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Inverse Substrates. XV.[1] Spectrometric Properties of Fluorescence-labeled Acyl Trypsins

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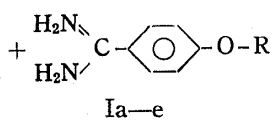
Acyl trypsins containing the 1-dimethylaminonaphthalene-5-sulfonyl (DNS) fluorophore were prepared in a very specific manner by employing substrates of a new type. The properties of isolated acyl trypsins were spectrofluorometrically analyzed. The deacylation rate of the acyl trypsin, which no longer retains a cationic site-specific group on the acyl group, was enhanced by the addition of alkylammonium ions. This kinetic behavior was shown to be reflected in the fluorescence spectra. These results are discussed on the basis of conformational change of the trypsin active site.

Keywords——trypsin substrate; reporter group; fluorescence labeling; catalytic efficiency; active site structure; acyl trypsin; conformational change

Affinity labeling has been a valuable tool in the study of the structure and function of enzymes. Tos–Lys–CH_2Cl (TLCK) and Tos–Phe–CH_2Cl (TPCK) are well-known specific labeling reagents for trypsin and chymotrypsin, respectively.[2] These derivatives were successfully applied for the identification of amino acid residues which are essential for the enzymatic action.[3,4] Similar affinity labels combined with a reporter group such as a spin-label[5] or a fluorescent molecule[6] have been used as probes of the microenvironment of enzyme active sites. In these instances, the modified enzymes always contain a certain site-specific group for the enzyme other than the reporter group.

Previously we reported that esters of *p*-amidinophenol (I) which possess a cationic center in the leaving group undergo efficient and specific tryptic hydrolysis, and termed "inverse substrates" for trypsin.[7] These "inverse substrates" provide a facile means for the specific introduction of an acyl group with a non-specific structure into the trypsin active site as acyl enzyme intermediate. Reporter groups carrying a spin-label and fluorophore have been used to explore the active site of trypsin by this method.[8,9] In these studies, acyl groups were simply designed to consist of a reporter group.

It is of special interest to analyze the spectral properties of acyl trypsins with an α-amino acid function. α-Amino acyl trypsin derivatives are the actual intermediates of tryptic catalysis. The purpose of the present work was to prepare acyl trypsins carrying both an α-amino acid function and a fluorescent reporter group and to analyze their spectral behavior as a model of actual acyl trypsin intermediates to demonstrate the applicability of "inverse substrates."



Ia—e

Ia: R=Dns-Gly
Ib: R=Dns-L-Ala
Ic: R=Dns-D-Ala
Id: R=Dns-L-Phe
Ie: R=Dns-D-Phe

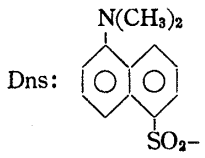


Chart 1

Experimental

Materials——Bovine trypsin was purchased from Worthington Biochemical Corp. (lot TRL), and purified by affinity chromatography on ST-Sepharose[10] to give enzyme that was 92% active toward *p*-

nitrophenyl *p'*-guanidinobenzoate.[11] Synthesis of *p*-amidinophenyl esters was carried out following the procedure reported previously.[12] *dl*-2-Amines were purchased from Aldrich Chemical Co. Resolution of racemic amines was accomplished by the use of *d*-tartaric acid (or *l*-tartaric acid). The specific rotations of prepared chiral amines are in good accord with reported values.[13–15] *p*-Nitrophenyl α-*N*-benzyloxycarbonyl-L-lysinate (ZLysONP) was obtained from Aldrich Chemical Co.

Preparation of Acyl Trypsins and Determination of Content of the Acyl Group introduced——Trypsin (39 mg) was dissolved in 10 ml of 0.1 M morpholinoethanesulfonate containing 0.02 M $CaCl_2$ (pH 6.0). To this solution, 10 equivalents of substrate in 0.2 ml dimethylformamide was added, and the mixture was kept at 25°C for 2 min. The pH was lowered to 2.0 by the addition of 1 N HCl, and the whole was gel-filtered (Sephadex G-25 with 5 mM HCl) and lyophilized. The acyl enzyme preparations were subjected to analysis of the catalytic activity. The measurements were performed using ZLysONP in 0.05 M citrate buffer (pH 3.0) following the reported procedure.[16] Reactivation of acyl enzyme resulting from deacylation was carried out by incubation of the preparation in 0.05 M Tris–0.02 M $CaCl_2$ (pH 8.0) at 25°C for 1 h. Residual activity of the incubate was analyzed by using ZLysONP as described above. In order to determine the acyl group content, the isolated acyl trypsins were incubated for 1 h in 0.05 M Tris–0.02 M $CaCl_2$ (pH 8.0) until deacylation was complete (1 h). The acyl group content (mol/mol of enzyme) was calculated according to the following equation:[18]

$$\text{acyl group content} = \frac{\varepsilon^{E}_{280}}{0.92[(\varepsilon^{H}_{325} \cdot A_{280}/A_{325}) - \varepsilon^{H}_{280}]}$$

where 0.92 is the molarity of the purified trypsin on the absorbancy basis and ε^{E}_{280} (36700), ε^{H}_{280} (1800), and ε^{H}_{325} (4800) are the molar extinction coefficients of trypsin and of the hydrolyzed acid, at 280 nm or 325 nm, as indicated.

Measurements of Fluorescence Spectra——Fluorescence spectra were obtained with a Hitachi 650-60 spectrofluorometer equipped with a corrected spectrum accessory. The concentration of acyl enzyme was 5 μM, where the optical density at the excitation wavelength was less than 0.1, to ensure linearity in fluorescence response. It was confirmed that during the period of spectral measurements no appreciable deacylation was observed. In the energy transfer study, tryptophan residue(s) were excited at 295 nm in order to avoid the contribution of tyrosine residue to the observed emission. The efficiency of energy transfer (T) between the tryptophan residue(s) of trypsin (donor) and the attached Dns group (acceptor) was measured by employing corrected emission spectra as follows:[17]

$$T = 1 - (F/F_0)$$

where F_0 is the intensity of the corrected emission spectrum of native trypsin at 330 nm and F is that of trypsin modified with fluorescent residue. The efficiency of such transfer (T) is related to the separation distance (R) between the two dipoles by the expression:[18]

$$T = R_0^6/(R_0^6 + R^6)$$

where R_0 is the "critical distance" at which T is 0.5, as described in the literature.[18]

Results and Discussion

Preparation of Acyl Trypsins

Fluorescent acyl trypsins were successfuly prepared from "inverse substrates" Ia, Ic and Ie following the general procedure reported previously.[7] As shown in Table I, in these compounds the acylation rate constants are much larger than deacylation rate constants. In contrast, attempts to isolate acyl trypsin from the L-enantiomers (Ib and Id) were unsuccessful. The relationship $k_2 \gg k_3$, which is required for the accumulation of the acyl enzyme intermediate, is not satisfied for the compounds Ib and Id. The remaining activities of the isolated acyl trypsins toward ZLysONP were found to be less than 6%. The recovery of enzymatic activities as a result of deacylation on incubation at pH 8.0 for 1 h is almost complete, as shown in Table II. Spectrometric quantification of the acyl group introduced into the trypsin molecule was carried out as described in the experimental section. This is in reasonable accordance with the results of kinetic analysis.

It is well known that enzymatic reaction are very stereo-specific. This stereo-specificity is known to be due to the acylation rather than to the binding process. For example, benzoyl-L-arginine ethyl ester is hydrolyzed efficiently by trypsin but its D-enantiomer is not susceptible to enzymatic catalysis. The D-enantiomer can be bound to trypsin as effectively as the L-

enantiomer, but the resulting molecular array is unfavorable to the subsequent acylation stage.[19] In contrast, "inverse substrates" are quite distinct from the conventional substrates, as discussed previously.[7] Since the achiral *p*-amidinophenoxycarbonyl linkage fits the active site structure well, the acyl moiety of a *p*-amidinophenyl ester is specifically transferred to the catalytic serine residue to give acyl enzyme even if the acyl moiety is a D-amino acid derivative. Thus, comparison of deacylation within enantiomeric pairs of α-amino acid derivatives is possible for the first time by the use of "inverse substrates." As shown in Table I, trypsin is capable of catalyzing the deacylation of D-alanine and D-phenylalanine derivatives. The rates for Ic and Ie are about one-tenth of those of the corresponding L-enantiomers.

Table I. Kinetic Parameters for Trypsin-Catalyzed Hydrolysis of "Inverse Substrates" in 0.1 M Tris–0.02 M $CaCl_2$ Buffer at pH 8.0, 25°C[a]

"Inverse substrate"	k_2 (s^{-1})	K_s (10^{-5} M)	k_2/K_s ($10^5 s^{-1} M^{-1}$)	k_3 ($10^{-1} s^{-1}$)	$k_{spont.}$ ($10^{-4} s^{-1}$)
Ia	28.6±0.9	0.73±0.14	39.2	7.56±0.07	3.72±0.11
Ib	>1.39	—	—	13.9	2.50±0.07
Ic	2.44±0.03	2.65±0.19	0.92	1.95±0.06	2.30±0.05
Id	>2.34	1.41±0.05[b]	1.66	16.7 ±0.1	1.20±0.03
Ie	1.02±0.14	5.96±1.30	0.17	1.87±0.02	1.31±0.03

a) Cited from the previous work (Ref. 20).
b) Apparent K_m.

Table II. Characteristics of Acyl Trypsin

Acyl trypsin	Activity lost[a] (%)	Recovered activity[b] (%)	Acyl group introduced[c] (mol/mol/enzyme)
Dns-Gly-trypsin	93	86	0.87
Dns-D-Ala-trypsin	96	92	0.83
Dns-D-Phe-trypsin	94	95	0.80

a) Determined by measuring the residual activity toward ZLysONP.
b) Deacylation was carried out by incubation in Tris buffer at pH 8.0, 25°C for 1 h.
c) Determined by spectrometric analysis.

Effects of Chiral Alkylammonium Ions on the Tryptic Catalysis of "Inverse Substrates"

The presence of a cationic compound such as amidinium or ammonium enhanced the steady-state hydrolysis of the fluorescent substrates. As reported previously,[21] the effect of these cationic ligands can be interpreted on the basis of a characteristic feature of "inverse substrates," as shown in Chart 2. It is expected that the chiral requirements of the active site also reflected in this activation process. In the present study, the effects of optically active 2-aminoalkanes were analyzed with a view to obtaining a refined structure of the active site.

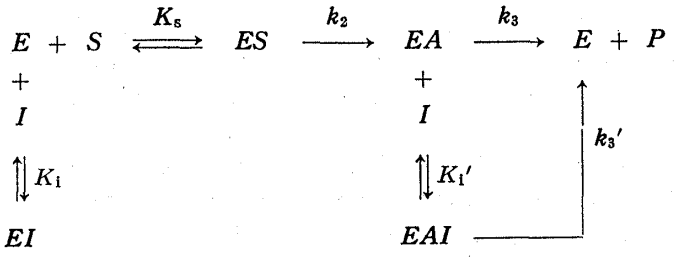


Chart 2

The dissociation constant of acyl enzyme-ligand complex, K_1', and the extent of enhancement in the deacylation, k_3'/k_3, were analyzed according to the previous work,[21] and the values are listed in Table III. The effects of chiral amines on this acceleration process were different within each enantiomeric pair. Each *l*-amine usually has (except in the case of aminooctane with Ia) a larger activation effect and shows less efficient binding than its *d*-enantiomer. This suggests that the sterically facile binding mode is not favorable for the induction of conformational transition of the active site. Spectrometric evidence for the conformational transition will be discussed in the following section.

Table III. Kinetic Parameters for Activation in the Tryptic Hydrolysis of "Inverse Substrates" at 25°C

Ligand	Substrate			
	Ia[a]		Ic[b]	
	k_3'/k_3	K_1', (10^{-2} M)	k_3'/k_3	K_1', (10^{-2} M)
2-Aminopentane				
l	5.38±0.53	8.34±1.34	1.79±0.07	4.61±0.68
d	3.45±0.13	3.49±0.33	1.0	—
2-Aminoheptane				
l	4.70±0.02	9.62±0.07	2.34±0.19	9.21±2.08
d	4.24±0.17	5.13±0.40	2.16±0.09	7.84±1.09
2-Aminooctane				
l	6.11±0.49	14.9 ±1.7	2.44±0.08	13.5 ±1.1
d	7.55±0.77	12.9 ±1.8	2.31±0.06	7.30±0.58

a) At pH 4.95.
b) At pH 6.60.

Analysis of Fluorescence Spectra of Acyl Trypsin

(a) **pH Dependency**——The pH dependency of fluorescence spectra of Dns–Gly-trypsin and Dns–D-Ala-trypsin was studied. As shown in Fig. 1, increasing the hydrogen ion concentration caused a red shift of the emission maximum along with a decrease of fluorescence intensity. In the analysis of the spectra of Dns–GlyOMe and Dns–D-AlaOMe, the fluorescence intensities were shown to decrease with increasing hydrogen ion concentration in the same manner as those of acyl trypsins, whereas emission maxima were shown to be constant with change of pH. This suggests that the observed shift of emission maximum reflects the change of microenvironments of the fluorophore in the polymer matrix induced by the change in hydrogen ion concentration. It is known that the Dns group is a sensitive probe responding to solvent polarity. The effects of solvent polarity (ethanol–water) on the emission maxima of model compounds, Dns–GlyOMe and Dns–D-AlaOMe, were determined, and the results are

Table IV. Effect of Solvent on the Fluorescence Spectra of Model Compounds

Solvent	Z	Dns-GlyOMe $\bar{\nu}_f$, Kcm^{-1}	Dns-D-AlaOMe $\bar{\nu}_f$, Kcm^{-1}
Ethanol: water			
100 : 0	79.6	19.3	19.4
90 : 10	82.5	19.0	19.2
80 : 20	84.8	18.9	19.0
60 : 40	87.9	18.6	18.8
40 : 60	90.5	18.3	18.5
20 : 80	92.6	18.0	18.2
0 : 100	94.6	17.7	17.8

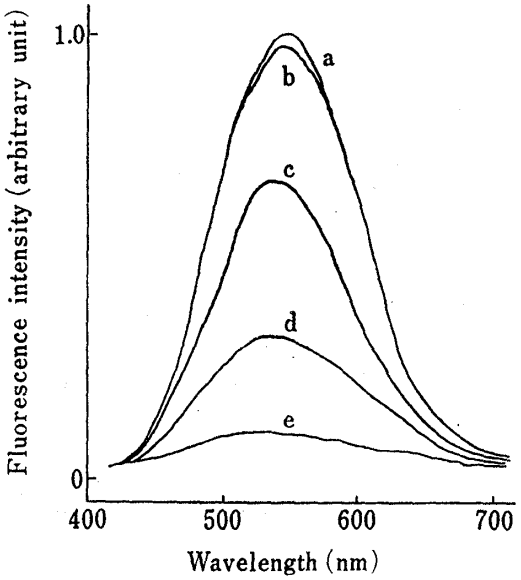


Fig. 1. Fluorescence Spectra of Dns-D-Ala-trypsin

Excitation was carried out at 339 nm. a, pH 6.0; b, pH 5.0; c, pH 4.0; d, pH 3.0; e, pH 2.0.

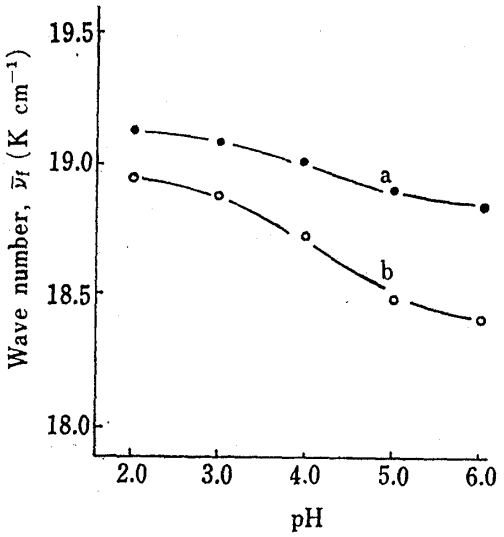


Fig. 2. Effects of pH on the Emission Maxima of Acyl Trypsins

Wave numbers at emission maxima were plotted as a function of pH. Excitation was carried out at 339 nm. a, Dns-Gly-trypsin; b, Dns-D-Ala-trypsin.

shown in Table IV together with Kosower's Z-value.[22] Using the values in Table IV as a measure of solvent polarity, the microenvironments of the acyl group in the trypsin active site at pH 6.0 were estimated to be 85 and 92 (Z-value scale) for Dns–Gly and Dns–D-Ala, respectively. Plots of emission maximum (polarity) as a function of pH which imply pH-induced conformational change of the active site vicinity are shown in Fig. 2.

(b) **Effects of Alkylamines**——The presence of alkylamines or 2-aminopentanes caused a blue shift of the emission spectra of acyl trypsins, as shown in Table V. These observations are ascribed to conformational transition of the trypsin active site induced by interaction with the amine because the fluorescence spectra of model compounds, Dns–GlyOMe and Dns–D-AlaOMe, are not affected by the presence of the amines. These conformational transitions reflected by the blue shifts are interpreted as being a movement of the active site to a less polar region. Changes of the fluorescence spectra were shown to be associated with enhancement of the deacylation rate, since their magnitudes are a linear function of amine concentration observed in these two methods, as reported previously.[8] The magnitude of conformational transition may be reflected in the k_3'/k_3 values listed in Table III. In the interaction of amines with acyl trypsins, it is generally observed that *l*-amines have a large effect relative to the *d*-enantiomers on both spectral shift and k_3'/k_3 values. *d*-Aminopentane, which is ineffective for tryptic catalysis of Ic, did not change the spectrum of Dns–D-Ala-trypsin.

Table V. Effects of 2-Aminopentanes on the Fluorescence Spectra of Acyl Enzyme at pH 5.0, 25°C[a]

Acyl enzyme	Emission maximum (nm)			
	l-Amine		*d*-Amine	
	None	0.2 M	None	0.2 M
Dns-Gly-trypsin	528	516	528	520
Dns-D-Ala-trypsin	540	536	540	540

a) Excitation wavelength: 325 nm.

(c) **Energy Transfer Studies**——Energy transfer can occur between the tryptophan residues and the bound Dns group within the acyl trypsin molecule. The efficiency was analyzed by measuring the emission spectra of acyl trypsin and of model compound excited at 295 nm on the basis of Förster's equation (experimental section). The mean transfer distances calculated from the observed efficiencies in Dns–Gly-trypsin and Dns–D-Ala-trypsin were 15.5 and 15.9 Å, respectively.

Several attempts for the specific introduction of fluorescent probe into serine enzymes have been reported. Site-specific affinity labeling has usually been done with amino acid and peptidyl chloromethanes which alkylate the active site histidine residue.[23] An example of modification of the active site serine residue, similar to ours, is seen in the case of Dns-trypsin.[6] Microenvironment polarity and energy transfer efficiency were analyzed in each case. It is of interest to compare our results with theirs. The Z-values, 85 and 92, in our study were close to that observed in Dns–Lys–CH_2(His-46)-trypsin($z=92$; the pH was not defined)[6] but were considerably larger than that observed in Dns-(Ser-195)-trypsin ($z<76.6$; the pH was not defined).[6] Presumably, the environment of the serine residue to which the Dns-group is directly attached would be more polar than that of the neighboring part where the Dns-group approaches through the spacer residue. Based on the efficiencies of energy transfer from tryptophan residue (s) to the fluorophore, the mean distance between the two groups was calculated to be 17.9 Å for Dns–Lys–CH_2(His-46)-trypsin and 19—19.3 Å for Dns–Ala–Lys–CH_2(His-46)-trypsin.[23] These results are consistent with ours, if the differences in the modifying residues (serine and histidine) and the differences in the structure of reporter groups are taken into account. In our previous report,[8] the distance was calculated to be 21.3 Å from the energy transfer efficiency for acyl trypsin derived from a Dns-aminomethylcyclohexane derivative (Dns–AMCHC-trypsin). In this case, the distance between the fluorophore and the carbonyl group is longer than that of Dns–Gly-trypsin or Dns–D-Ala-trypsin by four methylene linkage in the cyclohexane ring (*ca.* 4 Å). This geometric difference might reflect the distances obtained from the energy transfer efficiency. It might be expected that the acyl group of Dns–AMCHC-trypsin is extended in a direction away from the tryptophan residue(s). Dns–AMCHC and Dns-α-amino acyl residues are thus expected to report the microenvironments of different regions, presumably the outer limb and inner part of the active site hole, respectively. In our previous study the addition of cationic ligand caused a red shift of the emission spectra of Dns–AMCHC-trypsin, whereas the addition of the ligand caused blue shifts of the spectra of Dns–Gly- and Dns–D-Ala-trypsin in the present study. One of possible explanation for these results is as follows; conformational change induced by the cationic ligand would occur in such a way that the inner part of the active site hole is shifted to a less polar environment and its outer region is shifted to a more polar one.

The behavior of acyl trypsin carrying an aromatic ring at the β-carbon of the amino acid residue is a little different. In the preliminary analysis of Dns–D-Phe-trypsin, the presence of 2-aminopentane caused a slight rate decrease without affecting the emission maximum. It is possible to assume that in this case the induced conformation is a distorted one caused by the additional interaction through the bulky α-substituent.

Fluorescent "inverse substrates" provided valuable means for the investigation of structure–function relationships of trypsin. On the basis of their characteristic features, analysis of dynamic properties of trypsin which are related to the catalytic efficiency becomes feasible.

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