TRYPSIN SUBSTRATES DERIVED FROM BENZAMIDINE : APPLICATION TO ACTIVE SITE TITRATION AND ISOLATION OF ACYL-ENZYME INTERMEDIATE*

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In a series of works on the active center of trypsin, Shaw et al. (1965) first reported that benzamidine derivatives are very powerful competitive inhibitors of trypsin. Further, several derivatives of benzamidine or phenylguanidine containing ester group were recently prepared and important informations on the active center of the enzyme were obtained employing them (Shaw et al., 1967a, 1967b, 1967c).

In the course of studies of proteolytic enzymes, we found that β-naphthamidine is highly effective as a competitive inhibitor of trypsin (Tanizawa, Ishii and Kanaoka, 1966), and, in an effort to elucidate the interaction between these molecules and the enzyme, benzamidine derivatives containing hydrolyzable ester moiety were prepared and examined. The present commnuication describes the synthesis of ethyl p-amidinobenzoate hydrochloride (EAB) and p-nitrophenyl p-amidinobenzoate hydrochloride (NPAB), their kinetic behavior as trysin substrates, and the use of NPAB as a reagent for active site titration of trypsin together with the preparation of acyl-enzyme intermediate.

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Materials and Methods. Trypsin was a Worthington lot, TRL-6261. Ethyl p-amidinobenzoate (EAB): p-Cyanobenzoic acid was converted by treatment with dry HCl in ethanol solution to ethyl p-ethoxycarbonylbenzimidate hydrochloride, which on ammonolysis gave EAB as colorless pillars of mp 211-214° recrystllized from ethanol-ether. Anal. Calcd. for $C_{10}H_{12}N_2O_2 \cdot HCl$:C,52.52; H,5.47; N,12.35. Found: C,52.84; H,5.76; N,12.35.

p-Nitrophenyl p-amidinobenzoate (NPAB): EAB was hydrolyzed with aqueous NaOH, and the carboxylic acid was converted to the acid chloride with thionyl chloride as usual, which was coupled with p-nitrophenol in dimethylformamide solution in the presence of pyridine to give NPAB. Recrystllization from dimethylformamide gave pale yellow fine needles of mp 223-226°, which had a molecule of solvent of crystallization. Infrared spectrum (Nujol), $\nu_{max}(cm^{-1})$: 1755(ester C=O), 1675(amidine $C=N^+$), 1645(amide C=O). Anal. Calcd. for $C_{14}H_{11}N_3O_4 \cdot HCl \cdot (CH_3)_2NCHO$: C,51.75; H,4.83; N,14.18. Found: C,51.61; H,4.94; N, 13.90. By heating the above sample at 100° for 48 hr in vacuo, the hydrochloride free from the solvent was obtained; mp 234-237°; infrared spectrum, no amide band. Anal. Calcd. for $C_{14}H_{11}N_3O_4 \cdot HCl$: C,52.35;H,3.74; N,13.07. Found: C,52.37; H,4.01; N,12.93.

Kinetic and titration experiments with NPAB were carried out by observing the formation of p-nitrophenol at 405 or 320 mμ using a Hitachi EPS-3T recording spectrophotometer. Three milliliters of buffer solution (0.05M Tris, buffer, pH 5.4or 4.7) and 50 μl of substrate solution (in dimethylformamide) were pipetted into a 1-cm quartz cuvette placed in a thermostated cuvette holder at 25°. The enzymatic reaction was then initiated by introduction of aliquot (10∼50 μl) of enzyme solution in 0.001N HCl. The initial substrate concentration in the reaction mixture was $5 \times 10^{-7} \sim 10^{-3}$M. The actual concentration of nitrophenol produced was calculated taking 19,200 as $\varepsilon_{405m\mu}$ at pH 8.2 or 82,00 as $\varepsilon_{320m\mu}$ at pH 5.4 and 4.7. Kinetic experiments were also conducted using a Radiometer pH-stat titrator Model TTT1c. The

reaction was performed in 200 ml of 0.1M KCl containing 0.02M $CaCl_2$ at 25° and 0.02M NaOH was used as titrant.

Results and Discussion. The kinetic parameters for trypsin-catalyzed hydrolysis of the substrates are listed in Table 1, where $[E]_o$ estimated from the absorption at 280 mμ, with a optical factor (mg/ml/OD) of 0.65 and a molecular weight of 23,800, was used. In the case of NPAB, the data were obtained by two methods. The optical monitoring of p-nitrophenol production revealed an initial "burst", followed by a much slower linear increase. The burst was completed within 1 sec. As the k_{cat} values in the table were caluculated from this post-burst phase, they may be evaluated to represent close approximation to the rate constants (k_3) for deacylation of acyl-enzyme. The values are in good accord, in each pH condition, with those obtained by the pH-stat assay of acid production from the same substrate. On the contrary, k_{cat} value for EAB is only one sixth of the corresponding value for NPAB which has the same acyl moiety. The result indicates that the rate-determining step of trypsin-catalyzed hydrolysis of EAB is not the deacylating step although it is a substrate of ester-type (for a precedent in the chymotrypsin substrate, see Bender and Zerner, 1961).

Although p-nitrophenyl p-guanidinobenzoate has been reported to be a very good titrant for the active-site determination of trypsin (Chase and Shaw,

Table 1. Kinetic parameters of trypsin-catalyzed hydrolysis of ethyl p-amidinobenzoate(EAB) and p-nitrophenyl p-amidinobenzoate (NPAB) at 25°.

substrate	K_m(app.)	k_{cat}	spont.hydrolysis	condition
		2.4 min^{-1}	1.2 x 10^{-2} min^{-1}	pH 8.2 *a
		2.5	0.7 x 10^{-2}	pH 8.2 *b
NPAB	$\ll$ 5 x 10^{-7} mole/l	0.15	not observed	pH 5.4 *c
		0.14	not observed	pH 5.4 *b
		0.047	not observed	pH 4.7 *c
EAB	1.4 x 10^{-4}	0.40	not observed	pH 8.2 *b

*a: Optical assay in 0.05M Tris, 0.02M $CaCl_2$.
*b: pH stat assay in 0.1M KCl, 0.02M $CaCl_2$. The k_{cat} value at pH 8.2 was corrected for the alkali-uptake by p-nitrophenolate ion (pKa= 7.2).
*c: Optical assay in 0.1M acetate

1967a), NPAB may also be suitable for the same purpose, because (i) it has a small K_m value (immeasurable as far as the range of substrate concentration mentioned above was used), (ii) $k_2 \gg k_3$ and (iii) the post-burst phase can be easily traced with an usual recording spectrophotometer even at pH 8.2, the pH region optimal for activity of trypsin. The normality of trypsin was actually determined by extrapolating the spectrometer trace back to time zero, and measuring the initial burst. The specimen used in the present study was found to be 67 % active on an absorbancy basis and 54 % on a weight basis. The value was independent of pH and of substrate concentration. A control experiment using DIP-trypsin showed no reactivity with NPAB, excluding the possibility of non specific acylation on the functional groups of trypsin other than the active site.

Chymotrypsin was found to react with NPAB, giving a slower burst followed by fairy rapid turnover (k_3 = 4.1 min^{-1}) at pH 8.2. Because of the diminished reactivity at the lower pH, however, the normality of chymotrypsin determined at pH 4.7 became only 2.5 % on an absorbancy basis (optical factor = 0.50, molecular weight = 25,000).

For the preparation of acyl-enzyme intermediate, 81mg (3.4 μmole) of trypsin was dissolved in 4 ml of 0.1M acetate buffer, pH 4.4, to which a solution of 25 μmole of NPAB in 1 ml of dimethylformamide was added at 25°. After 5 minutes, pH of the solution was adjusted to 2 by addition of dilute HCl. Gel-filtration through Sephadex G-25 in HCl solution (pH 2) and subsequent lyophilization gave a preparation of 97.8 % inactivated trypsin. The remaining activity of the preparation was determined by using p-nitrophenyl N^2-benzyloxycarbonyl-L-lysinate as the rate assay substrate (Bender et al., 1965, 1966). The substrate operates at pH 3, where the acyl-trypsin is completely stable. Fig 1 shows the time course of restoration of activity to the preparation by incubating it as a solution (1.44×10^{-5}M) in 0.1M acetate buffer pH 4.7 at 25°. Quantitative recovery was not observed presumably due to denaturation of the enzyme during the course of experiments.

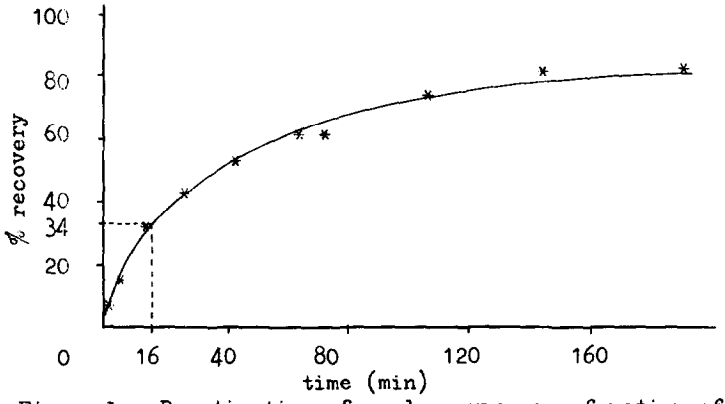

Figure 1. Reactivation of acyl enzyme as a function of time in pH 4.7 0.1M acetate buffer at 25°; Recovery of activity was measured by p-nitrophenyl N^2-benzyloxycarbonyl-L-lysinate hydrochloride in 0.05M citrate buffer pH 3.0 at 25°.

From Fig. 1, the rate constant of the deacylation is calculated to be 0.040 min^{-1} using the half-life time method ($\tau = \ln 2/k$). This value is quite reasonable as compared with k_{cat} (0.047 min^{-1}) in Table 1 obtained in experiment *in situ* without isolation of the intermediate acyl-enzyme.

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