Benzyl *p*-guanidinothiobenzoate hydrochloride, a new active-site titrant for trypsin and trypsin-like enzymes

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Benzyl *p*-guanidinothiobenzoate hydrochloride was synthesized and demonstrated to be useful for active-site titration of bovine trypsin, bovine thrombin, human lung tryptase, bovine activated protein C, human Factor XIIa fragment and bovine Factor Xa₂. The titration is based on rapid formation of a stable acyl-enzyme with a stoichiometric release of benzyl thiol. Thiol production is measured quantitatively by including 4,4'-dithiodipyridine in the reaction mixture and measuring the increase in absorbance at 324 nm. Ellman's reagent has also been successfully employed, allowing measurement at 410 nm. Unlike *p*-nitrophenyl *p*'-guanidinobenzoate, the thioester titrant reacts slowly with chymotrypsin A₁ thus eliminating interference by this enzyme in most titrations. Advantages of this reagent as a titrant include: flexibility in detection of the released thiol, selectivity between trypsin and chymotrypsin-like enzymes, minimal pH-dependence of the ϵ of the absorbing species, relative stability of the reagent under titration conditions, and high ϵ at pH 7.2 with either 4,4'-dithiodipyridine or Ellman's reagent. The reagent should prove useful as an alternative to *p*-nitrophenyl *p*'-guanidinobenzoate hydrochloride for the determination of active-site concentrations of the enzymes employed, as well as of other related enzymes.

In the course of kinetic investigations involving a large number of trypsin-like enzymes, especially those involved in the blood-clotting system, we have become aware of the need for routine accurate determinations of the concentrations of these enzymes. On the basis of the thorough work by Bender *et al.* (1966), active-site titration of hydrolytic enzymes has emerged as the most valuable method for such determinations. An active-site titration involves the stoichiometric interaction of a relatively small well-characterized molecule with the enzyme, ideally occurring only at the active site (Bender *et al.*, 1966; Kézdy & Kaiser, 1970). Active-site titrations have been shown to be generally superior to rate assays, primarily because the latter depend on a pure enzyme as a standard. Pure enzyme

standards are difficult if not impossible to obtain for most enzymes, and are less easily characterized. Furthermore, rate assays suffer from unpredictable interferences owing to the presence of impurities that may act on the substrate at variable rates.

Chase & Shaw (1967) introduced what has become the titrant of choice for trypsin and many trypsin-like enzymes, *p*-nitrophenyl *p*'-guanidinobenzoate hydrochloride (GBz-ONp). It has been used successfully to titrate trypsin, plasmin, thrombin (Chase & Shaw, 1969) and bovine Factor Xa (Smith, 1973). On interaction with an appropriate enzyme, it gives rise to an initial stoichiometric production of 4-nitrophenol, which can be conveniently measured down to the micromolar range in a spectrophotometer. The result is a 'burst' of absorbance (at 410 nm). Generally, pH 8.3 is employed to form the *p*-nitrophenolate anion, which has a higher ϵ than the protonated form (at 8.3, $\epsilon = 16.8 \text{ mm}^{-1} \cdot \text{cm}^{-1}$; at pH 7.5, $\epsilon = 12.6 \text{ mm}^{-1} \cdot \text{cm}^{-1}$). The fact that the titration results are highly pH-dependent and that a relatively high pH is required for the optimal ϵ value are drawbacks to the use of GBz-ONp. The pH-dependence tends to diminish accuracy in proportion to the uncertainty in pH. In addition, GBz-ONp is un-

Abbreviations used: GBz-SBzl, benzyl *p*-guanidinothiobenzoate hydrochloride; GBz-ONp, *p*-nitrophenyl *p*'-guanidinobenzoate hydrochloride; HS-Bzl, benzyl thiol; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Z-Arg-SBu¹, benzyloxycarbonyl-L-arginine iso-thiobutyl ester; GBz-Fl, fluorescein mono-*p*-guanidinobenzoate hydrochloride; GBz-MU, 4-methylumbelliferyl 4-guanidinobenzoate hydrochloride.

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stable towards hydrolysis at higher pH values, and this can introduce errors into the measurements.

In the present paper we report the synthesis, characterization and use of a new titrant for trypsin and enzymes of trypsin-like specificity: benzyl *p*-guanidinobenzoate hydrochloride (GBz-SBzl). This compound is similar in structure to GBz-ONp (Fig. 1), but, since it is a thioester, it is significantly different in terms of stability and enzymic reactivity. The method of detection of the released thiol (benzyl thiol, HS-Bzl) involves the inclusion in the assay of a compound that reacts quickly with the thiol to produce an absorbing species. As in previous reports with proteinase thioester substrates, 4,4'-dithiodipyridine has been used (McRae *et al.*, 1981; Castillo *et al.*, 1979). However, we have also demonstrated that 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) may be used similarly. Theoretically, any chromogenic or fluorogenic thiol-detecting reagent could be used, given a sufficient rate of reaction with HS-Bzl under the titration conditions. The absorbing species produced with 4,4'-dithiodipyridine is a thiopyridone, which exhibits a high ϵ ($19.8 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ at 324 nm) that is nearly independent of pH over a wide range (Grassetti & Murray, 1967). These features plus the rapidity with which the reaction takes place render 4,4'-dithiodipyridine an excellent choice for use in GBz-SBzl titrations.

Materials and methods

Materials

Bovine pancreas trypsin ($2\times$ crystallized) was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. For the k_2 and K_s evaluation experiment it was purified by passage through a Sephadex G-100 column equilibrated with 1.0 mM-HCl. Bovine thrombin was prepared as previously described (Fujikawa *et al.*, 1973). Bovine pancreas chymotrypsin A_{α} , purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A., was dissolved in 1 mM-HCl solution and stored at -20°C . Bovine activated protein C was graciously provided by Dr. Walter Kisiel of the University of Washington, Seattle, WA, U.S.A. It was purified by preparative electrophoresis after activation with Russell's-viper venom. It was pure by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and was stored at -70°C until use. Human activated protein C was also provided by Dr. Kisiel and was similarly prepared. The preparation contained a negligible amount of β -activated protein C by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Human lung tryptase was provided by Dr. David Johnson, East Tennessee State University, Johnson City, TN, U.S.A. Human Factor Xa_β was provided by Dr. Kotoku Kurachi of the University of Washington, Seattle, WA, U.S.A.

Human Factor XIIa fragment (M_r 28 000) was also provided by Dr. Kisiel and was purified similarly to bovine and human protein C. The 4-nitrophenol was obtained from Eastman Kodak Chemicals, Rochester, NY, U.S.A., and was recrystallized twice from ethanol/0.1 M-HCl and dried *in vacuo*. HS-Bzl, 5,5'-dithiobis-(2-nitrobenzoic acid) and 4,4'-dithiodipyridine (aldrithiol) were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. GBz-ONp was purchased from Sigma, and *p*-guanidinobenzoic acid from Research Organics, Elk Grove Village, IL, U.S.A.

Synthesis of GBz-SBzl

GBz-SBzl was synthesized by using either the dicyclohexylcarbodi-imide or the carbonyldi-imidazole coupling method. The carbonyldi-imidazole method was judged to be superior, since in the dicyclohexylcarbodi-imide method an *N*-acylurea by-product was found to contaminate the product even after column chromatography.

Carbonyldi-imidazole method

To dry dimethylformamide (10 ml) was added *p*-guanidinobenzoic acid (2.6 g, 10 mmol) and carbonyldi-imidazole (1.6 g, 10 mmol). The solution was stirred at room temperature for 35 min as CO_2 was liberated. HS-Bzl (1.29 ml, 11 mmol) was then added dropwise, and the mixture was stirred for 15 min. The solvent was removed under reduced pressure, and the resulting oil was purified by silica-gel chromatography. Elution was accomplished with methanol/ethyl acetate (1:4, v/v). The product was crystallized with methanol/ethyl acetate and recrystallized with methanol/ethyl acetate/diethyl ether. The product (1.6 g, 50% yield) was a white crystalline solid, showing one spot (R_F 0.80) on t.l.c. with chloroform/methanol (2:1, v/v). Its melting point was $154\text{--}155^\circ\text{C}$ (uncorr.). N.m.r. δ 4.4 p.p.m., singlet (2 protons); δ 7.0–8.0 p.p.m., singlet superimposed on a quartet (9 protons). U.v. λ_{max} 263 nm (0.10 M-Tris, pH 8.3). Calc. for $\text{C}_{15}\text{H}_{16}\text{ClN}_3\text{OS}$, $\frac{1}{2}\text{H}_2\text{O}$: C, 53.72; H, 5.26; N, 12.53; S, 9.56. Found: C, 53.52; H, 5.02; N, 12.55; S, 9.50%.

Hydrolytic stability of GBz-SBzl

The hydrolysis of the thioester was followed by monitoring the decrease in absorbance at 294 nm, where the hydrolysis products have negligible absorbance. The experiment was run in Tris buffer at pH values 7.5 and 8.5.

Titration procedure

A typical titration for trypsin consisted of the following steps. To 2.0 ml of buffer (0.10 M-Hepes, pH 7.5, containing 5 mM- CaCl_2) in a 1 cm quartz

cuvette was added 50 μ l of a 5.0 mM solution of 4,4'-dithiodipyridine in dimethyl sulphoxide. The cuvette was placed in a Beckman 35 spectrophotometer and 'balanced' against an identical reference cuvette. To each cell was then added 50 μ l of a 5.0 mM solution of GBz-SBzl in dimethyl sulphoxide and a stable baseline was established. Finally, 50 μ l of enzyme was added to the sample cuvette and 50 μ l of 1 M-HCl was added to the reference cuvette. The burst was recorded and the post-burst period monitored for 5 min.

In some cases microcuvettes were employed when the enzyme solution was relatively dilute (<10 μ M stock solution) or the enzyme supply was limited. In another modification, the titrant was added last to both reference and sample cells, giving the same results as when the titrant was added before the enzyme.

Determination of k_2 and K_s

The constants k_2 and K_s were determined for trypsin and thrombin by titrating the enzymes with small excesses of GBz-SBzl and recording the pre-steady-state portion of the reaction (see Fig. 2). The resulting curves were analysed as described by Bender *et al.* (1967).

Results

Synthesis

GBz-SBzl was synthesized by coupling HS-Bzl with *p*-guanidinobenzoic acid with the use of carbonyldi-imidazole. After purification, the compound was a white crystalline solid. Its structure and that of the related *p*-nitrophenyl ester are shown in Fig. 1.

Hydrolysis of GBz-SBzl

The hydrolysis rate constant of GBz-SBzl in 0.10 M-Tris buffer, pH 8.5, was found to be

$0.11 \times 10^{-4} \text{ s}^{-1}$; the half-life under these conditions is $1.0 \times 10^3 \text{ min}$. Under similar conditions (0.10 M-Tris buffer, pH 8.3) GBz-ONp was found to have a hydrolysis rate constant of $1.1 \times 10^{-4} \text{ s}^{-1}$ (Chase & Shaw, 1967). Thus GBz-SBzl is about 10 times more stable than GBz-ONp in Tris at pH 8.5. In another experiment, with 0.10 M-Hepes buffer, pH 7.5, GBz-SBzl was found to be only 6% hydrolysed after 12 h. Therefore, under typical titration conditions, spontaneous hydrolysis of GBz-SBzl is a negligible factor.

Titration theory

According to titration theory as developed and presented by Bender *et al.* (1967), the reaction of a serine proteinase and GBz-SBzl may be represented by the following mechanism (R = *p*-guanidinophenyl):

where K_s is the equilibrium dissociation constant for the adsorptive complex, k_2 is the first-order acylation rate constant, and k_3 is the deacylation rate constant. On the basis of this scheme, the observed burst of absorbance corresponding to HS-Bzl production (II) may be derived from eqn. (2):

$$\Pi = [E]_0 [k_2 / (k_2 + k_3)]^2 / (1 + K_{m(\text{app.})} / [S]_0)^2 \quad (2)$$

There are three basic requirements that a titrant must fulfil for it theoretically to allow enzyme concentration determination (i.e. $\Pi = [E]_0$) at a single titrant concentration: (a) $k_2 \gg k_3$; (b) $[S]_0 \gg K_{m(\text{app.})}$; (c) Π must result from reaction only at the active site of the enzyme. The first two requirements emerge directly from eqn. (2). If (a) is met but not (b), then Π will depend on the titrant concentration. If (b) is met and not (a), the burst will be replaced by a linear increase in absorbance representing enzymic turnover. To verify the validity of (a) and (b) for a particular enzyme, it is best to determine the kinetic constants to allow direct comparison.

The constants k_2 and K_s were evaluated as described by Bender *et al.* (1967). Fig. 2 illustrates the pre-steady-state portion of the reaction of GBz-SBzl with thrombin where the $[S]_0/[E]_0 = 3$ (this allows the pre-steady-state portion to be visualized more clearly). For evaluation of the steady-state rate constant and the determination of k_2 and K_s , $[S]_0/[E]_0$ ratios of 4.2–21 were used for thrombin and 3–17 were used for trypsin. The k_2/k_3 is so large that even ratios less than 10 result in only slight deviations in the steady-state rate constant.

Fig. 1. Structural formulae for GBz-SBzl and GBz-ONp

Fig. 2. Approach to steady state of the thrombin-catalysed reaction with GBz-SBzl. Thrombin ($1 \mu\text{M}$) was allowed to react with GBz-SBzl ($3.0 \mu\text{M}$). For full experimental details see the text.

Bovine trypsin and thrombin

First, to show that the burst varied linearly with trypsin and thrombin concentrations, various concentrations of the enzymes were titrated at a single titrant concentration. For example, when various concentrations of thrombin (0.60 – $3.0 \mu\text{M}$) were titrated with GBz-SBzl (0.20 mM), a plot of the burst ΔA_{324} versus thrombin concentration gave a straight line (correlation coefficient, 0.9987 ; slope, $2.0 \times 10^4 \Delta A_{324}/\mu\text{M}$; ordinate intercept, $0.072 \mu\text{M}$). Next, a single concentration of trypsin or thrombin was titrated with various concentrations of GBz-SBzl. The variation in burst size was only $\pm 5\%$ for each enzyme over a 20-fold range of GBz-SBzl concentration (3 – $60 \mu\text{M}$).

The kinetic constants for the reactions between trypsin or thrombin and GBz-SBzl are presented in Table 1. Included in Table 1 for comparison are the previously determined constants for ethyl *p*-guanidinobenzoate (GBz-OEt), GBz-ONp, and fluorescein mono-*p*-guanidinobenzoate hydrochloride (GBz-FI). The last-mentioned compound is a recently introduced fluorogenic titrant of serine proteinases (Melhado *et al.*, 1982), and is itself an ester of *p*-guanidinobenzoic acid. Since the acyl-enzyme for each substrate is the same, k_3 for a particular enzyme for all of the titrants will be the same. In the present paper we have used the previously reported k_3 values of $3.4 \times 10^{-5} \text{ s}^{-1}$ for trypsin and $98 \times 10^{-5} \text{ s}^{-1}$ for thrombin.

To verify the third requirement, i.e. that the active site of the enzyme is involved, trypsin was allowed to react with GBz-SBzl and its activity was monitored as a function of time with a good substrate, Z-Arg-SBu¹. The results of this experiment are shown in Fig. 4. GBz-SBzl was clearly reacting at the active site of the enzyme.

The ϵ value to be used in the titration with the

Fig. 3. Typical burst for a thrombin titration with GBz-SBzl.

The experiment was performed by placing in a 1 cm cuvette 2.0 ml of buffer (0.10 M -Hepes, pH 7.2, containing 20 mM -NaCl), $50 \mu\text{l}$ of GBz-SBzl (10 mM) in dimethyl sulphoxide, and $50 \mu\text{l}$ of thrombin. The reference cuvette was treated identically except for the addition of buffer instead of enzyme. The burst (Π) was determined as illustrated. The cuvette concentration of thrombin was $30 \mu\text{M}$.

thioester was determined and verified as follows. First, to a $100 \mu\text{M}$ solution of 4,4'-dithiodipyridine in 50 mM -Tris buffer, pH 7.5, was added a portion of HS-Bzl (3.4 mM in dimethyl sulphoxide). The maximum increase in absorbance was reached before mixing was complete even when the concentration of HS-Bzl in the cuvette was $5 \mu\text{M}$ (the lowest con-

Table 1. *Kinetic constants for the reaction of trypsin and thrombin with titrants derived from p-guanidinobenzoic acid*
 The data for GBz-OEt, GBz-ONp and GBz-FI are respectively from Mares-Guia & Shaw (1967), Chase & Shaw (1969) and Melhado *et al.* (1982). The k_3 values of $3.4 \times 10^{-5} \text{ s}^{-1}$ for trypsin (Mares-Guia & Shaw, 1967) and $98 \times 10^{-5} \text{ s}^{-1}$ for thrombin (Chase & Shaw, 1969) determined with GBz-ONp were used with GBz-SBzl.

Enzyme	Titrant	$k_2 \text{ (s}^{-1}\text{)}$	$K_s \text{ (M)}$	$k_3 \text{ (s}^{-1}\text{)}$	$K_{m(\text{app.})} \text{ (M)}$	$k_2/K_s \text{ (M}^{-1} \cdot \text{s}^{-1}\text{)}$
Bovine trypsin	GBz-OEt	0.0046	5.36×10^{-4}	3.4×10^{-5}	4.0×10^{-6}	8.6
	GBz-ONp	1.95	0.61×10^{-6}	3.4×10^{-5}	1.1×10^{-11}	3.1×10^6
	GBz-SBzl	0.49	13.9×10^{-6}	3.4×10^{-5}	96×10^{-11}	3.5×10^4
	GBz-FI	0.194	6.58×10^{-8}	1.7×10^{-5}	5.63×10^{-12}	2.95×10^6
Bovine thrombin	GBz-OEt	0.0019	0.0073	98×10^{-5}	0.0033	0.16
	GBz-ONp	0.13	3.95×10^{-6}	98×10^{-5}	3.0×10^{-8}	3.2×10^4
	GBz-SBzl	0.13	66.0×10^{-6}	98×10^{-5}	48×10^{-8}	2.0×10^3
	GBz-FI	0.0602	2.17×10^{-5}	178×10^{-5}	6.42×10^{-7}	2.77×10^3

centration tested). Under these conditions the ϵ of the thiopyridone was found to be $19.2 \pm 0.1 \text{ mm}^{-1} \cdot \text{cm}^{-1}$. The ϵ determined for GBz-SBzl under the same conditions was $2.10 \pm 0.05 \text{ mm}^{-1} \cdot \text{cm}^{-1}$. Thus, for titration purposes, the appropriate ϵ is the difference between these two: $17.1 \pm 0.2 \text{ mm}^{-1} \cdot \text{cm}^{-1}$. Verification of this value for trypsin was accomplished by using GBz-ONp for comparison. The ϵ of 4-nitrophenol at 410 nm was determined to be $11.6 \pm 0.5 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ under the above-mentioned conditions. A solution of trypsin was titrated with GBz-ONp and found to give an average concentration of $25.9 \pm 1.1 \mu\text{M}$. Titration with GBz-SBzl was then performed with 4,4'-dithiodipyridine at 324 nm. On the basis of the observed bursts and the known trypsin concentration, an ϵ of $17.1 \pm 0.7 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ was again obtained. Thus this value is the ϵ of choice for titrations with the thioester reagent in conjunction with 4,4'-dithiodipyridine.

Finally, a trypsin solution that was $50 \mu\text{M}$ by GBz-ONp and GBz-SBzl (4,4'-dithiodipyridine) titrations was shown to give comparable results with GBz-SBzl and Ellman's reagent. The burst was measured at 412 nm, where the absorbing species has an ϵ of $13.6 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (pH 8.0).

Chymotrypsin A_α

In the original study of GBz-ONp by Chase & Shaw (1967) they reported that the reagent reacted with chymotrypsin A_α to give a burst followed by fairly rapid turnover. These results were confirmed by us. However, on interaction of GBz-SBzl with chymotrypsin A_α , no burst was observed. The enzyme did hydrolyse GBz-SBzl, but at a relatively low rate: 6 times slower than with GBz-ONp under the same conditions. Trypsin may therefore be selectively titrated by GBz-SBzl in the presence of chymotrypsin A_α ; its only effect would be to increase the rate of post-burst hydrolysis. Fig. 5 depicts the difference in behaviour between GBz-SBzl and GBz-ONp with chymotrypsin A_α . Since the chymotrypsin-catalysed hydrolysis of GBz-SBzl

is quite slow, trypsin could easily be titrated in the presence of equimolar concentration of chymotrypsin without encountering difficulties in determining the burst by extrapolation.

Human lung tryptase

A solution of human lung tryptase was found to produce a burst of absorbance with both GBz-ONp and GBz-SBzl. With the use of microcuvettes for titrations, the concentration of the stock solution was found to be $3 \mu\text{M}$ by GBz-SBzl ($\epsilon = 17.1 \text{ mm}^{-1} \cdot \text{cm}^{-1}$, 324 nm) and $4 \mu\text{M}$ by GBz-ONp ($\epsilon = 12.6 \text{ mm}^{-1} \cdot \text{cm}^{-1}$, 410 nm). The titrations were performed in 0.10 M-Tris/HCl buffer, pH 7.5, containing 5 mM-CaCl₂. Silylated glassware was used throughout. One reason for the discrepancy in these results is the difference in post-burst hydrolysis observed with the two titrants. With GBz-SBzl it was negligible, whereas with GBz-ONp it was quite significant ($3.33 \times 10^{-5} A$ units/s, $[\text{GBz-ONp}]_{\text{cuvette}} = 0.14 \text{ mM}$). This introduces more error into the GBz-ONp titration. The difference in post-burst hydrolysis rates may be explained by the fact that the thioester is more stable and it is probably not as prone to hydrolysis catalysed by other parts of the enzyme.

In another experiment, GBz-SBzl was shown to be interacting at the active site, as previously described for trypsin. Again, Z-Arg-SBu¹ was employed as the substrate.

Limited quantities of enzyme precluded investigation of the kinetic constants associated with the reaction, but the results are strongly indicative of an accurate titration. The requirement that $[\text{S}]_0 \gg K_{m(\text{app.})}$ has not been proven, but $K_{m(\text{app.})}$ would have to be $>20 \mu\text{M}$ for the titration to be significantly affected by $[\text{S}]_0$ (on the basis of a typical cuvette $[\text{S}]$ of $200 \mu\text{M}$).

Bovine protein C

A sample of bovine activated protein C was found to be titratable by GBz-SBzl. In two trials it was found to give an average concentration of

Fig. 4. Loss of activity of trypsin as a function of time during reaction with GBz-SBzl as measured with the substrate Z-Arg-SBu¹

The enzyme (70nM) was incubated at room temperature with various quantities of titrant (A–D) and assayed for activity at various times. Curve A, 520-fold excess of titrant; curve B, 10-fold excess of titrant; curve C, 5.0-fold excess of titrant; curve D, equivalent concentrations of trypsin and titrant; curve E, trypsin alone.

$4.7 \pm 0.2 \mu\text{M}$ for a particular stock solution. This compares with a calculated concentration of $5.0 \mu\text{M}$ (based on A_{280}). Again, this sample was too dilute for a determination of kinetic constants.

Human Factor XIIa and Bovine Factor Xa_β

Both enzymes were found to give bursts with GBzl-SBzl. Human factor XIIa was titrated twice and found to give a concentration of $7.5 \pm 0.2 \mu\text{M}$. This was 50% of the calculated concentration (based on A_{280}). Bovine Factor Xa_β was titrated with GBz-ONp and found to give an average concentration of $23 \mu\text{M}$ (81% of the calculated concentration based on A_{280}), and titration with GBz-SBzl gave an average concentration of $19 \mu\text{M}$ (68% of that based on A_{280}). It should be noted that these titrations were performed in microcuvettes and only repeated twice each, such that the discrepancy in the two results is probably attributable to these factors.

Fig. 5. Reaction of chymotrypsin A_α with GBz-SBzl and GBz-ONp monitored at 324 nm and 410 nm respectively. The enzyme concentration was $1.3 \mu\text{M}$ in each case and the concentration of each titrant was $90 \mu\text{M}$. These are typical conditions for titration of a trypsin-like enzyme. GBz-ONp produced a burst (Π) with this enzyme whereas GBz-SBzl did not. The ϵ value for GBz-SBzl is 1.4-fold higher than that for GBz-ONp, so the difference in hydrolysis rates is larger than is apparent from the graph.

Discussion

The usefulness of an enzyme titrant such as GBz-ONp or GBz-SBzl is dependent on the following characteristics, which have been noted previously by Bender *et al.* (1966): (1) a high k_2/k_3 ratio such that acylation is much faster than deacylation; (2) a high $[S]/K_{m(\text{app.})}$ ratio, so that the titration is independent of titrant concentration; (3) a high ϵ for the absorbing species produced in the titration at the pH employed (preferably near neutrality); (4) a low rate of spontaneous hydrolysis of the titrant in the buffer; (5) maximum specificity of the titrant, such that one enzyme may be titrated in the presence of another. The titrant GBz-ONp has been shown to be highly effective in most of these areas. The titrant introduced in the present paper, GBz-SBzl, likewise has been shown to be an excellent titrant that, while retaining many of the useful aspects of GBz-ONp, possesses some unique advantages of its own.

The compound itself is an easily handled white

crystalline compound, which is also easily synthesized in one step from commercially available reagents. During a titration with GBz-SBzl, the absorbing species is not released directly as with GBz-ONp, but is produced in a second, coupled, reaction. This endows GBz-SBzl with flexibility in its use. For example, two procedures are illustrated in the present paper, one involving 4,4'-dithiodipyridine at 324 nm and one involving Ellman's reagent at 410 nm. Thus the titration may be accomplished in the u.v. or visible spectral regions. Further, as new thiol-detecting reagents become available, the flexibility and sensitivity of GBz-SBzl could increase.

In conjunction with 4,4'-dithiodipyridine in particular, GBz-SBzl titrations have several advantages. Since the resulting thiopyridone has a high ϵ value that is relatively pH-independent over the range 6.0–9.0, pH considerations are significantly diminished as compared with GBz-ONp. GBz-SBzl produces the same acyl-enzyme as GBz-ONp and therefore benefits from the same extraordinarily low k_3 value, the most important feature of both titrations. Although GBz-SBzl does not bind to trypsin or thrombin as well as does GBz-ONp (see Table 1), the resulting $K_{m(\text{app.})}$ values are still comfortably low to satisfy the requirement $[S]_0 \gg K_{m(\text{app.})}$. Perhaps because GBz-SBzl is more stable to nucleophilic attack than GBz-ONp, GBz-SBzl does not react to produce a burst with chymotrypsin A_α but instead turns it over slowly. This specificity is a clear advantage of GBz-SBzl in comparison with GBz-ONp, which does produce a burst with chymotrypsin A_α .

Another advantage of GBz-SBzl is its increased stability as compared with GBz-ONp. It is at least 10 times more stable in Tris buffer at pH 8.5 than is GBz-ONp. Thus stock solutions may be stored longer and spontaneous hydrolysis presents no interference during k_2 and K_s determinations, which in some instances (e.g. with thrombin) took 30 min to reach the steady state.

Although GBz-SBzl is a convenient and stable titrant, several more sensitive fluorogenic titrants for trypsin-like enzymes have been reported. These include GBz-MU (Jameson *et al.*, 1973) and GBz-Fl (Melhado *et al.*, 1982). Trypsin can be titrated with these reagents at concentrations as low as 6 and 0.3 nM respectively, and GBz-SBzl is suitable for titration in the 1–10 μM range with 4,4'-dithiodipyridine or Ellman's reagent. However, for most routine titrations high sensitivity is not required and spectrophotometers are more available than fluorimeters. With thrombin, both the oxygen esters GBz-MU and GBz-Fl are less suitable for titrations owing to the high turnover rates and the difficulty in determining the burst accurately by extrapolation. It is important to note that GBz-SBzl has the potential to be used fluorogenically should a fast-reacting

fluorogenic thiol-detecting reagent be discovered. Possibilities for such reagents include the recently reported difluorescein disulphide (Wingender & Arellano, 1982) and fluorescence-quenched thiol probes (Teale & Constable, 1981). Use of such reagents with GBz-SBzl would couple the sensitivity of fluorescence and the stability and low turnover rates of the thioester. The usefulness of GBz-SBzl could also be extended by use of more sensitive chromogenic thiol reagents such as 4,4'-bis(dimethylamino)diphenylcarbinol (Rohrbach *et al.*, 1973). One disadvantage to GBz-SBzl is the inability to use it with any proteinase that contains a free thiol group unless appropriate controls are run to correct for its reaction with the thiol-detecting reagent.

In summary, GBz-SBzl possesses the following advantages over presently available titrants: (1) flexibility in mode and wavelength of detection; (2) stability; (3) specificity. And, in conjunction with 4,4'-dithiodipyridine, one may add: (4) high ϵ at neutral pH; (5) minimal pH-dependence of ϵ . It is hoped that, in light of these features, GBz-SBzl will be a useful alternative to GBz-ONp and other titrants for the determination of concentrations of trypsin-like serine proteinases.

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