Registry No. (±)-**1**, 83588-18-5; (+)-**1**, 83648-33-3; (2*S*,3*R*)-**1**, 83648-34-4; **2**, 6829-42-1; (±)-**3**, 83588-19-6; (2*S*,3*S*)-**3**, 83648-35-5; (2*R*,3*R*)-**3**, 83648-36-6; **5**, 72594-19-5; **6**, 61898-56-4; **7**, 68702-74-9; (±)-**8**, 83648-37-7; (−)-**8**, 83588-20-9; **9**, 83588-21-0; PLE, 9016-18-6; (−)-*threo*-2,4-dimethyl-3-hydroxypentanoic acid, 78655-80-8; *erythro*-2,4-dimethyl-3-hydroxypentanoic acid, 77341-63-0; methacrylaldehyde, 78-85-3; methyl 2-bromopropionate, 5445-17-0; (−)-*threo*-2,4-dimethyl-3-hydroxypent-4-enoic acid, 83588-22-1.

p-Guanidinobenzoic Acid Esters of Fluorescein as Active-Site Titrants of Serine Proteases

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Abstract: Two active-site titrants of serine proteases, fluorescein mono-*p*-guanidinobenzoate hydrochloride (FMGB·HCl) and fluorescein di-*p*-guanidinobenzoate dihydrochloride (FDGB·2HCl), have been synthesized, purified, and chemically and enzymatically characterized. Electronic absorption and fluorescence emission spectra, fluorescence lifetimes and quantum yields, solubilities, and rates of spontaneous hydrolysis at pH 7–10 are reported. Macroscopic and microscopic kinetic constants for interaction of FMGB·HCl with trypsin, urokinase, plasmin, and thrombin have been determined. FMGB·HCl, which rapidly releases fluorescein upon formation of a stable acyl-enzyme intermediate with trypsin and other trypsin-like enzymes, is the most sensitive active-site titrant for serine proteases yet described.

Serine proteases are involved in a wide variety of physiological processes,[1] including ovulation,[2-4] blood coagulation,[5] embryogenesis,[6] processing of hormones,[7,8] inactivation of the λ repressor,[9] and activation of digestive enzymes.[10] Sensitive substrates are essential for accurate assay of serine proteases because such enzymes are often present in very small amounts and because, as proteases capable of self-degradation, they must be assayed at low concentrations. Active-site titrants are especially useful substrates for serine proteases because they form enzymatically inactive acyl-enzyme intermediates, thus causing the rate of self-proteolysis to decrease during assay. However, since the acyl-enzyme intermediate of an active-site titration does not turn over, the detectable product must be unusually easy to monitor. An ideal active-site titrant would react rapidly to form a stable, enzymatically inactive intermediate as it released a product capable of detection at extremely low concentration.

Recently, we reported the synthesis and biochemical characterization of FDE[11] (fluorescein diester[12]), an active-site titrant for serine proteases designed to fulfill these criteria. Efforts to purify this compound and to establish its structure by chemical and physical methods, especially to determine whether it is a mono- or a diester, have not yet been rewarded. Part of the difficulty stems from the controversy over the relationship between the electronic and fluorescence spectra of fluorescein (**1**) and its derivatives and their molecular structures[13-18] and part appears to arise from the presence of the thioureido bridge and the terminal carboxyl group in FDE. Because FDE has nevertheless proven quite useful,[19,20] we decided to make simpler analogues and to establish their structures unequivocally. Here we report the synthesis, purification, and chemical, physical, and enzymatic characterization of two such analogues,[21] fluorescein mono-*p*-guanidinobenzoate hydrochloride (FMGB·HCl, **2**) and fluorescein di-*p*-guanidinobenzoate dihydrochloride (FDGB·2HCl, **3**). These substrates are the most sensitive active titrants for serine proteases yet described.

Experimental Section

Melting points were determined on a Büchi melting point apparatus and are corrected. Electronic absorption spectra were measured on a Beckman Acta Model VI spectrophotometer with matched silica cells having 1-cm pathlength. Field desorption mass spectra (FDMS) were obtained on a Varian-MAT 731 spectrometer equipped with a Varian-MAT combination electron impact-field desorption ion source. Microanalyses were performed by Mr. Josef Nemeth and his staff at the University of Illinois, who also weighed samples for quantitative electronic absorption spectra and for determination of the purities of FMGB·HCl (**2**) and FDGB·2HCl (**3**) by hydrolysis with base and with trypsin.

Materials. *p*-Guanidinobenzoic acid hydrochloride (PGBA·HCl, **4**) was purchased from Sigma. Fluorescein (**1**), dicyclohexylcarbodiimide (DDC), and benzamidine hydrochloride hydrate (**5**) were purchased from

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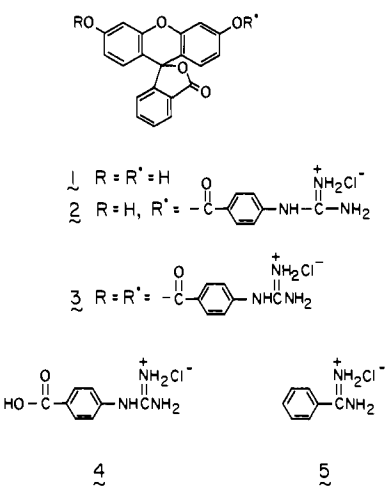
Aldrich. Stock solutions of **5** were prepared in phosphate-buffered saline (PBS), which contained 0.137 M NaCl, 2.68 mM KCl, 8.08 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.91 mM $CaCl_2$, and 0.49 mM $MgCl_2$ at pH 7.2. Bovine pancreatic trypsin, 3 times crystallized, was purchased from Worthington. Stock solutions were prepared in 1 mM HCl and stored at −20 °C. Reference standard human urokinase was purchased from Leo Pharmaceuticals. Stock solutions were prepared in PBS and stored at −20 °C. The preparation of dog plasmin was described previously.[19] Human thrombin, a gift from Dr. Robert Rosenberg, was stored in 0.1 M sodium phosphate, pH 7.0, and 0.9 M NaCl. Dry *N,N*-dimethylformamide (DMF)[22] was stored over anhydrous magnesium sulfate. Dry pyridine[22] was stored over Linde 4-Å molecular sieves and was periodically redistilled. Stock solutions of fluorescein (**1**), FMGB·HCl (**2**), FDGB·2HCl (**3**), and PGBA-HCl (**4**) were prepared in dry DMF and were stored at 4 °C for 1–2 months, except for spectroscopic studies, for which fresh stock solutions were prepared. Buffers at pH 8.2 (0.1 M



sodium barbital)[23] and pH 10.15 (0.025 M sodium bicarbonate)[24] were prepared according to published directions.

Chromatography. Analytical thin-layer chromatography (TLC) was performed on Brinkmann silica gel plates with fluorescent indicator. Visualization was under 254- and 365-nm light, before and after exposure to ammonia vapor. Preparative TLC was carried out on 20 × 20 cm Brinkmann silica gel plates, 2.0 mm thick, with fluorescent indicator. Visualization was under 254- and 365-nm light. The adsorbent for column chromatography was Brinkmann silica gel, 0.2–0.05 mm.

Fluorescence Lifetime and Quantum Yields. Fluorescence lifetimes were measured on a cross-correlation, phase-modulation fluorometer[25] with improved electronics from SLM Instruments, Inc., Urbana, IL. The exciting light was modulated at 30 MHz. The excitation wavelength was 491 nm. Emission was observed through a Corning 3-68 cutoff filter. Fluorescence emission spectra were recorded on a ratiometric spectrofluorometer equipped with digital electronics[26,27] and a Hamamatsu R928 photomultiplier tube. The excitation wavelength was 452 nm, and the emission was scanned from 460 to 700 nm. A 4-nm bandwidth was used on both the excitation and emission monochromators. After subtraction of base line for PBS, the recorded emission spectra were traced using a Hewlett-Packard Model 9864 digitizer board and a Hewlett-Packard Model 9825A computer. The emission spectra were then corrected for variations in the response of the photomultiplier tube at different wavelengths. The corrected intensities of fluorescence were plotted as a function of wavenumber, and the spectra were integrated. Fluorescence quantum yields were calculated relative to a quantum yield of 0.92 for fluorescein in 0.1 N sodium hydroxide.[28]

Fluorescence Assay. Active-site titrations were performed in PBS, pH 7.2, unless otherwise stated. The increase in fluorescence was monitored with a Perkin-Elmer MPF 44-A fluorescence spectrophotometer equipped with a Perkin-Elmer universal digital-readout meter (Model UDR 1) connected to a Hewlett-Packard Model 7015 X-Y recorder with time base. The excitation and emission wavelengths were 491 and 514 nm, respectively. The bandwidth of both monochromators was 4 nm. The fluorescence spectrophotometer was standardized each day by using a polymethacrylate block embedded with rhodamine B so that the relative fluorescence was comparable in different experiments.

Table I. TLC Characteristics of Starting Materials and Products

compound	R_f's in solvents[a]				appearance[b] under light at	
	A	B	C	D	254 nm	365 nm
fluorescein (**1**)	0.87	0.77	0.24	0.10	yellow	yellow-green
FMGB·HCl (**2**)	0.67	0.48	0.74	0.66	black	faint orange
FDGB·2HCl (**3**)	0.44	0.29	0.54	0.27	black	faint orange
PGBA·HCl (**4**)	0.71	0.35	0.29	0.05	black	black

[a] A = 1-butanol/HOAc/H_2O, 4/1/1; B = $CHCl_3$/absolute EtOH/HOAc, 50/50/1; C = $CHCl_3$/MeOH, 2/1; D = $CHCl_3$/MeOH, 4/1. [b] Before hydrolysis with ammonia vapor.

Analysis of Titration Curves. The kinetic parameters of each titration curve were determined with a Hewlett-Packard Model 9825A computer interfaced to a Model 9864A digitizer board and 9872 plotter. The kinetic profile of a titration curve was traced with the digitizer and recorded into computer memory. The computer then calculated the *Y*-axis intercept of the extrapolated steady-state line, *B*, and the slope of the steady-state line, *A*. Values for Be^{-bt} were determined at approximately 50 time points along the pre-steady-state portion of the curve by subtracting the experimental curve from the extrapolated portion of the steady-state line. The first-order rate constant for each substrate concentration, *b*, was then obtained by a computer-performed linear-regression treatment of the equation $\ln (Be^{-bt}/B) = -bt$. The analysis was confirmed visually by having the computer direct the plotter to graph the digitized titration curve, the extrapolated steady-state line, and a plot of $-\ln (Be^{-bt}/B)$ vs. time.

Synthesis of FMGB·HCl (2) and of FDGB·2HCl (3). To a dry 100-mL round-bottomed flask containing a stir bar and 3.23 g of PGBA·HCl (**4**, 1.5 × 10^{-2} mol) was added 10 mL of dry DMF and 10 mL of dry pyridine. The stoppered flask was immersed in an ice bath, and the contents were stirred until solution was complete. To this was added 3.09 g of DCC (1.5 × 10^{-2} mol) dissolved in 10 mL of dry DMF and 10 mL of dry pyridine. After 15 min, 3.32 g of fluorescein (**1**, 1 × 10^{-2} mol) was added. Stirring was continued at 0 °C for 2 h and then at room temperature overnight. A few drops of glacial acetic acid was added, and the mixture was stirred an additional 0.5 h. The white precipitate was removed by suction filtration and washed with methanol. The filtrate and washings were concentrated on a rotary evaporator at room temperature under reduced pressure. The resulting orange oil was filtered, the residue was washed with methanol, and the filtrate and washings were evaporated again. The orange oil so obtained was diluted with a few milliliters of methanol and poured slowly with stirring into 350 mL of 0.6 N HCl and 50 g of ice. As soon as the ice melted, the yellow precipitate was collected by suction filtration, washed with 0.6 N HCl, and dried overnight at room temperature over anhydrous calcium chloride in an evacuated desiccator. The crude product, weighing 6.12 g, was dissolved in 400 mL of water by stirring at room temperature for 0.5 h. The orange solution was filtered and extracted with four 100-mL portions of ethyl acetate. The organic layers were discarded, and the aqueous phase was extracted with five 100-mL portions of 1-butanol. The combined 1-butanol extracts were mixed with 100 mL of 0.6 N HCl. The 1-butanol was removed as an azeotrope by evaporation at 35 °C under reduced pressure, during which 0.6 N HCl was added until the odor of 1-butanol had disappeared. The residual orange gum was triturated under 0.6 N HCl, yielding a yellow powder, which was collected by suction filtration and washed with 0.6 N HCl. After being dried overnight in an evacuated desiccator at room temperature over anhydrous calcium chloride, the partially purified product was an orange powder, weighing 3.317 g [62%, based on FMGB·HCl (**2**)].

Purification of FMGB·HCl (2) and of FDGB·2HCl (3). Method A. Purification of FMGB·HCl (**2**) and FDGB·2HCl (**3**) was achieved by column chromatography, with a ratio of silica gel to loaded product of 3000/1 and an eluant consisting of either 1-butanol/water/acetic acid, 8/2/1, or chloroform/methanol/acetic acid, 4000/1000/5. The partially purified product (0.500 g), obtained from the extraction procedure described above, was dissolved in 50 mL of methanol and adsorbed onto 10 g of silica gel by evaporating the slurry to dryness at room temperature under reduced pressure. After the adsorbed product was loaded onto the column (110 × 3.5 cm), 7-mL fractions were collected at maximum flow rate. The progress of the separation was monitored by examining the

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column with a hand-held UV lamp and by analytical TLC (see Table I). Fast-running, highly fluorescent fractions were discarded. Slower-running, less-fluorescent fractions containing FMGB·HCl (**2**) were immediately pooled and concentrated under reduced pressure at room temperature. When the volume had been reduced 10-fold, the yellow solution was diluted with an equal volume of 0.6 N HCl, and evaporation was continued until a yellow-orange oil was obtained. Addition of a few milliliters of 0.6 N HCl to the oil caused precipitation of a yellow solid, which was collected by suction filtration, washed with 0.6 N HCl, and dried overnight at room temperature over anhydrous calcium chloride in an evacuated drying pistol. By this method 0.124–0.376 g of pure FMGB·HCl (**2**) was obtained as a bright yellow-orange powder (25–75% recovery). Anal. Calcd for $C_{28}H_{19}N_3O_6 \cdot HCl \cdot H_2O$: C, 61.38; H, 4.05; N, 7.67. Found: C, 61.12; H, 3.90; N, 7.36.

After collection of FMGB·HCl (**2**), the eluant was switched from 1-butanol/acetic acid/water, 8/2/1, to ethanol/acetic acid, 1/1, or from chloroform/methanol/acetic acid, 4000/1000/5, to methanol/acetic acid, 1000/5. Fractions (100 mL) were collected until a slow-running, faintly fluorescent peak was eluted. Appropriate fractions were immediately pooled and treated as described above for the 7-mL fractions. By this method 0.068–0.079 g of pure FDBG·2HCl (**3**) was obtained as a pale-yellow powder (14–16% recovery). Anal. Calcd for $C_{36}H_{26}N_6O_7 \cdot 2HCl \cdot H_2O$: C, 58.00; H, 4.06; N, 11.27; Cl, 9.51. Found: C, 57.96; H, 4.05; N, 11.50; Cl, 9.43.

Method B. Pure FMGB·HCl (**2**) was also obtained by preparative TLC. Partially purified product (0.100 g) obtained from the extraction procedure described above was dissolved in a minimum volume of methanol, streaked onto the plate, and dried with an air blower at room temperature. Fluorescein (**1**) and PGBA·HCl (**4**) markers were spotted onto the plate. The plate was eluted with absolute ethanol/chloroform/acetic acid, 50/50/1, and dried with an air gun at room temperature after the solvent front had run 10–12 cm. The appropriate streak was scraped from the plate and eluted with 50 mL of absolute ethanol/acetic acid, 100/1. After the silica gel was removed by filtration and washed with 3 20-mL portions of absolute ethanol/acetic acid, 100/1, the combined filtrate and washings were concentrated 10-fold by evaporation at room temperature under reduced pressure. About 10 mL of 0.6 N HCl was added, and the mixture was evaporated to dryness, giving a yellow residue, which was collected by filtration after the addition of 2–3 mL of 0.6 N HCl. The yellow solid was washed with 0.6 N HCl and dried overnight at room temperature over anhydrous calcium chloride in an evacuated drying pistol. This procedure gave 0.059 g of FMGB·HCl (**2**) (59%), which was identical with the material obtained by column chromatography.

Results

Synthesis. FMGB·HCl (**2**) and FDGB·2HCl (**3**) were prepared in dry DMF/pyridine from fluorescein (**1**) and PGBA·HCl (**4**) by condensation with DCC, a reaction accompanied by formation of dicyclohexylurea (DCU). Despite variations in time, temperature, solvent, and amount of DCC used, TLC showed the crude product to be a mixture of fluorescein (**1**), PGBA·HCl (**4**), FMGB·HCl (**2**), FDGB·2HCl (**3**), and DCU. Fortunately, adjustment of the ratios of fluorescein (**1**)/PGBA·HCl (**4**)/DCC allowed some control over the ratio of FMGB·HCl (**2**)/FDGB·2HCl (**3**) found in the product, with a ratio of 2/3/3 affording crude product that was predominately FMGB·HCl (**2**).

Purification. Preliminary purification of the crude product by liquid–liquid extraction removed most residual PGBA·HCl (**4**), fluorescein (**1**), and DCU. Although this procedure reduced the yield, it was difficult to obtain FMGB·HCl (**2**) or FDGB·2HCl (**3**) free of DCU and PGBA·HCl (**4**) when this step in the purification was eliminated. Final purification was achieved by column chromatography or by preparative TLC, both on silica gel. Although preparative TLC restricted the scale of purification more sharply than column chromatography, it was faster, cheaper, and more reproducible.

The purity of FMGB·HCl (**2**) and of FDGB·2HCl (**3**) was assessed by TLC on silica gel in four solvent systems (see Table I). Exposure of the plates to ammonia vapor revealed no evidence of PGBA·HCl (**4**) or fluorescein (**1**) in either product (**2** and **3**) and no evidence of cross-contamination of the mono- and diesters (**2** and **3**).

These fluorescein mono- and diesters are stable as the hydrochloride salts (**2** and **3**). Since they undergo rapid decomposition when converted to the free bases, however, the compounds must be kept acidic during purification and storage. When stored as solids in a desiccator at −20 °C, FMGB·HCl (**2**) and FDGB·2HCl (**3**) showed no decomposition for at least a year.

Assignment of Structures to FMGB·HCl (2) and FDGB·HCl (3). The structures assigned to FMGB·HCl (**2**) and FDGB·2HCl (**3**) on the basis of elemental analysis and method of synthesis were corroborated by FDMS. Under the harsh experimental conditions required to generate adequate ion current from these compounds, FMGB·HCl (**2**) produced a peak at m/e 494, corresponding to loss of chloride, a peak at m/e 451, corresponding to further loss of NH_2—C=NH_2 accompanied by transfer of hydrogen, and another peak at m/e 333, resulting from cleavage of the ester C—O bond accompanied by loss of chloride and migration of two hydrogens. FDGB·2HCl (**3**) gave a peak at m/e 334, attributable to cleavage of both ester C—O bonds with loss of two chlorides and migration of four hydrogens, and another peak at m/e 286, resulting from loss of both chlorides and both NH_2—C=NH_2 moieties with transfer of four hydrogens. Under similar conditions, fluorescein (**1**) gave a signal at m/e 333 ($M^+ + 1$). PGBA·HCl (**2**) generated peaks at m/e 179, corresponding to loss of chloride, in addition to peaks at m/e 75 and 74, attributable to complete loss of functional groups from the phenyl ring with migration of one or two hydrogens, respectively. The observed spectra for these compounds (**1**–**4**) are compatible with the known mass spectral behavior of aromatic acids and esters[29] and derivatives of guanidine.[30]

Exposure of TLC plates spotted with fluorescein (**1**), FMGB·HCl (**2**), FDGB·2HCl (**3**), and PGBA·HCl (**4**) to ammonia vapor for 10 min prior to elution provided further evidence that FMGB·HCl (**2**) and FDGB·2HCl (**3**) are respectively the mono- and diesters of fluorescein (**1**) and PGBA·HCl (**4**). This partial cleavage of FMGB·HCl (**2**) gave three spots, having R_f's and spectral characteristics under 254- and 365-nm light that corresponded to PGBA·HCl (**4**), FMGB·HCl (**2**), and fluorescein (**1**). For FDGB·2HCl (**3**), the procedure afforded four spots, which were attributable to PGBA·HCl (**4**), FDGB·2HCl (**3**), FMGB·HCl (**2**), and fluorescein (**1**) by the same criteria. Complete hydrolysis of FMGB·HCl (**2**) and FDGB·2HCl (**3**) by exposure to ammonia vapor for 40 min prior to elution gave, for both compounds, only two spots, corresponding to fluorescein (**1**) and PGBA·HCl (**4**).

Since FMGB·HCl (**2**) and FDGB·2HCl (**3**) both contain an asymmetric carbon at the spiro position and since the method of synthesis used here requires the product to be a 50/50 mixture of enantiomers, it is possible that enzymes might distinguish between these two forms, preferentially cleaving one. In fact, this does not appear to happen. As shown below with trypsin, 100% of the monoester is consumed with no change in kinetics as the reaction proceeds. Likewise, 100% of the diester is consumed, with no change in kinetics observed besides that due to the shift in cleavage of the diester to the monoester as the reaction proceeds. Apparently, the distance between the C–O ester bond and the asymmetric carbon is so great that trypsin is insensitive to the configuration of the substrate.

Electronic Absorption Spectra and Confirmation of Purity. Electronic absorption of fluorescein (**1**), FMGB·HCl (**2**), FDGB·2HCl (**3**), and PGBA·HCl (**4**) are displayed in Figure 1 and summarized in Table II. The occurrence of the longest wavelength absorption band for fluorescein (**1**) at 489 nm rather than 491 nm and the observance of 77 049 M^{-1} cm^{-1} for the extinction coefficient rather than 89 320 M^{-1} cm^{-1} result from these spectra being recorded at pH 7.2 instead of under the standard condition for fluorescein (**1**),[28] 0.1 N sodium hydroxide, in which FMGB·HCl (**2**) and FDGB·2HCl (**3**) are unstable. The present values agree with the known pH dependence of the position and intensity of this absorption peak for fluorescein (**1**).[13,14] Moreover, when we recorded the electronic absorption spectrum of fluorescein (**1**) in 0.01 N sodium hydroxide, we observed a λ_{max} at 490 nm

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Table II. Physical Constants of Starting Materials and Products

compound	M_r	melting point, °C	solubility,[a] μM	electronic absorption spectra,[a] λ_{max}, nm (log ε)	fluorescence[a] λ_{em}, nm[b]	Φ[b,c]	τ, ns[d]
fluorescein (1)	332.32	315–317		489 (4.89) 464[g] (4.53) 318 (3.86) 282 (4.04)	515	0.75	4.7
FMGB·HCl (2)	547.95	228[e,f]	25	475[g] (4.16) 452 (4.29) 430[g] (4.21) 346 (4.06) 264 (4.49)	557	0.03	1.7
FDGB·2HCl (3)	745.58	237[e,f]	15	273 (4.54)	527	0.02	2.1
PGBA·HCl (4)	215.64	272–274		254[g] (4.03)			

[a] In PBS, pH 7.2. [b] λ_{exc} = 452 nm. [c] Relative to Φ = 0.92 for fluorescein in 0.01 N sodium hydroxide.[7] [d] Phase determination, λ_{exc} = 491 nm. [e] Foamed. [f] Evacuated, sealed tube. [g] Shoulder.

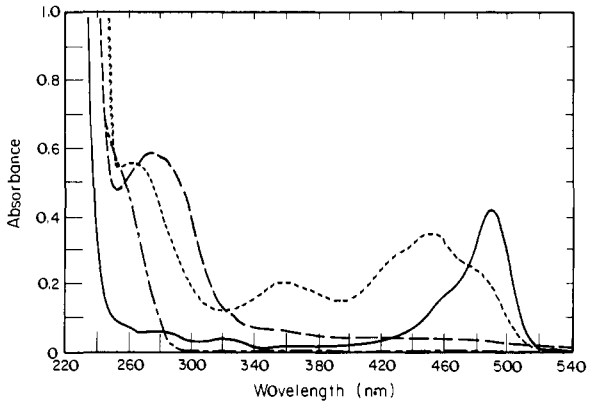

Figure 1. Electronic absorption spectra of fluorescein (**1**), —, 5.49 × 10^{-6} M; FMGB·HCl (**2**), ---, 1.82 × 10^{-5} M; FDGB·2HCl (**3**), – – –, 1.70 × 10^{-5} M; and PGBA·HCl (**4**), – – –, 5.23 × 10^{-5} M, in PBS, pH 7.2.

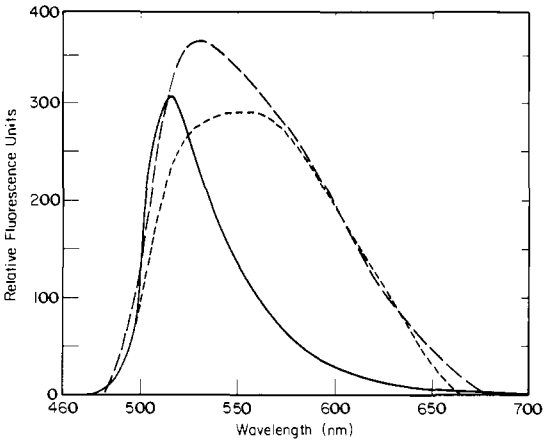

Figure 2. Corrected fluorescence emission spectra for fluorescein (**1**), —, 1.26 × 10^{-8} M; FMGB·HCl (**2**), ---, 6.08 × 10^{-7} M; and FDGB·2HCl (**3**), – – –, 9.35 × 10^{-6} M, in PBS, pH 7.2, λ_{exc} = 452 nm.

and an extinction coefficient of 89 125 M^{-1} cm^{-1}.

The similarity of the electronic absorption spectra for fluorescein (**1**), FMGB·HCl (**2**), and FDGB·2HCl (**3**) in the region 350–500 nm to spectra reported previously[13,14] for fluorescein (**1**) and for its mono- and dimethyl ethers at pH's near 7 may reflect similarities between the structures of the chromophores in the esters and in the ethers. This observation does not illuminate the structure of the ester chromophores, however, since the structure of the species responsible for the absorption of fluorescein and its ethers at different pH's and in different solvents remains controversial.[13-16]

The distinctive appearances of the electronic absorption spectra for fluorescein (**1**), FMGB·HCl (**2**), and FDGB·2HCl (**3**) and the sensitivity of the mono- and diesters **2** and **3** to base allow electronic absorption spectroscopy to be used to determine purity of samples of FMGB·HCl (**2**) and FDGB·2HCl (**3**). When these compounds were completely hydrolyzed in 0.1 N sodium hydroxide, the spectra of the mono- and diesters **2** and **3** were identical with that of fluorescein (**1**). The extinction coefficients of the hydrolyzed samples indicated that FMGB·HCl (**2**) and FDGB·2HCl (**3**) were both >98% pure.

Fluorescence Lifetimes, Emission Spectra, and Quantum Yields. For fluorescein (**1**), we observed lifetimes of 4.5 ns in 0.01 N sodium hydroxide and 4.7 ns at pH 7.2, in excellent agreement with the literature value of 4.6 ns 0.1 N sodium hydroxide.[31] Both the lifetimes and the fluorescence emission spectra (see Table II and Figure 2) observed for fluorescein (**1**), FMGB·HCl (**2**), and FDGB·2HCl (**3**) at pH 7.2 demonstrate that the fluorescence of FMGB·HCl (**2**) and FDGB·2HCl (**3**) is characteristic of these compounds and not due to residual fluorescein (**1**).

Quantum yields were not measured at λ_{exc} = 491 nm, the exciting wavelength used during assays, because at this wavelength the scatter peak overlaps the emission spectra for all three fluorophores (**1–3**). For intensely fluorescent fluorescein, the scatter peak is small enough to be subtracted from the emission spectrum, but for weakly fluorescent FMGB·HCl (**2**) and FDGB·2HCl (**3**), subtraction introduces unacceptably large errors. Instead, a lower wavelength absorption maximum for FMGB·HCl (**2**), 452 nm, was chosen for λ_{exc}.

As shown in Table II, the quantum yields of FMGB·HCl (**2**) and FDGB·2HCl (**3**) at pH 7.2 are much less than the quantum yield of fluorescein (**1**) at the same pH. The pH dependence of the quantum yield for fluorescein (**1**) observed here agrees closely with that reported by Guyot and coworkers.[18] The low quantum yields of FMGB·HCl (**2**) and FDGB·2HCl (**3**) are compatible with previous reports that mono- and dialkanoyl esters of fluorescein (**1**) are nonfluorescent.[32-36]

Because the quantum yields, which for fluorescein (**1**) at pH 5–7 are known to depend upon the excitation wavelength,[18,37] were measured at 452 nm and because they reflect the relationship between adsorption at the excitation wavelength and intensity of fluorescence integrated over the entire emission spectrum, the quantum yields do not provide an accurate picture of the ability of a fluorescence assay to discriminate between fluorescein (**1**), FMGB·HCl (**2**), and FDGB·2HCl (**3**). Under the conditions of

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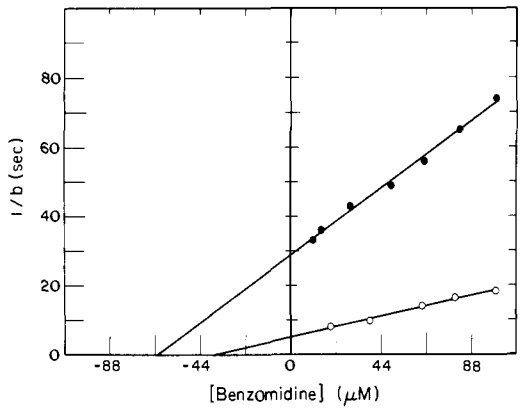

Figure 3. Determination of the microscopic kinetic constants, K_s and k_2, for the hydrolysis of FMGB·HCl (**2**) and FDGB·2HCl (**3**) by trypsin. The kinetics of hydrolysis of FMGB·HCl (**2**), 0.14 μM (○), or of FDGB·2HCl (**3**), 0.30 μM (●), in PBS by trypsin, 8 nM, were measured in the presence of the indicated concentrations of benzamidine (data not shown) and the apparent first-order rate constants, b, determined (see Experimental Section).

the present assay (λ_{exc} = 491 nm, 4-nm slits, pH 7.2), on an equimolar basis, fluorescein (**1**), FMGB·HCl (**2**), and FDGB·2HCl (**3**) exhibit relative fluorescence at 514 nm of 1.00, <0.1, and <0.01, respectively.

The Kinetics of Hydrolysis of FMGB·HCl (2) and FDGB·2HCl (3) by Trypsin. Serine proteases hydrolyze ester and amide bonds by way of an acyl-enzyme intermediate according to the reaction sequence:[38]

$$E + S \overset{K_s}{\rightleftarrows} E{\cdot}S \overset{k_2}{\rightarrow} \underset{+}{ES'} \overset{k_3}{\rightarrow} E + P_2$$
$$P_1$$

where E is trypsin; S is FMGB·HCl (**2**) or FDGB·2HCl (**3**); E·S is the adsorptive enzyme–substrate complex; ES′ is the covalent acyl–enzyme intermediate, *p*-guanidinobenzoyl-trypsin; P_1 is either fluorescein (**1**) or FMGB·HCl (**2**); P_2, the other product of the reaction, is *p*-guanidinobenzoate; K_s is the equilibrium dissociation constant; k_2 is the first-order rate constant for acylation; and k_3 is the first-order rate constant for deacylation.

The kinetics of hydrolysis of FMGB·HCl (**2**) and FDGB·2HCl (**3**), as monitored by the appearance of P_1, are biphasic. An initial burst of P_1 is followed by a slow linear increase in P_1. The initial burst or pre-steady-state phase is due to the rapid formation of the covalent acyl-enzyme intermediate, *p*-guanidinobenzoyl–trypsin, and the concomitant release of a stoichiometric amount of the highly fluorescent fluorescein (**1**) or the weakly fluorescent FMGB·HCl (**2**). Thereafter, the postburst or steady-state phase is reached, during which deacylation of the enzyme occurs very slowly.

Determination of the Microscopic and Macroscopic Kinetic Constants for FMGB·HCl (2) and FDGB·2HCl (3) with Trypsin. The microscopic constants K_s, k_2, and k_3 and the macroscopic constant $K_{m(app)}$ were determined by using the kinetic theory derived by Bender and coworkers.[38] Assuming $k_2 >> k_3$, we obtained K_s and k_2 under conditions in which $[S]_o >> [E]_o$, $[S]_o >> K_m$, and a competitive inhibitor benzamidine (**5**) was present to decrease the rate of the reaction. The apparent first-order rate constant for acylation was measured (see Experimental Section) at several inhibitor concentrations (data not shown). The reciprocal of the apparent first-order rate constant for acylation at a particular substrate concentration, b, is plotted vs. the competitive inhibitor concentration, [I] (Figure 3). The relationship between b and [I] is defined as follows: where K_i is the equilibrium

$$\frac{1}{b} = \frac{K_s}{k_2 K_i [S]_0}[I]_0 + \frac{K_s + [S]_0}{k_2 [S]_0}$$

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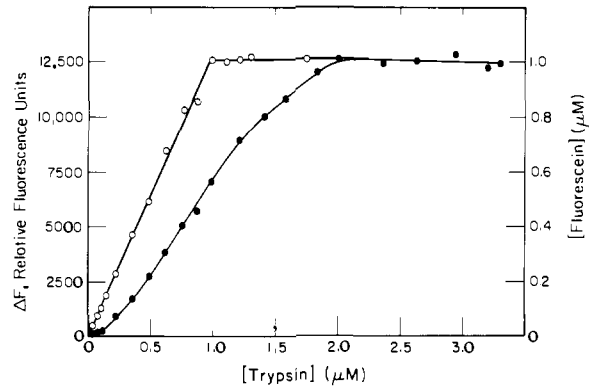

Figure 4. Titration of FMGB·HCl (**2**) and FDGB·2HCl (**3**) by increasing concentrations of trypsin. One-milliliter solutions of PBS containing 1 μM FMGB·HCl (**2**) or FDGB·2HCl (**3**) were incubated with the indicated concentrations of trypsin. The kinetics of hydrolysis were then monitored as described in Experimental Section. The steady-state lines were extrapolated to time zero to obtain ΔF, which is plotted vs. the trypsin concentration. The ordinate on the right correlates molar concentrations of fluorescein (**1**), determined by weight, with ΔF, the ordinate on the left. The concentrations of FMGB·HCl (**2**) and FDGB·HCl (**3**) were determined by weight. The concentration of trypsin was determined by an active-site titration with NPGB. FMGB·HCl (**2**) (○) and FDGB·2HCl (**3**) (●).

dissociation constant for the competitive inhibitor. The K_i for the competitive inhibitor benzamidine (**5**) with trypsin under these conditions had been shown to be 18.2 μM.[11] The slopes of the lines in Figure 3 yield a k_2 of 0.194 s^{-1} for FMGB·HCl (**2**) and of 0.035 s^{-1} for FDGB·2HCl (**3**) and the intercepts yield a K_s of 65.8 nM for FMGB·HCl (**2**) and of 84.8 nM for FDGB·2HCl (**3**). The first-order rate constant for deacylation,[11] k_3, with trypsin under these conditions had been shown to be 1.66×10^{-5} s^{-1}. Since the $K_{m(app)} = K_s k_3/(k_2 + k_3)$, the $K_{m(app)}$ for FMGB·HCl (**2**) is 5.63×10^{-12} M and for FDGB·2HCl (**3**) is 4.05×10^{-11} M.

Confirmation of the Structure and Purity of FMGB·HCl (2) and of FDGB·2HCl (3) by Enzymatic Analysis. FMGB·HCl (**2**) should be a monofunctional active-site titrant and FDGB·2HCl (**3**) a bifunctional active-site titrant. The product of an active-site titration of FMGB·HCl (**2**) should exhibit the properties of fluorescein (**1**). One mole of FMGB·HCl (**2**) should be titratable by 1 mol of enzyme. With FDGB·2HCl (**3**), the product of an active-site titration at a low enzyme to substrate ratio should exhibit the properties of FMGB·HCl (**2**). When the molar concentration of enzyme and substrate are equal, both FMGB·HCl (**2**) and fluorescein (**1**) should be present. In the limit, 1 mol of FDGB·2HCl (**3**) should be titratable by 2 mol of enzyme.

To determine whether FMGB·HCl (**2**) is a monofunctional substrate and whether fluorescein (**1**) is the product of an active-site titration, we titrated 1 μM solutions of **2** with increasing concentrations of trypsin (Figure 4). The ΔF (final fluorescence minus initial fluorescence) increases linearly with the trypsin concentration until a plateau is reached. At higher trypsin concentrations, no further increase in ΔF is observed, indicating that all of the titratable FMGB·HCl (**2**) has reacted. The minimum amount of trypsin required to titrate 1 equiv of FMGB·HCl (**2**) is 1 equiv. Thus, FMGB·HCl (**2**) is a monofunctional substrate. That the product of an active-site titration of FMGB·HCl (**2**) is fluorescein (**1**) can be deduced from the line correlating ΔF with low concentrations of trypsin. Since the line is straight, a ΔF per mole of trypsin can be calculated. This ΔF per mole of trypsin is the same as the ΔF per mole of fluorescein (**1**) that was obtained by measuring the fluorescence of solutions of fluorescein (**1**), under similar conditions. The relationship between ΔF and moles of fluorescein (**1**) is defined by the scales on the two ordinates in Figure 4.

To determine whether FDGB·2HCl (**3**) is a bifunctional substrate and whether fluorescein (**1**) is the ultimate product of an active-site titration, we also titrated 1 μM solutions of **3** with increasing concentrations of trypsin (Figure 4). The ΔF increases

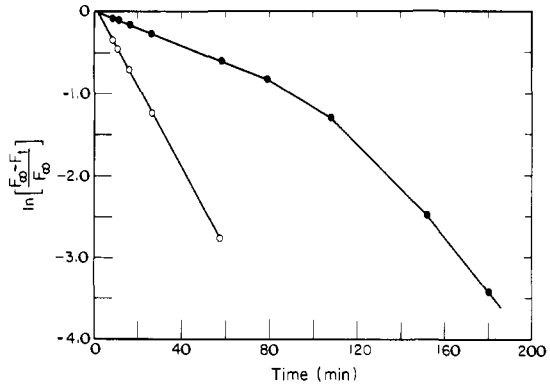

Figure 5. The rate of spontaneous hydrolysis of FMGB·HCl (**2**) and of FDGB·2HCl (**3**) at pH 10.15. Solutions of 0.0125 M $NaHCO_3$, pH 10.15, containing 0.1 μM FMGB·HCl (**2**) (○) or FDGB·2HCl (**3**) (●) were incubated at room temperature and the fluorescence was determined after the indicated time intervals, F_t. The fluorescence at infinite time, F_∞, was determined by hydrolyzing FMGB·HCl (**2**) and FDGB·2HCl (**3**) with 1 N NaOH, diluting an aliquot with PBS, and measuring the fluorescence.

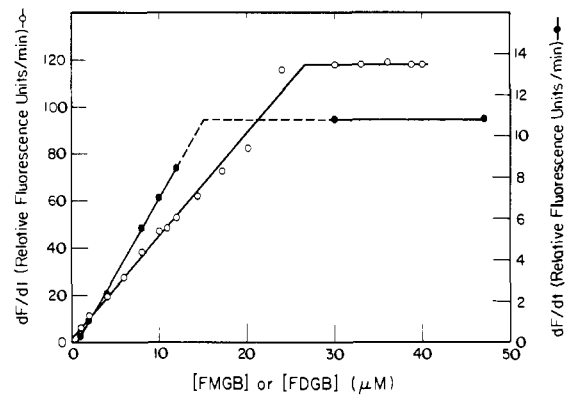

Figure 6. Determination of the solubility limit of FMGB·HCl (**2**) and FDGB·2HCl (**3**) in PBS. Five-microliter aliquots were withdrawn from solutions of FMGB·HCl (**2**) and FDGB·2HCl (**3**) in DMF whose concentrations ranged from 1 to 60 μM and were added to 1-mL solutions of PBS. Each solution was centrifuged at 8000*g* for 4 min to remove any precipitate. The increase in fluorescence was then monitored for 1 h. The rate of spontaneous hydrolysis $d(F)/dt$ is plotted vs. the amount of FMGB·HCl (**2**) (○) or FDGB·2HCl (**3**) (●) added to PBS.

nonlinearly with the trypsin concentration until a plateau is reached. The sigmoidal shape of the curve indicates that the ΔF per mole of trypsin is not constant and implies that there are at least two species of substrate present. Since the minimum amount of trypsin required to titrate 1 equiv of FDGB·2HCl (**3**) is 2 equiv, FDGB·2HCl (**3**) must be a bifunctional substrate. Since the final ΔF corresponds to 1 equiv of fluorescein (**1**), the final product of an active-site titration must be fluorescein (**1**).

The titration curve of FDGB·2HCl (**3**) by trypsin in Figure 4 is sigmoidal because of the differences in extinction coefficients and quantum yields between FMGB·HCl (**2**) and fluorescein (**1**). At low concentrations of trypsin relative to FDGB·2HCl (**3**), the major product is FMGB·HCl (**2**). As the concentration of trypsin increases, the concentration of FMGB·HCl (**2**) increases relative to the concentration of FDGB·2HCl (**3**). When FMGB·HCl (**2**) reacts with trypsin to yield fluorescein (**1**), the absolute increase in fluorescence is much greater than when FDGB·HCl (**3**) reacts with trypsin to yield FMGB·HCl (**2**).

The titration curves in Figure 4 also confirm the purity of FMGB·HCl (**2**) and FDGB·2HCl (**3**). Extrapolation of both of the plateau lines to the left ordinate yields a ΔF of 12 700. When 1 μM solutions, determined by weight, of FMGB·HCl (**2**) and FDGB (**3**) are hydrolyzed by alkali, the ΔF is 12 800. The ΔF of a 1 μM solution of fluorescein (**1**), determined by absorbance, is 12 740. Thus both FMGB·HCl (**2**) and FDGB·2HCl (**3**) are greater than 99% pure.

Rate of Spontaneous Hydrolysis of FMGB·HCl (2) and FDGB·2HCl (3). Although both derivatives are relatively stable in aqueous buffer near neutral pH, their ester bonds do spontaneously hydrolyze. The rate of spontaneous hydrolysis is proportional to the concentration of ester bonds and to the concentration of hydroxyl ions. The apparent first-order rate constant for the spontaneous hydrolysis of FMGB·HCl (**2**), $k_{(spont)}$, at constant pH is defined:

$$\ln ([FMGB]_t/[FMGB]_0) = -k_{spont}t$$

where $[FMGB]_t$ is the concentration of FMGB·HCl (**2**) at time t and $[FMGB]_0$ is the concentration of FMGB·HCl (**2**) at time zero. $[FMGB]_t = [FMGB]_0 - [F]_t$, where $[F]_t$ is the concentration of hydrolyzed FMGB·HCl (**2**), i.e., fluorescein (**1**), at time t. The apparent first-order rate constant can be determined by incubating **2** or **3** in PBS and monitoring the initial increase in fluorescence as a function of time.

The pseudo-first-order rate constant, $k_{(spont)}$, for the spontaneous hydrolysis of FMGB·HCl (**2**) to fluorescein (**1**) is the same as for the hydrolysis of FDGB·2HCl (**3**) to FMGB·HCl (**2**). An example of the data obtained at pH 10.15 is shown in Figure 5. The initial rates of decay of FMGB·HCl (**2**) and FDGB·2HCl (**3**) are constant. Their slopes differ because of the differences in their extinction coefficients and quantum yields. At longer times the slope of the FDGB·2HCl (**3**) curve begins to change, as the concentration of FMGB·HCl (**2**) increases and begins to hydrolyze to fluorescein (**1**). If the initial rates of decay of FMGB·HCl (**2**) and of FDGB·HCl (**3**) are normalized to moles of ester bonds instead of relative fluorescence units, the two rates become equal. The $k_{(spont)}$ at pH 7.2 is 2.55×10^{-6} s^{-1}, at pH 8.2 is 1.53×10^{-5} s^{-1}, and at pH 10.15 is 1.98×10^{-3} s^{-1}. Since $k_{(spont)} = k\,[OH^-]$, the second-order rate constant, k, is 12.7 M^{-1} s^{-1}. The similarity of the values of $k_{(spont)}$ at pH 7.2 for FMGB·HCl (**2**) and FDGB·HCl (**3**) with that for *p*-nitrophenyl-*p*-guanidinobenzoic acid (NPGB) and for FDE (2.0×10^{-6} s^{-1} and 5.1×10^{-6} s^{-1}, respectively)[11] indicates that the rate of spontaneous hydrolysis for these *p*-guanidinobenzoic acid esters is not very sensitive to the identity of the leaving chromophore or fluorophore.

Solubility of FMGB·HCl (2) and of FDGB·2HCl (3) in PBS. Since the rate of spontaneous hydrolysis is proportional to the concentration of derivative in solution, a saturated solution can be defined as one in which the rate of spontaneous hydrolysis does not increase upon addition of more derivative. The solubility of FMGB·HCl (**2**) in PBS was determined by preparing a series of solutions to which increasing amounts of FMGB·HCl (**2**) were added. The solutions were centrifuged to remove any insoluble material, and the increase in fluorescence of the supernatant with time was monitored for 1 h. Because only a small amount of the derivative hydrolyzed during that time interval, the kinetics of spontaneous hydrolysis appeared pseudo zero order (data not shown). These initial velocities are plotted vs. the amount of FMGB·HCl (**2**) added to each solution (Figure 6). Since the rate of spontaneous hydrolysis of FMGB·HCl (**2**) is first order with respect to FMGB·HCl (**2**), the rate of increase in fluorescence is proportional to the amount of added FMGB·HCl (**2**) below the solubility limit. Above that limit, the rate of increase of fluorescence is independent of added FMGB·HCl (**2**). The solubility limit of FMGB·HCl (**2**) is, therefore, the concentration at the intersection of the two extrapolated lines in Figure 6. The solubility limit of FMGB·HCl (**2**) in PBS is 25 μM and of FDGB·2HCl (**3**) in PBS is 15 μM.

Active-Site Titrations with FMGB·HCl (2) of Plasmin, Urokinase, and Thrombin. We next explored the usefulness of FMGB·HCl (**2**) as an active-site titrant of three major proteases important in hemostasis. Each of these enzymes was incubated in 3 μM solutions of FMGB·HCl (**2**) in PBS and the increase in fluorescence measured as a function of time (Figure 7). The burst with plasmin is completed quickly, in less than 10 s. The slope of the steady-state line is extremely small, indicating little turnover of enzyme. Urokinase and thrombin react more slowly with FMGB·HCl (**2**). The pre-steady-state phases take about 2 min to complete. The slopes of their steady-state lines indicate an

Table III. Kinetic Constants for Active-Site Titrations of Serine Proteases with FMGB·HCl (2)

	k_2, s^{-1}	k_3, s^{-1}	K_s, M	$K_{m(app)}$, M	k_2/K_s, M^{-1} s^{-1}
trypsin	0.194	1.66×10^{-5}	6.58×10^{-8}	5.63×10^{-12}	2.95×10^{6}
plasmin	0.936	6.27×10^{-6}	7.26×10^{-6}	4.86×10^{-11}	1.49×10^{5}
urokinase	0.482	3.64×10^{-4}	4.87×10^{-5}	3.68×10^{-8}	9.89×10^{3}
thrombin	0.0602	1.78×10^{-3}	2.17×10^{-5}	6.42×10^{-7}	2.77×10^{3}

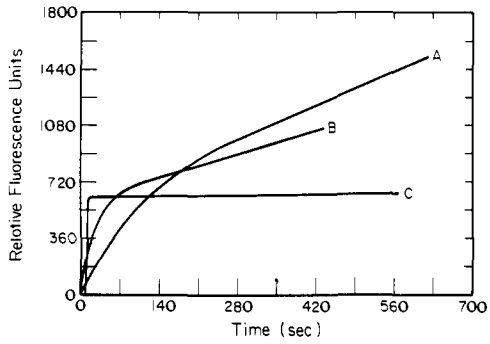

Figure 7. An active-site titration of plasmin, urokinase, and thrombin by FMGB·HCl (2). One-milliliter solutions of PBS containing 3 μM FMGB·HCl (2) and 50 nM dog plasmin (C), human urokinase (B), or human thrombin (A) were incubated at room temperature and the increase in fluorescence measured as a function of time.

appreciable rate of deacylation. Since each enzyme was present at the same concentration and since each of the steady-state lines extrapolates to nearly the same point on the ordinate (±5%), the concentration of active sites for each enzyme can be determined from a single titration curve with FMGB·HCl (2). However, this is valid theoretically only if $k_2 >> k_3$ and $[S]_o >> K_{m(app)}$. The failure of the steady-state lines for thrombin, urokinase, and plasmin to extrapolate to exactly the same point on the ordinate is probably a reflection of experimental error, although for thrombin $[S]_0$ is only 5-fold greater than the $K_{m(app)}$ and thus does not quite meet the criterion $[S]_o >> K_{m(app)}$. These titrations were done before kinetic parameters for all three enzymes had been accurately determined, and the concentrations of enzyme and substrate were chosen semiempirically. After determining the kinetic constants, we repeated the titration for thrombin at $[S]_0$ 22-fold greater than $K_{m(app)}$ and obtained a slightly higher extrapolation point, as anticipated.

The microscopic kinetic constants k_2, K_s, and k_3 for FMGB·HCl (2) with plasmin, urokinase, and thrombin were determined (data not shown). These results, along with the calculated macroscopic kinetic constant $K_{m(app)}$, are listed in Table III. The first-order rate constants for acylation, k_2, differ by 15-fold, with plasmin the fastest and thrombin the slowest. The first-order rate constant for deacylation, k_3, for plasmin is small and corresponds to a half-life of 30.70 h. Consistent with the appreciable slopes of their steady-state lines in Figure 7, the half-lives for deacylation of urokinase and thrombin are much shorter, 31.73 and 6.49 min, respectively. The K_s values for the three enzymes exhibit a 200-fold range. The only K_s values close to the solubility limit of FMGB·HCl (2) are those for urokinase and thrombin. With all three enzymes, the concentration of active sites can easily be determined from a single titration curve.

Discussion

FMGB·HCl (2) and FDGB·2HCl (3) were designed as active-site titrants for serine proteases on the basis of several unusual properties of esters of PGBA (4) and of fluorescein (1). Esters of PGBA (4) react quickly with many serine proteases. For example, the first-order rate constant for acylation, k_2, with NPGB and trypsin is 0.370 s^{-1}.[11] The acylated enzyme, *p*-guanidinobenzoyl-trypsin, is extremely stable at pH 7.2, exhibiting a half-life for deacylation of 11.6 h.[11] Furthermore, NPGB does not undergo appreciable spontaneous hydrolysis at pH 7.2, the first-order rate constant for this reaction being 2.0×10^{-6} s^{-1}.[11] Fluorescein (1) was chosen as the fluorogenic indicator of acylation because it possesses a large extinction coefficient and quantum yield in the visible region at neutral to basic pH.[13,14,18,37] Our earlier choice of a derivative of fluorescein isothiocyanate for this purpose[11] created multiple possibilities for byproduct formation. Fortunately, the terminal carboxyl group in FDE, which had been included in an attempt to make the titrant impermeable to membranes, is unnecessary for most applications, making fluorescein (1) a suitable alternative.

Here we have shown FMGB·HCl (2) to be a useful, sensitive active-site titrant of serine proteases. We know that FMGB·HCl (2) reacts specifically with active sites rather than nonspecifically with nucleophilic groups on the periphery of the enzyme because kinetic constants exhibited in a titration with FMGB·HCl (2) are similar to those exhibited with other active-site titrants, e.g., NPGB[23,39] and FDE.[11] Moreover, competitive inhibitors known to interact at the active site, such as benzamidine,[39] slow the rate of enzyme-catalyzed hydrolysis of FMGB·HCl (2). The sensitivity of FMGB·HCl (2) as an active-site titrant is illustrated by a comparison with NPGB. The lowest concentration of trypsin that can be titrated with NPGB with an accuracy of greater than 2%, based on the absorbance of *p*-nitrophenol anion at 400 nm, is in the micromolar range, whereas the lowest concentration of trypsin that can be titrated with a similar degree of accuracy by FMGB·HCl (2), based on the fluorescence intensity of fluorescein (1) at 514 nm, is approximately 3×10^{-10} M, an increase in sensitivity greater than 3000-fold. Plasmin can also be routinely measured at this concentration, but the less favorable kinetic constants for thrombin and urokinase raise the limit of routine detection of these enzymes by 1 order of magnitude. Special techniques can be employed to reduce limits of detection for all four enzymes 20-fold.[40] FMGB·HCl (2) is especially useful because the concentration of active sites can be determined from a single titration curve. Since $k_2 >> k_3$ and since titrations can be performed under conditions in which $[S]_0 >> k_{m(app)}$, extrapolation of the steady-state line yields the molar concentration of active sites. FMGB·HCl (2) would be much less useful if these two criteria were not fulfilled, because the enzyme concentration would have to be determined from a series of titrations in which the substrate concentration was varied.

Esters between fluorescein (1) and alkanoic acids[32-36] and between a derivative of fluorescein isothiocyanate and PGBA (4)[11] have been reported, but the spectral properties of these compounds have not been carefully investigated. Although mono- and dialkanoic esters with fluorescein (1) have been described as nonfluorescent,[32-36] we find that FMGB·HCl (2) and FDGB·2HCl (3) possess slight but measurable fluorescence. Under the conditions of the assay for serine proteases described here, both substrates exhibit a 10-fold increase in fluorescence upon cleavage of a single ester bond. Nevertheless, FMGB·HCl (2) is the better active-site titrant because it obviates complications present with FDGB·2HCl (3) due to subsequent cleavage of the second ester bond.

Until now, the most sensitive active-site titrants of serine proteases have been the 4-methylumbelliferyl ester of *p*-guanidinobenzoic acid[40] and FDE.[11] The fluorescein esters (2 and 3) described here have several advantages over these active-site titrants, however. Since fluorescein (1) can be detected at much lower concentrations than 4-methylumbelliferone,[11] the limit of detection of trypsin in an active-site titration with FMGB·HCl (2), 3×10^{-10} M, is approximately 20-fold lower than the limit

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of detection with the 4-methylumbelliferone ester of *p*-guanidinobenzoic acid, 6×10^{-9} M. In addition, unlike fluorescein (**1**), 4-methylumbelliferone undergoes rapid photolytic decomposition, which makes kinetic analysis extremely difficult, since the titration reaction must be monitored intermittently instead of continuously. The main advantage of the fluorescein esters **2** and **3** over FDE is that the structures of FMGB·HCl (**2**) and FDGB·2HCl (**3**) have been established, whereas the structure of FDE is still under investigation. Considering that it takes 1 equiv of FDE to titrate 1 equiv of trypsin under conditions in which FDE is limiting,[11] it now appears likely that FDE is a monoester, not a diester. The structure determination of FMGB·HCl (**2**) and FDGB·2HCl (**3**) and the spectral behavior of the compounds reported here guide our attempts to clarify the structure of FDE and to design substrate analogues for serine proteases that exhibit high enzymatic specificity as well as high sensitivity. At present, FMGB·HCl (**2**) is the best active-site titrant for serine proteases available.

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Registry No. 1, 2321-07-5; **2**, 83616-10-8; **3**, 83616-11-9; **4**, 42823-46-1; serine protease, 37259-58-8; trypsin, 9002-07-7; plasmin, 9001-90-5; urokinase, 9039-53-6; thrombin, 9002-04-4.

Biosynthetic and Structural Studies on Pheomelanin

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Abstract: Pheomelanin, the red-brown or yellow polymeric pigment present in the hair and skin of fair-skinned humans, is produced by the tyrosinase-mediated copolymerization of L-3,4-dihydroxyphenylalanine (L-dopa) and L-cysteine. A reinvestigation of the structure using radiotracer biosynthetic methodology is reported. Carbon-14 and tritium-labeled dopas and sulfur-35-labeled cysteine were used to demonstrate that, contrary to earlier reports, (1) pheomelanin is not a 1:1 copolymer of dopa and cysteine, (2) during the course of pheomelanin biosynthesis up to 50% of the dopa residues suffer decarboxylation, and (3) hydrogen peroxide, produced in situ during the course of the biosynthesis, does not play a role in modification of the pheomelanin polymer. Methods for isolation and purification of synthetic pheomelanin and the use of trichochrome F as a marker for monitoring the progress of the polymerization are reported.

Melanin pigments and their distribution in human skin are generally believed to be the most important factor in the protection of human skin from the biochemical devastation caused by chronic exposure to solar radiation.[2] Epidermal melanin pigmentation is thought to be composed of intimate mixtures of two separate but biogenetically related pigments: the black-brown eumelanins and the yellow and red-brown pheomelanins.[3] The two pigments exhibit different photochemistries, and it has been proposed that the facile photodegradation of pheomelanin coupled with concomitant production of superoxide radicals is responsible for the increased susceptibility of fair-skinned Caucasians to the detrimental effects of sunlight.[4]

Epidermal melanins consist of a polymeric chromophore covalently bound to a protein fraction. Extensive biosynthetic and degradative studies have demonstrated that the eumelanin chromophore is an amorphous, irregular polymer composed of several major monomers (see partial structure **1**).[5] Much less work has been done on the pheomelanin polymer; however, chemical degradative and biosynthetic studies indicate that the pheomelanin chromophore, a product of tyrosinase-catalyzed copolymerization of L-3,4-dihydroxyphenylalanine (dopa) and L-cysteine,[6] is mainly composed of 6,7,8,9-tetrahydro-4-hydroxythiazolo[4,5-*h*]isoquinoline-7-carboxylic acid (**2**) monomers.[7] However, the chemical degradative methods used were sufficiently harsh that one must question whether the products isolated were artifacts and whether the structure deduced from such products accurately represents the intact polymer. We therefore decided to reinvestigate the structure of pheomelanin using radiotracer biosynthetic methodology and herein report our initial findings.

Materials and Methods

Materials. Electrophoresis grade acrylamide, ammonium persulfate, *N,N,N',N'*-tetramethylenediamine (TEMED), and sodium dodecyl sulfate ($NaDodSO_4$) were purchased from Bio-Rad Laboratories. Electrofocusing gel was purchased from Pharmacia Fine Chemicals. Ampholytes were prepared synthetically as previously described.[8] Tyrosinase (EC 1.14.18.1) and catalase (EC 1.11.1.6) were purchased from Sigma Chemical Co. and assayed by literature methods.[9,10] All other biochemicals were purchased from Sigma Chemical Co. Radioactive compounds were purchased from Amersham, organic chemicals were from Aldrich, and liquid scintillation cocktail was from Beckman. Absorbance spectra were obtained on a Varian Cary 219 spectrophotometer.

Preparation of Synthetic Pheomelanin. An adaption of an earlier method was employed for preparation of pheomelanin.[11] L-dopa (70 mg) was added to a stirred 50-mL solution of 0.1 M phosphate buffer (pH 6.8) at 37 °C. To this solution was added 10 mg of tyrosinase (1200 eu/mg), and when a pink color developed (ca. 30 s), 85.6 mg of L-cysteine (dissolved in the minimum amount of phosphate buffer) was added. The solution had turned dark brown after 3 h; however, the yield of pigment was maximized if the reaction was allowed to continue for an additional 21 h. The reaction was quenched after 24 h by adjusting the pH to 1.5

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