p-NITROPHENYL-p'-GUANIDINOBENZOATE HCl: A NEW ACTIVE SITE TITRANT FOR TRYPSIN*

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Studies on relations between structure and activity in the enzyme trypsin, which are currently proceeding in a number of laboratories, have been hampered by autodigestion or denaturation which make it difficult to secure and maintain fully active preparations. Furthermore, the isolation of active forms of trypsin** which differ considerably in their esterase and amidase activities (Schroeder and Shaw, 1967) indicates that the use of a rate assay to establish the purity of trypsin is not desirable. In a recent paper on the determination of the concentration of solutions of hydrolytic enzymes, Bender et al. (1966) emphasized the advantages over rate assays of specific titration procedures. These have as absolute standard an easily purified small organic molecule rather than "pure enzyme," and avoid the uncertainties resulting from the large number of variables involved in rate assays. However, the best previously available titrant for trypsin, p-nitrophenyl N^{α}-benzyloxycarbonyl-L-lysinate HCl (Bender, Killheffer, and Roeske, 1965; Bender, Kézdy and Feder, 1965), is not particularly satisfactory because of rather rapid deacylation of the acyl-enzyme, even at pH 3, which necessitates a careful extrapolation. In addition, at this low pH the sensitivity

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**In this paper, β-trypsin refers to the single chain form and α-trypsin to a form with one peptide bond split.

of the method suffers since the extinction coefficient of the product, p-nitrophenol, is less than 40% of the value at pH 8. Preliminary reports of other reagents for titrating trypsin have appeared (Elmore and Smyth, 1967), but currently available information does not permit full evaluation. In view of the observation of Mares-Guia and Shaw (1967) that ethyl p-guanidinobenzoate is a partial substrate for trypsin, resulting in a stable acyl-enzyme compound, it seemed desirable to investigate the possible use of the corresponding p-nitrophenyl ester as a titrant for trypsin. In this paper, the preparation of this material is described together with a demonstration of its usefulness.

Materials and Methods. Bovine trypsin, twice crystallized, salt-free, lyophilized, was purchased from Worthington Biochemical Corp. and purified by sulfoethyl-Sephadex chromatography as described by Schroeder and Shaw (1967). α-Chymotrypsin was purchased from the same source. Diisopropylphospho-trypsin (DIP-trypsin) was prepared by treatment of 10^{-4} M β-trypsin with 0.005 M diisopropyl fluorophosphate at room temperature until all benzoyl-arginine p-nitroanilide hydrolysis activity had disappeared (15 min), followed by dialysis for 24 hours against two changes of 10^{-3} M HCl. p-Nitrophenol (Fisher Scientific Co.) was used for synthesis without further purification, but was recrystallized from ethanol-water for use as a spectrophotometric standard. p-Guanidinobenzoic acid HCl was prepared by the method of Beyerman and Bontekoe (1953). Other compounds used were of best reagent grade. Water was distilled water passed through a Barnstead demineralizer and stored in glass.

Synthesis of p-nitrophenyl p'-guanidinobenzoate HCl (NPGB): p-Guanidinobenzoic acid HCl (1.075 g), dicyclohexylcarbodiimide (1.083 g) and p-nitrophenol (0.725 g) were dissolved in 15 ml of 1:1 pyridine:dimethylformamide and let stand overnight at room temperature. After filtering off dicyclohexylurea the solvent was removed in vacuo. The residue was taken up in 25 ml 0.1 N HCl and the slurry extracted three times with 25 ml aliquots of ethyl acetate, separating the phases by centrifugation. Collection by filtration of the solid from the aqueous phase and recrystallization from glacial acetic acid

yielded a product suitable for use as a titrant, though only 92-94% pure by measurement of the p-nitrophenoxide produced on hydrolysis in 0.1 N NaOH. A purer product may be obtained by dissolving the extracted slurry in 300 ml t-amyl alcohol, saturating the solution with 0.1 N HCl and extracting twice with 0.1 N HCl to remove free p-guanidinobenzoic acid. The t-amyl alcohol solution is then filtered, evaporated to dryness, and the residue crystallized from glacial acetic acid; yield, 68-72%, giving 98-99% of the expected amount of p-nitrophenoxide on hydrolysis. The initial optical density at 410 mμ of a 10^{-4} M solution, pH 8.3, was 0.002 to 0.015, indicating a content of free p-nitrophenol not greater than 0.1-0.9% (because of the probable presence of traces of free nitrophenol it cannot be determined whether the ester has any absorbance of its own at 410 mμ). Analysis (by Alfred Bernhardt, Mulheim, Germany): calculated for $C_{14}H_{13}N_4O_4Cl$ (m.w. 336.74): C 49.93, H 3.89, N 16.63, Cl 10.52. Found: C 49.70, H 4.16, N 16.19, Cl 10.37. The compound showed some discoloration and decomposition at about 260°, but did not melt up to 300° except when heated rapidly.

Production of p-nitrophenol was followed in the Cary 15 double-beam recording spectrophotometer. The ϵ_{402} of p-nitrophenoxide ion in 0.1 M NaOH was 17,850 ± 240 in this instrument (Bender and Nakamura (1962) report ϵ_{402} = 18,300), and ϵ_{410} of a p-nitrophenol solution in 0.1 M veronal buffer, pH 8.3, was 16,595 ± 145. 0.1 M veronal buffer was prepared by acidification of an 0.1 M sodium barbital solution with conc. HCl at the Beckman pH meter.

Results. As shown in Fig. 1, addition of NPGB to a solution of trypsin results in an immediate "burst" of p-nitrophenol, followed by a very slow, linear, further production. With purified α- or β-trypsin (Schroeder and Shaw, 1967) the "burst" is complete in the time necessary to mix the reagent with the cell contents and place the cell in the instrument (10 sec), although with partially autolyzed trypsin solutions, apparently containing slow-reacting components, the tail end of the burst can be seen. The post-burst production of p-nitrophenol might be expected to represent deacylation

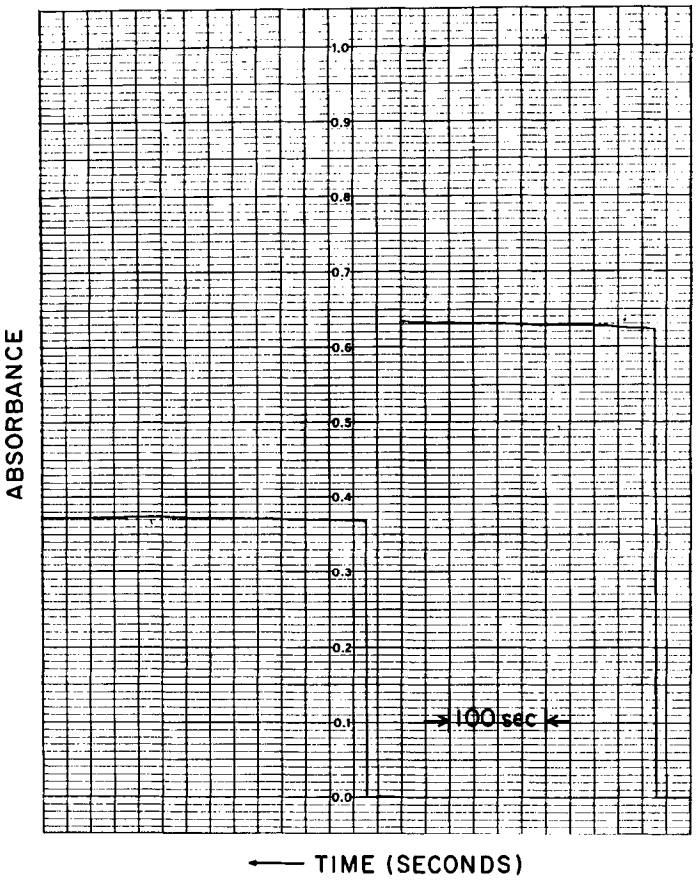

Fig. 1. Titration of (left) 0.05 ml and (right) 0.08 ml of a solution of β-trypsin. The procedure is described in the text. Extrapolation of the absorbance to time zero, which is the chart line just to the right of the initial rise in absorbance, corresponds to enzyme concentrations of 2.22 x 10^{-5} M and 3.76 x 10^{-5} M, respectively. The stock solution is thus 4.58 ± 0.13 x 10^{-4} M.

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of the acyl enzyme followed immediately by reaction with a second molecule of the reagent, but p-nitrophenol is produced at the same rate by the same concentration of DIP-trypsin, which shows no burst whatsoever, and the reaction is first order in both trypsin (or DIP-trypsin) and NPGB (second-order rate constant 0.82 M^{-1} sec^{-1}). Hence this slow post-burst production of p-nitrophenol is believed to represent nonspecific esterolysis catalyzed by other parts of the protein. Actual deacylation at the normal enzymatic site must be at least an order of magnitude slower (<7 x 10^{-6} sec^{-1}).

The procedure developed for titration of active trypsin is as follows: an aliquot of trypsin solution (in 10^{-3} M HCl - 0.02 M $CaCl_2$) is diluted with 0.1 M veronal buffer, pH 8.3, 0.02 M in $CaCl_2$ to give 0.99 ml of 1-5 x 10^{-5} M trypsin. This, in a 1-ml cuvette, is placed in the sample cell position and the instrument balanced against a reference cell containing the same buffer. Ten µl of a 0.01 M solution of NPGB in dimethyl formamide is added to the reference cuvette, the contents mixed, and the cell replaced in the instrument; the same procedure is followed with the sample cuvette and the instrument turned on. The optical density is followed long enough to permit an extrapolation to zero time, if necessary, and the burst of p-nitrophenol calculated: O.D. x 6.025 x 10^{-5} = molarity of nitrophenol produced = molarity of active trypsin present. In a typical titration of a solution of α-trypsin determined by amino acid analysis to be 6.03 x 10^{-4} M in total protein as trypsin, NPGB titration of three aliquots indicated the molarity of active trypsin to be 5.12 ± 0.08 x 10^{-4} M, or 84.6 ± 1.4% pure.

As shown by Bender et al. (1966), the observed burst π is equal to the actual enzyme concentration multiplied by a factor $\left(\frac{k_2}{k_2 + k_3}\right)^2 \Big/ \left(1 + \frac{K_{m(app)}}{[S]_o}\right)^2$, so that π equals the actual enzyme concentration only if $k_2 \gg k_3$ and $[S]_o \gg K_{m(app)}$; if not, the observed burst will depend on the titrant concentration and the actual enzyme concentration is best determined as the intercept of a plot of $1/\sqrt{\pi}$ vs $1/[S]$. In the case of NPGB, no concentration dependence was observed down to the lowest substrate concentration practicable, titration of 1.8 x 10^{-6} M trypsin with 2 x 10^{-6} M NPGB, indicating that the assumptions $k_2 \gg k_3$ and $[S] \gg K_{m(app)}$ are fulfilled very well. Hence a correction of the type used with p-nitrophenyl N^{α}-benzyloxycarbonyl-L-lysinate (Bender, Kézdy and Feder, 1965) is not necessary.

Veronal was chosen as the buffer for the titration because the breakdown of the reagent is much slower in this buffer than in amine buffers: k = 0.10 x 10^{-4} sec^{-1}, as against 1.1 x 10^{-4} sec^{-1} in Tris, 3.9 x 10^{-4} sec^{-1} in glycinamide, and 0.85 x 10^{-4} sec^{-1} in Tricine, all 0.1 M, pH 8.3, 0.02 M in

$CaCl_2$, 20°. pH 8.3 was chosen as sufficiently far from the pKa of p-nitrophenol (7.04) so that a small inaccuracy in the pH of the titration would not have a large effect on the color yield.

High concentrations of the competitive inhibitor, benzamidine, slow the burst enough to make it possible to follow the pre-steady state reaction, indicating that NPGB does react at the normal active site of trypsin; this technique has been utilized to derive an approximate value for the rate constant, k_2, as will be described in a later publication.

NPGB does react with α-chymotrypsin, giving a "burst" followed by a fairly rapid turnover (k_3 = 0.01 sec^{-1}); it is therefore not suitable for determination of trypsin in presence of chymotrypsin.

Discussion. Bender et al. (1966) have listed the following characteristics for an optimal enzyme titrant: that it "(1) be a specific substrate, (2) give a titration in a few minutes in order to obviate denaturation, and for convenience, (3) give a titration around neutrality in order to reproduce best physiological conditions, (4) be a stable, available and soluble reagent whose stoichiometrical reaction is easily detectable, and (5) give a titration over a wide range of enzyme concentrations." NPGB is significantly suboptimal on only the first of these counts, since it also reacts with chymotrypsin and presumably with other proteolytic enzymes. The titration is essentially complete immediately, though highest precision is obtained by following the post-burst production of p-nitrophenol for several minutes and extrapolating back to zero time (10 sec, before turning on the instrument). pH 8.3 is not precisely neutrality, but much closer than the pH (2.2 to 3.4) of most of the titrations described by Bender et al. (1966), and correspondingly more sensitive (by a factor of about 2.5); if the corresponding 2,4-dinitrophenyl ester is sufficiently stable it could be used at pH 7.0. The synthesis of NPGB is not difficult; the dry compound appears to be completely stable, and dimethylformamide solutions are usable for at least two weeks if stored at 4°; the solubility in water (saturated solution in 0.1 M veronal, pH 8.3, = 9 x 10^{-4} M) is quite

sufficient for the titration. The titration gives accurate results with trypsin concentrations from 2 x 10^{-6} M to 10^{-4} M. Consequently, we consider it to be the best available titrant for trypsin. Further studies, on the individual rate constants in the reaction, reactions of related compounds, and reaction of NPGB with other proteolytic enzymes, will be included in a later paper.

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