Hers, H. G. (1963), *Biochem. J. 86*, 11.
Hug, G., Garancis, J. C., Schubert, W. K., and Kaplan, S. (1966), *Amer. J. Dis. Child. 111*, 457.
Huijing, F. (1964), *Clin. Chim. Acta 9*, 269.
Jeffrey, P. L., Brown, D. H., and Brown, B. I. (1970a), *Biochemistry 9*, 1403.
Jeffrey, P. L., Brown, D. H., and Brown, B. I. (1970b), *Biochemistry 9*, 1416.
Lejeune, N., Thinès-Sempoux, D., and Hers, H. G. (1963), *Biochem. J. 86*, 16.
Lowry, O., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Rosenfeld, E. L., and Belenki, D. M. (1968), *Bull. Soc. Chim. Biol. 50*, 1305.
Steinitz, K., and Rutenberg, A. (1967), *Israel J. Med. Sci. 3*, 411.
Witzleben, C. L. (1969), *Lab. Invest. 20*, 424.
Zellweger, H., Brown, B. I., McCormick, W. F., and Tu, J.-B. (1965), *Ann. Paediat. 205*, 413.

Determination of Trypsin–Inhibitor Complex Dissociation by Use of the Active Site Titrant, *p*-Nitrophenyl *p'*-Guanidinobenzoate*

James C. Zahnley and John Gorton Davis

ABSTRACT: The active site titration of trypsin with *p*-nitrophenyl *p'*-guanidinobenzoate was used to determine the extent of reaction of three naturally occurring inhibitors with trypsin. The equilibrium between free and inhibitor-bound trypsin was determined by measuring the initial burst of *p*-nitrophenol liberated with and without inhibitor. Dissociation of the trypsin–inhibitor complex was followed by measuring the subsequent increase of *p*-nitrophenol liberated with time. Hence, both kinetic and equilibrium data were obtained. All protein inhibitors tested reduced the initial burst. The presence of excess ovoinhibitor eliminated the initial burst. Rates of dissociation of the trypsin–inhibitor complexes increased in the order: soybean trypsin inhibitor $\simeq$ chicken ovomucoid $<$ chicken ovoinhibitor $\ll$ benzamidine. Competitive inhibition by ovoinhibitor could be shown directly at $\leq 10^{-5}$ M *p*-nitrophenyl *p'*-guanidinobenzoate. Dissociation of the trypsin$_1$–ovoinhibitor complex followed apparent first-order kinetics up to at least 65% completion. Inhibition curves obtained by active site titration agree with those obtained by rate assay. These data suggest that this active site titration can be used to determine trypsin in the presence of an excess of dissociable endogenous inhibitors.

Rate assays of remaining trypsin activity are commonly used to assay naturally occurring trypsin inhibitors (Laskowski and Laskowski, 1954; Vogel *et al.*, 1968). In using rate assays to study the action of ovoinhibitors from chicken egg white (Davis *et al.*, 1969), we observed progressive increase in trypsin esterase activity at high inhibition (unpublished data). The effect was usually small, and it resembled the displacement observed by Green (1953) but with less substrate. The curvature could not be attributed entirely to substrate activation (Trowbridge *et al.*, 1963) because use of benzoyl-L-arginine ethyl ester as substrate instead of tosyl-L-arginine methyl ester did not completely eliminate the effect. Hence, use of a direct assay method based on stoichiometric titration of the free-trypsin active sites (all-or-none assay) available at the time of addition of substrate (titrant) seemed desirable. The advantages of such all-or-none assays of enzyme concentration over rate assays of enzyme activity have been pointed out by Koshland *et al.* (1962) and by Bender *et al.* (1966).

Our objective was to assay inhibitors by all-or-none assay of the active trypsin remaining, as a suitable titrant, *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride (NPGB)[1] is available (Chase and Shaw, 1967). Since soybean trypsin inhibitor prevents the reaction of trypsin with another active site titrant (Elmore and Smyth, 1968), and the competitive inhibitor, benzamidine, slows the reaction between trypsin and NPGB (Chase and Shaw, 1967, 1969), we might be able to determine the stoichiometry, the equilibrium, and the kinetics (dissociation of inhibitor–trypsin complex) of the reaction between ovoinhibitor and trypsin. In addition, trypsin has high affinity for NPGB and *p*-guanidinobenzoyl-trypsin deacylates very slowly (Chase and Shaw, 1967, 1969). (NPGB can be viewed here as either a substrate with a very low rate of deacylation or as a nearly irreversible inhibitor.)

The results reported in this paper show that NPGB titration can be used to assay protein inhibitors of trypsin. Ovoinhibitor is differentiated directly from chicken ovomucoid and soybean trypsin inhibitor in this assay by the more rapid postburst hydrolysis of NPGB by trypsin in the presence of ovoinhibitor.

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[1] Abbreviations used are: NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate·HCl; OI, chicken egg white ovoinhibitor; OM, chicken egg white ovomucoid.

TABLE I: Absorption Coefficients and Molecular Weights.

Protein	$E^{1\%}_{1\,cm}$	λ (mμ)	Molecular Weight	Reference
Trypsin	15.4	280	23,800	*a*
Soybean trypsin inhibitor	9.44	280	21,500	*b*
Chicken ovoinhibitor	6.75	278	47,600	*c*
Chicken ovomucoid	4.10	277.5	29,000	*d*

[a] Worthington (1967); [b] Wu and Scheraga, (1962); [c] Davis *et al.* (1969); [d] Chatterjee and Montgomery (1962); Donovan (1967).

Experimental Procedure[2]

Materials. *p*-Nitrophenyl *p'*-guanidinobenzoate hydrochloride was synthesized according to Chase and Shaw (1967). Benzamidine hydrochloride hydrate was obtained from Aldrich Chemical Co. Recrystallized *p*-nitrophenol (twice from acetone–water, Dr. A. K. Balls) was used. Other chemicals were commercial products.

Bovine trypsin (EC 3.4.4.4), twice-crystallized, TRL lot 8GA, and crystalline soybean trypsin inhibitor (chromatographically prepared, SI lot 8FA) were obtained from Worthington. Chicken ovomucoid was purified by the method of J. G. Davis as described in Donovan (1967). Chicken ovoinhibitor was the mixture prior to DEAE-cellulose chromatography (Davis *et al.*, 1969), except that it was further purified by redissolving in H_2O to give an absorbance of 1 OD/ml and reprecipitating with 0.285 saturated $(NH_4)_2SO_4$ at pH 6 and room temperature until starch gel electrophoresis of the supernatant showed no contaminating proteins present. (Usually 3–5 cycles were required.)

Methods. Buffer and salt solutions were filtered through Millipore type HA membranes before use to reduce scatter (Bender *et al.*, 1966). Trypsin was dissolved in cold 2 mM HCl, 0.02 M $CaCl_2$; inhibitors were dissolved or diluted in 0.02 M KCl (for soybean trypsin inhibitor or OM) or 0.10 M KCl (for OI). Protein solutions were centrifuged to remove insoluble matter. Protein concentrations were determined by ultraviolet absorption, using the optical factors and molecular weights given in Table I.

Assay conditions were modified from the Chase and Shaw (1967) procedure to permit addition of different volumes of trypsin and inhibitor. Final concentrations were normally 0.05 M sodium Veronal-HCl, pH 8.36 or 8.40 at 22°; 0.02 M $CaCl_2$; 0.10 M KCl; 0.2 mM HCl (from trypsin); 1×10^{-4} M NPGB; and 1% (v/v) dimethylformamide in a total reaction volume of 1.0 ml. Final pH of the mixture was above 8.25, and ϵ_{410} of *p*-nitrophenol was 16,370.

In a typical experiment, inhibitor and trypsin were allowed to equilibrate for 5–7 min (for soybean trypsin inhibitor or OM) or 10–12 min (for OI) before NPGB was added. When higher NPGB concentrations ($\geq 5 \times 10^{-5}$ M) were used, controls were run in the reference cuvet according to Chase and Shaw (1967). When lower NPGB concentrations ($1–2 \times 10^{-5}$ M) were used, corrections for nonenzymatic hydrolysis were obtained from separate control runs without trypsin, because the corrections were small under these conditions. Assays were run at room temperature (22 ± 1°), and cells were not thermostatted. The 0 to 0.1 absorbance slide-wire of a Cary 15 spectrophotometer was used to conserve inhibitor, and to have protein concentrations comparable with those used in rate assays with benzoyl-L-arginine ethyl ester and benzoyl-DL-arginine *p*-nitroanilide. Typical settings on the Cary were: dynode setting 1; amplifier sensitivity 1.5; slit width 0.1–0.2 mm. Absorption cells of 1-cm path length were used throughout.

Because inhibition experiments involving a number of assays require several hours to complete, stable trypsin solutions were essential if results with different amounts of enzyme or inhibitor were to be compared. Fresh trypsin solutions were 75–80% active enzyme by NPGB titration. After about 3 hr, the solutions had stabilized sufficiently (at 65% active enzyme by NPGB titration) to be used. This initial loss of activity has been described by Bender *et al.* (1966). All experiments are presented on the basis of active trypsin.

Amidase rate assays with benzoyl-DL-arginine *p*-nitroanilide as substrate were carried out according to Erlanger *et al.* (1961).

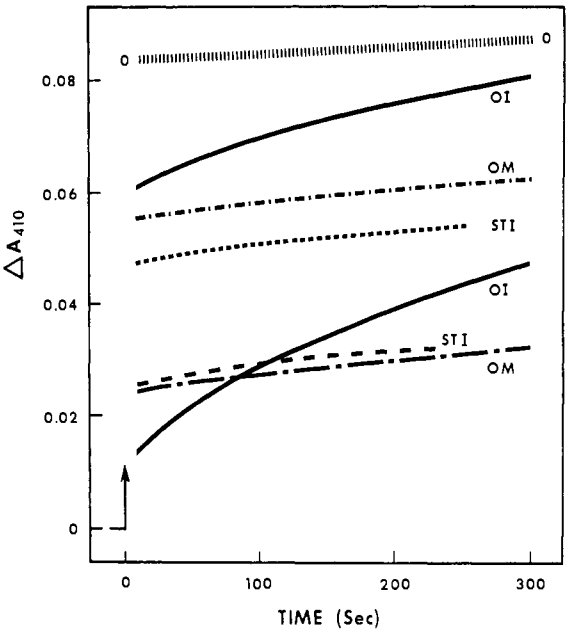

FIGURE 1: Time course of reaction of NPGB and trypsin with and without inhibitors. *p*-Nitrophenol release (ΔA_{410}) is shown for no inhibitor (0||||||0); and for two concentrations of chicken ovoinhibitor (——, OI); chicken ovomucoid (— – — –, OM); and soybean trypsin inhibitor (– – – – or — — —, STI). The vertical arrow indicates the time of addition of NPGB (see Methods).

Results

The initial burst of *p*-nitrophenol released when NPGB was added to trypsin solutions was reduced if the enzyme had been previously equilibrated with any of three naturally occurring inhibitors (Figure 1). The extrapolated zero-time absorbance is a measure of the free active trypsin present when NPGB is

[2] Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

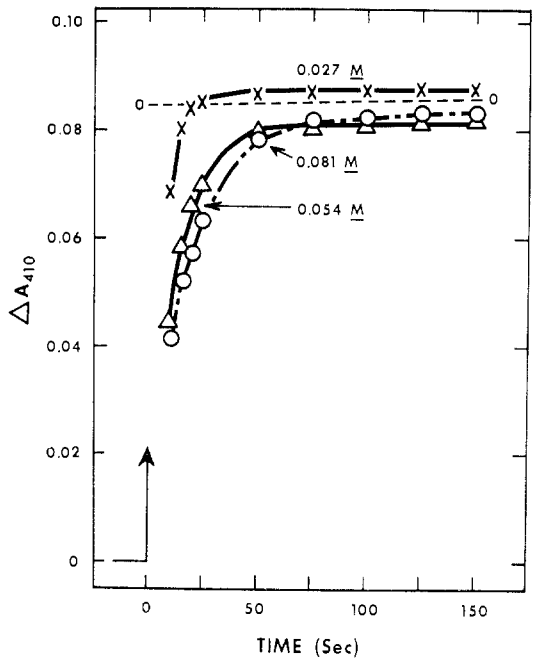

FIGURE 2: Trypsin–NPGB reaction in the presence of benzamidine. Points indicate times at which corrections for nonenzymatic hydrolysis of NPGB by benzamidine were calculated. Inhibitor: none (O-----O); 0.027 M (×——×), 0.054 M (Δ——Δ), and 0.081 M (O—O) benzamidine.

added (Chase and Shaw, 1967). Mixtures of trypsin with OM or soybean trypsin inhibitor show a slow postburst release of *p*-nitrophenol, as measured by the increase in ΔA_{410}. However, trypsin plus OI shows a more rapid release of *p*-nitrophenol with time, especially at higher OI:trypsin ratios. Commercial OM, which contains some OI, shows a more rapid postburst increase in *p*-nitrophenol release than the purified, OI-free preparation shown. (OI in the absence of trypsin does not catalyze significant hydrolysis of NPGB.) By comparison, even

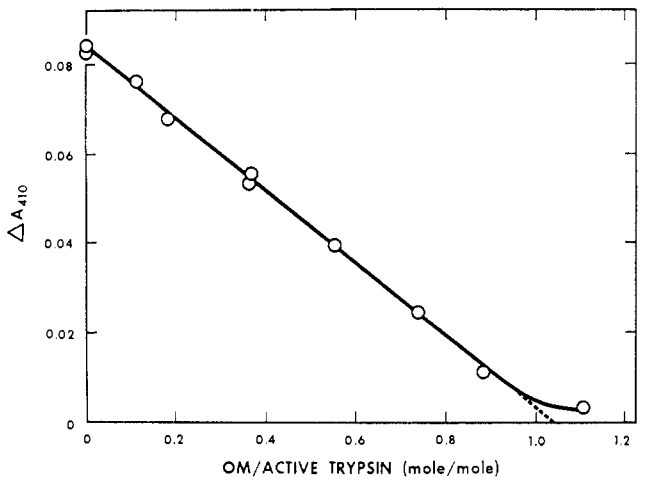

FIGURE 3: Inhibition of the trypsin–NPGB reaction by chicken ovomucoid. ΔA_{410} is the "burst" (obtained by extrapolation to zero time). The straight-line portion of the curve is an unweighted least-squares fit.

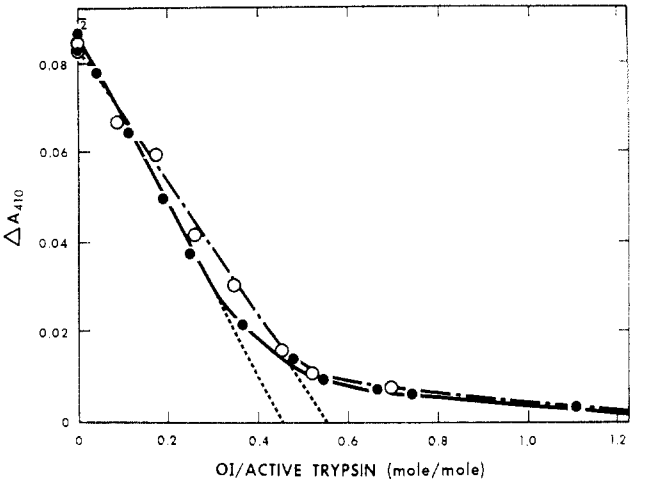

FIGURE 4: Inhibition of the trypsin–NPGB reaction by OI. NPGB (1×10^{-4} M, O— - —O; or 1×10^{-5} M, ●—●) was added to mixtures containing trypsin and OI (see Methods). See Figure 3 for other details.

high concentrations of benzamidine ($\geq 10^3 \times K_i$) are almost completely displaced in 60 sec or less (Figure 2). The failure of the lines to coincide after leveling off is due to small errors in the corrections used. The effect of benzamidine on the NPGB–trypsin reaction has been described in detail by Chase and Shaw (1969). Figures 1 and 2 show that the effect of OI is intermediate to the effects of benzamidine and of soybean trypsin inhibitor or OM.

When different concentrations of OM were added to a fixed concentration of trypsin, the initial burst of *p*-nitrophenol, when plotted as a function of inhibitor:enzyme ratio, yields an inhibition curve similar to that obtained from rate assays of residual enzyme activity (Figure 3). The extrapolated ratio for 100% inhibition is 1.04 ± 0.01 moles of OM/mole of active trypsin. (Standard deviations were obtained by dividing the probable error in the slope by 0.6745.) A similar curve was obtained for soybean trypsin inhibitor. The corresponding ratio was 0.95 ± 0.02 mole of soybean trypsin inhibitor/mole of active trypsin using the values listed in Table I, or 0.90 using the figures of Rackis *et al.* (1962).

Inhibition curves were obtained for OI plus trypsin at both 1×10^{-4} and 1×10^{-5} M NPGB (Figure 4). Inhibitor:enzyme ratios for 100% inhibition were 0.55 ± 0.02 and 0.47 ± 0.01 mole of OI per mole of trypsin, respectively. These results are consistent with evidence from rate assays (Feeney *et al.*, 1963; Tomimatsu *et al.*, 1966; Davis *et al.*, 1969) that 1 mole of OI binds 2 moles of trypsin. Dissociation constants can be calculated from the data presented in Figures 3 and 4. The dissociation constant calculated for OI–trypsin from Figure 4 is 4×10^{-8} M. This is in good agreement with the values of 1.5×10^{-8} M (competitive inhibition) or 5×10^{-8} M (noncompetitive inhibition) obtained by Davis *et al.* (1969) from rate assays. Similarly, the dissociation constants for OM–trypsin calculated from the data presented in Figure 3 is in good agreement with that given by Green (1953). Inhibition was linear with OI concentration up to about 80% inhibition at 1×10^{-4} M NPGB, but only up to about 70% inhibition at 1×10^{-5} M NPGB (Figure 4). The rate of postburst *p*-nitrophenol liberation from NPGB in the presence of OI plus

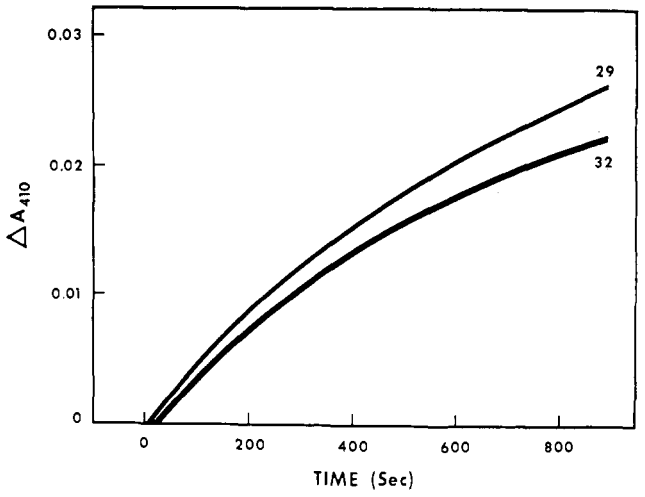

FIGURE 5: Release of *p*-nitrophenol from NPGB by OI–trypsin complex with excess OI present. Each assay contained 0.47 ml of eluate. A_{280}/ml values were 0.37 and 0.28 for fractions 29 and 32, respectively.

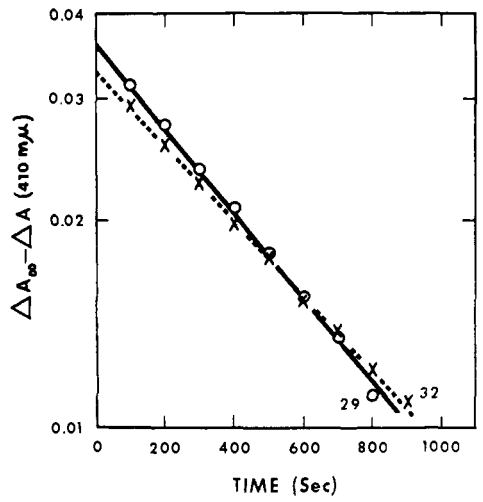

FIGURE 6: Semilogarithmic plots of $(\Delta A_\infty - \Delta A_t)$ *vs.* time of the data from Figure 5. Points indicate times at which differences were calculated for fractions 29 (○——○) and 32 (×---×).

trypsin increased as NPGB concentration was increased from 1×10^{-5} to 5×10^{-4} M. To see whether OI and NPGB compete for trypsin if added simultaneously, 9.5×10^{-6} M OI and 1×10^{-4} M NPGB were mixed, then 3.2×10^{-6} M active trypsin was added. No inhibition was observed; therefore, the enzyme is acylated by the guanidinobenzoyl group under these conditions faster than it can be inhibited by OI. These results, along with those in Figures 1 and 5, all indicate that inhibition of trypsin by OI is competitive, as expected.

When trypsin was reacted with OI and the proteins were separated by gel filtration, fractions consisting of OI–trypsin complex contaminated with free OI were obtained (J. G. Davis and J. C. Zahnley, manuscript in preparation). Aliquots of two fractions were tested with NPGB. No free trypsin was apparent initially, since no "burst" was observed (Figure 5). However, a trace of trypsin activity was detected by the amidase rate assay. Figure 5 shows that in the presence of excess OI, trypsin progressively becomes accessible to NPGB. Semilogarithmic plots of the differences between ΔA_t and an estimated ΔA_∞ are shown in Figure 6. The reaction with NPGB is apparently first-order up to at least 65% of complete dissociation of trypsin from the OI–trypsin complex. The observed rate constant is 1.3×10^{-3} sec^{-1}. Figure 4 shows that the excess of OI present is sufficient for OI–T_1 to predominate. From the dissociation constant given above, we can conclude that this rate constant is probably within an order of magnitude of the rate constant for dissociation of the OI–T_1 species.

Figure 7 shows the results obtained when trypsin concentration is varied and OI concentration is held constant. When no inhibitor is present, ΔA_{410} is directly proportional to active trypsin concentration. With inhibitor, the right-hand portions of the curves resemble those termed pseudoirreversible inhibition by Ackerman and Potter (1949). Significant dissociation is apparent at high OI : trypsin ratios (see also Figure 4).

Rates of postburst hydrolysis of NPGB by trypsin with different amounts of OI present were obtained from nine curves like those shown in Figure 1 and plotted in several ways to try to determine the kinetics of this apparent dissociation of the OI–trypsin complex. For data obtained with excess trypsin (low OI:trypsin ratios), semilogarithmic plots of inhibited trypsin ($\Delta A_{\text{uninhibited}} - \Delta A_{\text{inhibited}}$) against time were not straight lines. In contrast, data obtained with excess OI (high OI:trypsin ratios) showed simple first-order kinetics (Figures 5 and 6). These results are consistent with the mechanism shown in eq 1–3. Dissociation of the OI–T_2 complex occurs *via* two successive first-order reactions.

$$\text{OI–T}_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{OI–T} + \text{T} \qquad (1)$$

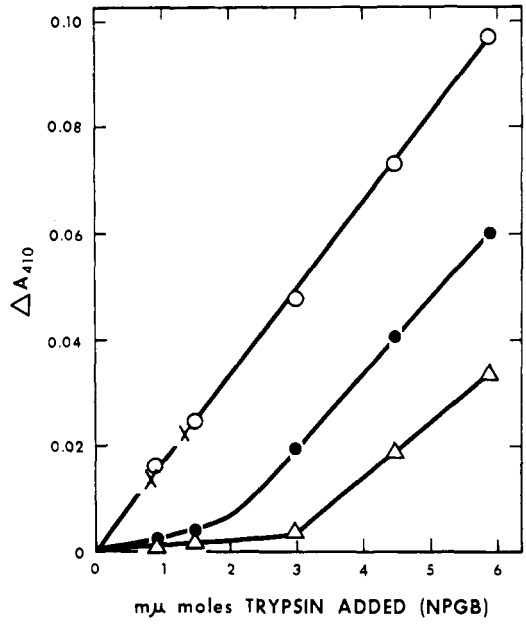

FIGURE 7: Effect of OI as a function of trypsin concentration. NPGB concentration was 1×10^{-5} M. Three separate runs were made at each trypsin concentration: (1) no inhibitor (○——○) or (—×—); (2) 0.955 mμmole (●——●) or (3) 1.91 mμmoles (△——△) OI. The abscissa is total active trypsin added. The ordinate shows the burst. Other details are given in Methods.

$$\text{OI–T} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{OI} + \text{T} \qquad (2)$$

$$\text{T} + \text{NPGB} \underset{}{\overset{K_s}{\rightleftharpoons}} \text{NPGB·T} \longrightarrow \text{GB–T} + \text{NP} \qquad (3)$$

Although the rate constants for association, k_{-1} and k_{-2}, are much larger than k_1 and k_2, respectively, the rate of reassociation is probably negligible under the conditions used. The high affinity of trypsin for NPGB, the NPGB concentration ($>10^2 \times K_s$ in this case), and the low free OI concentration all favor reaction of free trypsin with NPGB rather than with any free OI present. Using this approximation, we obtained estimates for the rate constants k_1 and k_2. (Note that dissociation is viewed as the forward reaction here.) The average of the data from nine curves closely fits a theoretical curve with $k_1/k_2 \cong 10$ and $k_2 \cong 1 \times 10^{-3}$ sec^{-1}. (Hence, $k_1 \cong 1 \times 10^{-2}$ sec^{-1}.) This value of k_2 agrees well with the observed rate constant, given above, obtained in the presence of excess OI (Figure 6). The extremes of the data fit curves having $k_1/k_2 \cong 10$ with $k_2 \cong 5 \times 10^{-4}$ sec^{-1} and $k_1/k_2 \cong 4$ with $k_2 \cong 3 \times 10^{-3}$ sec^{-1}. Uncertainties in the extrapolated burst probably account in part for the variations in the rate of trypsin liberation. The values given here are approximate, but they suggest that this method can be used to obtain rate constants for the dissociation.

Discussion

The complexes of trypsin with OM and soybean trypsin inhibitor appear more stable than the OI–trypsin complex, as judged by this assay method. This is consistent with previous measurements of the affinities of the inhibitors for trypsin (Green, 1953; Sri Ram *et al.*, 1954; Vithayathil *et al.*, 1961; Lebowitz and Laskowski, 1962; Chu and Hsu, 1965; Davis *et al.*, 1969). The high affinity of trypsin for NPGB facilitates direct detection of competitive inhibition (Green, 1953; Laskowski and Laskowski, 1954) by inhibitors such as OI which have intermediate affinities for trypsin (K_i values *ca.* 10^{-7}–10^{-8} M). Green (1953) demonstrated competitive inhibition of trypsin by displacing the inhibitors (OM, soybean trypsin inhibitor, and pancreatic trypsin inhibitor) with high concentrations of substrate (0.05 M benzoyl-L-arginine ethyl ester, [S] $\geq 10^3 \times K_m$) and by displacing the substrate with the inhibitors. Change in the rate of substrate turnover was a measure of change in free trypsin, and hence it was an indicator of displacement. Competitive inhibitors such as thionine or proflavine are also displaced by soybean trypsin inhibitors or ovomucoids (Glazer, 1965, 1967; Feinstein and Feeney, 1967). In the present method, free trypsin reacts once with NPGB to form a stable acyl-enzyme. Thus it is unnecessary to measure slopes from tangents to curves because the total *p*-nitrophenol released (ΔA_{410}) is equivalent to the total free enzyme.

According to the mechanism shown in eq 1–3, NPGB probably competes with OI for trypsin by reacting with free trypsin produced on dissociation of the OI–trypsin complex, thereby displacing the equilibrium. Estimates obtained when reasonable values for the rate constants are assumed suggest that essentially all of the trypsin reacts with NPGB and none with free OI. An alternative mechanism for these reactions is direct displacement of OI from the complexes by NPGB (eq 4 and 5).

$$\text{OI–T}_2 + \text{NPGB} \longrightarrow \text{OI–T} + \text{NPGB·T} \longrightarrow \text{OI–T} + \text{GB–T} + \text{NP} \qquad (4)$$

$$\text{OI–T} + \text{NPGB} \longrightarrow \text{OI} + \text{NPGB·T} \longrightarrow \text{OI} + \text{GB–T} + \text{NP} \qquad (5)$$

The slow appearance of *p*-nitrophenol from NPGB in the presence of OI plus trypsin could be due to decreased accessibility of the active site of trypsin in the OI–trypsin complex rather than to dissociation of the complex; however, substrate displacement results in rate assays and the small effect of NPGB concentration (Figure 4) do not support this explanation.

The NPGB concentration chosen should be high enough that depletion is negligible (*i.e.*, final [NPGB] $\simeq$ initial [NPGB]), but low enough that corrections for nonenzymatic hydrolysis are not excessive. (The delay between addition of NPGB to the reference and sample cuvets gives rise to significant errors at high inhibition, if [NPGB] is high.) As the [NPGB] is decreased, [S]/K_s is decreased. Unless [S] $\gg K_s^{app}$, the inhibitor may compete significantly with NPGB for free trypsin. If the inhibition is competitive but only slowly reversible, the apparent K_m and K_s for NPGB may be increased to the point where the titration is no longer stoichiometric (Bender *et al.*, 1966).

All-or-none assays or titrations measure the concentration of active sites directly; therefore, they are not subject to uncertainties in rate assays resulting from the presence of several forms having different enzymatic activities (Koshland *et al.*, 1962; Bender *et al.*, 1966) as is the case with trypsin (Ganrot, 1966; Maroux *et al.*, 1967; Schroeder and Shaw, 1968; Hruska *et al.*, 1969). Also, the NPGB assay bypasses the need for separate determination of active enzyme concentration, and it exploits the advantages of NPGB as an active site titrant for trypsin, as pointed out by Chase and Shaw (1967, 1969). A wide range of enzyme concentrations can be used without changes in volume (Chase and Shaw, 1967); therefore, possible changes in the inhibitor–trypsin equilibrium with protein concentration can be studied easily by using both 0.1 and 1.0 absorbance scales.

Reaction of NPGB with OI–trypsin complex in the presence of excess OI (Figure 4) suggests that this may be a useful method for detecting trypsin in the presence of endogenous inhibitors, if the inhibition is reversible. If suitable titrants can be designed, this approach should be applicable to other enzymes (see Bender *et al.*, 1966). The NPGB assay also provides a convenient way to discriminate between OI and OM from chicken egg white. Furthermore, these studies provide an experimental basis for highly specific assay of naturally occurring dissociable trypsin inhibitors. The different dissociation kinetics of the trypsin–inhibitor complexes afford the opportunity for simultaneous determination of more than one inhibitor *in situ* without separating them physically, *i.e.*, by performing assays at two different temperatures.

Various factors can affect the reaction between trypsin and NPGB. The immediate burst observed here for commercial trypsin without inhibitor or with OM or soybean trypsin inhibitor is observed with purified α- or β-trypsin (Chase and Shaw, 1967; Hruska *et al.*, 1969), but not with commercial trypsin at pH 4 (Hruska *et al.*, 1969). (Information presented in the recent paper by Hruska *et al.* (1969)

is not sufficient to permit comparison of the data at pH 8 for the same lot of commercial trypsin as we used.) The presence of autolysis products, slow-reacting forms of trypsin, or competitive inhibitors (*e.g.*, benzamidine) slows the burst (Chase and Shaw, 1967, 1969; Hruska *et al.*, 1969), but slowly reversible inhibitors interfere with titration of inhibited enzyme (Bender *et al.*, 1966). OI appears to fit this last category.

Added in Proof

Sealock and Laskowski (1969) recently showed, by measuring the change in the rate of hydrolysis of tosyl-L-arginine methyl ester with time at pH 5, that complexes of trypsin with soybean trypsin inhibitors having arginine or lysine at the reactive site dissociate at different rates. Laskowski and Duran (1966) previously obtained dissociation rate constants for the complexes of trypsin with OM and soybean trypsin inhibitor by the same method.

Acknowledgments

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References

Ackerman, W. W., and Potter, V. R. (1949), *Proc. Soc. Exp. Biol. Med. 72*, 1.

Bender, M. L., *et al.* (1966), *J. Amer. Chem. Soc. 88*, 5890.

Chase, T., Jr., and Shaw, E. (1967), *Biochem. Biophys. Res. Commun. 29*, 508.

Chase, T., Jr., and Shaw, E. (1969), *Biochemistry 8*, 2212.

Chatterjee, A. K., and Montgomery, R. (1962), *Arch. Biochem. Biophys. 99*, 426.

Chu, T. H., and Hsu, C.-L. (1965), *Sheng Wu Hua Hsueh Yu Sheng Wu Wu Li Hsueh Pao 5*, 431; (1966), *Chem. Abstr. 64*, 5373*f*.

Davis, J. G., Zahnley, J. C., and Donovan, J. W. (1969), *Biochemistry 8*, 2044.

Donovan, J. W. (1967), *Biochemistry 6*, 3918.

Elmore, D. T., and Smyth, J. J. (1968), *Biochem. J. 107*, 103.

Erlanger, B. F., Kokowsky, N., and Cohen, W. (1961), *Arch. Biochem. Biophys. 95*, 271.

Feeney, R. E., Stevens, F. C., and Osuga, D. T. (1963), *J. Biol. Chem. 238*, 1415.

Feinstein, G., and Feeney, R. E. (1967), *Biochemistry 6*, 749.

Ganrot, P. O. (1966), *Acta Chem. Scand. 20*, 175.

Glazer, A. N. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 171.

Glazer, A. N. (1967), *J. Biol. Chem. 242*, 3326.

Green, N. M. (1953), *J. Biol. Chem. 205*, 535.

Hruska, J. F., Law, J. H., and Kézdy, F. J. (1969), *Biochem. Biophys. Res. Commun. 36*, 272.

Koshland, D. E., Jr., Strumeyer, D. H., and Ray, W. J., Jr. (1962), *Brookhaven Symp. Biol. 15*, 101.

Laskowski, M., Jr., and Duran, R. (1966), *Fed. Proc. 25*, 752.

Laskowski, M., and Laskowski, M., Jr. (1954), *Advan. Protein Chem. 9*, 203.

Lebowitz, J., and Laskowski, M., Jr. (1962), *Biochemistry 1*, 1044.

Maroux, S., Rovery, M., and Desnuelle, P. (1967), *Biochim. Biophys. Acta 140*, 377.

Rackis, J. J., Sasame, H. A., Mann, R. K., Anderson, R. L., and Smith, A. K. (1962), *Arch. Biochem. Biophys. 98*, 471.

Schroeder, D. D., and Shaw, E. (1968), *J. Biol. Chem. 243*, 2943.

Sealock, R. W., and Laskowski, M., Jr. (1969), *Biochemistry 8*, 3703.

Sri Ram, J., Terminiello, L., Bier, M., and Nord, F. F. (1954), *Arch. Biochem. Biophys. 52*, 451.

Tomimatsu, Y., Clary, J. J., and Bartulovich, J. J. (1966), *Arch. Biochem. Biophys. 115*, 536.

Trowbridge, C. G., Krehbiel, A., and Laskowski, M., Jr. (1963), *Biochemistry 2*, 843.

Vithayathil, A. J., Buck, F., Bier, M., and Nord, F. F. (1961), *Arch. Biochem. Biophys. 92*, 532.

Vogel, R., Trautschold, I., and Werle, E. (1968), Natural Proteinase Inhibitors, New York, N. Y., Academic, pp 2–8.

Worthington Enzymes and Enzyme Reagents (1967), Worthington Biochemical Corp., Freehold, N. J.

Wu, Y. V., and Scheraga, H. A. (1962), *Biochemistry 1*, 698.