New Thioester Substrates for Fibrinoligase (Coagulation Factor $XIII_a$) and for Transglutaminase. Transfer of the Fluorescently Labeled Acyl Group to Amines and Alcohols†

K. N. Parameswaran and L. Lorand*

ABSTRACT: Four new fluorescent thiocholine esters were synthesized, with a dansyl group attached to a different acyl moiety in each molecule. [Dansyl-(*N*-methyl-β-alanyl)]thiocholine and [3-(4-(dansylamido)phenyl)propionyl]thiocholine as well as [ε-[(dansylsarcosyl)amino]caproyl]thiocholine proved to be good substrates for human fibrinoligase (i.e., thrombin- and Ca^{2+}-activated blood coagulation factor XIII or factor $XIII_a$) and also for transglutaminase. However, (dansylsarcosyl)thiocholine, probably on account of branching so close to its reactive carbonyl group, showed no measurable turnover with these enzymes. [Dansyl-(*N*-methyl-β-alanyl)]-thiocholine was selected for detailed analysis with human fibrinoligase, using a previously described fluorescent kinetic system [Lorand, L., Gray, A., Brown, K., Credo, R. B., Curtis, C. G., Domanik, R. A., & Stenberg, P. (1974) *Biochem. Biophys. Res. Commun. 56*, 914; Stenberg, P., Curtis, C. G., Wing, D., Tony, Y. S., Credo, R. B., Gray, A., & Lorand, L. (1975) *Biochem. J. 147*, 155]. Steady-state constants were obtained for the enzyme-catalyzed transfer of the acyl group from the ester to amines and alcohols. The k_{cat} values were similar for both types of nucleophiles, indicating that the reactions are acylation rate limiting. There was a large difference, however, in the apparent K_M's for amines and alcohols (i.e., at pH 7.5, K_M for methanol:K_M for methylamine ≈ 30). The kinetic system was useful for studying the specificity of amines, and the measured order of affinity, (mesitylenesulfonyl)cadaverine > *t*-Boc-cadaverine > *n*-butylamine > methylamine, was in accord with our earlier results using other procedures. The best substrate contains an apolar substituent attached to an alkylamine residue which resembles the lysine side chain in proteins. The reaction between thioester and methanol made it possible for the first time to obtain a meaningful pH–activity profile for the enzyme-catalyzed acyl transfer. The measured pK_a of 6.7 suggests the participation of the imidazole group of a histidine residue in the active site of the enzyme.

(β-Phenylpropionyl)thiocholine[1] is representative of a group of thioesters which display good substrate specificities toward fibrinoligase (thrombin- and Ca^{2+}-activated fibrin stabilizing factor, i.e., factor $XIII_a$) and other endo-γ-glutamine:ε-lysine transferases, such as transglutaminase, in general (Lorand et al., 1972a; Curtis et al., 1974a; Stenberg et al., 1975). The β-phenylpropionyl moiety apparently takes on the role of susceptible glutamine residues present in endo positions in the protein substrates of these enzymes. Actually, the use of thiocholine esters made it possible for the first time to measure the relevant steady-state kinetic constants for human fibrinoligase in an entirely synthetic substrate system and led to the conclusion that acyl group transfer from ester to amine substrates proceeded through an acyl-enzyme intermediate on the acylation–deacylation pathway.

From the point of view of kinetic analysis, the reaction between (β-phenylpropionyl)thiocholine and dansylcadaverine (Lorand et al., 1973; Stenberg et al., 1975) bears directly on the work to be described in the present paper. The ester and amine substrates were allowed to react with enzyme and Ca^{2+} in an aqueous medium (pH 7.5) under constant stirring in a cuvette in the spectrophotofluorometer, while the water-insoluble amide product was partitioned into an organic phase of heptane, where it could be measured by virtue of the dansyl group fluorescence: $C_6H_5(CH_2)_2COS(CH_2)_2N(CH_3)_3^+ + H_2N(CH_2)_5NH\text{-Dns} \rightarrow C_6H_5(CH_2)_2CONH(CH_2)_5NH\text{-Dns}$ (in heptane phase) + $HS(CH_2)_2N(CH_3)_3^+$.

By synthesis of fluorescent ester substrates, in which the dansyl group was incorporated into the acyl portions of the molecules, the above experimental approach could be broadened considerably for examining transfer reactions with a variety of nucleophiles. Of several compounds synthesized, [dansyl(*N*-methyl-β-alanyl)]thiocholine proved to be the most suitable substrate, and steady-state constants could be obtained for the human fibrinoligase catalyzed reactions of the ester with amines as well as with alcohols. Moreover, this system offered a novel means for evaluating the amine specificity of the enzyme.

Materials and Methods

Guinea pig liver transglutaminase was prepared by Dr. Y. S. Tong of this laboratory essentially by the procedure of Connellan et al. (1971) and was stored frozen in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA. This endo-γ-glutamine:ε-lysine transferase was employed for the screening of the newly synthesized fluorescent thiocholine ester substrates, using the thin-layer chromatographic analytical procedure given below.

Reaction mixtures of 0.1 mL contained 50 mM Tris-HCl buffer, pH 7.5, 1.0–3.0 mM fluorescent thiocholine ester, 0.2 mM dansylcadaverine, 0.4 μM guinea pig liver transglutaminase, and, finally, 10 mM calcium chloride. Depending

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[1] Abbreviations used: dansyl (Dns), 5-(dimethylamino)naphthalene-1-sulfonyl; *t*-Boc, *tert*-butyloxycarbonyl; mesitylene, 2,4,6-trimethylphenyl; cadaverine, 1,5-diaminopentane; thiocholine, (trimethylammonio)ethanethiol; factor XIII, the zymogen from blood plasma, precursor of the fibrin cross-linking enzyme (fibrinoligase or factor $XIII_a$); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonate).

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on the solubility of the thioesters, the reaction mixtures also contained up to 10% of acetonitrile. The mixtures were incubated at 37 °C, and 5-μL aliquots were withdrawn at intervals of 0, 10, 20, 40, and 60 min and spotted on silica gel sheets (Merck precoated silica gel sheets, 60 F-254, 0.25-mm layer thickness) 1.5 cm apart from each other. Chromatography was performed in chloroform/2-propanol/glacial acetic acid (17:1:1 v/v/v) until the solvent front travelled to a distance of 10 cm. After hot air drying, the chromatograms were viewed under a UV hand lamp (Minerallight UVL-22) for locating the greenish fluorescent spots. Synthetic reference markers for the fluorescent thioesters, for the amine substrate, for the fluorescent acid byproducts resulting from the solvolysis of the thioesters, and for the doubly fluorescent amide products were spotted and chromatographed on the same chromatography sheet for each reaction studied.

The fibrin-stabilizing factor zymogen (factor XIII) was prepared by Dr. R. B. Credo and T. Janus of this laboratory from citrated, outdated blood-bank human plasma by published procedures (Curtis & Lorand, 1976). The purified zymogen was stored at 4 °C in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA. Protein molarity was calculated on the basis of $E^{1\%}_{1cm}$ = 13.8 at 280 nm and by assuming a molecular weight of 326 000 for an a_2b_2 tetrameric unit (Schwartz et al., 1973). The functional purity of factor XIII was measured as 80–90% by titration with iodoacetamide, as described by Curtis et al. (1974b). Human α-thrombin (2162 NIH units/mg) was a gift of J. W. Fenton III, Division of Laboratories and Research, New York State Department of Health, Albany, NY.

Hydrolytic activation of the factor XIII zymogen was carried out on the day of the experiment by incubation for 30 min (25 °C) with thrombin (3.75 NIH units/mg of factor XIII) in 0.2–0.3-mL solutions of the buffers designated for each experiment, and the activated zymogen was kept at 4 °C.

Stock solutions of [dansyl-(*N*-methyl-β-alanyl)]thiocholine iodide (i.e., compound IV) were prepared freshly each day in 5% aqueous *N*,*N*-dimethylformamide and the concentration of this acyl substrate solution was deduced from measuring the characteristic absorbancy of the dansyl chromophore (ϵ_{327nm} = 4.67 × 10^3 L mol^{-1} cm^{-1} in 20% aqueous dioxane; Deranleau & Neurath, 1966).

Kinetic measurements were carried out in an Aminco-Bowman ratio spectrophotofluorometer equipped with a thermostatically controlled (27 °C) cell holder and a magnetic stirring device. Formations of fluorescent amide or ester products, as catalyzed by the thrombin- and Ca^{2+}-activated factor XIII, were followed through continuous extraction of the 0.1-mL aqueous reaction phase into 2.0 mL of heptane (spectral grade, Fisher H-340) and by measurement of the fluorescence (excitation 340 nm; emission 460 nm) in the upper organic phase (Lorand et al., 1974; Stenberg et al., 1975). The measured increments in relative fluorescence intensities were converted to actual micromolar concentrations of the dansylated product by referring to a standard curve which was obtained with an authentic sample of dansyl-*N*-methylamide in heptane solution under the same settings of the spectrofluorometer as in the experiments. Linearity of fluorescence intensity values for this standard was observed in the concentration range 0–2.5 μM.

The lower 0.1-mL aqueous phase, in addition to the buffers specified, contained thrombin-activated factor XIII (functional concentration, E_0, as indicated in Table II), varying concentrations of [dansyl-(*N*-methyl-β-alanyl)]thiocholine iodide (0.35–2.0 mM), and a fixed concentration of one of the following as second substrate. For studying aminolysis, methylamine (5–20 mM), *n*-butylamine (2.5–10 mM), *t*-Boc-cadaverine (0.25–2.5 mM), or (mesitylenesulfonyl)cadaverine (0.025–0.25 mM) was selected. For alcoholysis, methanol (0.1–1.24 M), ethanol (0.15–1.22 mM), or 1-butanol (0.22–0.55 M) was chosen. Reactions were initiated by the addition of calcium chloride to the aqueous phase at a final concentration of 30 mM.

The various amines and alcohols used were the purest available quality (Aldrich and Mallinckrodt chemicals). (Mesitylenesulfonyl)cadaverine was prepared as previously described (Lorand et al., 1979a). Dansyl chloride was purchased from Pierce; *N*-methyl-β-alaninenitrile and other reagents were from Aldrich.

The following solvent systems were employed for thin-layer chromatographic (TLC) analysis: (A) chloroform/2-propanol/glacial acetic acid (17:1:1), (B) absolute ethanol/anhydrous ether/concentrated ammonium hydroxide (14:4:2), and (C) 1-butanol/glacial acetic acid/water (4:1:1 v/v/v).

Results

(1) Synthesis of New Substrates (I–V) and of Reference Compounds (VI–IX). *[3-(4-(Dansylamido)phenyl)-propionyl]thiocholine Iodide (I).* The catalytic reduction of *p*-nitrocinnamic acid gave 3-(4-aminophenyl)propionic acid which was dansylated to give 3-(4-(dansylamido)phenyl)-propionic acid. The latter was then coupled to 2-(dimethylamino)ethanethiol by using the mixed anhydride procedure to give the title thioester as follows.

A mixture of 2.9 g (15 mmol) of *p*-nitrocinnnamic acid in 80 mL of absolute ethanol, 120 mL of dry tetrahydrofuran, and 0.9 g of 10% Pd/C catalyst was hydrogenated at 50 psi of H_2 in a Parr shaker for 6.5 h. The catalyst was filtered off through Celite and washed with anhydrous methanol, and the filtrate was concentrated to a small volume (3 mL) under reduced pressure. Anhydrous ether (100 mL) and petroleum ether (50 mL) were added to the above concentrate and cooled (4 °C) for several hours. The precipitated product was filtered off and recrystallized from a mixture of 20 mL of absolute ethanol, 20 mL of benzene, and 60 mL of petroleum ether to give 1.12 g (45%) of a tan solid: mp 129–131 °C [lit. mp 130–131 °C (Skinner et al., 1959); mp 128–131 °C (Cummings, 1958)]; one UV and ninhydrin-positive spot in TLC with an R_f of 0.33 in solvent system A, as compared to an R_f 0.67 (UV positive, ninhydrin negative) for the *p*-nitrocinnamic acid starting material.

To a stirred and cooled (4 °C) solution of 400 mg (2.4 mmol) of the above 3-(4-aminophenyl)propionic acid in 15 mL of water and 10 mL of tetrahydrofuran were added 0.7 mL (5.0 mmol) of triethylamine and then a solution of 540 mg (2.0 mmol) of dansyl chloride in 25 mL of dry tetrahydrofuran in 30 min. After 2 h, another 0.2-mL (1.4 mmol) portion of triethylamine was added to adjust the pH of the reaction mixture to 10.0. The reaction mixture was protected from light, stirred at room temperature for 24 h, and evaporated under reduced pressure. The residue was mixed with 50 mL of cold water and 20 mL of saturated sodium bicarbonate solution, and the mixture was extracted once with 50 mL of anhydrous ether. The aqueous layer was acidified gradually with 6 N hydrochloric acid to pH 2.0, and the mixture was extracted with dichloromethane (4 × 40 mL). The organic extract was washed with water (1 × 70 mL) and saturated sodium chloride (2 × 70 mL) and dried over anhydrous sodium sulfate. The filtered extract was then concentrated to a volume of 1.0 mL. To this were added 5 mL of anhydrous ether and 35 mL of petroleum ether and cooled to give 600 mg (75%)

of cream-colored crystals: mp 148–150 °C; one greenish fluorescent spot in TLC with an R_f of 0.63 in system A and 0.77 in C.

To a stirred and cooled (0 °C) solution of 200 mg (0.5 mmol) of the above 3-(4-(dansylamido)phenyl)propionic acid in 10 mL of dry tetrahydrofuran was added 0.07 mL (0.5 mmol) of triethylamine followed by 0.066 mL (0.5 mmol) of isobutyl chloroformate. After 25 min, a preneutralized solution of 75 mg (0.53 mmol) of 2-(dimethylamino)ethanethiol hydrochloride, prepared in 1 mL of absolute ethanol and 0.07 mL (0.5 mmol) of triethylamine followed by dilution with 6 mL of dry tetrahydrofuran, was added to the above mixed anhydride. The reaction mixture was stirred for a further 30 min in an ice bath (4 °C) and then at room temperature for 7.5 h. After addition of 30 mL of anhydrous ether to the mixture, the precipitated triethylamine hydrochloride was filtered off, and to the filtrate was added 0.16 mL (2.56 mmol) of methyl iodide. The reaction mixture was stirred for 65 h in a stoppered flask. Upon addition of 50 mL of anhydrous ether and cooling, the precipitated product was filtered off and was twice recrystallized from absolute ethanol/methanol/anhydrous ether and finally from 2-propanol/acetone/anhydrous ether to give 205 mg (65%) of light yellow crystals, showing one greenish fluorescent spot in TLC with an R_f of 0.1 in system A and 0.2 in C.

(Dansylsarcosyl)thiocholine Iodide (II). Sarcosine (2.4 mmol) was allowed to react with 2.0 mmol of dansyl chloride in the presence of 5 mmol of triethylamine in aqueous tetrahydrofuran essentially as described above to give 1.55 mmol (78%) of dansylsarcosine as an amorphous yellow solid: one spot in TLC with an R_f of 0.62 in system A and 0.14 in B; the dicyclohexylammonium salt derivative melted at 161–163 °C.

A mixed anhydride coupling of the above dansylsarcosine (0.485 g; 1.5 mmol), with 2-(dimethylamino)ethanethiol using equimolar quantities of reagents was performed essentially as above followed by the reaction with methyl iodide (0.4 mL; 6.4 mmol) for 72 h at room temperature. The precipitated product was filtered off, washed with ether, and recrystallized from anhydrous methanol/anhydrous ether to give 498 mg (0.91 mmol; 73% overall yield) of the product, showing one greenish fluorescent spot in TLC with an R_f of 0.12 in system A and 0.17 in C.

[ε-[(Dansylsarcosyl)amino]caproyl]thiocholine Iodide (III). Methyl ε-aminocaproate was acylated with (dansylsarcosyl)-thiocholine iodide (II) under anhydrous conditions, and the resulting methyl ε-[(dansylsarcosyl)amino]caproate was saponified under mild conditions to give ε-[(dansylsarcosyl)-amino]caproic acid, which was then coupled to 2-(dimethylamino)ethanethiol. Synthetic steps were carried out as follows.

To a solution of 91 mg (0.5 mmol) of methyl ε-aminocaproate hydrochloride (mp 124–125 °C; moving in TLC with an R_f of 0.1 in system A and 0.62 in B) in 15 mL of anhydrous chloroform and 0.07 mL (0.63 mmol) of *N*-methylmorpholine was added 110 mg (0.2 mmol) of (dansylsarcosyl)thiocholine iodide (II). The reaction mixture was stirred at room temperature for 40 h. When a thin-layer chromatographic examination of the reaction mixture showed unreacted components, 0.07 mL (0.5 mmol) of triethylamine and 3.0 mL of dry dimethylformamide were added, and the mixture was refluxed for 5 h. The reaction mixture was evaporated to dryness under vacuum. To the residue were added 25 mL of chloroform, 20 mL of water, and 10 mL of saturated sodium chloride. The aqueous layer was acidified to pH 2.0 with 1 N hydrochloric acid, and the mixture was extracted with chloroform (3 × 25 mL). The chloroform extract was washed with 0.2 N hydrochloric acid (1 × 50 mL) and saturated sodium chloride (2 × 50 mL) and dried over anhydrous sodium sulfate. The filtered extract was evaporated under reduced pressure to give an oily residue which showed one greenish fluorescent spot in TLC with an R_f of 0.81 in system A and 0.91 in B. This oily product, methyl ε-[(dansylsarcosyl)-amino]caproate, was dissolved in a mixture of 3.0 mL of 95% ethanol and 4 mL of 1 N sodium hydroxide and stirred at room temperature for 1 h. The mixture was evaporated to remove ethanol, diluted with 25 mL of water, and then extracted with ether (1 × 20 mL). The basic aqueous layer was acidified with 3 N hydrochloric acid to pH 2.0 and extracted with chloroform (4 × 50 mL), and the chloroform extract was subjected to a similar workup as above. The chloroform extract was evaporated, and the residue was mixed with 20 mL of petroleum ether and cooled. The oily product was separated from the supernatant by decantation and dried under vacuum to give 80 mg (92%), giving in TLC one greenish fluorescent spot with an R_f of 0.63 in system A and 0.13 in B.

The above ε-[(dansylsarcosyl)amino]caproic acid (80 mg; 0.184 mmol) was coupled with 2-(dimethylamino)ethanethiol hydrochloride (0.2 mmol neutralized with an equivalent amount of triethylamine) by the mixed anhydride procedure above, followed by reaction with methyl iodide (0.07 mL, 1.12 mmol). Following a similar workup, an oily product was obtained from anhydrous methanol, absolute ethanol, and anhydrous ether. This was repeatedly recrystallized from acetone–anhydrous ether and dried under vacuum to give 46 mg (38%) of a light yellow solid which showed in TLC one greenish fluorescent spot with an R_f of 0.1 in system A.

[Dansyl-(N-methyl-β-alanyl)]thiocholine Iodide (IV). The basic hydrolysis of *N*-methyl-β-alaninenitrile to *N*-methyl-β-alanine, followed by dansylation of the latter to give dansyl-*N*-methyl-β-alanine, was carried out as follows.

To a hot (90 °C) solution of 32 g (0.1 mol) of barium hydroxide in 100 mL of water was added 10 mL of *N*-methyl-β-alaninenitrile (0.108 mol) in 15 min. The reaction mixture was refluxed on a steam bath for 3 h and evaporated at 60 °C under reduced pressure to remove ammonia resulting from the saponification of the nitrile. The alkaline reaction mixture (pH 12) was filtered to remove a small amount of white precipitate ($BaCO_3$), and to the filtrate was added a solution of 7 mL (50 mmol) of *tert*-butyloxycarbonyl azide in 50 mL of peroxide-free dioxane in 1 h. The reaction mixture was stirred for a further 16 h at 30 °C and filtered. The alkaline filtrate was extracted with ether (2 × 100 mL) and dichloromethane (1 × 100 mL), and the aqueous alkaline layer was gradually acidified under ice cooling with cold 3 N hydrochloric acid to pH 2.0. The mixture was quickly extracted with dichloromethane (3 × 100 mL), and the organic extract was washed with saturated sodium chloride and dried over anhydrous sodium sulfate. The extract was evaporated under reduced pressure to give an oily residue. A portion of this residue was allowed to crystallize from a mixture of 10 mL of anhydrous ether and 50 mL of petroleum ether (bp 35–60 °C) in a freezer (−20 °C) to give white crystals of *t*-Boc-*N*-methyl-β-alanine: mp 62–64 °C; one spot in TLC with R_f of 0.58 in system A and 0.19 in B (ninhydrin positive upon prolonged heating at 100 °C, chlorine/tolidine positive). Anal. Calcd for $C_9H_{17}NO_4$: C, 53.18; H, 8.43; N, 6.89. Found: C, 53.00; H, 8.38; N, 6.68. The above *t*-Boc derivative was deblocked with 10 mL of a 1:1 mixture of 2-propanol/concentrated hydrochloric acid for 4 h at room temperature; the reaction mixture was diluted with anhydrous ether (100 mL),

cooled, and filtered to give white crystals of *N*-methyl-β-alanine hydrochloride: yield, 3.74 g (25%); mp 97–99 °C; one ninhydrin-positive spot in TLC with an R_f of 0.06 in system A and 0.05 in B. Anal. Calcd for $C_4H_{10}ClNO_2$: C, 34.42; H, 7.22; N, 10.04. Found: C, 34.24; H, 7.17; N, 9.90.

Dansyl-*N*-methyl-β-alanine was prepared from 210 mg (1.5 mmol) of *N*-methyl-β-alanine hydrochloride by a procedure described above under compound I, using sodium carbonate (500 mg; 4.7 mmol) as the base. Dansyl chloride (425 mg; 1.55 mmol) dissolved in 25 mL of dry tetrahydrofuran was added dropwise in 1 h. After a workup as described for compound I, but using chloroform to extract the product, dansyl-*N*-methyl-β-alanine was obtained as an oil: yield, 0.48 g (95%); one major greenish fluorescent spot in TLC with an R_f of 0.56 in system A, 0.19 in B, and 0.78 in C and a negligible trace of fluorescent trailing spot with an R_f of 0.32 in A, 0.10 in B, and 0.68 in C. The dicyclohexylammonium salt prepared by adding dicyclohexylamine to an ether solution of the above oil, followed by dilution with petroleum ether, melted at 104–107 °C.

A mixed anhydride coupling of the oily dansyl-*N*-methyl-β-alanine (0.48 g; 1.43 mmol) with 2-(dimethylamino)-ethanethiol was carried out as under compounds I and II with equimolar quantities of reagents. The reaction mixture was stirred in an ice bath for 2 h and then at room temperature for 14 h. Upon addition of 50 mL anhydrous ether and cooling, the precipitated triethylamine hydrochloride was filtered off. To the filtrate was added 0.5 mL (8 mmol) of methyl iodide, and the reaction mixture was stirred in a stoppered flask in an ice bath for 1 h followed by 54 h at room temperature. Anhydrous ether (50 mL) was added to the reaction mixture and cooled. The precipitated product was filtered off, washed with anhydrous ether (3 × 10 mL), and twice recrystallized by dissolving in a warm (60 °C) mixture of 10 mL of 2-propanol and 10 mL of acetone and by adding 20 mL of anhydrous ether to give 380 mg (47% overall yield) of creamy crystals which gave one fluorescent spot in TLC with an R_f of 0.06 in system A and 0.11 in C.

N-(tert-Butyloxycarbonyl)-1,5-diaminopentane Hydrochloride or N-(t-Boc)cadaverine Hydrochloride (*V*). The reported procedure for the synthesis of mono-Boc-1,4-diaminobutane hyrdochloride (Geiger, 1971) was modified as follows. To a stirred solution of 1,5-diaminopentane (cadaverine, 3.5 mL, 30 mmol) in 25 mL of peroxide-free dioxane and 25 mL of water was added *tert*-butyloxycarbonyl azide (9.0 mL, 64 mmol) followed by triethylamine (9.5 mL, 68 mmol) in 15 min. The reaction mixture was stirred for 48 h at room temperature and diluted with 100 mL of cold water. After cooling (4 °C), the precipitated product was filtered off, washed with water, and dried. The precipitate was dissolved in 200 mL of dichloromethane, and the organic extract was washed successively with 5% sodium bicarbonate, water, 10% citric acid, water, and saturated sodium chloride and then dried over anhydrous sodium sulfate. The filtered extract was concentrated under reduced pressure at 50 °C and diluted with petroleum ether. After cooling (4 °C), the precipitated crystals were filtered off, washed with petroleum ether, and dried to give 8.1 g (89%) of white needles, mp 76–78 °C. Recrystallization from dichloromethane and petroleum ether gave *N,N'*-bis(*tert*-butyloxycarbonyl)-1,5-diaminopentane: mp 88–90 °C; single spot in TLC with an R_f of 0.90 in system B and 0.82 in C. Anal. Calcd for $C_{15}H_{30}N_2O_4$: C, 59.57; H, 10.00; N, 9.27. Found: C, 59.40; H, 10.20; N, 9.22.

A solution of 3.22 g (10.6 mmol) of *N,N'*-bis(*tert*-butyloxycarbonyl)-1,5-diaminopentane in 50 mL of 2 M hydrogen chloride in anhydrous ether was stirred at room temperature for 5 h. After addition of 50 mL of anhydrous ether, the mixture was cooled (4 °C), and the precipitated product was filtered off and washed with anhydrous ether to give 1.72 g (68%) of white crystals: mp 111–113 °C; TLC showed two spots, indicating contamination from minor amounts of completely deblocked product, 1,5-diaminopentane. The acidic ethereal filtrate was washed with cold water (2 × 50 mL), and the ether extract was concentrated to give a 40% recovery of unreacted *N,N'*-bis(*tert*-butyloxycarbonyl)-1,5-diaminopentane. The acidic aqueous layer was combined with the precipitated product above, and the solution was made basic (pH 12) with cold 4 N sodium hydroxide. The mixture was extracted with chloroform (5 × 50 mL). The chloroform extract was washed with saturated sodium bicarbonate, water, and saturated sodium chloride and then dried over anhydrous sodium sulfate. The extract was evaporated under reduced pressure, and the oily residue was redissolved in a mixture of 2 mL of 2-propanol and 20 mL of anhydrous ether and cooled. To this was added anhydrous ether saturated with HCl gas until precipitation of the hydrochloride derivative was complete. After cooling, the precipitate was filtered off, washed with anhydrous ether, and dried to give 1.38 g (54%) of white crystals of *N*-(*tert*-butyloxycarbonyl)-1,5-diaminopentane hydrochloride: mp 112–114 °C; one ninhydrin and chlorine/tolidine positive spot in TLC with an R_f of 0.69 in system B and 0.52 in C. Anal. Calcd for $C_{10}H_{23}ClN_2O_2$: C, 50.29; H, 9.71; N, 11.73. Found: C, 50.32; H, 9.70; N, 11.78.

N-Dansyl-N'-[3-(4-(dansylamido)phenyl)propionyl]-1,5-diaminopentane [*Dns-4-NHC*$_6$*H*$_4$*CH*$_2$*CH*$_2$*CONH(CH*$_2$*)*$_5$*-NH-Dns*] (*VI*). A mixed anhydride was prepared from 0.2 mmol of 3-(4-(dansylamido)phenyl)propionic acid and equimolar quantities of triethylamine and isobutyl chloroformate as described under compound I above. To this was added 0.25 mmol of the free base form of dansylcadaverine (Lorand et al., 1968), and the reaction mixture was stirred at room temperature for 26 h. After evaporation of the mixture under reduced pressure, the product was extracted with chloroform, and the extract was washed successively with 0.5 N hydrochloric acid, water, saturated sodium bicarbonate, water, and saturated sodium chloride and dried over anhydrous sodium sulfate. The extract was then concentrated under reduced pressure, and petroleum ether was added to cloudiness. The precipitate was filtered off and recrystallized from chloroform and petroleum ether to give a 38% yield of product: mp 197–199 °C; one fluorescent spot in TLC with an R_f of 0.35 in system A and 0.76 in B. Anal. Calcd for $C_{38}H_{45}N_5O_5S_2$: C, 63.75; H, 6.34; N, 9.78. Found: C, 63.79; H, 6.49; N, 9.75. Similarly prepared were the following. *N-Dansyl-N'-(dansylsarcosyl)-1,5-diaminopentane* [*Dns-N(CH*$_3$*)CH*$_2$*CONH-(CH*$_2$*)*$_5$*NH-Dns*] (*VII*): yield, 48%; one fluorescent spot in TLC with an R_f of 0.6 in system A; mol wt calcd, 639; found, 639 (mass spectrum). *N-Dansyl-N'-[ε-[(dansylsarcosyl)-amino]caproyl]-1,5-diaminopentane* [*Dns-N(CH*$_3$*)-CH*$_2$*CONH(CH*$_2$*)*$_5$*CONH(CH*$_2$*)*$_5$*NH-Dns*] (*VIII*): yield, 23%; one major fluorescent spot in TLC with an R_f of 0.23 in system A; mol wt calcd, 752; found, 752.5 (mass spectrum). *N-Dansyl-N'-[dansyl-(N-methyl-β-alanyl)]-1,5-diaminopentane* [*Dns-N(CH*$_3$*)CH*$_2$*CH*$_2$*CONH(CH*$_2$*)*$_5$*NH-Dns*] (*IX*): yield, 76%; one major fluorescent spot in TLC with an R_f of 0.55 in system A; mol wt calcd, 653; found, 653 (mass spectrum).

(*2*) *Survey of the New Thioesters as Enzyme Substrates.* Four new fluorescent thiocholine esters were synthesized (Table I) with a dansyl group attached to a different acyl moiety in each molecule. The thin-layer silica gel chroma-

Table I: Analytical Data and Properties of New Thiocholine Ester Substrates

compd	R	formula	calcd (found) (%) C	H	N	I	mp (°C)	substrate of transglutaminase[a]	substrate of fibrinoligase[b]
I	-4-NHC$_6$H$_4$CH$_2$CH$_2$-	C$_{26}$H$_{34}$IN$_3$O$_3$S$_2$	49.75 (49.51)	5.46 (5.58)	6.70 (6.47)	nd[c]	167–169	+	+
II	-N(CH$_3$)CH$_2$-	C$_{20}$H$_{30}$IN$_3$O$_3$S$_2$	43.55 (43.14)	5.48 (5.38)	7.62 (7.57)	23.01 (23.36)	160–162	−	−
III	-N(CH$_3$)CH$_2$C(=O)NH(CH$_2$)$_5$-[d]	C$_{26}$H$_{41}$IN$_4$O$_4$S$_2$	46.98 (46.75)	6.22 (6.40)	8.43 (7.91)	nd[c]	78–80	+	nd[c]
IV	-N(CH$_3$)CH$_2$CH$_2$-	C$_{21}$H$_{32}$IN$_3$O$_3$S$_2$	44.60 (44.59)	5.70 (5.85)	7.43 (7.34)	nd[c]	154–156	+	+

[a] Guinea pig liver transglutaminase. [b] Human, thrombin- and Ca^{2+}-activated coagulation factor XIII (factor XIII$_a$). [c] Not determined. [d] Unstable on prolonged storage.

tographic procedure, described under Materials and Methods and employing chloroform/2-propanol/acetic acid (17:1:1), permitted the rapid evaluation of the aminolytic reactivities of these compounds with dansylcadaverine. In this chromatographic system, the thioesters as well as dansylcadaverine remained at the origin. R_f values for the acids arising from the solvolysis of thioesters, as verified with the aid of known substances, were between 0.6 and 0.7 for esters I, III, and IV and 0.43 for II. The mobilities for the amide products, again checked with synthetic reference materials, were 0.35 for I, 0.6 for II, 0.23 for III, and 0.55 for IV. Thus the amides which, incidentally, were doubly fluorescent on account of two dansyl groups, could be readily distinguished from the acid byproducts.

The progressive, time-dependent intensification of the amide spots in samples from the enzyme-containing mixtures over nonenzymatic controls showed that [3-(4-(dansylamido)-phenyl)propionyl]thiocholine (I), [ε-[(dansylsarcosyl)-ε-amino]caproyl]thiocholine (III), and [dansyl-(*N*-methyl-β-alanyl)]thiocholine (IV) were all good enzyme substrates but that (dansylsarcosyl)thiocholine (II) was not.

(*3*) *Kinetic Analysis of the Fibrinoligase (Factor XIII$_a$) Catalyzed Aminolysis and Alcoholysis of [Dansyl-(N-methyl-β-alanyl)]thiocholine (IV).* Considering the relative lability of compound III, of the two remaining enzyme substrates, [dansyl-(*N*-methyl-β-alanyl)]thiocholine (IV) was selected for detailed kinetic studies. The choice was dictated by the fact that this compound is more soluble in water than compound I. Since the kinetic identity of the two electrophoretic forms of transglutaminase (A and B), which are present even in highly purified guinea pig liver preparations (Lorand et al., 1979b), is still an open question, evaluation of the steady-state constants was carried out for human fibrinoligase (i.e., thrombin- and Ca^{2+}-activated coagulation factor XIII).

The two-phase continuous, fluorescent assay system (Lorand et al., 1974; Stenberg et al., 1975) was particularly suitable for studying the reactions of the thioester substrate with any amine or alcohol which gave rise to a heptane-soluble product.

Measurements of the linear initial velocities (v) for the formation of fluorescent products, at fixed enzyme (E_0) concentration, but at several concentrations of the thioester as first substrate (S_1) and at different concentrations of the amines or alcohols as second substrates (S_2), yielded double-reciprocal lines for v^{-1} vs. $(S_1)^{-1}$ which converged to a single point on the negative abscissa (Figure 1). From this point of intersection, the apparent Michaelis constant for S_1 could be calculated. A plot of the velocity intercepts against reciprocal concentrations of second substrates (see insert to Figure 1) permitted the evaluation of apparent Michaelis constants for S_2 as well as the maximal velocities (V_{max}) of the transfer reactions. Turnover numbers were then calculated: $k_{cat} = V_{max}/E_0$.

Table II summarizes the measured steady-state kinetic constants for the reactions of [dansyl-(*N*-methyl-β-alanyl)]-thiocholine with various amines (methyl- and butylamine and *t*-Boc- and (mesitylenesulfonyl)cadaverine) and alcohols (methanol, ethanol, and butanol). K_M values for S_1 were found to be independent of the nature of second substrate [(0.9–1.1) × 10^{-3} M for amines; (0.6–1.5) × 10^{-3} M for alcohols], and essentially the same may be said with regard to the turnover number, k_{cat} (0.18–0.8 s^{-1} for amines; 0.15–1.14 s^{-1} for alcohols). There are major differences, however, in K_M values for S_2. Solubility difficulties precluded measurements with alcohols which could have been used as analogues for the more specific amines (i.e., *t*-Boc- and (mesitylenesulfonyl)cadave-

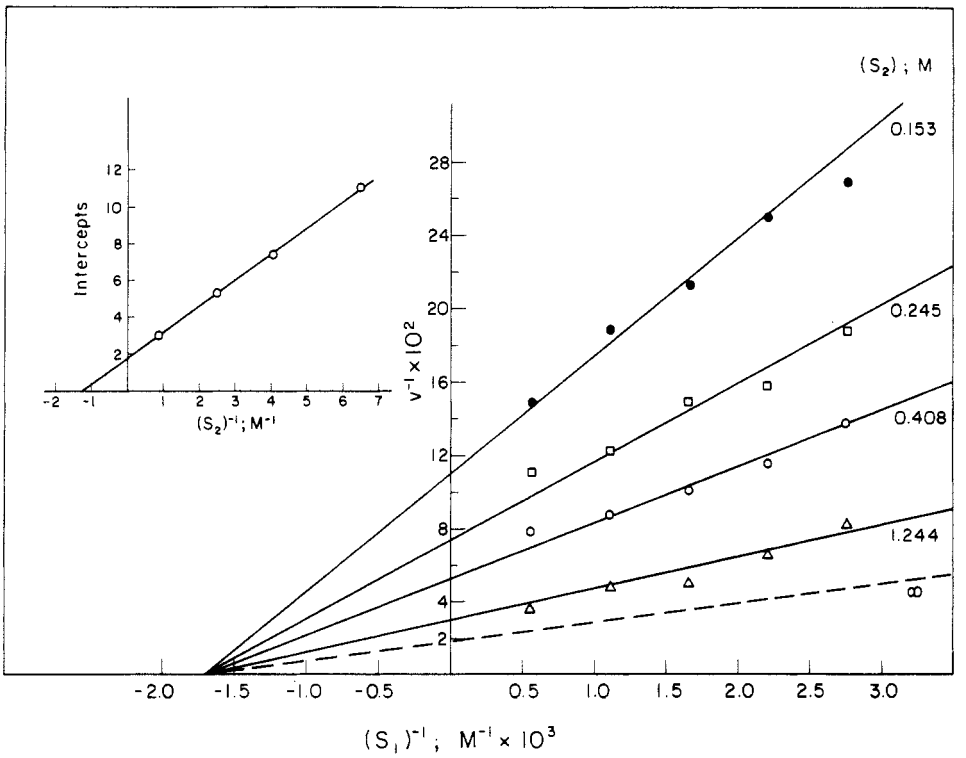
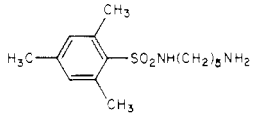


FIGURE 1: Lineweaver–Burk plots of the kinetic data of steady-state formation of product of ethanolysis reaction with [dansyl-(*N*-methyl-β-alanyl)]thiocholine iodide (S_1) catalyzed by fibrinoligase. (Inset) Replot of intercepts vs. reciprocal concentration of ethanol (S_2). The 0.1-mL aqueous reaction mixtures, maintained at 27 °C, contained 0.1 M sodium acetate, pH 7.0, varying concentrations (0.36–1.8 mM) of S_1, 0.5% *N*,*N*-dimethylformamide, fixed initial concentrations of ethanol as indicated, thrombin-activated human plasma factor XIII (E_0 = 0.36 μM), and, finally, 30 mM calcium chloride.

Table II: Steady-State Kinetic Constants for the Fibrinoligase (Human Plasma Factor $XIII_a$; $E_0 = (0.3–0.72) \times 10^{-6}$ M) Catalyzed Reactions of [Dansyl-(*N*-methyl-β-alanyl)]thiocholine Iodide (S_1) with Amines or Alcohols as Second Substrates (S_2) in the Presence of 30 mM $CaCl_2$ (27 °C; 0.5% Dimethylformamide)

S_2	K_M (M × 10³) for S_1	K_M (M × 10³) for S_2	k_{cat} (s^{-1})	k_{cat}/K_M (for S_2) (s^{-1} M^{-1})
amines				
CH_3NH_2	1.1[a]	9.1[a]	0.45[a]	50[a]
	1.1[b]	12.5[b]	0.5[b]	40[b]
	1.1[c]	13.3[c]	0.18[c]	14[c]
$CH_3(CH_2)_3NH_2$	1.1[a]	7.7[a]	0.8[a]	104[c]
$(CH_3)_3COC(=O)NH(CH_2)_5NH_2$ *t*-Boc-cadaverine	1.1[a]	0.95[a]	0.64[a]	674[a]
 (mesitylenesulfonyl)cadaverine	0.9[a]	0.05[a]	0.21[a]	4200[a]
	K_M (M × 10³)	K_M (M)		
alcohols				
CH_3OH	1.5[d]	0.4[d]	0.72[d]	1.8[d]
	1.3[e]	0.4[e]	1.14[e]	2.85[e]
CH_3CH_2OH	0.6[d]	0.8[d]	0.2[d]	0.25[d]
	1.3[e]	1.1[e]	0.48[e]	0.44[e]
	0.7[f]	0.8[f]	0.35[f]	0.45[f]
	1.4[g]	1.1[g]	0.37[g]	0.34[g]
$CH_3(CH_2)_3OH$	0.9[d]	0.65[d]	0.15[d]	0.23[d]

[a] 0.05 M Tris-HCl, pH 7.5, at μ = 0.15. [b] 0.1 M sodium acetate, pH 7.5, at μ = 0.21. [c] 0.05 M *N*-methylmorpholine hydrochloride, pH 7.5, at μ = 0.14. [d] 0.1 M sodium acetate, pH 7.0, at μ = 0.19. [e] 0.05 M *N*-methylmorpholine hydrochloride, pH 7.5, at μ = 0.12. [f] 0.05 M Pipes-Na^+, pH 7.5, at μ = 0.2. [g] 0.05 M Pipes-Na^+, pH 7.0, at μ = 0.2.

rines). Nevertheless, a comparison of methylamine with methanol or butylamine with butanol reveals that amines are considerably better substrates for the enzyme than alcohols and that the difference is mainly due to the smaller apparent Michaelis constants for the amines (e.g., K_{M,CH_3OH}:K_{M,CH_3NH_2} = 30).

Good water solubility allowed exploration with a much wider range of primary amines. The data in the right-hand column of Table II (i.e., k_{cat}/K_M for S_2) clearly show an order of specificity of (mesitylenesulfonyl)cadaverine > *t*-Boc-cadaverine > butylamine > methylamine. Thus, in terms of this "aminolytic index", there is a more than 80-fold difference

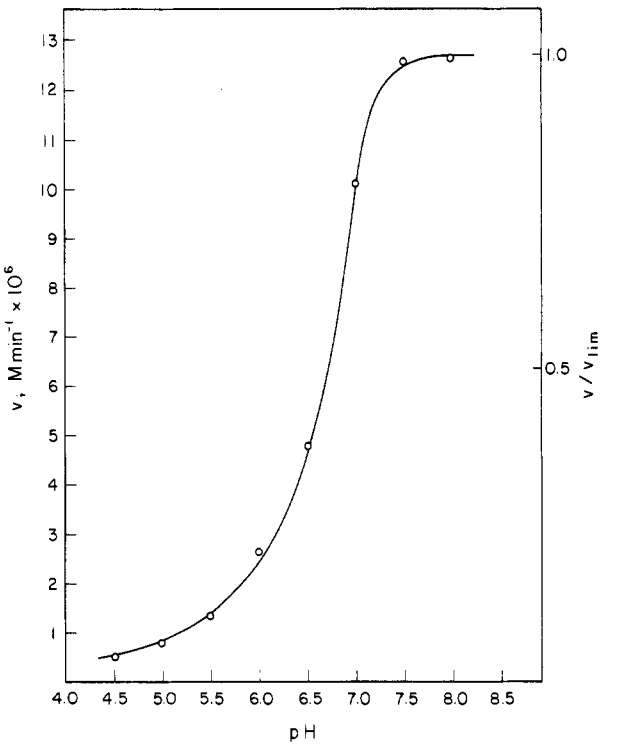


FIGURE 2: Effect of pH on the steady-state velocity of methanolysis of [dansyl-(*N*-methyl-β-alanyl)]thiocholine iodide (S_1) by fibrinoligase in 0.1 M sodium acetate, 27 °C, and 30 mM calcium chloride in the pH range 4.5–8.0. The reaction was carried out in 0.1 mL of aqueous phase containing 0.1 M sodium acetate, 1.86 mM S_1, 1.24 M methanol, 0.5% *N*,*N*-dimethylformamide, thrombin-activated plasma factor XIII (E_0 = 0.36 μM), and finally 30 mM calcium chloride.

between the first and last compounds which obviously can be attributed to differences of the apparent Michaelis constants of these amines.

Using alcohol as a second substrate, it was possible to obtain a pH–activity profile for the fibrinoligase-catalyzed transfer reaction. The results in Figure 2 pertain to the reaction of [dansyl-(*N*-methyl-β-alanyl)]thiocholine (1.86 mM) with methanol (1.24 M). A value of half-limiting velocity was obtained at about pH 6.7. With a slight correction for the residual velocity remaining at pH 4 ($v = 0.4 \times 10^{-6}$ M min^{-1}), the pH dependency of log v (or log v/v_{lim}) obeys the equation for a single ionization [see Dixon & Webb (1964)] and indicates that an acid with a pK_a of 6.7 has to be deprotonated in order to catalyze the enzymatic reaction. Since the concentrations of both substrates were well above their respective K_M values, the results suggest that these K_M's are independent of pH and that the observed pK_a of 6.7 pertains to k_{cat}.

Discussion

(β-Phenylpropionyl)thiocholine and related thioesters are among the best synthetic substrates for enzymes of the endo-γ-glutamine:ε-lysine transferase type, such as fibrinoligase (i.e., factor $XIII_a$) and transglutaminase (Lorand et al., 1972a, 1974; Curtis et al., 1974a,b; Stenberg et al., 1975). The acyl moieties of these substrates seem to mimic the reactive glutamine side chains of proteins, while the thiocholinium residue provides water solubility and also serves as a good leaving group. These esters (RCOSR′) already enabled us to study the kinetic properties of human fibrinoligase, which is also the enzyme of interest in the present work. The pathway of catalysis, apparently involving the active-center cysteine (Curtis et al., 1973, 1974b) of the enzyme (ESH), was postulated to proceed through an acyl-enzyme (RCOSE) intermediate (i.e., step 1). Deacylation could then take place by

$$\mathrm{RCOSR' + ESH} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{(RCOSR'{\cdot}ESH)} \xrightarrow{k_2} \mathrm{RCOSE + R'SH} \quad (1)$$

$$\mathrm{RCOSE + H_2O} \xrightarrow{k_3} \mathrm{RCOOH + ESH} \quad (2)$$

$$\mathrm{RCOSE + H_2NR''} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{(RCOSE{\cdot}H_2NR'')} \xrightarrow{k_5} \mathrm{RCONHR'' + ESH} \quad (3)$$

alternate routes of hydrolysis (step 2) and aminolysis (step 3), if an amine (H_2NR'') substrate was present.

Examination of step 3 was greatly aided by the availability of dansylcadaverine, i.e., *N*-(5-aminopentyl)-5-(dimethylamino)-1-naphthalenesulfonamide. The water-insoluble coupling product (RCONHR″), formed by the reaction of this amine with thioesters, could be followed directly through fluorescence measurements, using a continuous extraction of the product into a layer of heptane placed above the enzymatic mixture (Lorand et al., 1974; Stenberg et al., 1975).

The present research broadens the kinetic scope of this two-phase experimental design. When esters of thiocholine in which a fluorigenic group is attached to the acyl moiety are prepared, the same method could be used for studying the enzyme-catalyzed reactions of esters with a variety of amines and, interestingly, also with alcohols. The latter is represented as

$$\mathrm{RCOSE + HOR'''} \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} \mathrm{(RCOSE{\cdot}HOR''')} \xrightarrow{k_7} \mathrm{RCOOR''' + ESH} \quad (4)$$

As shown in Table I, four dansyl group containing thioesters were synthesized for this study. [3-(4′-(Dansylamido)-phenyl)propionyl]thiocholine (I), [ε-[(dansylsarcosyl)-amino]caproyl]thiocholine (III), and [dansyl-(*N*-methyl-β-alanyl)]thiocholine (IV) all proved to be substrates for guinea pig transglutaminase and also for human fibrinoligase. Remarkably, however, (dansylsarcosyl)thiocholine (II), which differs from compound IV only by the lack of a methylene group in between the *N*-methyl branching and the carbonyl function, showed essentially no reactivity with either enzyme. It is possible that branching so close to the susceptible carbonyl group is detrimental for the binding of this compound (II) into the narrow cleft which, as postulated for transglutaminase, is thought to accommodate the glutamine side chains of substrates (Gross & Folk, 1973). Because of stability[2] and solubility considerations, [dansyl-(*N*-methyl-β-alanyl)]thiocholine (IV) was singled out for detailed kinetic analysis with human fibrinoligase.

Regarding the scheme given in steps 1–4 and by making the reasonable assumptions that $k_{-1} \gg k_2$, $k_{-4} \gg k_5$, and $k_{-6} \gg k_7$, rate laws for the steady-state velocities of amide ($v_{RCONHR''}$) and *O*-ester products ($v_{RCOOR'''}$) are given by

$$v_{RCONHR''} = [k_2k_5/(k_2 + k_5)]E_0/\{1 + [k_5/(k_2 + k_5)](K_a/a) + [(k_2 + k_3)/(k_2 + k_5)](K_b/b) + [k_3/(k_2 + k_5)](K_aK_b/ab)\} \quad (5)$$

$$v_{RCOOR'''} = [k_2k_7/(k_2 + k_7)]E_0/\{1 + [k_7/(k_2 + k_7)](K_a/a) + [(k_2 + k_3)/(k_2 + k_7)](K_c/c) + [k_3/(k_2 + k_7)](K_aK_c/ac)\} \quad (6)$$

[2] We found that thiocholine esters could be more readily synthesized from the acid precursors which contained dansyl *N*-methylsulfonamide groups rather than just a dansyl sulfonamide group. Furthermore, sulfonamide N-methylated thioesters proved to be more stable on storage.

where E_0 is the number of enzymatic sites, a, b, and c represent the concentrations of the thioester (RCOSR'), amine (H_2NR''), and alcohol (HOR''') substrates, $K_a = k_{-1}/k_1$, $K_b = k_{-4}/k_4$, and $K_c = k_{-6}/k_6$, k_2 is the rate constant for acylation of the enzyme, and k_3, k_5, and k_7 are deacylation rate constants for the hydrolysis, aminolysis, and alcoholysis reactions, respectively.

At infinite concentrations of the first ($a \rightarrow \infty$) and of the second substrates ($b \rightarrow \infty$ or $c \rightarrow \infty$), the limiting maximal velocities are given by

$$V_{RCONHR''} = [k_2k_5/(k_2 + k_5)]E_0$$

$$V_{RCOOR'''} = [k_2k_7/(k_2 + k_7)]E_0$$

from which the molar turnover numbers for formation of either the amide or the *O*-ester product can be calculated:

$$k_{cat,RCONHR''} = k_2k_5/(k_2 + k_5)$$

and

$$k_{cat,RCOOR'''} = k_2k_7/(k_2 + k_7)$$

From the data in Table II, it is seen that k_{cat} values for amines and alcohols are very similar, indeed. This can best be interpreted by suggesting that $k_2 \ll k_5$ and also that $k_2 \ll k_7$; i.e., both the amide and *O*-ester forming transfer reactions are acylation rate limiting: $k_{cat,RCONHR''} = k_{cat,RCOOR'''} = k_2$.

As illustrated in Figure 1, plots of the reciprocal initial velocities for forming the amide or *O*-ester products against the reciprocal concentrations of [dansyl-(*N*-methyl-β-alanyl)]thiocholine, when examined at different concentrations of amines or alcohols, give a family of lines which intersect each other in a single point on the negative abscissa.[3] These points of intersections, given as their negative inverse (i.e., K_M for S_1) in Table II, were found to be independent of the chemical nature of the amine or alcohol employed as second substrate. Thus, these K_M values may be taken to represent K_a, the dissociation constant of the Michaelis complex between enzyme and thioester.

From secondary plots such as shown by the insert to Figure 1, the apparent Michaelis constants for the various amines and alcohols could also be calculated (Table II). It is immediately obvious that these constants express specificities for the second substrate and that the spread of values found within the amine series reflects on the relative ease with which the acyl-enzyme intermediate can be saturated by a given amine. The order of affinity shown, i.e., (mesitylenesulfonyl)cadaverine > *t*-Boc-cadaverine > *n*-butylamine > methylamine, is in accord with previous data from this laboratory regarding the amine specificities of human fibrinoligase and of guinea pig liver transglutaminase, obtained either by inhibition kinetics on protein substrates (Lorand et al., 1968, 1979a) or by the enhancement of thiocholine production from thiocholine esters (Curtis et al., 1974). As demonstrated by the example of (mesitylenesulfonyl)cadaverine, again it is evident that the best synthetic amine contains a large apolar substituent attached to an alkylamine residue which resembles the lysine side chain of proteins.

When the fibrinoligase-catalyzed reaction of [dansyl-(*N*-methyl-β-alanyl)]thiocholine with alcohols is discussed, it may be relevant to review some findings in regard to transesterification kinetics with α-chymotrypsin, which constitutes perhaps the best similar case in the literature. McDonald & Balls (1956) noted some degree of specificity for alcohols in chymotrypsin-catalyzed reactions. Bender & Glasson (1960), studying the methanolysis of acetyl-L-phenylalanine methyl ester, concluded that the kinetic results could not be interpreted in terms other than the independent binding of both the ester and methanol to chymotrypsin. However, further efforts by Bender et al. (1964) and by Inward & Jencks (1965) still failed to show a saturation phenomenon with alcohols, and, in fact, in no case did the plot of rate against the concentration of alcohol indicate a decrease in slope which would be expected as the concentration of the alcohol approached the K_S value for its binding to a site. Inward & Jencks (1965) thus suggested that the most reasonable conclusion would be that there is a site of low specificity, with K_S values which are considerably larger than those which can be reached with experimentally accessible concentrations of alcohols without denaturing the enzyme. By contrast, our results (Figure 1 and Table II) show that in the transesterification reaction with human fibrinoligase the alcohol clearly displays a saturation behavior which is consistent with a binding site to the acyl-enzyme intermediate. Nevertheless, apparent affinity for the alcohol is some 30 times less favorable than for the corresponding amine. Solubility difficulties precluded the use of alcohols higher than 1-butanol in our system, and, as such, we cannot comment on the specificity of alcohols in general.

The reaction between [dansyl-(*N*-methyl-β-alanyl)]thiocholine and methanol permitted the generation of a pH–activity profile for the human fibrinoligase catalyzed transfer (Figure 2). The pH value pertaining to half-limiting velocity suggests that a group with an apparent pK_a of about 6.7 is involved in the acylation step ($k_{cat} = k_2$) of catalysis. This pK_a is significantly different from that obtained for the titration of the active center cysteine with iodo[^{14}C]acetamide under quite similar conditions (Curtis et al., 1974b). Therefore, it is likely that the pH–activity profile reflects on the behavior of a prototropic group on the enzyme other than a cysteine thiol and suggests the involvement of an imidazole side chain in catalysis. This is the first indication that fibrinoligase, and possibly also its transglutaminase relative, may function by "cysteine–histidine" active-site mechanisms.

Regarding future research, the fluorescent ester substrates offer some interesting possibilities. The introduction of an environmentally sensitive probe, which is capable of undergoing turnover at the catalytic center, should be useful in studying the acyl-enzyme intermediate (RCOSE). It might also be possible to employ the new compounds for the site-specific acylation of ε-lysine amino groups in protein substrates (H_2NP) which react with factor $XIII_a$ or with transglutaminase: RCOSE + $H_2NP \rightleftharpoons$ (RCOSE; H_2NP) → RCONHP + ESH. These reagents could become a counterpart of dansylcadaverine which, as it may be recalled, has already been widely used for the enzyme-directed labeling of γ-glutamine functionalities in proteins (i.e., in fibrin, fibronectin, and rhodopsin; Lorand et al., 1968, 1972b; Mosher, 1975; Pober et al., 1978). There are only two examples for the acylation of reactive lysines by endo-γ-glutamine:ε-lysine transferases, and, thus far, neither has been analyzed by determining the exact position of substitutions. Kornguth & Waelsch (1963) reported the transglutaminase-dependent labeling of β-lactoglobulin and globin by reaction with benzyloxycarbonylglutaminylvaline ethyl ester. Myhrman (1973) demonstrated the Homarus clotting enzyme-mediated transfer of the isotope-containing acyl group from *trans*-cinnamoylthio[^{14}C]choline

[3] From eq 1 and 2, for the formation of amides and *O*-esters, $K_{m,app} = K_a[k_5 + k_3(K_b/b)]/[k_2 + k_5 + (k_2 + k_3)K_b/b]$ and $K_a[k_7 + k_3(K_b/b)]/[k_2 + k_7 + (k_2 + k_3)K_b/b]$ are obtained, respectively. There are two sets of circumstances in which Michaelis constants for the first substrate become independent of b: when $k_3 = k_5 = k_7$ or when $k_2 \ll k_3$, k_5, and k_7. In the latter case, $K_{m,app} = K_a$.

to fibrinogen in the same species. The availability of the new fluorescent substrates should facilitate a more rigorous examination of protein modifications of this type.

Acknowledgments

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References

Bender, M. L., & Glasson, W. A. (1960) *J. Am. Chem. Soc. 82*, 3336.
Connellan, J. M., Chung, S. I., Whetzel, N. K., Bradley, L. M., & Folk, J. E. (1971) *J. Biol. Chem. 246*, 1093.
Cummings, W. A. W. (1958) *J. Chem. Soc.*, 2058.
Curtis, C. G., & Lorand, L. (1976) *Methods Enzymol. 45*, 177.
Curtis, C. G., Stenberg, P., Chou, C.-H. J., Gray, A., Brown, K. L., & Lorand, L. (1973) *Biochem. Biophys. Res. Commun. 52*, 51.
Curtis, C. G., Stenberg, P., Brown, K. L., Baron, A., Chen, K., Gray, A., Simpson, I., & Lorand, L. (1974a) *Biochemistry 13*, 3257.
Curtis, C. G., Brown, K. L., Credo, R. B., Domanik, R. A., Gray, A., Stenberg, P., & Lorand, L. (1974b) *Biochemistry 13*, 3774.
Deranleau, D. A., & Neurath, H. (1966) *Biochemistry 5*, 1413.
Dixon, M., & Webb, E. C. (1964) *Enzymes, 2nd Ed.*, 132.
Geiger, R. (1971) *Justus Liebigs Ann. Chem. 750*, 165.
Gross, M., & Folk, J. E. (1973) *J. Biol. Chem. 248*, 1301.
Inward, P. W., & Jencks, W. P. (1965) *J. Biol. Chem. 240*, 1986.
Kornguth, S. E., & Waelsch, H. (1963) *Nature (London) 198*, 188.
Lorand, L., Rule, N. G., Ong, H. H., Furlanetto, R., Jacobsen, A., Downey, J., Oner, N., & Bruner-Lorand, J. (1968) *Biochemistry 7*, 1214.
Lorand, L., Chou, C.-H. J., & Simpson, I. (1972a) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2645.
Lorand, L., Chenoweth, D., & Gray, A. (1972b) *Ann. N.Y. Acad. Sci. 202*, 155.
Lorand, L., Gray, A., Bropwn, K., Credo, R. B., Curtis, C. G., Domanik, R. A., & Stenberg, P. (1974) *Biochem. Biophys. Res. Commun. 56*, 914.
Lorand, L., Parameswaran, K. N., Stenberg, P., Tong, Y. S., Velasco, P. T., Jonnson, N. A., Mikiver, L., & Moses, P. (1979a) *Biochemistry 18*, 1756.
Lorand, L., Siefring, G. E., Jr., Tong, Y. S., Bruner-Lorand, J., & Gray, A., Jr. (1979b) *Anal. Biochem. 93*, 453.
McDonald, C. E., & Balls, A. K. (1956) *J. Biol. Chem. 221*, 993.
Mosher, D. F. (1975) *J. Biol. Chem. 250*, 6614.
Myhrman, R. V. (1973) Doctoral Dissertation, Northwestern University, Evanston, IL (University Microfilm Order No. DCJ73-30672).
Pober, J. S., Iwanij, V., Reich, E., & Stryer, L. (1978) *Biochemistry 17*, 2163.
Schwartz, M. L., Pizzo, S. V., & Hill, R. L. (1973) *J. Biol. Chem. 248*, 1395.
Skinner, W. A., Gram, H. F., Mosher, C. W., & Baker, B. R. (1959) *J. Am. Chem. Soc. 81*, 4639.
Stenberg, P., Curtis, C. G., Wing, D., Tong, Y. S., Credo, R. B., Gray, A., & Lorand, L. (1975) *Biochem. J. 147*, 155.

Isolation and Characterization of a Photoaffinity-Labeled Peptide from the Catalytic Site of Prenyltransferase†

David N. Brems,‡ Eveline Bruenger, and Hans C. Rilling*

ABSTRACT: Previously we presented evidence for the selective modification of the catalytic site of prenyltransferase by photoaffinity labeling with *o*-azidophenylethyl pyrophosphate [Brems, D. N., & Rilling, H. C. (1979) *Biochemistry 18*, 860]. In the present work, we report the isolation and characterization of a CNBr fragment of 30 amino acid residues from the photoaffinity-labeled enzyme. This CNBr fragment contains over 80% of the total label attached to prenyltransferase as a result of photoaffinity labeling. Several lines of evidence indicate that a number of residues in this CNBr fragment have been modified. First, Edman degradation of this labeled peptide demonstrates that at least 16 of the 30 amino acids have been modified by the photoaffinity reagent. The two most extensively modified amino acids are a specific arginine and alanine. Second, two-dimensional chromatography of Pronase digestions of the labeled CNBr fragment indicates that at least 11 different products resulted from photoaffinity labeling. Third, peptide maps of a trypsin digest of this CNBr fragment show that the attached affinity label is distributed among at least three of the resulting products of tryptic hydrolysis. Finally, comparison of amino acid analysis of this CNBr fragment with that of its counterpart isolated from native enzyme is consistent with the modification of a number of amino acids rather than a few by the photoaffinity labeling process.

Prenyltransferase (farnesyl pyrophosphate synthetase, EC 2.5.1.1) is an enzyme of molecular weight 86 000. It consists of two identical subunits with one catalytic site per subunit. Prenyltransferase catalyzes two consecutive head-to-tail condensations between isopentenyl pyrophosphate and an allylic pyrophosphate.

The reaction catalyzed by prenyltransferase is unique since it is the first biochemical reaction unequivocally shown to proceed via a carbonium ion (Poulter & Rilling, 1976, 1978; Poulter & Satterwhite, 1977; Poulter et al., 1977; Brems & Rilling, 1977; Rilling, 1979). The mechanism of this reaction

† From the Department of Biochemistry, University of Utah College of Medicine, Salt Lake City, Utah 84108. *Received July 18, 1980; revised manuscript received February 12, 1981.* This investigation was supported by National Institutes of Health Grants AM 13140 and AM 21745.

‡ Present address: Department of Biochemistry, University of Iowa, Iowa City, IA 52242.

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