A Novel "Induced-Fit" Phenomenon. Evidence for Conformational Changes Closely Associated with Efficient Hydrolysis of Acyl Trypsin[1]

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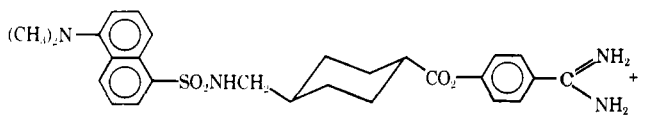
Abstract: An acyl trypsin containing the Dns fluorophore was prepared in a very specific manner by employing a substrate of a new type, an "inverse substrate". Properties of the isolated acyl trypsin have been kinetically and spectrofluorometrically studied. The deacylation rate of the acyl-enzyme, which no longer retains a positive charge on the acyl group, is noticeably enhanced by addition of aromatic amidinium ions. In the course of the accelerated deacylation, the cationic compounds affect some fluorescence characteristics of the acyl-enzyme. The spectral analyses revealed that the acyl group shifts toward a more polar environment and that the solvent (water) accessibility increased in the vicinity of the acyl group to be hydrolyzed. These fluorescence spectral changes closely associated with the enhancement of the rate of deacylation explain the conformational change of the acyl-enzyme, induced by the site-specific cationic compounds, providing a refined example of an "induced-fit" phenomenon.

Esters of *p*-amidinophenol which possess a cationic center in the leaving group have been found to undergo efficient and specific tryptic hydrolysis, and have been termed "inverse substrates" for trypsin.[2-4] It has also been pointed out that these substrates may be applied to the preparation of a wide range of acyl trypsins. In the study of the structure and function of biological macromolecules, one can use organic fluorescent compounds as reporters capable of providing a variety of information.[5] Thus, a logical extension of the concept of "inverse substrates" would be the introduction of a suitable fluorescent acyl group into the active site of trypsin.

Interestingly, we have observed that the deacylation rates of certain acyl trypsins derived from "inverse substrates" are enhanced by addition of positively charged organic compounds,[3,4] in accord with observations reported previously.[6-8] By utilizing a fluorescent group as a sensitive reporter molecule, it is now possible to investigate the correlation between the functional and structural characteristics of the enzyme. In the present article, we wish to report the analysis of the function of trypsin on the basis of kinetic and spectrofluorometric studies of the behavior of the fluorescent acyl trypsin, which is prepared by employing a fluorescent inverse substrate, *p*-amidinophenyl *trans*-*N*-(1-dimethylaminonaphthalene-



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5-sulfonyl)aminomethylcyclohexanecarboxylate (Dns-AMCHC-OAm) (**1**).

Results and Discussion

Preparation of the Acyl-Enzyme. When trypsin was treated with a tenfold molar excess of the fluorescent inverse substrate **1**, a strong increase in absorbance at 305 nm (ϵ_{305} 16 700 M^{-1} cm^{-1} at pH 8.0[2]) was observed, and an acyl trypsin with the covalently and stoichiometrically introduced fluorophore (0.98 mol/mol of enzyme) was isolated after gel filtration. The remaining activity of the isolated acyl trypsin (Dns-AMCHC-trypsin) toward *p*-nitrophenyl α-*N*-benzyloxycarbonyl-L-lysinate (ZLysONP)[9] was 4%, and it recovered almost complete activity (98%) as a result of deacylation on incubation of the acyl-enzyme at pH 8.0 for 1 h.

Effect of Amidinium Ions on the Deacylation Rates. The deacylation rate of the acyl-enzyme (Dns-AMCHC-trypsin) was enhanced by the presence of aromatic amidinium ions such as *p*-amidinophenol and benzamidine (Table I), both of which are competitive inhibitors of trypsin for specific substrates.[2,10] At pH 8.0, 20 mM *p*-amidinophenol increases the rate more than 6-fold, and 20 mM benzamidine causes a 1.4-fold increase (Figure 1). The increase with *p*-amidinophenol was greater at pH 8.0 than at pH 6.0.

This different efficiency of *p*-amidinophenol at the two pH values might be ascribed to more efficient activation by the ionized form of the phenolic hydroxyl group of *p*-amidinophenol than by the un-ionized form ($pK_a \simeq 8$[11]). However, *p*-aminobenzamidine at a concentration of 20 mM increases the rate 3.9-fold at pH 8.0 and 2.0-fold at pH 6.0, though the amino group of this compound is in its un-ionized form at both pH values (pK_a = 3.7, unpublished observation by us). Therefore, the efficiency variation of the amidinium ions on activation between pH 6.0 and 8.0 may be ascribed not to different mechanisms operating, but to a difference in the extent with which a single conformational change occurs.

In the activation phenomenon, the reactions involved can be represented by the following scheme (eq 1), where an amidinium ion (A) can bind an acyl-enzyme (E′) and free enzyme (E) to form two complexes, (E′·A) and (E·A), respectively. At the very initial stage of the deacylation where the binding of A with E is negligible ([E′·A] ≫ [E·A]), the observed deacylation rate should be correlated with the dissociation constant (K_A) and the concentrations of amidinium ion.

$$\begin{array}{ccc} E' & \xrightarrow{k} & E + \text{product} \\ K_A \Big\| {\scriptstyle +A} & & K_i \Big\| {\scriptstyle +A} \\ E'\cdot A & \xrightarrow{k_a} & E\cdot A + \text{product} \end{array} \qquad (1)$$

The concentrations of E′·A and E′ are related by:

$$\frac{[E'][A]}{[E'\cdot A]} = K_A \qquad (2)$$

The observed rate (v) is represented by:

$$v = \frac{[E']v_0 + [E'\cdot A]v_a}{[E'] + [E'\cdot A]} \qquad (3)$$

where the rate of deacylation of the acyl-enzyme with the amidinium ion bound or not bound is denoted as v_a or v_0, respectively. From eq 2 and 3, the following equation is derived, where v_r is v divided by v_a:

$$v_r = -K_A(v_r - 1)/[A] + v_a/v_0 \qquad (4)$$

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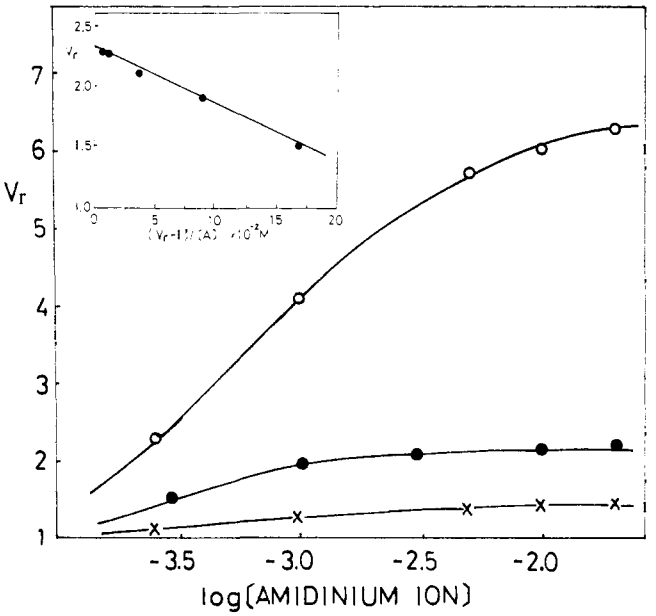

Figure 1. Effects of *p*-amidinophenol at pH 8.0 (○) and pH 6.0 (●) and of benzamidine at pH 8.0 (×) on the relative deacylation rate, v_r, of Dns-AMCHC-trypsin. The insert is a plot according to eq 4 for the increase of the deacylation rate of the acyl trypsin by *p*-amidinophenol at pH 6.0.

Table I. Effect of Amidinium Ions on First-Order Rate Constants for Deacylation of Dns-AMCHC-trypsin[a]

concn of the additive, mM	*p*-amidinophenol k_{1st}, 10^{-4} s^{-1}		benzamidine, k_{1st}, 10^{-4} s^{-1}
	pH 6.0[b]	pH 8.0[c]	pH 8.0[c]
0	1.55 ± 0.18	11.4 ± 1.2	11.4 ± 1.2
0.25		25.7 ± 3.0	12.5 ± 0.8
0.3	2.35 ± 0.20		
1	2.95 ± 0.31	46.2 ± 4.2	14.3 ± 2.1
3	3.27 ± 0.37		
5		65.2 ± 7.1	15.4 ± 2.6
10	3.50 ± 0.32	68.0 ± 7.5	16.0 ± 1.9
20	3.55 ± 0.38	72.2 ± 13.0	16.2 ± 2.3

[a] After the deacylation reaction was terminated by addition of 0.1 M citrate buffer (pH 3.0), recovery of enzymatic activity of the acyl trypsin toward ZLysONP was measured. [b] 0.1 M Mes-0.04 M $CaCl_2$ at 25 °C. [c] 0.1 M Tris-0.04 M $CaCl_2$ at 25 °C.

A plot of v_r against $(v_r - 1)/[A]$ is shown in Figure 1, insert, for *p*-amidinophenol at pH 6.0. The plot gives a straight line, and the K_A value, the dissociation constant of the acyl trypsin-*p*-amidinophenol complex, is obtained as 4.5×10^{-4} M. This value is ten times larger than K_i, the dissociation constant of the free trypsin-*p*-amidinophenol complex (the K_i value was determined to be 4.7×10^{-5} M using α-*N*-benzoyl-DL-arginine *p*-nitroanilide as substrate at pH 6.0).

Activation phenomena in trypsin-catalyzed reactions in the presence of cationic compounds have been reported by several groups.[3,6-8] Inagami and Murachi reported that the dissociation constant (K_A) of alkylamines in the process of activating the hydrolysis of ethyl acetylglycinate[6] is approximately equal to the inhibition constant (K_I) obtained from a competition experiment with a specific substrate. Seydoux et al. also found that alkylamines act as activators in the hydrolysis of *p*-nitrophenyl acetylglycinate.[8] In that case, the dissociation constants for the binding of several amines to the acyl-enzyme were larger than those for free enzyme. Our observation coincides with the latter. The amidinium ions may be bound in the vacant specific pocket[21] of the neutral acyl-enzyme with lower affinity than that for the native enzyme. This lowered affinity may be due to partial interference of the large acyl group introduced in the acyl-enzyme with binding of amidinium ions in the pocket, or to a conformational change near the active site as a result of acylation.

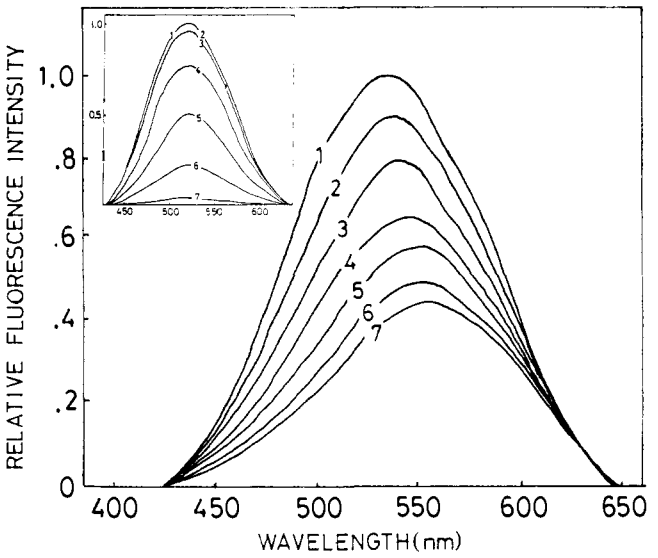

Figure 2. Effect of *p*-amidinophenol on the fluorescence spectra of Dns-AMCHC-trypsin and Dns-AMCHC-OMe (insert) at pH 6.0.[12] Numbers indicate the concentrations of *p*-amidinophenol at which the spectra were recorded: (1) 0; (2) 0.1 mM; (3) 0.3 mM; (4) 1 mM; (5) 3 mM; (6) 10 mM; (7) 20 mM.

Effect of *p*-Amidinophenol on the Fluorescence Characteristics of Dns-AMCHC-trypsin. (a) Maximum Wavelength and Intensity of Fluorescence. Figure 2 (insert) shows that *p*-amidinophenol quenched the fluorescence of Dns-AMCHC-OMe at the concentrations higher than 1 mM; phenols are known to be effective fluorescence quenchers.[14] Interestingly, the compound progressively quenched the fluorescence of Dns-AMCHC-trypsin, accompanied in this case by a shift of its maximum wavelength (538 nm) to longer wavelength (550 nm) as the concentration was increased (Figure 2). Phenol itself brought about only 7% fluorescence quenching of Dns-AMCHC-trypsin, but no shift in its maximum wavelength even at 20 mM concentration. These observations suggest that *p*-amidinophenol affects the fluorescence of the acyl-enzyme by a mechanism different from its simple quenching of the fluorescence of Dns-AMCHC-OMe and from the case of the nonspecific compound, phenol. The changes in the fluorescence spectrum of the acyl-enzyme occurring on addition of *p*-amidinophenol, involving the red shift and the decrease in intensity, indicate that the binding of *p*-amidinophenol, a specific ligand, changed the polarity of the environment of the acyl group, causing it to be more polar.[13]

Figure 3 shows the double reciprocal plot of the change of fluorescence intensity vs. concentrations of the amidinium ion. A significant negative deviation from linearity is observed at higher concentrations. In view of the quenching effect of *p*-amidinophenol at higher concentrations, a straight line can be drawn, based on the linear relationship of $[E']/(F_0 - F)$ vs. $[p\text{-amidinophenol}]^{-1}$ at concentrations lower than 1 mM, and the value of K_f is obtained as 4.1×10^{-4} M. This value is in good agreement with the dissociation constant, K_A, estimated from the activation phenomenon. This observed coincidence provides strong evidence that the conformational change suggested by the fluorescence data is directly related to the enzyme activation.

(b) Effect of D_2O on the Quantum Yields of Fluorophores. The magnitude of the effect of substituting H_2O in the medium with D_2O on the quantum yield of a given fluorophore, incorporated into a macromolecule, is taken as a measure of solvent accessibility of the fluorophore.[15] The quantum yield of

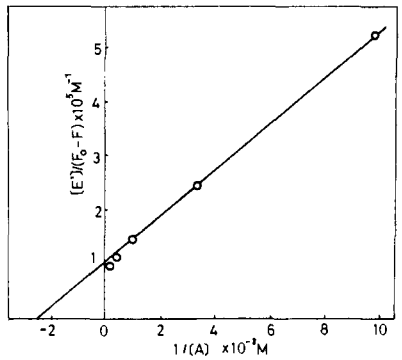

Figure 3. Double reciprocal plots of the change of fluorescence intensity of Dns-AMCHC-trypsin at maximum wavelength vs. concentrations of *p*-amidinophenol at pH 6.0, according to the equation described in the Experimental Section.

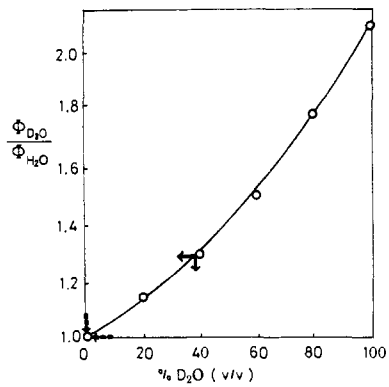

Figure 4. Effect of D_2O on the fluorescence quantum yields of Dns-AMCHC-OMe. A solution of the compound contained 1% dimethylformamide. Superimposed on the curve are the Φ_{D_2O}/Φ_{H_2O} ratios for Dns-AMCHC-trypsin (90% D_2O, v/v) in the absence (- - -) and presence (—) of 10 mM *p*-aminophenol at pH 6.0.

Dns-AMCHC-trypsin in D_2O increased 1.3-fold on addition of *p*-amidinophenol at a concentration of 10 mM. It is therefore suggested that the accessibility of the Dns group in the acyl-enzyme to solvent changed from a buried state to a 40%-exposed one, on the basis of the scale obtained from the quantum yield of Dns-AMCHC-OMe, a model compound, in various contents of D_2O, as shown in Figure 4. This increase in solvent accessibility is substantially consistent with the polarity change of the microenvironment of the acyl-enzyme measured by the above fluorescence shift.

In conjunction with the activation phenomenon, the observed fluorescence changes of the acyl-enzyme may be interpreted as follows: The neutral acyl-enzyme, Dns-AMCHC-trypsin, appears to be incapable of exhibiting its full catalytic function in the deacylation step, unless the additional positively charged amidinium ion induces certain necessary conformational changes of the acyl-enzyme for proper alignment of the cationic entities, as the "induced-fit" theory suggests.[16] The "induced" favorable conformation for the deacylation clearly involves, in this particular case, a shift of the acyl group toward a more polar environment and an increase in the solvent (water) accessibility in the vicinity of the acyl group to be hydrolyzed. The conformational changes of trypsin, which occur subsequent to the acylation step in the course of the reactions with specific substrates,[17] have now been spectrofluorometrically witnessed by employing the inverse substrates, which sever a cationic moiety and neutral acyl groups and artificially slow down the fast process of normal enzymatic reactions in the deacylation step.

Energy Transfer Study. The excitation spectrum shows that energy is transferred from tryptophan residues to the Dns group (Figure 5). The results of an energy transfer study according to Förster's equation (Experimental Section) are summarized in Table II. The average distance between the Dns group and the tryptophan residue(s) in the acyl trypsin is calculated to be 21.3 Å. On the three-dimensional crystal-structure model of DIP-trypsin at 2.7-Å resolution,[21] Trp-237, one of four tryptophan residues, is located at the back side of the active-site cleft in the globular molecule of trypsin and thus cannot interact with the Dns group. Trp-51 is oriented perpendicular to the surface of the trypsin molecule and imbedded in it in such a fashion that only one edge of the ring is exposed to the solvent. Since a face-to-face contact between the donor and acceptor ring is required for energy transfer, it is unlikely that Trp-51 is a donor molecule. Trp-141 is completely buried and may be situated behind the binding region of leaving groups of ordinary substrates, and be excluded. The exposed Trp-215 near the active-site cleft is the most probable candidate as the donor molecule in the energy-transfer process.

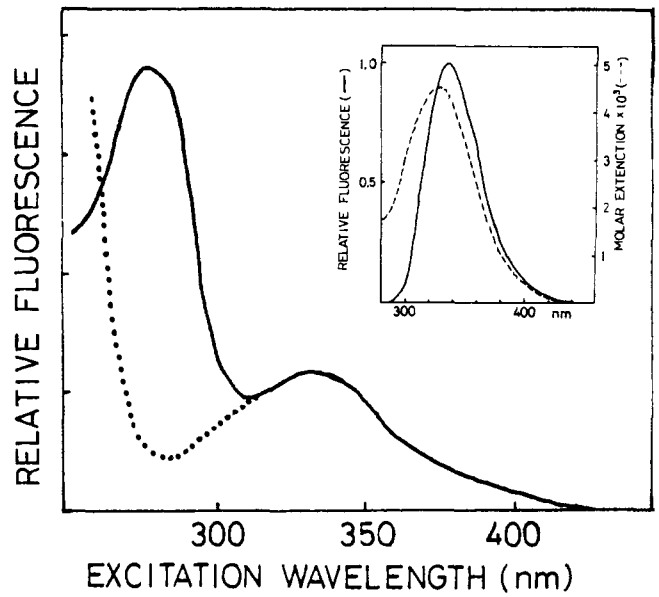

Figure 5. Fluorescence excitation spectra of Dns-AMCHC-trypsin (—) and Dns-AMCHC-OMe (• • •) at pH 6.0. Emission at 530 nm. The insert depicts the spectral overlap of the emission spectrum of Trp residues in native trypsin (solid) and the difference absorption spectrum of the Dns group in Dns-AMCHC-trypsin (broken).

Vaz and Schoellmann calculated the distance between Trp-215 and a Dns group in His-46 modified trypsin prepared by reaction with Dns-lysine chloroketone as 17.9–18.8 Å on the basis of energy transfer and three-dimensional model-building studies on the enzyme.[15] The Dns-AMCHC group in the acyl-enzyme, when assumed to be oriented in a similar fashion to that of the Dns-lysine group, would be extended along the region of Ser-214 and Trp-215 at the active-site cleft, and the distance between Trp-215 and the Dns group of the acyl-enzyme is tentatively calculated as being somewhat shorter than 18 Å by comparing the two modified trypsins. The observed longer distance, 21.3 Å, suggests that the neutral acyl group is flexible at the cleft and the transfer efficiency is lower than that expected. The flexibility may be due both to the lack of a cationic charge in the acyl moiety to interact with the anionic specific pocket of trypsin,[21] and to the bulkiness of the AMCHC group, which may affect how it fits in the specific side-chain pocket.

In summary, the deacylation rate of the neutral acyl-enzyme is enhanced by the addition of cationic amidinium ions resulting in a limiting deacylation rate similar to that of a normal cationic acyl-enzyme. The observation is essentially similar to those obtained in the study of "inverse substrates" of some

Table II. Energy Transfer between the Protein Trp Residues and a Single Dns Group in Dns-Trypsins

Dns-trypsins	overlap integral, $J_{AD} \times 10^{15}$, $cm^6\ M^{-1}$	critical distance, R_0, Å	transfer efficiency, T, %	calcd R, Å	ref
Dns-AMCHC-trypsin[a]	4.3	18.8	32	21.3	this work
DLCM-trypsin[b]	3.7	17.7	48	17.9	14
			41	18.8	14

[a] At pH 6.0 with 0.1 M Mes–0.2 M NaCl and emission at 530 nm. [b] His-46-modified trypsin by Dns-lysine chloroketone.

acyl moieties such as acetyl and propionyl.[3] However, the present results on activation, by employing an *isolated* neutral acyl-enzyme, explain the activation mechanism more clearly without considering such complicating factors as the intricate binding of neutral substrate and amines.[8] Moreover, the changes of some fluorescence characteristics closely associated with the activation process shed light on the induced conformational changes of neutral acyl-enzymes, providing a refined example of an "induced-fit" phenomenon. This approach using the "inverse substrates" is expected to offer further information about the detailed aspects of enzyme–substrate intermediates of trypsin and the related enzymes.

Experimental Section

Materials. *p*-Amidinophenol *p*-toluenesulfonate (**2**),[2] benzamidine hydrochloride,[22] and *p*-aminobenzamidine dihydrochloride[23] were prepared as described in the literature. *trans*-Aminomethylcyclohexanecarboxylic acid (**3**) was the kind gift of Daiichi Seiyaku Co. 1-Dimethylaminonaphthalene-5-sulfonyl chloride (**4**) was prepared according to the literature procedure.[24] *trans*-*N*-(1-Dimethylaminonaphthalene-5-sulfonyl)aminomethylcyclohexanecarboxylic acid (**5**) was preprepared from **3** and **4** by a similar method described previously;[25] the melting point was 184–186° dec. The acid chloride hydrochloride of **5** was prepared by treating **5** with an excess of thionyl chloride in the presence of a catalytic amount of pyridine at room temperature overnight.

The *p*-toluenesulfonate salt of *p*-amidinophenyl *trans*-*N*-(1-dimethylaminonaphthalene-5-sulfonyl)aminomethylcyclohexanecarboxylate (**1**) was synthesized by the reaction of the corresponding acid chloride hydrochloride (1.7 g) and **2** (1.2 g) in the presence of triethylamine (0.8 g) in dimethylformamide (20 mL) at 0 °C for 1 h, followed by allowing it to stand at room temperature for 3 h. After the addition of ether, the precipitated oil was purified by column chromatography on silica gel using chloroform and ethanol (5:2) as solvent. Recrystallization from acetonitrile gave yellow prisms, mp 168–170 °C (18% yield); IR (Nujol) 1740, 1670 cm^{-1}; UV (MeOH) 330 nm (ϵ 4300). Anal. Calcd for $C_{34}H_{40}N_4O_7S_2$: C, 59.98; H, 5.92; N, 8.23; S, 9.42. Found: C, 59.86; H, 5.82; N, 8.01; S, 9.22.

The methyl ester of **5** (Dns-AMCHC-OMe) was prepared by treating **5** (0.8 g) with an excess of diazomethane in tetrahydrofuran (20 mL) at 0 °C overnight. After evaporation, the residual oil solidified in the refrigerator. Recrystalization from ether and *n*-hexane gave pale yellow prisms, mp 84–85.5 °C dec (80% yield); IR (Nujol) 1725 cm^{-1}; UV (MeOH) 330 nm (ϵ 4200). Anal. Calcd for $C_{21}H_{28}N_2O_4S$: C, 62.36; H, 6.98; N, 6.93; S, 7.91. Found: C, 62.21; H, 6.98; N, 6.93; S, 7.94.

p-Nitrophenyl α-*N*-benzyloxycarbonyl-L-lysinate hydrochloride (ZLysONP) was purchased from Aldrich Chemical Co. All other chemicals used were products of Nakarai Chemicals, analytical grade. Trypsin (2× crystallized, lot TRL) was purchased from Worthington Biochemical Corp. and purified by affinity chromatography on ST-Sepharose[26] to give 95% active enzyme toward *p*-nitrophenyl *p'*-guanidinobenzoate.[27]

Preparation of Acyl-Enzyme and Determination of the Content of the Acyl Group Introduced. The purified trypsin (12 mg) was dissolved in 10 mL of 0.05 M Tris–0.02 M $CaCl_2$ (pH 8.0). To this solution 3.5 mg of **1** in 0.5 mL of dimethylformamide was added, and the mixture was kept at 25 °C for 3 min. The pH was lowered to 2.5 by the addition of 1 N HCl, and the resultant solution was gel-filtered (Sephadex G-25 with 5 mM HCl, at 4 °C) and lyophilized.

In order to determine the acyl group content, the isolated acyl trypsin was incubated for 1 h in 0.05 M Tris–0.02 M $CaCl_2$ (pH 8.0) until deacylation was completed (below), as followed by measuring absorbancies at 325 nm (A_{325}) and 280 nm (A_{280}). The acyl group content (moles/mole of enzyme) was calculated according to the following equation:

$$\text{acyl group content} = \frac{\epsilon^{E}_{280}}{0.95[\epsilon^{H}_{325}A_{280}/(A_{325} - \epsilon^{H}_{280})]}$$

where 0.95 is the normality of the purified trypsin and ϵ^{E}_{280} (36 700), ϵ^{H}_{280} (1800), and ϵ^{H}_{325} (4800) are the molar extinction coefficients of trypsin and of the product acid **5** at 280 and 325 nm.

Kinetics of the Deacylation of the Acyl-Enzyme. The deacylation rate was monitored by measuring the recovery of enzymatic activity of the isolated acyl trypsin toward ZLysONP.[9] To 0.1 M Mes–0.04 M $CaCl_2$ (pH 6.0) or 0.1 M Tris –0.04 M $CaCl_2$ (pH 8.0) buffer solution containing 0.2 M NaCl in the presence of 0–22 mM amidinium ions, 0.01 mL of a 3×10^{-4} M solution of the acyl-enzyme in 1 mM HCl was added, and the reactivation reaction was initiated. To the incubation mixture in a 1-cm cuvette, 3 mL of 0.1 M citrate buffer (pH 3.0) was added at various time intervals to terminate the deacylation reaction. A 3×10^{-2} M solution of ZLysONP in CH_3CN containing 20% 1 mM HCl was then added. The reaction velocity for the hydrolysis of ZLysONP determined from the slope of the initial, linear portion of the absorbance vs. time curve is used here as a measure of the construction of reactivated enzyme. All reactions were carried out at 25 °C. The first-order rate constants for reactivation were determined from the slopes of linear plots of log (activity$_t$ − activity$_0$) vs. time. In order to calculate the dissociation constant for E'A (K_A), we determined the rate constants for reactivation at pH 6.0 measuring the initial velocity during the very beginning of the reaction (within 10 min) where less than 15% of the activity had been recovered. Determination of K_A at pH 8.0 was not accomplished because the reactivation was so fast that practical analysis by eq 3 was not feasible.

Measurements of Fluorescence Spectra. All fluorescence spectra were obtained at 25 °C using a 1-cm cuvette on a Shimadzu RF 502 spectrofluorometer equipped with a corrected spectrum accessory. To 3 mL of 0.1 M Mes (pH 6.0) buffer solution containing 0.2 M NaCl in the presence of various concentrations of *p*-amidinophenol, 0.05 mL of 3×10^{-4} M solution of the acyl trypsin in 1 mM HCl or 0.025 mL of 2.4×10^{-4} M solution of Dns-AMCHC-OMe in dimethylformamide was added. The optical density at the excitation wavelength was less than 0.1 to ensure linearity in fluorescence response.

The dissociation constant for the acyl trypsin–*p*-amidinophenol complex (K_f) was determined fluorometrically by the following equation:[28]

$$\frac{[E']}{(F_0 - F)} = \frac{1}{K_f}\frac{[E'\cdot A]}{(F_0 - F)}\frac{1}{[A]} + \frac{[E'\cdot A]}{(F_0 - F)}$$

where $(F_0 - F)$ is the decrease in fluorescence of the acyl-enzyme on addition of *p*-amidinophenol at maximum wavelength, and [A], [E'], and [E'·A] indicate concentrations of *p*-amidinophenol, the acyl-enzyme, and the complex, respectively.

When the effect of D_2O on fluorescence was investigated, the solutions were prepared in the following manner: Dns-AMCHC-OMe solution was prepared in dimethylformamide and diluted with D_2O or H_2O as necessary to give final concentrations of 0–100% D_2O (v/v). In all cases the dimethylformamide concentration was 1% (v/v). In the case of acyl trypsin, a concentrated solution of the acyl-enzyme in 1 mM HCl (0.05 mL) was diluted with D_2O (2.7 mL) and 0.5 M Mes containing 2 M NaCl (pH 6.0) (0.3 mL) in the presence and absence of 10 mM *p*-amidinophenol, to a final concentration of 90% D_2O (v/v). For comparison with the quantum yield of the acyl trypsin

in H_2O, the dilution was done with distilled water. Emission spectra in D_2O and H_2O showed no qualitative differences in maximum wavelength, and so the fluorescence intensities at maximum wavelength of the spectra were directly compared without correction.

In the energy-transfer study, tryptophan residues were excited at 295 nm in order to avoid, or at least to reduce, the contribution of tyrosine residues to the observed emission. The efficiency of excitation energy transfer (T) between the tryptophan residue(s) of tyrpsin (donor) and the attached Dns group (acceptor) was measured by employing corrected excitation spectra[29] as follows:

$$T = \left(\frac{E_{295}}{E_{350}} - \frac{\epsilon_{295}^{Dns}}{\epsilon_{350}^{Dns}}\right)\frac{\epsilon_{350}^{Dns}}{\epsilon_{295}^{enz}}$$

where E_{295} and E_{350} are the magnitudes of the corrected excitation spectrum at 295 and 350 nm, and ϵ_{295}^{Dns} (3300), ϵ_{350}^{Dns} (3300), and ϵ_{295}^{enz} (9000) are the molar extinction coefficients of the Dns-AMCHC group at 295 and 350 nm, and trypsin alone at 295 nm, respectively. The efficiency of such transfer (T) is related to the separation distance (R) between the two dipoles by the expression:

$$T = \frac{1}{1 + (R/R_0)^6}$$

where R_0, the "critical distance" at which T is 0.5, is given[30] by:

$$R_0 = (9.79 \times 10^3)(J_{AD}\kappa^2\Phi_D\eta^{-4})^{1/6}$$

where a quantum yield, Φ_D, of 0.124 was used for trypsin,[18] κ^2, the orientation factor, was assumed to be 0.476[19] (a fixed acceptor and a fixed array of randomly oriented donors), η, the refractive index, was taken to be that of the solvent, 1.5,[20] and J_{AD}, the spectral overlap integral, was calculated from the spectrum shown in Figure 5 (insert) according to the expression:

$$J_{AD} = \frac{\int F_D(\lambda)\epsilon_A(\lambda)\lambda^4\,d\lambda}{\int F_D(\lambda)\,d\lambda}$$

Here $F_D(\lambda)$ and $\epsilon_A(\lambda)$ are the fluorescence intensity of the donor and the molar extinction coefficient of the acceptor, respectively, at the wavelengths shown in Figure 5. The computer program used in the calculation of J_{AD} was kindly written for Facom 230-75 by Dr. Y. Aizawa.

Acknowledgments. We are grateful to Professor Emil T. Kaiser for helpful advice and reading of the manuscript. We also wish to thank Professor S. Ishii for advice in using ST-Sepharose and Dr. Y. Aizawa for computer programming. This work was supported in part by grants from the Ministry of Education, Science and Culture (Grant-in-Aid for Scientific Research, No. 311730 and 447120) and the Yamada Science Foundation.

References and Notes

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