(29) E. Caspi, J. P. Moreau, and P. J. Ramm, *J. Am. Chem. Soc.*, **96**, 8107 (1974).
(30) J. P. Moreau, P. J. Ramm, and E. Caspi, *Eur. J. Biochem.*, **56**, 393 (1975) (see Table 3).
(31) J. Sliwowski, V. R. Reddy, and E. Caspi, *J. Neurochem.*, in press.
(32) R. C. Ebersole, W. O. Godfredsen, S. Vangedal, and E. Caspi, *J. Am. Chem. Soc.*, **96**, 6499 (1974).
(33) J. K. Sliwowski and E. Caspi, *Chem. Commun.*, 196 (1976).
(34) Reference 1, pp 69, 70; J. W. Cornforth, G. T. Phillips, B. Messner, and H. Eggerer, *Eur. J. Biochem.*, **42**, 591 (1974).
(35) J. M. A. Al-Rawi, J. A. Elvidge, R. Thomas, and B. J. Wright, *Chem. Commun.*, 1031 (1974).
(36) H. J. Brodie, M. Hayano, and M. Gut, *J. Am. Chem. Soc.*, **84**, 3766 (1962).
(37) E. Caspi, R. C. Ebersole, L. J. Mulheirn, W. O. Godtfredsen, and W. Daehne, *J. Steroid Biochem.*, **4**, 433 (1973).
(38) D. C. Wilton, K. A. Munday, S. J. M. Skinner, and M. Akhtar, *Biochem. J.*, **106**, 803 (1968).
(39) E. Caspi, J. P. Moreau, and P. J. Ramm, *J. Am. Chem. Soc.*, **96**, 8107 (1974) (microfilm edition).

"Inverse Substrates" for Trypsin. Efficient Enzymatic Hydrolysis of Certain Esters with a Cationic Center in the Leaving Group[1]

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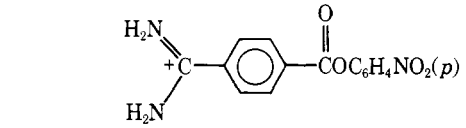
Abstract: The kinetics of the reaction of trypsin with several esters derived from *p*- and *m*-amidinophenol have been studied. These esters are characterized by their linkage, i.e., the specific group for the enzyme (charged amidinium) is not involved in their carbonyl groups but in the leaving portion. Esters of *p*-amidinophenol were demonstrated to undergo efficient and specific tryptic hydrolysis whereas the meta derivatives exhibited only low reactivity. The selectivity and efficiency of the *p*-amidinophenyl esters were compared to those of normal-type substrates, and it was concluded that these esters are almost comparable to normal type substrates both in binding and in acylation. A slow deacylation process was also demonstrated. The process may be governed by the inherent nature of nonspecific acyl residues although it had been introduced specifically. All these enzymatic processes were found identical with those of substrates with normal linkages, together with the effects of competitive inhibitor. These compounds were named "inverse substrates" with respect to these properties. A facile procedure for the preparation of acyl-enzymes carrying nonspecific residue was described and the potential usefulness of the "inverse substrate" concept was discussed.

It is generally believed that only esters and amides containing a specificity-determining residue in their carbonyl portions function as good substrates of the endopeptidase, trypsin, and chymotrypsin.[2] Very few attempts have been made to question this empirical rule to which exceptions have not yet been found.[3] Previously, we have reported that the esters of *p*-amidinobenzoic acid (**1**) were efficiently hydrolyzed by trypsin.[4] In an extension of that investigation we have found that several acyl derivatives of *p*-hydroxybenzamidine (**2**), which are related "inverted" types of ester substrates having an amidine function in the leaving group, were hydrolyzable as well by trypsin.[5] At the same time Markwardt et al.[6] reported the inhibitory action of a series of *p*-amidinophenyl benzoates on trypsin and thrombin as well as chymotrypsin. These observations both suggest that the compounds in which the arrangement of the site-specific group is of a type "inverse" to that of the normal substrates may also act as specific substrates if they are properly designed to fit the active site.



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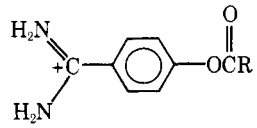

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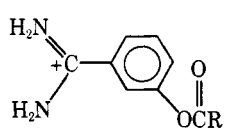

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a, R = CH_3; **b**, R = $C(CH_3)_3$; **c**, R = C_6H_5

In order to confirm whether these "inverse" esters are "true substrates" or not, a series of aliphatic esters of *p*-amidinophenol (**2a** and **2b**) and *m*-amidinophenol (**3a** and **3b**) has been prepared, and detailed kinetic studies on the individual steps of their tryptic hydrolysis were conducted. Benzoyl esters (**2c** and **3c**) are also included for comparison. In this work we wish to present evidence which indicates that these esters **2** behave like the normal substrates and may be regarded as "inverse substrates".

Results and Discussion

Kinetic parameters for the trypsin-catalyzed hydrolysis of the amidinophenol esters are listed in Table I. The acylation rate constants, k_2, and Michaelis constants, K_s, were obtained by following the formation of the hydrolysis products, the amidinophenols, which absorb at longer wavelength than the substrates. As shown in Table I, all the compounds in the series **2** and **3** were found to have a strong affinity to trypsin, with K_s values ranging around 10^{-5} M. These K_s values are comparable to those for normal-type specific substrates, e.g., *p*-nitrophenyl amidinobenzoate (**1**) and *p*-nitrophenyl α-*N*-benzyloxycarbonyl-L-lysinate (ZLysONP)[7] whose parameters are shown in Table II. In contrast, the K_s value for the nonspecific substrate, *p*-nitrophenyl acetate (AcONP),[8] is approximately three orders of magnitude larger than those of **2** and **3**.

The rates of the acylation of trypsin are strongly dependent on the nature of the leaving groups of the substrates. Since the Hammett σ_p values for nitro and amidino substituents are not markedly different, the acylation rates for **2a–c** can be reasonably compared with those for normal substrates which have

Table I. Kinetic Characteristics of the Reactions of Trypsin with "Inverse Substrates"[a]

Compd	K_s, M ($\times 10^5$)	k_2, s^{-1}	$k_3(k_{cat})$, s^{-1} ($\times 10^4$)	$k_{spont.}$, s^{-1} ($\times 10^6$)	N, %[b]
2a[c]	3.87 ± 0.29	17.0 ± 3.4	92.6 ± 9.7 [93][g]	26.0 ± 2.7	58 ± 5
3a	3.03 ± 0.78	30.1 ± 3.8 × 10^{-3}	(49.8 ± 5.0)[d]	15.8 ± 2.9	
2b[c]	16.2 ± 4.2	11.5 ± 2.2 × 10^{-1}	2.59 ± 0.43 [2.9][g]	2.27 ± 0.24	56 ± 4
3b	8.02 ± 0.98	16.7 ± 0.9 × 10^{-4}	(2.10 ± 0.60)[d]	1.87 ± 0.20	
2c[c]	3.30 ± 0.26	36.7 ± 0.8 × 10^{-1} [e]	8.09 ± 1.20[f]	14.6 ± 2.5	57 ± 2
3c	6.09 ± 1.21	32.0 ± 2.0 × 10^{-4}	(3.96 ± 0.35)[d]	5.82 ± 0.37	

[a] pH 8.0, 0.05 M Tris–0.02 M $CaCl_2$ at 25 °C. Parameters were calculated using values of $[E]_0$ obtained by active-site titration, and error factors were computed as outlined in the Experimental Section. [b] Calculated as the percent of the active $[E]_0$ with the total enzyme concentration determined from absorbance measurement taking ϵ_{280nm} 39 000. [c] The stopped-flow technique was employed. [d] Overall k_{cat} ($k_2 \gg k_3$). [e] A value of 0.045 s^{-1} was reported for the inactivation rate constant at pH 7.2.[6] [f] A value of 18 × 10^{-4} s^{-1} was reported for the reactivation rate constant at pH 7.2.[6] [g] Calculated from the rate of reactivation of isolated acyltrypsin, pH 8.0 (cf. Figure 1).

Table II. Kinetic Parameters for the Trypsin-Catalyzed Hydrolysis of Normal Type Substrates

Compd	K_s, M ($\times 10^5$)	k_2, s^{-1}	k_3, s^{-1} ($\times 10^4$)	k_{spont}, s^{-1} ($\times 10^6$)	N, %	pH	Ref
1	0.503 ± 0.043	30.4 ± 0.6	653 ± 13	217 ± 10	58 ± 5	8.0[a]	This work
AcONP	2100	1.5	130		64	≃8.0[b]	8, 19
ZLysONP	79.5	0.395	14.3		57.5	2.66[c]	11, 19

[a] 0.05 M Tris–0.02 M $CaCl_2$ at 25 °C. [b] The acylation rate constant corresponds to k(lim.), the rate constant measured on the plateau of the sigmoid curve dependent on a pK_a value of 6.9 to 7.4. [c] 0.05 M citrate, 25 °C.

p-nitrophenyl as the leaving group. The σ_p values of amidino and nitro groups are reported to be +0.65[9] and +0.778,[10] respectively. In spite of the fact that in its nonenzymatic hydrolysis **2a** is somewhat less reactive than **1** in accord with the respective σ_p values, the acylation rate for **2a** is nearly identical with that for the normal type substrate **1,** and significantly larger than that for AcONP, a nonspecific substrate. The acylation rate for **2a** is exceedingly small if compared to that for ZLysONP at neutral pH. The k_2 value for ZLysONP extrapolated to pH 8.0 was calculated to be 7860 s^{-1} using a pK_a value 7.0 for the histidine present in the active site of trypsin.[11] However, it must be noted that this enhanced value for ZLysONP is obviously due to the total assistance of the α-acylamido group and the asymmetric carbon existing in ZLysONP, and that extent was estimated to be three orders of magnitude.[12] If this is taken into account, the corrected value, of the k_2 for **2a** would well reach to the level of the value for the natural α-amino acid substrate, ZLysONP, e.g., 7860 s^{-1}. Unfortunately one cannot easily design any "inverse substrate" which accommodates an α-acylamido group and an asymmetric carbon unit. The smaller values of k_2 for **2b** and **2c,** which are still comparable to that for AcONP, are probably due to steric and other factors intrinsic to these acyl groups, pivaloyl and benzoyl. The kinetic parameters K_s and k_2 can be used to evaluate substrates critically. The former can provide information on the strength of the binding of the substrate which is a characteristic of the enzymatic process, while the latter directly reflects the accessibility of the carbonyl function of the substrate molecule to the catalytic residue(s) of enzyme in the ES complex. The value of k_3 for **2a,** 9.3 × 10^{-3} s^{-1}, is very close to the value 1.3 × 10^{-2} s^{-1} for AcONP, the tryptic hydrolysis of which is known to proceed by way of acetyl-enzyme.[8,13] This value will not depend on the nature of the leaving group if the condition $k_2 \gg k_3$ is satisfied, and must represent the deacetylation rate constant for the intermediate acetyl-enzyme. In fact, the reactivation rates of isolated acetyl- and trimethylacetyltrypsin are in good accord with the respective k_3 values as shown in Table I. This slow rate of the deacylation process may be due to the inherent nature of the noncationic acid residues.

There are a number of reports which suggest that certain small substrates are bound to enzymes primarily in a "non-productive" mode. "Nonproductive" binding may be characterized by unexpectedly small K_m values and compensatory decreases in k_{cat}.[14] Such binding is unlikely in the present system involving the *p*-amidinophenyl esters for the following reasons. (1) The K_s values for all of the *p*-amidinophenol esters together with the normal type substrate **1** are reasonably small and are in good accord with K_i values of the parent amidinophenols, specific inhibitors of trypsin (Table III). (2) The acylation of trypsin by *p*-acetoxybenzamidine is definitely more efficient than by AcONP, and the observed K_s value for the former substrate is much smaller. (3) When the *m*-amidinophenol esters are compared, the slight difference in the K_s values for these isomers cannot compensate for the extraordinary difference in k_2 values.

Table III. Inhibition Constant K_i for Hydroxybenzamidines Measured in the Trypsin-Catalyzed Hydrolysis of Benzoyl-DL-arginine *p*-Nitroanilide[a]

Inhibitor	K_i, M
Para	2.7 × 10^{-5}
Meta	2.4 × 10^{-5}

[a] 0.05 M Tris–0.02 M $CaCl_2$, pH 8.2, 25 °C.

The preparation of acyl-enzymes was successfully carried out with **2a** and **2b.** The activities observed for solutions of trimethylacetyl- and acetyltrypsin were less than 1% of that of the original enzyme, while almost complete reactivation (>98%) as a result of deacylation was observed on incubation of the acyl-enzymes at pH 8.0 for appropriate times as shown in Figure 1. The deacylation rate constants thus obtained for the isolated acyl-enzymes, which are in good agreement with those measured by rate assays, are listed in Table I.

Our observations all lead to the conclusion that the hydrolysis of these "inverse substrates" **2** proceeds through specific binding with efficient acylation leading to the formation of acyl-enzyme intermediates followed by their deacylation, a pathway essentially identical with those followed by normal type substrates **1.** Furthermore, the inhibition of the hydrolysis of **2a** with benzamidine was fully competitive and an inhibition constant of 9.8 × 10^{-6} M, determined by measuring the rate of acylation with **2a,** was in good agreement

with the value 1.8×10^{-5} M[15] obtained by measuring the k_{cat} value for the hydrolysis of benzoyl-DL-arginine *p*-nitroanilide. Finally, **2a** was specific for trypsin and neither a burst reaction nor a turnover process was observed for chymotrypsin. In fact, **2a** and **2b** are useful as titrants for the determination of the operational normalities of trypsin solution. As shown in Tables I and II, there was no difference in observed normalities found with the normal-type substrate **1** and the "inverse" type substrates (**2a** and **2b**) supporting the idea that trypsin *does not differentiate* "inverse substrates" from normal ones. In contrast, all the meta derivatives **3** have much slower acylation rates ($^1/_{1000}$ of those of the para derivatives) although there are no differences in the strength of their binding. These different results with seemingly subtle variation in the structure of the substrates are most probably due to the strict stereochemical requirements of the active site: i.e., inaccessibility of the enzyme catalytic residue(s) to the unfavorably positioned carbonyl carbon of the meta isomers though the binding itself is tight and specific.[16] At present, however, the possibilities cannot be eliminated that the meta derivatives **3**, unlike the para counterparts **2**, bind in a rather "nonproductive" mode[14] in view of the fact that they have lowered k_{cat} values, even though their K_s values are not different from the K_i values of their parent compound, *m*-amidinophenol.

It is perhaps worth reconsidering the status of conventional substrates in terms of the "inverse" concept. For example, *p*-nitrophenyl acetate, a well-known substrate for chymotrypsin, could be considered to be an inverse substrate. A substrate such as 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone[17] would come midway between normal and inverse ones. With respect to trypsin and related enzymes, application of the *p*-amidinophenol esters may potentially be very useful. This approach provides a general method for the efficient synthesis of acyl-enzymes[18] without recourse to cation-containing acyl compounds, which could lead to the discovery of novel features of enzymatic reaction mechanisms by virtue of the preparation of various new acyl-enzymes.[20] In addition, any desired reporter groups might be specifically introduced into the enzyme active sites. Systematic studies on scope and limitations of the "inverse" substrate concept are in progress.

Experimental Section

Materials. Benzamidine hydrochloride was prepared as described in the literature.[21] *p*-Hydroxybenzamidine hydrochloride was prepared from *p*-cyanophenol in a manner analogous to that used for the synthesis of benzamidine hydrochloride. Recrystallization from an ethanol–ether mixture gave colorless prisms, mp 223–224 °C (65% yield). Anal. Calcd for $C_7H_9O_1N_2Cl$: C, 48.71; H, 5.25; N, 16.22. Found: C, 48.75; H, 5.20; N, 16.23.

m-Hydroxybenzamidine hydrochloride was prepared from *m*-cyanophenol in a similar manner. Recrystallization from an ethanol–ether mixture gave colorless prisms, mp 185–187 °C (60% yield). Anal. Calcd for $C_7H_9O_1N_2Cl$: C, 48.71; H, 5.25; N, 16.22. Found: C, 48.76; H, 5.28; N, 16.29.

p- and *m*-hydroxybenzamidine *p*-toluenesulfonates were prepared by treating the hydrochlorides with *p*-toluenesulfonic acid. Recrystallization from an ethanol–ether mixture gave colorless prisms, mp 215–217 °C (para) and 148–150 °C (meta).

p-Acetoxybenzamidine *p*-toluenesulfonate (**2a**) was prepared by heating 637 mg of *p*-hydroxybenzamidine hydrochloride and 200 mg of *p*-toluenesulfonic acid monohydrate in 60 mL of acetic anhydride at 80 °C for 7 h followed by the addition of ether and collection of the precipitate. Recrystallization from an ethanol–ether mixture gave colorless needles, mp 194–197 °C (98% yield). Anal. Calcd for $C_{16}H_{18}O_5N_2S$: C, 54.85; H, 5.17; N, 8.00. Found: C, 54.61; H, 5.08; N, 7.85.

m-Acetoxybenzamidine *p*-toluenesulfonate (**3a**) was synthesized in the manner described for the para isomer. Recrystallization from an ethanol–ether mixture gave colorless leaflets, mp 173–175 °C (85% yield). Anal. Calcd for $C_{16}H_{18}O_5N_2S$: C, 54.85; H, 5.17; N, 8.00. Found: C, 54.61; H, 5.13; N, 7.86.

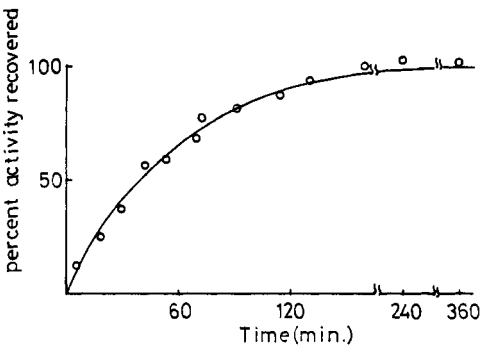

Figure 1. Time course for the reactivation of trimethylacetyltrypsin observed on incubation at pH 8.0 (see Experimental Section). The curve is a theoretical one calculated from the pseudo-first-order equation taking $k_3 = 2.9 \times 10^{-4}$ s^{-1}.

p-Trimethylacetoxybenzamidine *p*-toluenesulfonate (**2b**) was synthesized by heating 1.8 g of *p*-hydroxybenzamidine *p*-toluenesulfonate in mixture of pivalic chloride (4 g) and pivalic acid (3 g) at 120 °C for 10 h. Ether was added and the resultant precipitate was collected. The product was converted completely to the *p*-toluenesulfonate by addition of *p*-toluenesulfonic acid. Recrystallization from ethanol gave colorless leaflets, mp 267–269 °C dec (quantitative yield). Anal. Calcd for $C_{19}H_{24}O_5N_2S$: C, 58.15; H, 6.16; N, 7.14. Found: C, 58.14; H, 6.18; N, 6.92.

m-Trimethylacetoxybenzamidine *p*-toluenesulfonate (**3b**) was prepared in the manner described for the para derivative. Recrystallization from an ethanol–ether mixture gave colorless leaflets, mp 217–219 °C (quantitative yield). Anal. Calcd for $C_{19}H_{24}O_5N_2S$: C, 58.15; H, 6.16; N, 7.14. Found: C, 57.89; H, 6.13; N, 7.12.

p-Benzoxybenzamidine perchlorate (**2c**) was prepared by heating *p*-hydroxybenzamidine (840 mg) with 50 mL of benzoyl chloride and 0.8 g of concentrated H_2SO_4 at 80 °C for 3 h, followed by addition of ether, filtration of the precipitate, and conversion to the perchlorate. Recrystallization from water gave colorless needles, mp 233–235 °C (51% yield). Anal. Calcd for $C_{14}H_{13}N_2O_6Cl$: C, 49.35; H, 3.85; N, 8.22. Found: C, 49.11; H, 3.86; N, 8.08.

m-Benzoxybenzamidine *p*-toluenesulfonate (**3c**) was prepared in the manner described for the para isomer. Recrystallization from water gave colorless needles, mp 147–148 °C (44% yield). Anal. Calcd for $C_{14}H_{13}N_2O_6Cl$: C, 49.35; H, 3.85; N, 8.22. Found: C, 49.08; H, 3.84; N, 8.06.

p-Nitrophenyl *p*-amidinobenzoate hydrochloride (**1**) was prepared as described previously.[4]

p-Nitrophenyl α-*N*-benzyloxycarbonyl-L-lysinate hydrochloride was prepared following the literature procedure.[7] Ethyl *N*-benzoyl-L-argininate hydrochloride and *N*-benzoyl-DL-arginine *p*-nitroanilide hydrochloride were purchased from Protein Research Foundation (Osaka, Japan).

All other chemicals used were Koso Chemical and Nakarai Chemicals, analytical grade.

Trypsin (twice crystallized lot, TRL) was purchased from the Worthington Biochemical Corp.

Methods. The rates for the rapid acylation of trypsin by compounds in the series of **2** and **1** were determined by measurement of the formation of *p*-amidinophenol and *p*-nitrophenol using a Union Giken Corp. stopped-flow spectrophotometer. The rates for the slow acylation of trypsin by compounds in the **3** series were measured by observation of the liberation of *m*-amidinophenol using a Shimazu UV 200 double beam spectrophotometer. The following experimentally determined values were used for the calculation of kinetic parameters and operational normalities at pH 8.0: *p*-nitrophenol, $\Delta\epsilon_{405nm}$ 19 000; *p*-amidinophenol, $\Delta\epsilon_{305nm}$ 16 700; and *m*-amidinophenol, $\Delta\epsilon_{305nm}$ 2770. Deacylation rates were determined spectrophotometrically and potentiometrically (Radiometer TTT 2 pH stat), and they were in good agreement.

The determination of k_2 and K_s was carried out in 0.05 M Tris–0.02 M $CaCl_2$, containing 0.83% ethanol, at 25 °C. These kinetic parameters were determined both under conditions where the enzyme concentration was much less than substrate concentration (condition I) and where the substrate concentration was much less than enzyme

concentration (condition II) as described in the literature.[22] Kinetic parameters obtained under both sets of conditions were consistent with each other. In the case of compounds **3a–c** which acylate trypsin slowly, condition II was principally applied in order to minimize experimental errors. For condition I, the enzyme concentration was 2–3 μM and substrate concentrations were in the range 21–310 μM. For condition II, enzyme concentrations were in the range 150–490 μM and the substrate concentrations were 2–3 μM. The first-order experimental rate constant, k_{expt1}, was determined from the slope of the linear plot of $\log(A_\infty - A_t)$ vs. t. The values of k_2 and K_s were then determined by the use of inverted (Lineweaver–Burk) plots: either $1/k_{expt1}$ vs. $1/[S]_0$ (condition I) or $1/k_{expt1}$ vs. $1/[E]_0$ (condition II). The deacylation rate constants were obtained spectrophotometrically under conditions where the enzyme concentration was much less than the substrate concentration. The enzyme concentrations were 6–10 μM and the substrate concentrations were in the range of 0.2–5 mM. The measured rate constants were found to be almost independent of the substrate concentrations. In the pH stat titration, the assays were performed in 0.1 M KCl–0.02 M $CaCl_2$, containing 0.5% ethanol, at 25 °C, with an end point of pH 8.0, using 0.05 M sodium hydroxide as the titrant under a stream of nitrogen. At pH 8.0, 1.6 equiv of hydroxide ion was found to be consumed in the hydrolysis of the para substrates due to additional ionization of amidinophenol. For the meta substrates, the corresponding value was determined to be 1.11. First-order rate constants for spontaneous hydrolysis were determined in the same manner as described for the deacylation reactions with the omission of the enzyme. Titrations to determine the operational normality of trypsin solutions were performed in the same way as described elsewhere.[4] The percentages of active sites present were calculated from the normality measurements and the $[E]_0$ determined on the basis of the absorbance taking ϵ_{280nm} to be 39 000.

Acyl-enzyme preparations were carried out as follows. Trypsin, 24 mg (10^{-6} mol), was dissolved in 2 mL of 0.05 M Tris–0.02 M $CaCl_2$ (pH 8.0). To this solution, 3.5 or 3.9 mg (10^{-6} mol) of **2a** or **2b** in 0.15 mL of ethanol was added and the mixture was kept at 25 °C for 5 min. The pH was adjusted to 2.0 by addition of 1 N HCl and the resultant solution was gel filtered (Sephadex G-25 using pH 2.0 HCl as the eluent) and lyophilized. The acyl-enzyme preparations were subjected to the analysis of the catalytic activity. The measurements were performed using *p*-nitrophenyl α-*N*-benzyloxycarbonyl-L-lysinate hydrochloride as the substrate in 0.05 M citrate buffer (pH 3.0) containing 0.8% acetonitrile, at 25 °C where the acyl-enzymes are sufficiently stable. In these measurements, substrate concentrations of 1.08×10^{-3} M and an acyl-enzyme concentration of 2.88×10^{-5} M were used.

Reactivation of acyl-enzymes resulting from deacylation was carried out by incubation of the preparation in 0.1 M KCl–0.02 M $CaCl_2$ medium at pH 8.0, 25 °C. The time course of reactivation was obtained by measuring the residual activities of the aliquots toward *p*-nitrophenyl α-*N*-benzyloxycarbonyl-L-lysinate hydrochloride. The reactivation rate constants were calculated from the slope of semilog plots. The activity of trypsin obtained by complete reactivation of the acyl-enzyme was also analyzed using *N*-benzoyl-L-arginine ethyl ester hydrochloride as the substrate in 0.1 M KCl–0.02 M $CaCl_2$ at 25 °C, pH 8.0, as described elsewhere.[23] The substrate concentration was 4.66×10^{-4} M and the acyl-enzyme concentration was 1.18×10^{-9} M.

The effect of benzamidine hydrochloride on the acylation of trypsin by **2a** was measured in the manner described for the determination of k_2. Substrate concentrations used were 1.60×10^{-5} and 7.42×10^{-5} M, the enzyme concentration was 2.34×10^{-6} M, and the benzamidine concentration was 1.03×10^{-5} to 3.44×10^{-4} M. The K_i value was determined by analysis of the acylation rates obtained, following the procedure which has been described.[24] The K_i values for *p*- and *m*-hydroxybenzamidine hydrochloride were obtained using *N*-benzoyl-DL-arginine *p*-nitroanilide as the substrate at pH 8.2, 25 °C, following the known procedure.[25]

The values listed in Tables I and II are given with their standard deviations and standard errors. Michaelis–Menten kinetic constants with standard errors were calculated using a digital computer program as discussed by Wilkinson.[26] The number of points used in determinations varied from 4 to 18 but was usually 7.

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References and Notes

(1) Proteolytic Enzymes. VII. For part VI see ref 15.
(2) M. L. Bender and F. J. Kézdy, *Annu. Rev. Biochem.*, **34,** 49 (1965); K. A. Walsh, *Methods Enzymol.*, **19,** 41 (1970); P. E. Wilcox, *ibid.*, **19,** 64 (1970); G. P. Hess, *Enzymes, 3rd* Ed., **3,** 213 (1971); T. Inagami, "Proteins", M. Funatsu, K. Hiromi, K. Imahori, T. Murachi, and K. Narita, Ed., Vol. 1, Kōdansha, Tokyo, Japan, 1971, p 1.
(3) M. Muramatsu, Y. Hayakumo, and S. Fujii, *J. Biochem. (Tokyo)*, **62,** 408 (1963); H. Hartmann and E. Hokker, *Eur. J. Biochem.*, **16,** 80 (1970); J. B. Jones, D. W. Sneddon, and A. J. Lewis, *Biochim. Biophys. Acta,* **341,** 284 (1974).
(4) K. Tanizawa, S. Ishii, and Y. Kanaoka, *Biochem. Biophys. Res. Commun.*, **32,** 893 (1968); K. Tanizawa, S. Ishii, and Y. Kanaoka, *Chem. Pharm. Bull.*, **18,** 2247 (1970).
(5) T. Kuga, Master Dissertation, Hokkaido University, 1972.
(6) F. Markwardt, G. Wagner, P. Walsmann, H. Horn, and J. Sturzebecher, *Acta Biol. Med. Ger.*, **28,** K 19 (1972).
(7) M. L. Bender, J. V. Killheffer, Jr., and R. W. Roeske, *Biochem. Biophys. Res. Commun.*, **19,** 161 (1965).
(8) J. A. Stewart and J. Ouellet, *Can. J. Chem.*, **37,** 751 (1959).
(9) C. Wang and E. Shaw, *Arch. Biochem. Biophys.*, **150,** 259 (1972).
(10) D. H. McDaniel and H. C. Brown, *J. Org. Chem.*, **23,** 420 (1958).
(11) M. L. Bender, F. J. Kézdy, and J. Feder, *J. Am. Chem. Soc.*, **87,** 4953 (1965).
(12) M. M. Nachalis, R. E. Plapinger, and A. M. Seligman, *Arch. Biochem. Biophys.*, **108,** 266 (1964); E. Shaw and J. Ruscica, *ibid.*, **145,** 484 (1971).
(13) It has been suggested that the acylation step in the chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate proceeds via a transient acyl-imidazole intermediate; C. D. Hubbard and J. F. Kirsh, *Biochemistry,* **11,** 2483 (1972). At present it is not certain whether this mechanism is involved in our system or not. If this were the case, the acylation rates would reflect the accessibility of the carbonyl carbon of "inverse substrates" not to Ser-183 but rather to His-46.
(14) P. M. Hinkle and J. F. Kirsch, *Biochemistry,* **10,** 2717 (1971); J. Fastrez and A. R. Fersht, *ibid.*, **12,** 1067 (1973).
(15) K. Tanizawa, S. Ishii, K. Hamaguchi, and Y. Kanaoka, *J. Biochem. (Tokyo)*, **69,** 893 (1971).
(16) Evaluating the effects of proximity, orientation, and other factors is a subject of intensive current interest; cf., for example, D. E. Koshland, Jr., *J. Theor. Biol.*, **2,** 85 (1962); D. R. Storm and D. E. Koshland, Jr., *J. Am. Chem. Soc.*, **94,** 5805, 5815 (1972).
(17) J. H. Heidema and E. T. Kaiser, *J. Am. Chem. Soc.*, **89,** 460 (1967); **90,** 1860 (1968).
(18) Preparations of substituted benzoyltrypsins by means of nonspecific *p*-nitrophenyl benzoates were reported.[9]
(19) M. L. Bender, M. L. Begué-Cantón, R. L. Blakeley, L. J. Brubacher, J. Feder, C. R. Gunter, F. J. Kézdy, J. V. Killheffer, Jr., T. H. Marshall, C. G. Miller, R. W. Roeske, and J. K. Stoops, *J. Am. Chem. Soc.*, **88,** 5890 (1966).
(20) Marked effects of positively charged substances on deacylation rates were observed. These are considered characteristics of inverse substrates which produce noncationic acyltrypsins. These observations will be reported elsewhere.
(21) A. W. Dox, "Organic Synthesis", Vol. 1, H. Gilman and A. H. Blatt, Ed., Wiley, New York, N.Y., 1932, p 5.
(22) F. J. Kézdy and M. L. Bender, *Biochemistry,* **1,** 1097 (1962).
(23) G. W. Schwert and M. A. Eisenberg, *J. Biol. Chem.*, **179,** 665 (1949).
(24) M. Dixon, *Biochem. J.*, **55,** 170 (1953).
(25) M. Mares-Guia and E. Shaw, *J. Biol. Chem.*, **240,** 1579 (1965).
(26) G. N. Wilkinson, *Biochem. J.*, **80,** 324 (1961).