described under Materials and Methods. The points shown were obtained with 0.6 mM MeOSucAAPVpNa. The amidolytic rates (v) were all normalized to the rate observed in the absence of oleate (v_0). (B) Replots of the data in Figure 3A at low oleate concentrations in the presence of (○) 0.6, (●) 0.3, (△) 0.15 mM MeOSucAAPVpNa. The line is a linear least-squares fit, the slope of which gives a value of $K_{i(app)} = K_i$ of 48 ± 3 nM. (C) Replots of the data in (A) at higher oleate concentrations in the presence of (○) 0.3 and (●) 0.6 mM substrate concentrations. The lines are the linear least-squares fit to the data.

hibition by the partition ratio method, we varied the ratios of oleic acid to HNE in amidolysis assays from 0.5 to 800. The dependence of inhibition on the ratio of inhibitor to enzyme is apparently biphasic (Figure 3A). At low concentrations of oleic acid, a partial inhibition can be detected (Figure 3B). This reduction in catalytic activity is apparently saturable (at ca. 1 μM oleate) but is independent of substrate concentration, and is therefore classified as partial noncompetitive (Segel, 1975); i.e., at any inhibitor concentration, the velocity of amidolysis can be described as

$$v = k_{cat}[ES] + \beta k_{cat}[ESI] \quad (1)$$

The amidolysis of MeOSucAAPVpNa by HNE in the absence of inhibitors conforms to simple Michaelis–Menten kinetics. The apparent value of K_m for this substrate under the conditions we have employed is 0.17 ± 0.02 mM (data not shown). We have determined the value of β from the dependence of the y intercept of double-reciprocal plots of $1/v$ vs $1/[S]$ on inhibitor concentration (data not shown). At [I] = 0, this intercept = $1/V_{max}$, and at high [I], the value of the intercept extrapolates to $1/\beta V_{max}$. From such plots, we compute a value of $\beta = 0.87 \pm 0.02$. In order to obtain an apparent value of K_i, we have used a modification of the Henderson plot for tightly bound partial inhibitors (Green & Work, 1953; Morrison, 1969; Henderson, 1972; Bieth, 1974; Empie & Laskowski, 1982):

$$\frac{[I]_0}{1 - v/v_0} = \frac{K_{i(app)}}{v/v_0 - \beta} + \frac{[E]_0}{1 - \beta} \quad (2)$$

In this expression, the fractional activity, v/v_0, ranges from 1 to β, rather than from 1 to 0 as in the case of simple inhibitors. Because the value of $K_{i(app)}$ for this mode of inhibition by oleate is independent of substrate concentration, the general expression gives a true value of K_i in this case. The value of K_i for this mode of inhibition by oleate computed from the data in Figure 3B is 48 ± 3 nM.

At higher oleate concentrations, a different mode of inhibition can be observed. The affinity of HNE for oleate bound in this inhibitory mode is relatively weak; at a ratio of inhibitor to enzyme of 50:1, amidolytic activity is reduced by only 10%. Accordingly, we have analyzed this inhibition in Figure 3C by a modified form of the Dixon plot (1953):

$$\frac{1}{1 - v/v_0} = \frac{K_{i(app)}}{[I]} + 1 \quad (3)$$

In this expression, the inhibitor concentration, [I], is rigorously defined as the concentration of free inhibitor, but since the total inhibitor concentration is ca. 2 orders of magnitude greater than that of the total enzyme concentration, the amount of bound inhibitor can be disregarded. As can be seen from Figure 3C, the slope of this plot, $K_{i(app)}$, is dependent on substrate concentration, and therefore reflects some competitive character. The data can be fit to a model of simple competitive inhibition:

$$K_{i(app)} = K_i(1 + [S]/S_m) \quad (4)$$

giving a value for K_i of 16 ± 1 μM.

Neither mode of inhibition by oleic acid can be attributed to a general effect of hydrophobic fatty acids on HNE. No inhibition of the enzyme was seen in the presence of concentrations of stearic acid up to 200 μM.

Inhibition of HNE by HPTSoleate. HPTSoleate is also a tightly bound inhibitor of HNE. The level of inhibition of HPTSoleate was independent of the order of addition of substrate and inhibitor within the limits of manual mixing times, justifying the assumption of equilibrium binding. Under conditions in which a significant fraction of the inhibitor is bound, the inhibition can be analyzed by the partition ratio method as discussed above (Figure 4). The inhibition is sensitive to substrate concentration, and the Henderson plots conform to a model of simple competitive inhibition (Figure 4B) (Green & Work, 1953; Morrison, 1969; Henderson, 1972; Bieth, 1974; Empie & Laskowski, 1982):

$$\frac{[I]_0}{1 - v/v_0} = \frac{K_i(1 + [S]/K_m)}{v/v_0} + [E]_0 \quad (5)$$

From plots at several different substrate concentrations, we have computed a value of K_i for HPTSoleate of 44 ± 3 nM. At concentrations of enzyme which are 10 times the K_i, addition of increasing concentrations of HPTSoleate gives rise to a titration curve with a roughly linear segment at the lower end of the range of inhibitor concentrations (Figure 5). The slope of the linear portion of the curve is 1.3 ± 0.1 and is consistent with a stoichiometry of inhibition of 1:1. The solid line in Figure 5 represents the nonlinear least-squares fit of

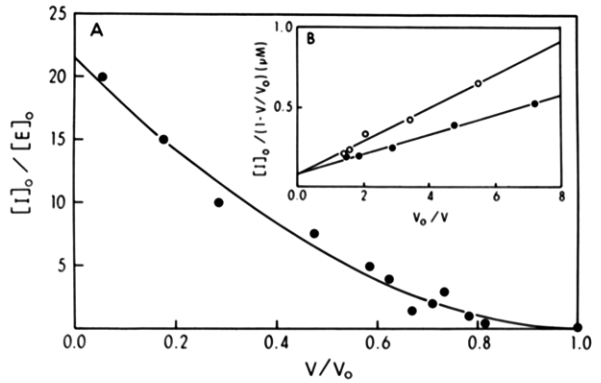

FIGURE 4: (A) Inhibition of HNE (30 mM) by various concentrations of HPTSoleate. After addition of HPTSoleate to the enzyme, the residual amidolytic activity was assayed as in Figure 3. The curve is a visually smoothed fit to the data points, which were obtained at 0.6 mM MeOSucAAPVpNa. All amidolytic rates (v) were normalized to the rate observed in the absence of HPTSoleate (v_0). (B) Replots of the data at (●) 0.15, and (○) 0.6 mM substrate concentrations. The lines are the linear least-squares fits to the data.

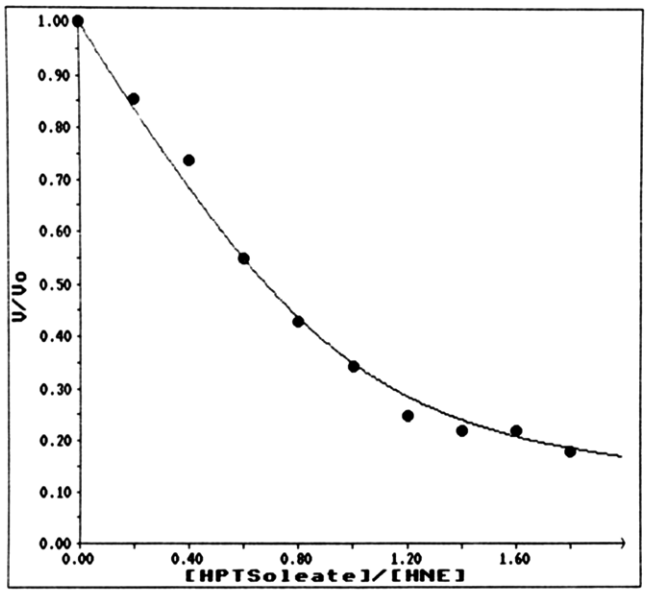

FIGURE 5: Stoichiometry of binding of HPTSoleate to HNE. Aliquots of a stock solution of HPTSoleate were equilibrated with 1.5 μM HNE, and residual amidolytic activity was determined by dilution with 0.3 mM MeOSucAAPVpNa in PBS, pH 7.4, containing 0.01% Triton X-100. The final enzyme concentration was 0.5 μM. All amidolytic rates (v) were normalized to rates obtained in the absence of HPTSoleate (v_0). (●) Experimentally determined normalized rates; the solid curve represents the nonlinear least-squares fit of the data to the equation of Bieth (1974) for tightly bound inhibitors.

eq 6 (Bieth, 1984) to the observed data, as computed by the Marquand method (Enzfitter, Elsevier). Under the conditions

$$v/v_0 = 1 - \{[E]_0 + [I]_0 + K_{i(app)} - \{([E]_0 + [I]_0 + K_{i(app)})^2 - 4[E]_0[I]_0\}^{1/2}\}/2[E]_0 \quad (6)$$

we have employed (0.5 μM HNE, 0.3 mM MeOSucAAPVpNa), at a 1:1 ratio of HNE to HPTSoleate, about 71% of the enzyme will be complexed with inhibitor, using the value of $K_{i(app)}$ of 58 nM computed from the least-squares fit.

HPTSoleate and Oleic Acid Are Competitive with Each Other. Since oleic acid appears to bind tightly to HNE as a partial noncompetitive inhibitor, whereas HPTSoleate binds tightly as a competitive inhibitor, we added both of these compounds at 10 times their apparent K_i values to 30 nM HNE to see if they compete with each other for the same site

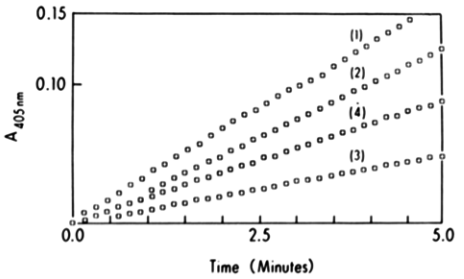

FIGURE 6: Competition between oleic acid and HPTSoleate as inhibitors of amidolysis. The inhibitors were added to 30 nM HNE, and the amidolytic assays were initiated by addition of 0.3 mM MeOSucAAPVpNa. The release of *p*-nitroaniline (absorbance at 405 nm) was recorded as a function of time. (1) After addition of substrate alone; (2) after addition of 0.4 μM oleic acid and substrate; (3) after addition of 0.4 μM HPTSoleate and substrate; (4) after addition of 0.4 μM oleic acid plus 0.4 μM HPTSoleate and substrate.

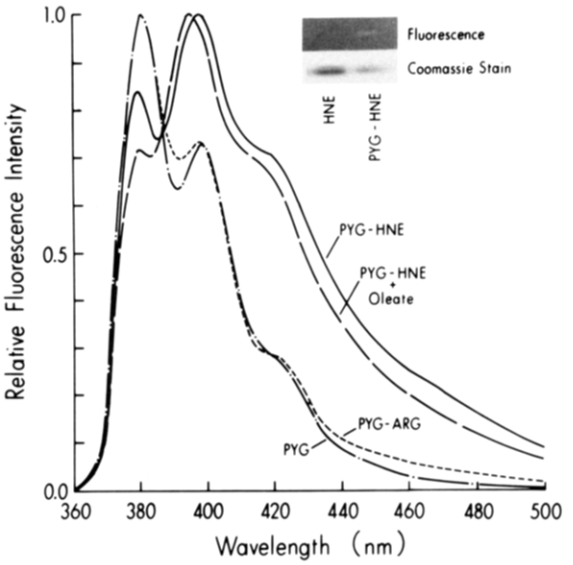

FIGURE 7: Fluorescence emission spectra of PYG (-·-). PYG–Arg (---), purified PYG–HNE complex (—), and PYG–HNE complex to which 0.2 mM oleic acid was added (--). The excitation wavelength was 340 nm. Excitation and emission slits were adjusted for 1.25- and 2.5-band-pass, respectively. Spectra were corrected for wavelength dependence of excitation energy and for contribution from buffer alone. The PYG–Arg spectrum was determined in the presence of a 10-fold excess of arginine (the reaction was completed within 30 min as measured by the change in absorbance at 30 nm) and was also corrected for emission from free arginine alone. All these spectra have been normalized so that the intensity of the highest peak in each spectrum is arbitrarily set at 1. This presentation reveals changes in ratios of intensities but not overall quenching or enhancement. The insert shows the visualization of samples of HNE and PYG–HNE run in parallel on 4.5% SDS–PAGE by fluorescence and by staining with Coomassie blue.

on the enzyme (Figure 6). In the presence of 0.4 μM oleate alone, the amidolysis of 0.3 mM MeOSucAAPVpNa is inhibited 12%, whereas in the presence of 0.4 μM HPTSoleate, the amidolysis is inhibited 87%. Amidolysis is inhibited 48% in the presence of a mixture of 0.4 μM oleate + 0.4 μM HPTSoleate, suggesting that both inhibitors do indeed compete for the same site on the enzyme.

Fluorescence of PYG–HNE. The fluorescence emission spectra of PYG alone, PYG plus a 10-fold excess of arginine, and the isolated PYG–HNE complex are shown in Figure 7. All three spectra have the characteristic features of monomeric pyrene derivatives when excited at 340 nm (Rawitch et al., 1696). However, after reaction of PYG with HNE, the two maxima seen at 381 and 398 nm in PYG alone and in the PYG–Arg complex are blue-shifted about 3 nm, to 378 and

395 nm, respectively. Furthermore, the intensity of the band at the shorter wavelength is decreased while that of the band at the longer wavelength is increased. The blue shift and the decreased ratio of the intensities at 378 vs 395 nm are both consistent with transfer of the pyrene ring to a more hydrophobic environment after reaction with HNE (Ollman et al., 1987). Reaction with arginine has no significant effect on the emission spectrum of PYG. Low concentrations of oleic acid have no effect on the emission spectrum of the PYG–HNE complex, but at 200 μM oleate (over 10 times the apparent low-affinity K_i of 16 μM), the emission maxima are further blue-shifted by an additional 2 nm, and the ratio of the intensities at 376 vs 393 nm is still further decreased. Concentrations of stearic acid up to 200 μM have no effect on the emission spectrum of PYG–HNE, confirming that the effect of oleic acid is not due to nonspecific binding of a hydrophobic anion.

Fluorescence of HPTSoleate Bound to HNE. The emission spectrum of HPTSoleate alone has two maxima at 388 and 410 nm. To ensure that a major fraction of this noncovalent inhibitor was bound to HNE, we determined the emission spectrum of a mixture of 1 μM HNE and 50 nM HPTSoleate; under these conditions, 95% of the inhibitor is associated with the enzyme. The maximum at the longer wavelength is shifted about 2 nm to shorter wavelengths, and the ratio of the intensities of the maxima at 388 vs 408 nm is decreased after compex formation, again consistent with a transfer of the pyrene ring to a more hydrophobic environment upon binding to HNE.

Effect of Oleate Binding on HNE Tryptophan Fluorescence. The three tryptophan residues in HNE give rise to a characteristic emission spectrum with a maximum around 334 nm with excitation at 295 nm. The effect of oleic acid on this endogenous fluorescence was studied over a range of fatty acid concentrations to include effects which might be ascribed to binding with high affinity (K_i = 48 nM) or low affinity (K_i = 16 μM). In the presence of 1 μM oleate, the tryptophan fluorescence of 0.1 μM HNE was shifted about 5 nM to shorter wavelengths. No further shift could be induced by increasing concentrations of oleic acid up to 200 μM. Concentrations of stearic acid up to 200 μM had no effect on the endogenous fluorescence spectrum of HNE.

Discussion

Neutrophil elastase is implicated as a major contributor to tissue damage in both acute and chronic inflammatory processes (Sandborg et al., 1988; Travis, 1988) and has therefore been targeted by many researchers for development of potent and specific inhibitors (Bonney et al., 1989; Hassall et al., 1985; Nick et al., 1988; Fournel et al., 1988; Trainor, 1987). Many of the most effective of the low molecular weight inhibitors reported in the literature have both hydrophobic and anionic substituents which appear to enhance both specificity and binding affinity (Harper et al., 1985; Doherty et al., 1986; Lentini et al., 1987; Cook & Ternai, 1988). Analysis of the three-dimensional structure of HNE shows that the extended substrate binding site is filled with a number of hydrophobic residues, while only 2 of the 19 arginine residues, Arg-217 and Arg-177, lie within 10–15 Å of serine-195, at the catalytic center (Bode et al., 1986). The hypothetical role of Arg-217 in binding anionic inhibitors has been suggested previously, but no experimental evidence for the role of the guanidino side chain has been reported (Bode et al., 1989; Cook & Ternai, 1988).

We present here evidence that at least one of two guanidino side chains has some role in maintaining HNE in a catalytically active state. Chemical modification of arginines by the hydrophobic vicinal dione PYG results in a complete loss of amidolytic activity associated with modification of only two side chains. The loss of activity is directly proportional to the fraction of residues modified, and we have observed only one modified species of PYG–HNE by isoelectric focusing, leading us to suspect that the modification of both residues may be concerted. We do not yet have direct evidence that the two modified residues are in fact Arg-217 and Arg-177. Adducts of glyoxals and arginines can undergo decomposition during peptide isolation and sequencing, making identification of the modified residues particularly difficult (Haining & McFadden, 1990). Nevertheless, the extensive array of hydrophobic side chains lining the extended substrate binding site in the vicinity of these two residues could certainly provide the hydrophobic environment which gives rise to the characteristic blue shifts and intensity ratio changes in the emission spectrum of PYG after reaction with HNE.

Other proteases have been reported to undergo some partial loss of catalytic activity after modification with other vicinal diones (Ozawa & Laskowski, 1966; Liu et al., 1968; Riordan, 1973; Vencent et al., 1975; Weber et al., 1975; Takahashi, 1977; Barhanin et al., 1981; Genov & Idakieva, 1983). Porcine pancreatic elastase loses up to 85% of its catalytic activity after modification of two arginine residues with cyclohexanedione, although the apparent affinity of the enzyme for amidolytic substrates is actually increased after modification (Davril et al., 1984). However, the location of the guanidino side chains in the pancreatic enzyme is not equivalent to that in the neutrophil-derived enzyme, and the general significance of this observations is limited. The rate of reaction of PYG with HNE is slowed in the presence of the oligopeptide substrate MeOSucAAPVpNa, suggesting that the substrate either directly interacts with a guanidino group or occupies a site which is otherwise filled by the bulky pyrene fused ring system. Arginine side chains have been implicated in binding of substrates to other proteases (Davril et al., 1984; Riordan, 1973, Takahashi, 1977; Genov & Idakieva, 1983), but since PYG–HNE has apparently lost all catalytic activity, we cannot provide direct assessment of the effects of modification of Arg-217 and/or Arg-177 on substrate binding.

Oleic acid has been recognized as an inhibitor of neutrophil elastase which binds to the enzyme with an affinity in the low micromolar range (Ashe & Zimmerman, 1977; Cook & Ternai, 1988). Our observations that the saturated fatty acid stearic acid has no effect either on spectral properties of native or modified HNE or on K_m or k_{cat} for amidolysis of MeOSucAAPVpNa are also consistent with these previous kinetic studies. The mode of inhibition by oleic acid has been described as noncompetitive by Ashe and Zimmerman (1977), and as mixed by Cook and Ternai (1988). Oleyl peptide derivatives lacking the free negatively charged carboxyl group, which appear to be simple competitive inhibitors (K_i = 4–9 μm), have been described by Horneback et al. (1985). Under our assay conditions, we observe that oleic acid appears to have two modes of binding to HNE: a high-affinity mode which results in a small reduction in catalytic activity and no alteration in substrate binding, and a second lower affinity mode which results in competitive inhibition. The high-affinity mode of binding is supported not only by kinetic analysis of amidolytic rates but also by a blue shift in tryptophan fluorescence, suggesting increased hydrophobicity in the environment of at least one of the three indole side chains. In the absence of additional spectral data, we cannot describe more completely the effect of the apparent high-affinity mode of binding of

oleate on the conformation of HNE. Concentrations of oleic acid which would be sufficient to saturate the apparent high-affinity binding site have no effect on the fluorescence of PYG in the modified enzyme, suggesting either that this binding site is not in the vicinity of the modified arginines or that modification of the arginines with PYG eliminates the high-affinity oleic acid binding site.

The lower affinity competitive inhibitory mode of binding of oleic acid which we observe may be similar to that reported by Cook and Ternai (1988), and by Ashe and Zimmerman (1977). Concentrations of oleate sufficient to saturate this apparent low-affinity site do perturb the fluorescence of PYG on the modified enzyme, increasing the apparent hydrophobicity of the environment of the pyrene ring(s). This result suggests that this low-affinity mode of binding may also be in the vicinity of the arginine guanidino side chains and, since the inhibition is competitive, may also involve part of the extended substrate binding site of HNE.

The noncovalent inhibitor HPTSoleate appears to possess a structure which is optimal for interactions with both hydrophobic and charged domains in HNE. The affinity of HNE for this amphiphilic compound is at least as great as that of the apparent high-affinity binding site for oleic acid. The apparent binding sites for HPTSoleate and for free oleate (high affinity) overlap, since the two inhibitors are competitive with one another. However, the effects of these two inhibitors on HNE are very different. We have already presented evidence that oleic acid bound in a high-affinity mode is a relatively weak partial noncompetitive inhibitor of HNE. On the other hand, once bound, HPTSoleate appears to prevent substrate binding to the enzyme; we are hindered from obtaining any evidence for effects of HPTSoleate on catalytic activity under these conditions since the competitive mode of inhibition is complete. We speculate that the negative charge arising from the three sulfonate groups on the pyrene ring of this inhibitor interacts with the centers of positive charge from at least one of the two guanidino groups which can also be chemically modified by PYG. The extended hydrophobic domain contributed by the oleyl side chain on this amnphiphilic inhibitor may also bind to HNE in a fashion similar to that of oleic acid when it binds as a competitive inhibitor of the enzyme. However, the double mode of interaction of HPTSoleate with HNE confers upon this inhibitor a much higher affinity for HNE than the free fatty acid.

We believe these observations support a rational basis for design of synthetic anionic hydrophobic protease inhibitors with high specificity and affinity for HNE. Evidence of interactions of such inhibitors with the enzyme through hydrophobic domains is seen in the characteristic changes in the emission spectra of bound environment-sensitive fluorescent inhibitors. An additional electrostatic mode of interaction apparently involves binding of anionic substituents on the inhibitors to at least one arginine side chain in HNE. These two foci of interaction between HNE and inhibitors are structurally very close, since probes which are specifically targeted to arginine guanidino groups also experience a hydrophobic environment once they react with HNE.

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