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Topographical Analysis of Trypsin Active Site Structure by Means of the "Inverse Substrate" Method

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Trypsin-specific substrate analogs, "inverse substrates," carrying the fluorescent dimethylaminonaphthalene group were synthesized. Preparation of acyl trypsins in which the fluorescent group is attached to the catalytic residue through a spacer group of various chain lengths was successfully carried out by use of these inverse substrates. The topographical structure of the trypsin active site vicinity was estimated on the basis of the fluorescence spectra of these acyl trypsins.

INTRODUCTION

In a previous report (*1*), it was shown that esters of *p*-amidinophenol (**1**; R = CH_3, $C(CH_3)_3$, C_6H_5) are specifically hydrolyzed by trypsin. In the esters the site-specific group for the enzyme, the charged amidinium group, is designed at their leaving portion. Accordingly, the site-specific group is liberated during acylation to produce an acyl enzyme intermediate composed of a nonspecific residue (Fig. 1). Kinetic analysis of these "inverted" esters showed that the binding specificity and the efficiency of the acylation process are comparable to those for normal-type substrates, and eventually a new term, "inverse substrates," was proposed for these esters. The design of such *p*-amidinophenyl esters was extended to those carrying a variety of reporter groups in the acyl part, such as fluorophore, optically active chromophore, and stable free radical (*2–4*). This approach, the inverse substrate method, is now accepted as a general method for preparing acyl enzymes of trypsinlike enzymes (*5*).

In this respect, inverse substrates are expected to be a useful tool for the structural analysis of the vicinity of the trypsin active site. In the present study, synthesis of *p*-amidinophenyl esters in which the dimethylaminonaphthalene (DNS) group is linked to methylene chains of different lengths (**1a–d**) is carried out. The preparation and spectrometric analysis of acyl enzymes are also presented.

RESULTS AND DISCUSSION

Synthesis of Fluorescent Inverse Substrates

The synthesis for **1a–d** is shown in Fig. 2. Aminoalcohols were treated with dimethylaminonaphthalenesulfonyl chloride (DNS-Cl) and the resulting fluorescent

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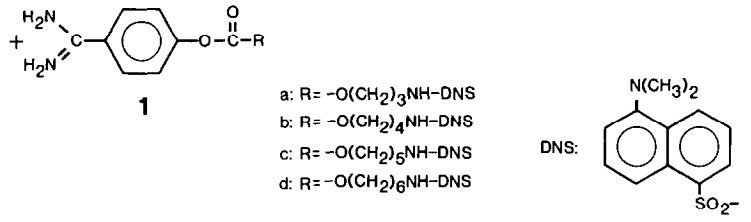


SCHEME 1

aminoalcohol derivatives were reacted with phosgene to give chlorocarbonates. Acylation of the hydroxy group of *p*-amidinophenol is generally difficult because of the diminished nucleophilicity of the hydroxy group. The coupling reaction was tested under various conditions, and finally a substantial amount of the ester was obtained when N-blocked *p*-amidinophenol and a biphasic solvent system were used. After the purification of the product by preparative thin-layer chromatography, the carbobenzyloxy (*Z*) group was deblocked by catalytic hydrogenation to give *p*-amidinophenyl ester (**1a–d**).

Kinetic Parameters for Trypsin-Catalyzed Reactions of ***1a–d***

Trypsin-catalyzed reactions of **1a–d** at pH 8.0, 25°C, were analyzed by a stopped-flow spectrophotometer by monitoring the liberation of *p*-amidinophenol. Dissociation constants of the enzyme–substrate complex, K_s, acylation rate constants, k_2, and deacylation rate constants, k_3, were determined as shown in Table 1. It is shown that carbonate esters (**1a–d**) are also specific substrates for trypsin

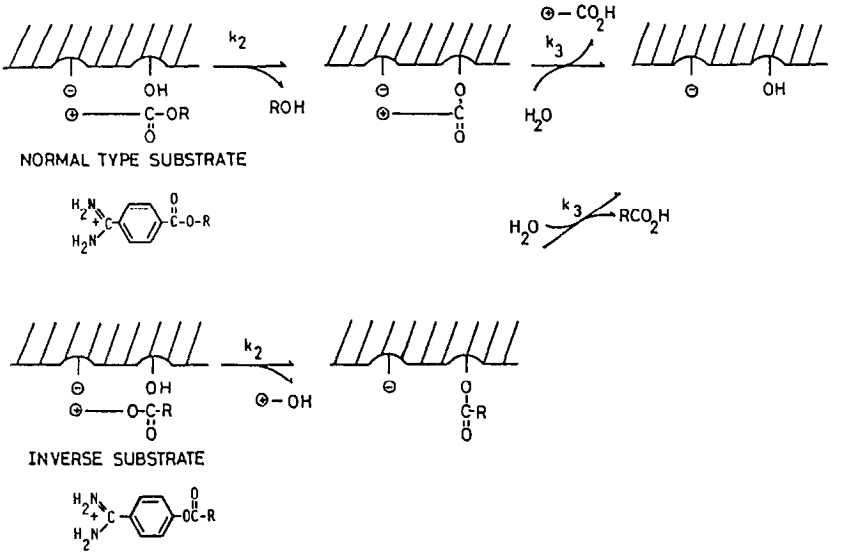


FIG. 1. Schematic representation of the reaction sequences of trypsin with normal and "inverse" substrates. The hydroxy group and the negative charge represent the catalytic residue Ser-195 and the binding residue Asp-189 at the active site of trypsin, respectively.

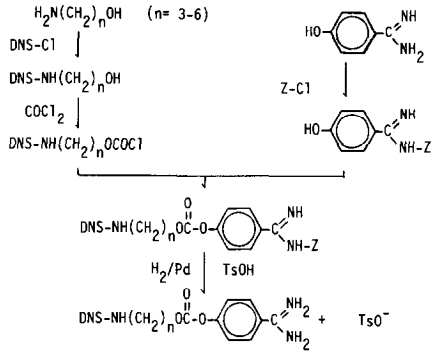


FIG. 2. Synthetic route for **1a–d**.

as well as carboxylic acid ester (**1**; R = CH_3, $C(CH_3)_3$, C_6H_5) (*1*). The K_s values for all of the carbonate esters, on the order of 10^{-6} M, exhibit strong binding affinity. These compounds are most suitable for the specific production of an acyl enzyme intermediate because their k_2/k_3 ratios are sufficiently large and they have small K_s values.

Preparation of Acyl Trypsins

Acyl trypsin was successfully prepared using the following general procedure: Trypsin was incubated with a 15–20 molar excess of ester for 2 min at pH 8.0, 25°C. After the pH was adjusted to 2.0 by addition of 1 M HCl, the reaction mixture was gel-filtered and lyophilized. The preparation was completely inactive as a result of acylation at the active site. The enzymatic activity was recovered after incubation for 2.5 h at pH 8.0 as a result of deacylation. These observations are summarized in Table 2 together with the stoichiometry of acylation determined spectrometrically.

Structural Analysis of Trypsin Active Center Vicinity by Means of Fluorescent Reporter Groups

Polarity of the microenvironment. Fluorescence spectra of native trypsin and an acyl trypsin derivative from **1c** are shown in Fig. 3. Excitation and emission

TABLE 1

KINETIC PARAMETERS FOR TRYPSIN-CATALYZED HYDROLYSIS OF **1a–d** AT PH 8.0 AND 25°C

Substrate	K_s (M)	k_2 (s^{-1})	k_3 (s^{-1})	k_2/k_3
1a	$1.86 \pm 0.18 \times 10^{-6}$	$8.36 \pm 0.11 \times 10^{-1}$	$2.50 \pm 0.18 \times 10^{-3}$	334
1b	$2.77 \pm 0.18 \times 10^{-6}$	1.44 ± 0.02	$4.18 \pm 0.09 \times 10^{-3}$	344
1c	$9.90 \pm 0.82 \times 10^{-7}$	$9.25 \pm 0.90 \times 10^{-3}$	$4.29 \pm 0.46 \times 10^{-3}$	216
1d	$2.48 \pm 0.13 \times 10^{-6}$	1.02 ± 0.02	$3.70 \pm 0.25 \times 10^{-3}$	519

TABLE 2

CHARACTERISTICS OF ACYL TRYPSINS

Acyl trypsin derived from	Remaining activity[a] (%)	Activity recovered after deacylation[a,b] (%)	Acyl group introduced (mol/mol enzyme)
1a	1>	82	0.83
1b	1>	77	0.82
1c	1>	84	0.89
1d	1>	99	1.04

[a] Catalytic activity was analyzed using N^{α}-carbobezyloxy-L-lysine-*p*-nitrophenyl ester as a substrate at pH 3.0.

[b] Deacylation was carried out at pH 8.0 for 2.5 h.

maxima of the acyl trypsins in which the DNS group is attached to the catalytic residue through spacer groups of different chain lengths were measured. At pH 5.0, emission maxima for **1a–c** were not very different from each other (554–557 nm), but the maximum for **1d** was observed at a shorter wavelength (546 nm). It is reasonable to assume that the difference in the emission maxima arose as a result of the different microenvironments in which the fluorophore resides, since fluorescence of the DNS group is very sensitive to the polarity of the medium used (*6*). In this respect the dependence of the emission maxima of the model compounds on the solvent polarity was studied. Emission maxima of the ethyl carbonates **2a,b** in aqueous ethanol were determined as shown in Table 3. *Z*-values, an empirical parameter of solvent polarity (*7*), are also listed. As shown in the table emission maxima shifted progressively to the shorter wavelengths (higher wavenumber) with increasing solvent polarity. In this respect the influence of chain length was negligible. Consequently, polarities of the different microenvironments in which the DNS group resides in the acyl trypsins are estimated on the polarity scale shown in Table 3 as $Z = 90$ for $n = 3, 4, 5$, and $Z = 85$ for $n = 6$,

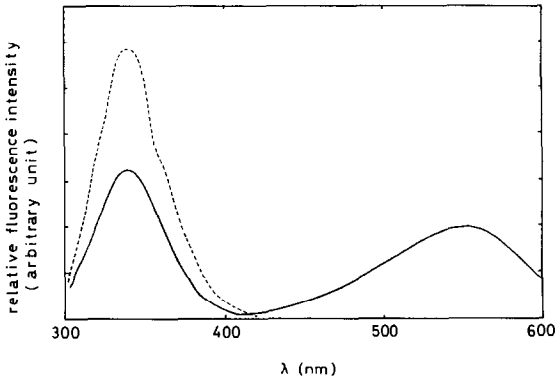
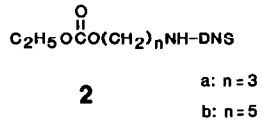


FIG. 3. Emission spectra of native trypsin (---) and trypsin modified with **1c** (——) excited at 295 nm.



SCHEME 2

respectively. The assumed polarity of the microenvironment at the region rather close to the active site reported by **1a–c** (n = 3–5) corresponds to that of 40% ethanol whereas the microenvironment at the region reported by **1d** (n = 6) is less polar (80% ethanol). Investigation of the microenvironment of the trypsin active site was carried out previously. Vaz and Shoellman (*8*) reported Z = 76.1 for modified trypsin in which the active site Ser-195 was directly sulfonylated by DNS–Cl and Z = 92.5 for the modification of His-57 with the N^{α}-DNS–Lys analog. It is noticeable that the modification of His-57 with the N^{α}-DNS–Lys analog nearly corresponds to the modification of Ser-195 with **1d** or **1e** from the topological perspective, deduced by the three-dimensional enzyme model based on X-ray diffraction data (*9*); Z-values obtained from the two experiments do not differ greatly. Many studies using fluorescent probes involve the noncovalent adsorption of the fluorophore to protein (*10*). Characterization of the dye interaction is fundamentally important in these cases since the results may include ambiguity of the stoichiometry and the site of dye binding to protein. Although site-directed covalent probes are more definitive in this respect, ambiguity due to the probe itself must be taken into account for the determination of the Z-value. As noted by Turner and Brand (*6*) the measure of the polarity is not always independent of the probe used. In some cases, the fluorescent character might be altered through an interaction with certain functional groups on the protein molecule, and this interaction is intrinsic to the chemical structure of the probe. In this study mapping of the active site with the Z-value scale is carried out using probes, **1a–d**, designed for a common fluorophore, DNS. Nevertheless it is also possible to assume that the different polarities observed with probes of different chain lengths

TABLE 3

SOLVENT EFFECT ON FLUORESCENCE OF **2**

		2a		**2b**	
Medium EtOH : H_2O	Z-value	λ_{max} (nm)	ν_{max} (k cm^{-1})	λ_{max} (nm)	ν_{max} (k cm^{-1})
100 : 0	79.6	527	19.0	525	19.0
90 : 10	82.5	535	18.7	536	18.7
80 : 20	84.8	545	18.3	544	18.4
60 : 40	87.9	549	18.2	549	18.2
40 : 60	90.5	558	17.9	554	18.1
20 : 80	92.6	564	17.7	563	17.8
0 : 100	94.6	572	17.5	574	17.4

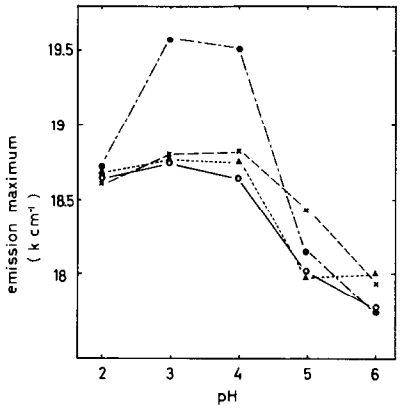


FIG. 4. Effect of pH on emission maxima of acyl trypsins. Acyl trypsins are from **1a** (○), **1b** (●), **1c** (▲), and **1d** (×).

are due to the different orientations of the acyl residue with respect to the dye–functional group interaction.

pH-Induced Conformational Changes

The effect of pH on the emission maxima was investigated for each of the acyl trypsins, as shown in Fig. 4. In each case the curve changes between pH 4 and 5, whereas emission maxima of the model compounds, ethylcarbonates, were pH-independent. This phenomenon could be explained by the conformational change of the enzyme which arises because of the change in polarity of the active site vicinity as reflected by the reporter group. On the basis of this assumption, a conformational change accompanying a polarity change is exhibited more intensely at the region reported by **1b** than those reported by **1a**, **1c**, and **1d**. A similar conformational transition of the enzyme at pH 3–5 was analyzed by absorption spectra (*11*) and circular dichroism spectra previously (*12, 13*). Therefore the pH-induced conformational transition which has not been characterized was defined as that involved in the active site vicinity by our study. These results are in accord with our previous observations with spin-labeled acyl trypsins (*4*).

Energy Transfer between Tryptophan and DNS Residues

As shown in Fig. 3 when excited at 295 nm fluorescence spectra of trypsin exhibit emission maxima at 330 nm due to intrinsic tryptophan residues. Because excitation maxima of the extrinsic DNS group of acyl trypsins is observed at 330 nm, excitation of tryptophan (295 nm) will eventually cause the emission of DNS group (550 nm). This energy transfer was analyzed, from Fig. 3, as follows: The fluorescence intensity of tryptophan residues at 330 nm is lower for acyl trypsin (F) than for native trypsin (F_0). The extent of the decrease is related to the energy transfer efficiency (T) given by Eq. [1] (*14*):

$$T = 1 - F/F_0. \qquad [1]$$

TABLE 4

ENERGY TRANSFER EFFICIENCY FROM TRYPTOPHAN RESIDUES TO EXTRINSIC DNS GROUP

Acyl trypsin derived from	T (%)
1a	36
1b	38
1c	39
1d	48

Values of energy transfer were determined for **1a–d** and are listed in Table 4. The efficiency of energy transfer is sensitive to the geometry of the donor and acceptor. A value for the case of **1d** remarkably larger than the values for **1a–c**, suggests that the DNS group linked with six methylene units can reach a position closer to the tryptophan residues. The value of 48% for **1a** is identical to a reported value (*8*) analyzed with a DNP–lysine analog for which polarity $Z = 92.5$ was determined as mentioned. As is known, the energy transfer efficiency depends not only on the distance but also on the orientations of the donor and the acceptor (*15*). Investigations in this field at present are based on the assumption that the array of donors and acceptors is the average of random orientations. Likewise, the analysis in this study is based on the approximation that the orientation factors are similar in **1a–d**.

EXPERIMENTAL PROCEDURES

Materials. Bovine trypsin obtained from Worthington Biochemical Corp. (twice-crystallized, salt-free, Code TRL) was purified through ST-Sepharose following the procedure in (*16*). The titrated normality of the enzyme preparation was determined to be 92% (*17*). N^{α}-Carbobenzyloxy-L-lysine *p*-nitrophenyl ester hydrochloride was purchased from Aldrich Chemical Co. All other chemicals for synthetic work were obtained from Tokyo Kasei Industry, Tokyo. Buffers and solvents were of the best grade available from commercial sources.

General procedure for the synthesis of ***1a–e.*** DNS–Cl (*18*) (10 mmol) in 150 ml acetone was added to a solution of aminoalcohol (10 mmol) in 2% sodium bicarbonate solution (100 ml) and kept at room temperature for 12 h. Acetone was removed *in vacuo*, extracted with ethyl acetate, and dried over magnesium sulfate, and the solvent was evaporated. The resulting DNS–aminoalcohol was recrystallized from benzene. A 10% solution of phosgene in 2 ml tetrahydrofurane (THF) was added to a solution of DNS–aminoalcohol (1 mmol) dissolved in THF (5 ml) at −20°C and kept for 12 h at room temperature. Evaporation of the solvent gave an amorphous solid of chloroformate. A solution of chloroformate (2 mmol)

in methylenechloride (5 ml) was added to a solution of *N*-carbobenzyloxy-*p*-amidinophenol (*19*) (2 mmol) in saturated sodium bicarbonate solution (7 ml) at 0°C with vigorous stirring. After standing for 12 h at room temperature the methylenechloride layer was dried over magnesium sulfate and evaporated. The resulting residue was purified by preparative thin-layer chromatography (silica gel; benzene : ethyl acetate, 2 : 1); the yield was 27–40%.

N-Carbobenzyloxy-*p*-amidinophenyl ester (2 mmol) and *p*-toluenesulfonic acid (4.2 mmol) were dissolved in methanol (50 ml) and hydrogenated in the presence of 10% Pd/C (370 mg). After removal of the catalyst by filtration, the solvent was evaporated. The residue was solidified by the addition of ether and recrystallized from the appropriate solvent.

1a · CH_3OH · $2C_7H_7SO_3H$: recrystallized from methanol : ether, mp 150–151.5°C (dec.). *Anal.* Calcd: C, 53.87; H, 5.47; N, 6.61, S, 11.36. Found: C, 54.10; H, 5.18; N, 6.82; S, 11.50.

1b · $C_7H_7SO_3H$: recrystallized from methanol : ether, mp 148–149°C (dec.). *Anal.* Calcd: C, 56.69; H, 5.53; N, 8.53, S, 11.50. Found: C, 56.65; H, 5.48; N, 8.34; S, 9.62.

1c · $C_7H_7SO_3H$: recrystallized from methanol : ether, mp 179–180°C (dec.). *Anal.* Calcd: C, 57.30; H, 5.71; N, 8.35; S, 9.56. Found: C, 57.07; H, 5.91; N, 8.40; S, 9.58.

1d · $2C_7H_7SO_3H$: recrystallized from ethanol, mp 182–183°C (dec.). *Anal.* Calcd: C, 56.06, H, 5.65; N, 6.54; S, 11.22. Found: C, 55.86; H, 5.72; N, 6.57; S, 11.32.

Synthesis of model compounds, **2.** A solution of ethylchloroformate (4.6 mmol) in THF (3 ml) at −5°C was added to a solution of DNS–aminoalcohol (4 mmol) and triethylamine (5 mmol) in THF (5 ml) and stirred at 0°C for 1 h and subsequently for 12 h at room temperature. After removal of precipitated triethylamine hydrochloride, the solvent was evaporated. The resulting residue was purified by preparative thin-layer chromatography (silica gel; benzene : ethyl acetate, 2 : 1) gave oil.

Determination of kinetic parameters. Kinetic parameters for trypsin-catalyzed hydrolysis of **1a–e** were determined using a Union Giken Corp. RA-401 stopped-flow spectrophotometer following the procedure reported in (*1*).

Preparation of acyl enzymes. Preparation of acyl enzymes was carried out following the procedure previously reported (*1*).

Determination of the remaining acyl trypsin activity. The assay was carried out using N^{α}-carbobenzyloxy-L-lysine *p*-nitrophenyl ester as substrate in 0.1 M citrate at pH 3.0 following the procedure in (*20*).

Spectrometric determination of number of acyl residues per mole of enzyme. The following experimentally determined values were used: DNS residue, $\varepsilon_{329\,nm}$ 4730, $\varepsilon_{280\,nm}$ 1680; trypsin, $\varepsilon_{280\,nm}$ 36,700.

Measurement of fluorescence spectra. Corrected fluorescence spectra were recorded on a Hitachi 650-60 spectrofluorometer using rhodamine B as standard. All the measurements were carried out at 25°C at an enzyme concentration of 0.2 mg/ml. The following buffers were used for the pH dependence experiments: 0.05 M AcONa–HCl (pH 2–3), 0.05 M citric acid–Na_2HPO_4 (pH 3–6).

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