Specificity of Guinea Pig Liver Transglutaminase for Amine Substrates†

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ABSTRACT: The amine specificity of guinea pig liver transglutaminase, a model enzyme for endo-γ-glutamine:ε-lysine transferases, was explored with the aid of synthetic substrates of high apparent affinities. As exemplified by dansyl- (5-dimethylamino-1-naphthalenesulfonyl), (2,4-dinitrobenzenesulfonyl)-, and (2,4,6-triisopropylbenzenesulfonyl)-cadaverines—each of which showed affinities of approximately 4×10^7 M^{-1}—the best amine substrates carried a large hydrophobic substituent attached to an alkylamine side chain of about 7.2 Å in length. Altogether, our results point to the importance of a hydrophobic binding region in the enzyme from where the alkyl side chain reaches into a narrow crevice toward the active center and positions the primary amine of the substrate for attacking the carbonyl group of the acyl enzyme intermediate.

Calcium ion dependent transamidases of the endo-γ-glutamine:ε-lysine transferase type (see Lorand & Stenberg, 1976; Folk & Finlayson, 1977) are rather widely distributed in nature. These enzymes catalyze the posttranslational cross-linking of protein substrates by γ-glutamyl-ε-lysine side-chain bridges, and several of them have already been shown to perform biologically important functions pertaining to extracellular as well as intracellular cross-linking processes. The stabilization of the fibrin structure during blood coagulation (see Lorand, 1972) and the clotting of seminal fluid (Williams-Ashman et al., 1972) are examples of the former; the stiffening of erythrocyte membrane (Lorand et al., 1976; Siefring et al., 1978) and the formation of the cornifying envelope in keratinocytes (Abernethy et al., 1977; Rice & Green, 1977) illustrate the significance of the latter. By competing with ε-amino groups of lysyl side chains and thus by becoming covalently incorporated into the amine acceptor sites of the protein substrates themselves, certain primary amines could be shown to inhibit the enzymatic cross-linking reactions quite effectively. Such inhibition studies, first performed on the fibrin cross-linking system, revealed a remarkably high degree of specificity requirement for the amine and led to the synthesis of *N*-(5-aminopentyl)-5-(dimethylamino)-1-naphthalenesulfonamide or dansylcadaverine (Lorand et al., 1966; Lorand et al., 1968). These earlier studies suggested that the amine specificity of these enzymes probably required the positioning of a suitable hydrophobic substituent at an optimal distance from the primary amino group of the alkylamine side chain, creating a situation similar perhaps to the one presented by the reactive lysyl residues in protein substrates.

In this study, we examine the amine specificity of guinea pig liver transglutaminase (Clarke et al., 1959) which, though still of unknown biological role, is a representative of the intracellular group of endo-γ-glutamine:ε-lysine transferases and which is readily obtainable in a pure and a rather stable form (Connellan et al., 1971). The present survey extends the scope of "template" mapping for this enzyme considerably beyond previous reports (Pincus & Waelsch, 1968; Gross et al., 1977). Among the various features of structure-affinity relationships, particular attention will be drawn to the importance of a hydrophobic subsite in determination of amine specificity.

Materials and Methods

Kinetic Studies. Guinea pig liver transglutaminase (Connellan et al., 1971) was stored frozen in 10 mM Tris–acetate buffer of pH 6.0, containing 1 mM EDTA and 0.16 M potassium chloride. *N,N'*-Dimethylcasein was prepared from Hammersten casein (Schwartz/Mann) by treatment with formaldehyde and sodium borohydride as described by Lin et al. (1969). [1,4-$^{14}C_2$]Putrescine (sp act. 63 mCi/mmol) was purchased from Amersham/Searle.

Apparent Michaelis constants for *N*-(5-aminopentyl)-5-(dimethylamino)-1-naphthalenesulfonamide (dansylcadaverine) and for *N*-(5-amino-3-thiapentyl)-5-(dimethylamino)-1-naphthalenesulfonamide (dansylthiacadaverine) were measured according to the procedure described by Lorand et al. (1974) and by Stenberg et al. (1975). The transglutaminase-catalyzed formation of fluorescent amide, the product of reaction between (β-phenylpropionyl)thiocholine iodide and the monodansylated diamine, was followed at 25 °C through the continuous extraction of the coupling product into 2.0 mL of *n*-heptane (Fisher H-340) and by measurement of the fluorescence in the organic phase in an Aminco-Bowman ratio spectrophotofluorometer (excitation 340 nm; emission 460 nm). The 0.1-mL aqueous phase, in which the reaction took place, contained 50 mM Tris–acetate buffer of pH 7.5, 1.1 mM (β-phenylpropionyl)thiocholine iodide, 0.163–0.815 mM of dansylcadaverine, or 0.038–0.189 mM of dansylthiacadaverine. Reactions were initiated by addition of calcium chloride to the aqueous phase to a final concentration of 10 mM. Concentrations of stock solutions of the dansyl compounds were deduced from measuring absorbancies at 327 nm [$\epsilon_{327} = 4.67 \times 10^3$ L mol^{-1} cm^{-1} in 20% aqueous *p*-dioxane; Deranleau & Neurath (1966)].

Inhibition studies were performed by the method of Lorand et al. (1972a) in reaction mixtures of 80 μL comprising 50 mM Tris-HCl buffer of pH 7.5, 5 mg/mL *N,N'*-dimethylcasein, 0.05 mg/mL enzyme [$A_{1cm}^{1\%} \approx 15.8$ at 280 nm (Folk & Cole, 1966)], 0.02–0.2 mM [1,4-$^{14}C_2$]putrescine, and also, depending on inhibitory potency, up to 2 mM of the synthetic amines. (With good inhibitors such as dansylcadaverine, the concentration of the amine was 0.1 mM or lower.) Following mixture of these constituents at 37 °C, the reactions were initiated by adding 10 μL of calcium chloride solution to a final

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concentration of 5 mM. Aliquots of 5 μL were withdrawn after 5 and 10 min and were spotted on Whatman 3 MM filter paper disks of 1-cm diameter. The reactions were terminated by immersing the filter papers in 10% ice-cold trichloroacetic acid. Free [1,4-$^{14}C_2$]putrescine was removed by repeated washings with 5% trichloroacetic acid (3 × 10 min), with a 1:1 mixture of acetone–95% ethanol (5 min), and, finally, with acetone (2 min). The filter papers were dried at 50 °C, and the remaining covalently protein-bound radioactivity was measured in a Packard 3375 liquid scintillation spectrometer with 10 mL of the scintillation fluid containing 11.4 g of 2,5-diphenyloxazole and 1.14 g of 1,4-bis[2-(5-phenyloxazoyl)benzene] in 3.8 L of toluene (Mallinckrodt reagent grade).

Spermine and spermidine were purchased from Calbiochem and *n*-butylamine was from Aldrich.

Synthetic Work. The sulfonyl chlorides used to prepare the compounds reported in this work, with a few exceptions, were purchased from commercial sources (Eastman, Aldrich, and Pierce Chemical Co). *N*-Methyl-2-anilinonaphthalene-6-sulfonyl chloride (mansyl chloride; used for preparing compound XXIII) was a gift from Professor Irvin Isenberg, Corvallis, OR. The sulfonyl chlorides needed for the synthesis of compounds XX, XXI, and XXXV were prepared as described below. 2-Aminoethyl sulfide (or 3-thiacadaverine) was from K & K Laboratories. 3,3-Dimethylcadaverine was prepared according to Yamamoto & Kimura (1967) and 3-thiacadaverine 3,3-dioxide and 3-thiacadaverine 3-oxide were prepared according to Barnett (1944). 1,5-Diaminopentane (cadaverine) and all other organic reagents were from Aldrich Chemical Co.

5-[(Dimethylamino)methyl]-1-naphthalenesulfonyl Chloride. This novel sulfonyl chloride was prepared as follows. 5-Aminonaphthalene-1-sulfonic acid (45 g, 200 mmol) dissolved in 100 mL of 2 N sodium hydroxide (200 mmol) was added dropwise simultaneously with sodium nitrite (14 g, 200 mmol) in 60 mL of water to 130 mL of concentrated hydrochloric acid to which crushed ice was added periodically to maintain the temperature at 5 °C. After stirring for a further 1 h, we added 200 g of sodium chloride, and the precipitate was filtered off, washed with cold brine, and dried to give a crude diazonium derivative. In the meantime a cuprous cyanide solution was prepared as follows. Sodium bisulfite (42 g) in 120 mL of water was warmed to 60 °C and added rapidly to a solution of cupric sulfate (150 g, 600 mmol) in 500 mL of water at 50 °C. After 1–2 min the mixture was treated with a solution (60 °C) of potassium cyanide (42 g) in 120 mL of water. The cuprous cyanide was filtered off after 10 min, washed four times with boiling water, pressed dry on the filter, and then dissolved in a solution of potassium cyanide (65 g) in 180 mL of water. Concentrated hydrochloric acid was then added to the cyanide solution until cloudy. The above diazonium derivative was added portionwise to the fresh cuprous cyanide solution at 60–70 °C with stirring. The mixture was warmed on a water bath for 1 h, acidified with concentrated hydrochloric acid, and filtered hot. The filter cake was washed with boiling water and the combined filtrate adjusted to pH 3–4. The mixture was cooled and filtered, the filtrate was taken to dryness in vacuo, the residue was leached repeatedly with hot ethanol, and the alcoholic solution was concentrated, clarified with active carbon, and chilled strongly. The precipitate was collected, washed with cold ethanol and ether, and dried to give 39.5 g (78%) of the crude sodium salt of 5-cyano-1-naphthalenesulfonic acid. The above intermediate (33 g, ca. 130 mmol) was dissolved in a mixture containing 130 mL of concentrated ammonia, 130 mL of methanol, and 30 mL of water. Raney nickel (2 teaspoons) was added, and the mixture was hydrogenated at 100 atm and 125 °C for 1.5 h. A few milliliters of water was added to the reaction mixture and warmed to dissolve the product. The mixture was filtered, and the filtrate was concentrated in vacuo and acidified with acetic acid to pH 6–7 and cooled. The white crystals were collected, washed successively with water and methanol, and dried to give 17 g (55%) of 5-(aminomethyl)-1-naphthalenesulfonic acid. Anal. Calcd for $C_{11}H_{11}NO_3S$: C, 55.7; H, 4.67; N, 5.90; S, 13.5. Found: C, 55.6; H, 4.60; N, 5.79; S, 13.3. This was dimethylated as follows. A mixture made up of the above 5-(aminomethyl)-1-naphthalenesulfonic acid (14.0 g, 59 mmol), formic acid (30 mL, 600 mmol), and formaldehyde solution (35%, 12 mL, 130 mmol) was heated on a steam bath for 4 h; by then gas evolution had ceased. After heating for a further 12 h, the reaction mixture was evaporated under reduced pressure. The residue was triturated with water and filtered, and the precipitate was washed with water and dried to give 14.1 g (90%) of light brown crystals. Recrystallization from 50% acetic acid gave white crystals of 5-[(dimethylamino)methyl]-1-naphthalenesulfonic acid. Anal. Calcd for $C_{13}H_{15}NO_3S$: C, 58.9; H, 5.70; N, 5.28; S, 12.1. Found: C, 58.7; H, 5.70; N, 5.16; S, 12.3. This was then converted to the sulfonyl chloride; thus, a mixture of 5-[(dimethylamino)methyl]-1-naphthalenesulfonic acid (26.5 g, 100 mmol) and finely powdered sodium hydroxide (4.0 g, 100 mmol) was warmed with methanol until a clear solution was obtained. The methanol was then evaporated in vacuo, the last traces being removed by codistillation first with benzene and then with diisopropyl ether, to give the sodium salt of the sulfonic acid. This was gently heated with phosphorus oxychloride (100 mL) whereupon a vigorous exothermic reaction ensued and all the material went into solution. After being warmed for 1 h on a water bath, the mixture was cooled to ca. 50 °C and diisopropyl ether was added cautiously. The precipitated product was collected, washed with diisopropyl ether, and dried to give 39 g of white powder consisting of a mixture of the title compound contaminated with inorganic material. This crude sulfonyl chloride was used as such in the next step for preparing compounds XX and XXXV. A purer sample of the sulfonyl chloride was prepared as follows. The crude product was added to ice water, the mixture was basified with sodium bicarbonate and extracted with methylene chloride, the extract was dried over anhydrous sodium sulfate, and the solvent was removed in vacuo to give an oily product. Trituration with petroleum ether gave crystals, mp 65–67 °C. This was converted to the hydrochloride derivative: mp >200 °C (chloroform–diisopropyl ether). Anal. Calcd for $C_{13}H_{14}ClNO_2S{\cdot}HCl$: C, 48.8; 8; H, 4.72; N, 4.37; S, 10.0; Cl, 22.1. Found: C, 49.0; H, 4.86; N, 3.83; S, 9.86; Cl, 21.7.

5-[3-(Dimethylamino)propyl]-1-naphthalenesulfonyl Chloride. First, 5-(3-aminopropyl)-1-naphthalenesulfonic acid was prepared via 5-(2-chloro-2-cyanoethyl)-1-naphthalenesulfonic acid and subsequent reduction of the latter as follows. A mixture of 5-amino-1-naphthalenesulfonic acid (46 g, 200 mmol), 2 N sodium hydroxide (100 mL, 200 mmol), and concentrated hydrochloric acid (120 mL) was diazotized at 0 °C with sodium nitrite (14.5 g, 210 mmol) in 30 mL of water in the usual manner. After cooling (0–5 °C) for 2 h, we poured the mixture into a solution made up of vinyl cyanide (13.2 g, 250 mmol), cupric chloride (8.5 g), acetone (100 mL), and water (20 mL). The mixture was then treated with sodium acetate (200 g, 1600 mmol), giving a pH of 3.5, and was allowed to warm-up spontaneously to 20 °C. After the gas

evolution had ceased (1.5–2 h), concentrated hydrochloric acid was added to give a pH of 2. A small amount of insoluble matter was filtered off, and the filtrate was evaporated. The residue was leached with hot methanol and filtered, and the filtrate was evaporated under reduced pressure. The residue was fractionally crystallized from a combination of methanol and ethanol. The fractions which were identical by IR were combined, thus giving 60 g (ca. 93%) of crude product. The above crude product (39 g, ca. 110 mmol) was hydrogenated in a mixture of methanol (100 mL) and concentrated ammonia (80 mL) at 90 atm and 120 °C for 2 h, with Raney nickel as catalyst. The cooled mixture was filtered, and the filtrate was concentrated in vacuo, acidified to pH 3 with hydrochloric acid, and cooled (4 °C) overnight. The crystals were collected, washed with cold water, and dried to give 6.8 g (24%) of pale orange-violet product. Recrystallization from hot water gave whitish crystals of 5-(3-aminopropyl)-1-naphthalenesulfonic acid containing 1 mol of water: mp >200 °C. Anal. Calcd for $C_{13}H_{15}NO_3S \cdot H_2O$: C, 55.1; H, 6.05; N, 4.94; S, 11.3. Found: C, 54.7; H, 6.30; N, 4.80; S, 11.0.

This intermediate was then dimethylated as follows. A mixture of the above 5-(3-aminopropyl)-1-naphthalenesulfonic acid (23 g, 80 mmol), formic acid (40 mL), and formaldehyde solution (37%, 16 mL, 180 mmol) was heated in a boiling water bath for 16 h. The reaction mixture was evaporated in vacuo, the residual gummy material was dissolved in methanol, decolorized with active carbon, and filtered, and the filtrate was evaporated to dryness in vacuo. The residue was triturated with absolute ether to give 21.2 g (85%) of crystals. Recrystallization from 90% ethanol gave white crystals of 5-[3-(dimethylamino)propyl]-1-naphthalenesulfonic acid containing 1 mol of water. Anal. Calcd for $C_{15}H_{19}NO_3S \cdot H_2O$: C, 57.9; H, 6.80; N, 4.50; S, 10.3. Found: C, 57.4; H, 6.80; N, 4.40; S, 10.4.

This sulfonic acid was converted to the title compound, 5-[3-(dimethylamino)propyl]-1-naphthalenesulfonyl chloride, essentially as described above for the analogous compound. The crude sulfonyl chloride was used as such to prepare compound XXI (Table II).

General Procedure for the Synthesis of Aminoalkylsulfonamides. Using the appropriate sulfonyl chlorides and diamines, we synthesized compounds IX, XI, XX, XXI, XXIII, XXVII–XXXV, and XXXVII–XXXIX according to the general procedure described below for XXVII (tosylthiacadaverine). Physicochemical data for this set of compounds are given in Table I.

N-(5-Amino-3-thiapentyl)-p-toluenesulfonamide Hemifumarate (Tosylthiacadaverine; Compound XXVII). A solution of *p*-toluenesulfonyl chloride (1.0 g, 5.2 mmol) in chloroform (25 mL) was added dropwise at room temperature during 2 h to a stirred solution of triethylamine (0.5 g, 0.7 mL, 5.2 mmol) and 2-aminoethyl sulfide (1.2 g, 10.4 mmol) in chloroform (10 mL). The chloroform extract of the product was successively washed with 5% aqueous sodium bicarbonate (3 × 50 mL), water (2 × 50 mL), and saturated aqueous sodium chloride (50 mL). The chloroform extract was dried over anhydrous sodium sulfate and filtered, and the filtrate was evaporated in vacuo. The residual oil was dissolved in absolute ethanol (10 mL) and then anhydrous ether (25 mL) was added to the solution. The fumarate salt was precipitated by addition of a saturated solution of fumaric acid in absolute ethanol: yield, 1.2 g (68%); mp 146–148 °C (from absolute ethanol–anhydrous ether). Anal. Calcd for $C_{11}H_{18}N_2O_2S_2 \cdot 0.5C_4H_4O_4$: C, 46.97; H, 6.07; N, 8.43. Found: C, 46.70; H, 6.47; N, 8.25.

For preparing the hydrochlorides, fumaric acid–ethanol was replaced by anhydrous hydrogen chloride in anhydrous ether. In the synthesis of IX, the above workup was slightly modified in order to facilitate separation of the desired IX from the bis(sulfonamide) byproduct, by taking advantage of the fact that the latter was insoluble in dilute aqueous hydrochloric acid, whereas the former was soluble.

N-(5-Aminopentyl)-2,4-dinitrobenzenesulfonamide Hydrobromide (Compound XII). First, the amino group blocked precursor, *N*-[5-[(benzyloxycarbonyl)amino]pentyl]-2,4-dinitrobenzenesulfonamide was prepared as follows. To a solution of *N*-(benzyloxycarbonyl)-1,5-diaminopentane hydrobromide (mono-Z-cadaverine; Clarke et al., 1959) (0.95 g, 3 mmol) and 2,4-dinitrobenzenesulfonyl chloride (0.8 g, 3 mmol) in *p*-dioxane (25 mL) and water (5 mL) was added an aqueous solution (5 mL) of sodium hydroxide (0.24 g, 6 mmol). The solution was stirred at room temperature for 30 min, and the solvent was then evaporated in vacuo. The residue was dissolved in chloroform (100 mL), and the solution was washed with water (2 × 100 mL), aqueous saturated sodium bicarbonate (2 × 100 mL), 1 N hydrochloric acid (1 × 100 mL), water (100 mL), and saturated aqueous sodium chloride (50 mL) and dried (anhydrous sodium sulfate). The solution was filtered, the filtrate was evaporated in vacuo, and the residue was recrystallized from absolute ethanol: yield, 1.2 g (86%); mp 108–110 °C. Anal. Calcd for $C_{19}H_{22}N_4O_8S$: C, 48.92; H, 4.76; N, 12.01. Found: C, 49.07; H, 5.14; N, 11.87.

The Z-group deblocking from the above precursor was carried out as follows. To a solution of *N*-[5-[(benzyloxycarbonyl)amino]pentyl]-2,4-dinitrobenzenesulfonamide (0.75 g, 1.6 mmol) in methylene chloride (8 mL) was added 3 mL of a 30% (3.7 M) solution of anhydrous hydrogen bromide in glacial acetic acid. After 1 h at room temperature, anhydrous ether (25 mL) was added, and after another 30 min the precipitated product was filtered off and washed carefully with anhydrous ether: yield, 0.6 g (90%) (77% overall); mp 159–161 °C (from absolute ethanol–anhydrous ether). Anal. Calcd for $C_{11}H_{16}N_4O_6S \cdot HBr$: C, 31.97; H, 4.15; N, 13.56. Found: C, 32.43; H, 4.05; N, 13.17.

ε-Aminocaproyl-p-nitroanilide Hydrobromide (Compound XIV). First, the blocked precursor, ε-(Z-amino)caproyl-*p*-nitroanilide, was prepared as follows. To a stirred, cooled (−15 °C) solution of 2.7 g (10 mmol) of ε-(Z-amino)caproic acid in 40 mL of dry tetrahydrofuran were added *N*-methylmorpholine (1.2 mL, 10 mmol) and isobutyl chloroformate (1.3 mL, 10 mmol). After 15 min a solution of *p*-nitroaniline (1.5 g, 11 mmol) in 20 mL of cold, dry tetrahydrofuran was added. The reaction mixture was stirred at −10 °C for 30 min, then at 0 °C for 3 h, and finally at room temperature for 46 h. The mixture was evaporated under reduced pressure at 45 °C to remove tetrahydrofuran. The product was extracted with ethyl acetate (4 × 50 mL) after addition of 50 mL of water. The organic extract was successively washed with 100 mL each of 1 N hydrochloric acid, 5% sodium bicarbonate, water, and saturated sodium chloride and dried over anhydrous sodium sulfate. The extract was filtered and the filtrate was concentrated to 30 mL volume and diluted with petroleum ether (40–60 °C) to give, upon chilling and filtration, 2.8 g (72%) of light yellow crystals, mp 95–97 °C. This crude precursor was deblocked as follows. The above blocked derivative (2.5 g, 6.5 mmol) was dissolved in a mixture of 2.5 mL of glacial acetic acid and 15 mL of a 31% solution of anhydrous hydrogen bromide in glacial acetic acid. After 1 h at room temperature the mixture was evaporated under

Table I: Analytical Data for Amine Derivatives[a]

R–X–CH_2CH_2–Y–$CH_2CH_2NH_2$

compd	R	X	Y	formula	calcd (%) C	calcd (%) H	calcd (%) N	found (%) C	found (%) H	found (%) N	mp[b] (°C)	yield (%)
IX	*p*-$NO_2C_6H_4$–	–SO_2NH–	CH_2	$C_{11}H_{17}N_3O_4S$·HCl	40.80	5.60	12.98	40.59	5.50	12.90	159–161	39
XI	2,4-$(NO_2)_2C_6H_3$-1–	–SNH–	CH_2	$C_{11}H_{16}N_4O_4S$·0.5$C_4H_4O_4$	43.57	5.06	15.63	44.09	5.30	15.49	160 dec	39
XV	C_6H_5–	–$SO_2N(CH_3)$–	CH_2	$C_{12}H_{20}N_2O_2S$·$C_4H_4O_4$[c]	51.6	6.49	7.52	51.5	6.52	7.55	157–162	60
XVI	C_6H_5–	–$SO_2N(C_2H_5)$–	CH_2	$C_{13}H_{22}N_2O_2S$·$C_4H_4O_4$[d]	52.8	6.78	7.25	52.5	6.68	7.25	149–150	50
XVII	C_6H_5–	–$SO_2N[CH(CH_3)_2]$–	CH_2	$C_{14}H_{24}N_2O_2S$·$C_4H_4O_4$[e]	54.0	7.05	7.01	53.8	6.93	6.66	142–146	40
XVIII	C_6H_5–	–$SO_2N(CH_2C_6H_5)$–	CH_2	$C_{18}H_{24}N_2O_2S$·$C_4H_4O_4$[f]	58.9	6.29	6.25	58.7	6.36	5.90	182–184	50
XX	5-$(CH_3)_2NCH_2$-1-naphthalene–	–SO_2NH–	CH_2	$C_{18}H_{27}N_3O_2S$·2HCl·0.5H_2O[g]	50.1	7.01	9.74	50.1	7.11	9.5	dec[n]	
XXI	5-$(CH_3)_2N(CH_2)_3$-1-naphthalene–	–SO_2NH–	CH_2	$C_{20}H_{31}N_3O_2S$·2HCl·3H_2O[h]	47.6	7.79	8.33	47.5	7.47	8.39	dec[n]	
XXIII	6-CH_3-N(Ph)-naphthalene-2–	–SO_2NH–	CH_2	$C_{22}H_{27}N_3O_2S$·0.5$C_4H_4O_4$·0.5H_2O	62.05	6.51	9.05	62.11	6.48	8.94	156–158	28
XXVIII	*p*-$H_3CC_6H_4$–	–SO_2NH–	$C(CH_3)_2$	$C_{14}H_{24}N_2O_2S$·$C_4H_4O_4$[i]	54.0	7.05	7.00	53.8	7.10	6.88	162	
XXIX	*p*-$H_3COC_6H_4$–	–SO_2NH–	S	$C_{11}H_{18}N_2O_3S_2$·0.5$C_4H_4O_4$	44.81	5.79	8.04	44.63	5.99	7.85	146–147	59
XXX	2,4,6-$(CH_3)_3C_6H_2$–	–SO_2NH–	CH_2	$C_{14}H_{24}N_2O_2S$·HCl	52.40	7.85	8.73	52.29	8.02	8.77	104–106	62
XXXI	2,4,6-(*i*-$C_3H_7)_3C_6H_2$–	–SO_2NH–	CH_2	$C_{20}H_{36}N_2O_2S$·HCl	59.30	9.21	6.92	59.21	9.44	7.12	195–197	72
XXXII	2,3,4-$Cl_3C_6H_2$–	–SO_2NH–	S	$C_{10}H_{13}N_2Cl_3O_2S_2$·0.5$C_4H_4O_4$	34.17	3.59	6.64	34.54	3.92	6.46	173–175	38
XXXIII	4-$C_6H_5N{=}NC_6H_4$	–SO_2NH–	S	$C_{16}H_{20}N_4O_2S_2$·0.5$C_4H_4O_4$	51.16	5.25	13.26	50.83	5.26	12.87	158–160	66
XXXIV	naphthalene-1–	–SO_2NH–	S	$C_{14}H_{18}N_2O_2S_2$·0.5$C_4H_4O_4$	52.15	5.47	7.60	51.88	5.68	7.24	137–140	45
XXXV	5-$(CH_3)_2NCH_2$-1-naphthalene–	–SO_2NH–	S	$C_{17}H_{25}N_3O_2S_2$·2HCl·0.5H_2O[j]	45.4	6.28	9.35	45.6	6.42	9.46	dec[n]	
XXXVII	5-$(CH_3)_2N$-1-naphthalene–	–SO_2NH–	$C(CH_3)_2$	$C_{19}H_{29}N_3O_2S$·$C_4H_4O_4$[k]	57.6	6.93	8.76	57.9	7.02	8.53	140–146 dec	
XXXVIII	5-$(CH_3)_2N$-1-naphthalene–	–SO_2NH–	SO	$C_{16}H_{23}N_3O_3S_2$ (free base)[l]	52.0	6.27	11.4	52.1	6.36	11.2	172–174	
XXXIX	5-$(CH_3)_2N$-1-naphthalene–	–SO_2NH–	SO_2	$C_{16}H_{23}N_3O_4S_2$·0.5$C_4H_4O_4$[m]	48.7	5.68	9.47	48.9	5.66	9.75	amorphous[n]	
	5-$(CH_3)_2N$-naphthalene-1-$SO_2NH(CH_2)_5$-$N(CH_3)_2$·0.5$C_4H_4O_4$			$C_{19}H_{29}N_3O_2S$·0.5$C_4H_4O_4$	59.83	7.41	9.93	59.77	7.05	9.65	150–152	82

[a] Synthesized by the general procedure given under Materials and Methods. [b] Recrystallized from absolute ethanol–anhydrous diethyl ether. Melting points (uncorrected) were determined with a Kofler micro-hot-stage apparatus. [c–m] The calculated/found values for S are respectively 8.61/8.77; 8.30/8.68; 8.01/7.92; 7.15/7.16; 7.43/7.67; 6.36/5.83; 8.01/7.86; 14.3/13.9; 6.69/6.54; 17.3/17.2; and 14.5/14.2. [g,h,j] The calculated/found values for Cl are respectively 16.4/16.3; 14.1/14.4; and 15.8/16.2. [n] No definite melting point.

reduced pressure at 45 °C to a final 5-mL volume. Upon addition of 50 mL of anhydrous ether, trituration, and cooling to 0 °C, a white solid separated, which was filtered off and washed with anhydrous ether to give 1.7 g (78%; 56% overall) of yellow needles, mp 200–202 °C (dec) (anhydrous methanol–ether). Anal. Calcd for $C_{12}H_{17}N_3O_3$·HBr: C, 43.38; H, 5.46; N, 12.65. Found: C, 43.36; H, 5.36; N, 12.63.

General Procedure for the Synthesis of Compounds V and XV–XVIII. These were prepared from 6-(benzenesulfonamido)hexanamide via the Hofmann rearrangement reaction of amides with bromine and alkali in the presence of methanol to give the corresponding methyl carbamates, the immediate precursors of the title compounds. Alkylation of the sulfonamide nitrogen was done with the appropriate alkyl iodide or alkyl bromide after completion of the Hofmann reaction. Alkaline hydrolysis of the methyl carbamates gave the title amine derivatives. The general procedure is described below for the synthesis of V.

5-(Benzenesulfonamido)pentylamine Fumarate (V). First, the precursor, methyl *N*-[5-(benzenesulfonamido)pentyl]-carbamate was prepared as follows. To a solution of sodium methoxide made up fresh from sodium (7 g) and anhydrous methanol (200 mL) was added 6-(benzenesulfonamido)-hexanamide (27 g, 100 mmol) [the latter was prepared from 6-(benzenesulfonamido)hexanoic acid via the usual method by conversion to the acid chloride with thionyl chloride followed by treatment with concentrated ammonia to give a 62% yield of the product: mp 100–103 °C]. Anal. Calcd for $C_{12}H_{18}N_2O_3S$: C, 53.3; H, 6.71; N, 10.4; S, 11.9. Found: C, 53.3; H, 6.74; N, 9.96; S, 12.2. The reaction mixture was cooled in an ice water bath, and a solution of bromine (16 g) in 10 mL of methanol was rapidly added. After the mixture was stirred for 1 h in a water bath, the mixture was acidified with AcOH and evaporated to dryness under reduced pressure. The residue was treated with chloroform and water, and the chloroform layer was separated, washed with 2 M sodium hydroxide solution and water, and dried (Na_2SO_4). The extract was then evaporated under reduced pressure to give 17 g (57%) of an oil which crystallized on standing. A sample recrystallized from a mixture of 2-propanol and diisopropyl ether melted at 88 °C. Anal. Calcd for $C_{13}H_{20}N_2O_4S$: C, 52.0; H, 6.71; N, 9.33; S, 10.7. Found: C, 52.3; H, 6.76; N, 9.32;, S, 10.8.

The above precursor, methyl *N*-[5-(benzenesulfonamido)pentyl]carbamate (7.5 g, 25 mmol), was dissolved in ethanol (50 mL). After adding KOH (5.6 g) the mixture was refluxed gently for 24 h, cooled, and filtered. The filtrate was evaporated to dryness under reduced pressure. Water was added, the pH was adjusted to about 10 (with N-disubstituted sulfonamides pH adjustment is not required), the oil that separated was taken up in methylene chloride, washed with water, and dried, and the solvent was removed. The residual oil was dissolved in ethanol, and a saturated ethanolic solution of fumaric acid was added to give the fumarate derivative: mp 175 °C (from ethanol); yield, 35%. Anal. Calcd for $C_{11}H_{18}N_2O_2S$·$C_4H_4O_4$: C, 50.3; H, 6.19; N, 7.82; S, 8.95. Found: C, 49.9; H, 6.31; N, 7.71; S, 9.22.

The above general procedure was used in the synthesis of compounds XV–XVIII with slight modification. *N,N*-Dimethylformamide was also added as a cosolvent in the preparation of the carbamate precursors for compounds XVII and XVIII. The alkylations of the sulfonamido nitrogen were achieved when in the above general procedure for the preparation of methyl carbamate precursors the appropriate alkyl iodides (or alkyl bromides) were added to the reaction mixtures and refluxed for three to several hours before workup of the reaction mixtures. These methyl *N*-[5-(*N*-alkylbenzenesulfonamido)pentyl]carbamates were used as such without further purification in the next step. Alkaline hydrolysis of these precursors was carried out essentially as described above for the synthesis of V to give the desired amine fumarate derivatives of XV–XVIII. The elemental analysis and other physical data for these compounds are given in Table I.

2-(5-Aminopentyl)-2,3-dihydro-1H-benz[de]isoquinoline Dihydrochloride (Compound XXV). First, the precursor, *N*-(4-cyanobutyl)-1,8-naphthalenedicarboximide, was prepared as follows. 1,8-Naphthalenedicarboximide (39.4 g, 200 mmol) in *N,N*-dimethylformamide (200 mL) was treated portionwise with sodium hydride (9.6 g, 50% in paraffin oil) with stirring until the evolution of hydrogen subsided. 5-Chloropentanenitrile (23.5 g, 0.2 mol) was added and the mixture stirred overnight on a water bath. The mixture was treated with ice and water and extracted with 3 × 50 mL of dichloromethane. After washing with water and drying (Na_2SO_4), we concentrated the solution. The solid thus obtained was recrystallized from ethanol: mp 140–142 °C. Anal. Calcd for $C_{17}H_{14}N_2O_2$: C, 73.4; H, 5.07; N, 10.1. Found: C, 73.2; H, 5.22; N, 9.85.

The above imide (15 g, 54 mmol) was placed in an extraction thimble and deposited in the cylinder of a Soxhlet extraction apparatus containing anhydrous ether (500 mL) and lithium aluminum hydride (6.15 g). The ether was refluxed for 2 days during which time the solid imide slowly dissolved. The excess hydride was decomposed with saturated aqueous sodium sulfate solution (40 mL), and the solid was filtered off and washed with ether. The ether phase was separated, washed with water, and dried (K_2CO_3). The dried extract was treated with saturated anhydrous hydrogen chloride in ether to give a colorless solid which was collected and crystallized from an ethanol–ether mixture: mp 243 °C (dec); yield, 9.8 g (54%). Anal. Calcd for $C_{17}H_{24}Cl_2N_2$·$0.5H_2O$: C, 60.7; H, 7.49; Cl, 21.1; N, 8.33. Found: C, 60.5; H, 7.51; Cl, 21.2; N, 8.28.

N-(5-Aminopentyl)-1,8-naphthalenedicarboximide Hydrochloride (Compound XXVI). 1,8-Naphthalenedicarboxylic anhydride (26.8 g, 135 mmol) and 1,5-diaminopentane (32 g, 270 mmol) were intimately mixed and heated to 200 °C until the evolution of water vapor ceased. The light brown oil thus obtained was treated with 2 M hydrochloric acid when a solid precipitated [later identified as 1,5-bis(1,8-naphthalenedicarboximido)pentane] and was filtered off. The desired product (XXVI) was isolated from the filtrate as follows. The acidic filtrate was made strongly alkaline and extracted with ether, and the ether extract was dried over anhydrous K_2CO_3. The hydrochloride derivative was prepared as above: mp 203 °C (dec) (after repeated crystallization from ethanol–ether); yield, 6.8 g. Anal. Calcd for $C_{17}H_{19}ClN_2O_2$: C, 64.0; H, 6.01; Cl, 11.1; N, 8.79. Found: C, 63.8; H, 5.93; Cl, 11.2; N, 8.74.

Results

In order to compare the kinetic specificities of amine substrates regarding transglutaminase, first we sought to establish some reference values by determining Michaelis constants for *N*-(5-aminopentyl)-5-(dimethylamino)-1-naphthalenesulfonamide (dansylcadaverine) and for the thia analogue, *N*-(5-amino-3-thiapentyl)-5-(dimethylamino)-1-naphthalenesulfonamide (dansylthiacadaverine), two often-used substrates of endo-γ-glutamine:ε-lysine transferases. A continuous rate assay described by Lorand et al. (1974) was employed for measuring the steady-state production of

fluorescent amides formed in the enzymatic reactions of (β-phenylpropionyl)thiocholine with either dansylcadaverine or dansylthiacadaverine. The concentration of the thiol ester substrate was set to 1.1 mM, corresponding to about three times the concentration necessary for half-saturation of the transglutaminase with this first substrate (Stenberg et al., 1975). From Lineweaver–Burk plots of the experimental data, uncorrected $K_{M,app}$ values of about 3×10^{-4} and 7×10^{-5} M were calculated for dansylcadaverine and dansylthiacadaverine, respectively. If, however, when the pK_a values of 10.6 and 9.6 for the primary ammonium ions of these two compounds are used (Ljunggren et al., 1974; Perrin, 1965), Michaelis constants are expressed in relation to the effective concentrations of unprotonated amine species existing at the pH of experiments (pH 7.5; 25 °C) rather than on the basis of the total (i.e., amine plus ammonium ion) concentration of substrates, a corrected $K_{M,app}$ of 3×10^{-7} M is obtained for dansylcadaverine and 7×10^{-7} M is obtained for dansylthiacadaverine. These figures provide quantitative proof that both of these amines are excellent substrates for transglutaminase and that the cadaverine derivative may actually be somewhat more specific than the thiacadaverine analogue.

As expected for an enzyme-catalyzed reaction proceeding through an acyl enzyme intermediate followed by aminolytic deacylation [see Curtis et al. (1974) and Stenberg et al. (1975)], the maximal rates obtained at saturating concentrations of amines were identical (k_{cat} = 0.8 s^{-1}) regardless of whether dansylcadaverine or dansylthiacadaverine was used as substrate.

Since the technique employed for obtaining the above data is restricted to a few fluorescent amine substrates, a more generally applicable procedure had to be used for comparing the relative affinities of amines of various types. Advantage was taken of the rapid filter paper assay developed by Lorand et al. (1972a) which allowed kinetic measurements for inhibition by any amine against the enzymatic incorporation of [^{14}C]putrescine into *N,N'*-dimethylcasein. The initial velocity of isotope incorporation (measured at 37 °C for 5 min), followed Michaelis kinetics, and, when inhibitory, the presence of a nonradioactive amine gave rise to an increase in the slope of the Lineweaver–Burk line, characteristic for competition. As a convenience for comparison of the relative affinities of amines, reciprocals of the calculated apparent inhibition constants [$K_{i,app}^{-1}$; see Segel (1976)] are expressed as the percentage of the same quantity obtained for dansylcadaverine. Thus, dansylcadaverine serves as reference (relative affinity 100%) for the 38 other amine substrates listed in Table II where, for reasons of structural relationships, they are subdivided into seven categories (A–G), and some are tabulated more than once for easier comparison.

The primary ammonium ion in most of the compounds in Table II can be assumed to have pK_a values very similar to that in dansylcadaverine and could thus be directly compared with this reference. However, when this pK_a departs from that of dansylcadaverine, a correction has to be introduced to account for the difference in effective amine concentrations at the pH of experiments, and the corrected relative affinity values are also presented in Table II. The uncorrected $K_{i,app}^{-1}$ for dansylcadaverine (compound III), given as the 100% reference for "measured relative affinity", is approximately 4×10^4 M^{-1}. This corresponds to a corrected $K_{i,app}^{-1}$ of about 4×10^7 M^{-1} for the unprotonated primary amine species existing at the pH of the measurements (7.5), which is represented as the 100% reference on the "corrected relative affinity scale" in Table II.

On the whole, the data in Table II pertain to amines of very much higher affinities than those observed for simple alkylamines. *n*-Butylamine (pK_a = 10.6; Perrin, 1965), for example, gave a relative affinity (uncorrected as well as corrected) of only about 0.5% of dansylcadaverine and, as such, it is not even included in the tabulation. It is noteworthy that the $K_{i,app}^{-1}$ value measured for *n*-butylamine in our system (2×10^2 M^{-1} uncorrected and 2×10^5 M^{-1} corrected) agrees well with that found by Gross et al. (1977) for the competition of *n*-butylamine against the transglutaminase-catalyzed incorporation of labeled methylamine into the oxidized and acetylated B chain of insulin.

The specificities of spermine [$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$] and spermidine [$H_2N(CH_2)_3NH(CH_2)_4NH_2$], two polyamines widely distributed in nature (see Cohen, 1971), were also examined and uncorrected relative affinities of 35 and 70% were obtained, respectively.[1] However, when allowance is made for the different pK_a values of primary ammonium ions [7.92 and 8.81 in spermine, Hirschman et al. (1967); 7.92 and 9.3 in spermidine; and 10.6 in dansylcadaverine], the corrected affinities of spermine and spermidine become similar to that of *n*-butylamine and reduce to about 0.1 and 0.2% of the value for dansylcadaverine.

Discussion

Endo-γ-glutamine:ε-lysine transferases are Ca^{2+}-activated enzymes which catalyze amide replacement reactions on the γ-glutaminyl side-chain functions of protein or peptide substrates in endo positions. Catalysis takes place by an acylation–deacylation pathway and, as it has been shown explicitly for guinea pig liver transglutaminase (Folk & Chung, 1973) and for human fibrinoligase (Curtis et al., 1974), deacylation can be effected either by hydrolysis or aminolysis.

$$\text{RCOY} + \text{HS–E} \rightleftharpoons [\text{RCOY; HS–E}] \rightarrow \text{RCOS–E} + \text{HY}$$

$$\text{RCOS–E} + \text{H}_2\text{O} \rightarrow \text{RCOOH} + \text{HS–E}$$

$$\text{RCOS–E} + \text{H}_2\text{NR}' \rightleftharpoons [\text{RCOS–E; H}_2\text{NR}'] \rightarrow \text{RCONHR}' + \text{HS–E}$$

In the transamidation reaction, the amine serves as the second substrate for the enzyme (E–SH); thus, when considering amine specificities, note must be made of the fact that the reaction of the amine occurs with the acyl enzyme intermediate (RCOS–E). By use of synthetic substrate pairs such as β-phenylpropionylthiocholine and dansylcadaverine (at close to saturating concentration of the ester), it is possible to obtain apparent K_M values for amines. Measuring the aminolytic enhancement on the velocity of elimination of HY (e.g., thiocholine) would also have permitted evaluation of an index of amine specificity (Curtis et al., 1974). However, for the purpose of comparison of a large number of different amines, as presented in this paper, testing inhibition against the enzymatic incorporation of [1,4-^{14}C]putrescine into *N,N'*-dimethylcasein was deemed to be the simplest kinetic procedure. The chemical modification of the lysine side chains in casein served the purpose of preventing the cross-linking of this protein substrate onto itself.

Of the 39 amines listed in Table II, 23 comprised a pentylamine side chain totally analogous to that found in dansylcadaverine (III) and, of the remaining 16, seven shared the thiacadaverine moiety present in dansylthiacadaverine

[1] Thus, from the point of view of possible biological reactions in cells and tissues, the polyamines should be regarded as very important substrates for transglutaminase and related enzymes.

Table II: Apparent Relative Affinities of Amines for Guinea Pig Liver Transglutaminase, as Determined by Competitive Inhibition Kinetics for the Incorporation of Labeled Putrescine into *N,N'*-Dimethylcasein at pH 7.5 and 37 °C[b]

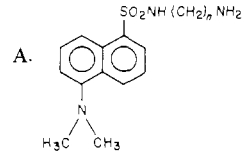
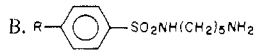
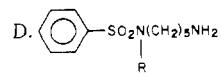
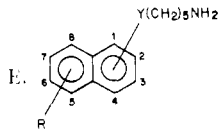
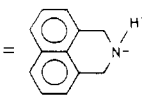
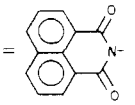
compd			lit. ref	rel affinity measured	rel affinity corrected[a]
					
I	$n = 3$		Nilsson et al. (1971)	2	2
II	$n = 4$		Nilsson et al. (1971)	60	60
III	$n = 5$		Lorand et al. (1968)	100	100
IV	$n = 6$		Nilsson et al. (1971)	44	44
					
V	R = -H			28	28
VI	R = $-CH_3$		Lorand et al. (1968)	25	25
VII	R = $-OCH_3$		Hoffmann et al. (1973)	23	23
VIII	R = -I		Hoffmann et al. (1973)	35	35
IX	R = $-NO_2$			34	34
	C. Ar-Y-$(CH_2)_5NH_2$				
VI	Ar = p-$H_3CC_6H_4$-	Y = SO_2NH		25	25
X	Ar = p-$H_3CC_6H_4$-	Y = NHCO	Stenberg et al. (1972)	6	6
XI	Ar = 2,4-$(NO_2)_2C_6H_3$-	Y = SNH		47	47
XII	Ar = 2,4-$(NO_2)_2C_6H_3$-	Y = SO_2NH		105	105
XIII	Ar = 2,4-$(NO_2)_2C_6H_3$-	Y = NH	Clarke et al. (1959)	20	20
IX	Ar = p-$NO_2C_6H_4$-	Y = SO_2NH		34	34
XIV	Ar = p-$NO_2C_6H_4$-	Y = NHCO		24	24
					
V	R = -H			28	28
XV	R = $-CH_3$			24	24
XVI	R = $-C_2H_5$			29	29
XVII	R = $-CH(CH_3)_2$			17	17
XVIII	R = $-CH_2C_6H_5$			31	31
					
XIX	R = -H	Y = 1-SO_2NH-	Stenberg et al. (1971)	37	37
III	R = 5-$N(CH_3)_2$	Y = 1-SO_2NH-		100	100
XX	R = 5-$(CH_3)_2NCH_2$-	Y = 1-SO_2NH-		41	41
XXI	R = 5-$(CH_3)_2N(CH_2)_3$-	Y = 1-SO_2NH-		54	54
XXII	R = -H	Y = 2-SO_2NH-	Stenberg et al. (1971)	40	40
XXIII	R = 6-CH_3-N(Ph)-	Y = 2-SO_2NH-		57	57
	F. $R(CH_2)_5NH_2$				
XXIV	R = $(C_6H_5CH_2)_2NH^+$-		Hoffmann et al. (1975)	156	16
XXV	R = 			422	42
XXVI	R = 			33	33
	G. R-$SO_2NHCH_2CH_2$-Y-$CH_2CH_2NH_2$				
VI	R = p-$H_3CC_6H_4$-	Y = CH_2		25	25
XXVII	R = p-$H_3CC_6H_4$-	Y = S		181	18
XXVIII	R = p-$H_3CC_6H_4$-	Y = $C(CH_3)_2$		2	2
VII	R = p-$H_3COC_6H_4$-	Y = CH_2		23	23
XXIX	R = p-$H_3COC_6H_4$-	Y = S		103	10
XXX	R = 2,4,6-$(CH_3)_3C_6H_2$-	Y = CH_2		63	63
XXXI	R = 2,4,6-$(i\text{-}C_3H_7)_3C_6H_2$-	Y = CH_2		95	95
XXXII	R = 2,3,4-$Cl_3C_6H_2$-	Y = S		198	20

Table II (*Continued*)

compd			lit. ref	rel affinity measured	rel affinity corrected[a]
	G. R-$SO_2NHCH_2CH_2$-Y-$CH_2CH_2NH_2$				
XXXIII	R = *p*-$C_6H_4N{=}NC_6H_4$-	Y = S		223	22
XIX	R = naphthalene-1-	Y = CH_2		37	37
XXXIV	R = naphthalene-1-	Y = S		214	21
XX	R = 5-$(CH_3)_2NCH_2$-naphthalene-1-	Y = CH_2		41	41
XXXV	R = 5-$(CH_3)_2NCH_2$-naphthalene-1-	Y = S		183	18
III	R = 5-$(CH_3)_2$N-naphthalene-1-	Y = CH_2		100	100
XXXVI	R = 5-$(CH_3)_2$N-naphthalene-1-	Y = S	Ljunggren et al. (1974)	767	77
XXXVII	R = 5-$(CH_3)_2$N-naphthalene-1-	Y = $C(CH_3)_2$		11	11
XXXVIII		Y = SO		21	2
XXXIX		Y = SO_2		20	2

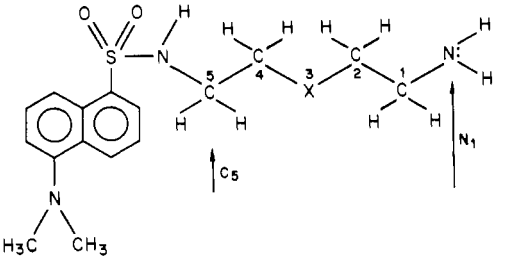
[a] Corrected for difference of the pK_a of the primary ammonium ion of the compound relative to that of dansylcadaverine. [b] Lorand et al., 1972a. Affinities are given in relation to dansylcadaverine (III) for which the reciprocal apparent inhibition constant ($K_{i,app}^{-1}$) is referred to as 100.

(XXXVI). Thus, the specificities of 22 compounds could be directly compared with dansylcadaverine and of six with dansylthiacadaverine, without the necessity of compensating for pK_a differences. However, when trying to relate the specificities of all amines to a single compound, we had to make corrections for differing pK_a values. Dansylcadaverine was chosen as standard and its $K_{i,app}^{-1}$ value of about 4×10^7 M^{-1} was taken as 100% on the affinity scale. In calculating corrected relative affinities, we made the following assumptions: (a) mono-*N*-acyl- and -*N*-sulfonylalkyldiamine derivatives which have a five methylene-carbon spacer between the primary amino group and the amide substituent have a pK_a for the primary amino group identical with that in dansylcadaverine [compound III; pK_a = 10.6 ± 0.1; Perrin (1965) and Ljunggren et al. (1974)]; (b) mono-*N*-acyl-3-thiacadaverine derivatives (where a $-CH_2-$ group in position 3 of the alkyl side chain is replaced by a –S–) have the same pK_a for the primary amino group as in dansylthiacadaverine [compound XXXVI; pK_a = 9.6 ± 0.1; Perrin (1965) and Ljunggren et al. (1974)], this being consistent with the observed acid strengthening effect of a vicinal S atom on the pK_a of a primary amino group in model compounds such as 2-(methylthio)ethylamine ($CH_3SCH_2CH_2NH_2$) with a pK_a of 9.2 instead of 10.6 as in butylamine (Jencks & Regenstein, 1970); (c) in *N*,*N*-bis(alkyldiamine) derivatives such as compounds XXIV and XXV of series F in Table II, because the tertiary amine is positively charged at the pH of the experiment, there will be a lowering of the pK_a of the primary amino group by about 1 pK_a unit compared to that in dansylcadaverine; a similar acid strengthening effect by a neighboring positive charge has been documented for simple alkylpolyamines, e.g., 4-(diethylamino)butylamine, $(C_2H_5)_2N(CH_2)_4NH_2$ (Perrin, 1965). Once corrections consistent with the above guidelines are made, relative affinities can be considered to reflect more accurately on the true chemical features of the amine specificity of the enzyme.

In discussing the results presented in Table II, it is convenient to refer to a primary and to a secondary binding site for the amine substrate.

Primary Binding Site. The data pertaining to compounds I–IV in Table II (series A) reveal that an optimal effect was obtained with an alkylamine side-chain length equivalent to five methylene groups similarly to what has been observed earlier with other endo-γ-glutamine:ε-lysine transferases (Lorand et al., 1968; Nilsson et al., 1971; Lorand & Nilsson, 1972; Myhrman, 1973). These findings are of fundamental significance in light of the specificity of the enzyme for lysyl side chains as expressed in the cross-linking of protein substrates by γ-glutamyl-ε-lysine bridges in biological systems (see Lorand, 1972; Williams-Ashman et al., 1972; Harding & Rogers, 1972; Asquith et al., 1974; Folk & Finlayson, 1977; Abernethy et al., 1977; Rice & Green, 1977; Siefring et al., 1978).

A comparison of the extended conformational length of amino alkyl side chains, undertaken with the help of CPK atomic models, shows that the approximate side-chain length between the primary amino group (N_1) and the C_5-methylene



X_3 = CH_2 or S

carbon is 7.2 Å in dansylcadaverine (compound III, X = CH_2) and 7.6 Å in dansylthiacadaverine (compound XXXVI, X = S). It should be emphasized that both of these compounds were found to be excellent substrates of the enzyme and that their side-chain length of 7.2–7.6 Å is comparable to the extended conformational distance between the N^{ϵ} and C^{α} atoms of lysyl residues (7.5 Å) in polypeptides. Dansylhexanediamine (IV) and dansylputrescine (II) with side-chain lengths of about 8.6 and 5.8 Å, respectively, show significantly lower affinities than dansylcadaverine (III).

Apart from the optimal requirement for side-chain length, this binding site on the enzyme seems to have rather narrow limits of tolerance with regard to the cross-sectional dimensions of the alkylamine moiety. Changing the bulk around position 3 while keeping the length of the side chain essentially unaltered permits the following conclusions. While substitution of $-CH_2-$ (approximate width 2.8 Å) by –S– (3.4 Å), as seen by comparison of compound III to XXXVI in the dansyl and compound VI to XXVII in the tosyl series, caused some drop in corrected affinities, introduction of the very much bulkier *gem*-dimethyl branching (width of about 4.7 Å) in the same position as in XXXVII for the dansyl and in XXVIII for the tosyl derivative (N_1–C_5 distance = 7.1 Å in both) produced a nearly 10-fold loss of affinity. A similar effect is seen in the dansylthiacadaverine series by substituting –SO– (compound XXXVIII) or $-SO_2-$ (XXXIX) for –S– (XXXVI)

where relative affinities for the former two dropped nearly 40-fold in comparison. These results suggest that the primary binding site for accommodating the amine substrate on the enzyme is a narrow crevice. The findings by Gross et al. (1977) regarding methyl branched aliphatic amine inhibitors, tested in conjunction with methylamine and acetylated B chain of oxidized insulin as substrates, lend themselves to a similar interpretation.

Data presented in series D of Table II show that, at a distance corresponding to about six atoms from the primary amino end of the substrate, the binding crevice on the enzyme can accommodate rather large substituents. The N-alkylation of the sulfonamide group in benzenesulfonylcadaverine did not adversely influence affinity even when the N substituent was a bulky benzyl group.

Secondary Binding Site. The significance of a hydrophobic locus in the extended amine binding domain of the enzyme can be best appreciated by considering the approximately 200-fold difference of affinity in favor of dansylcadaverine over *n*-butylamine. An illustration of the direct relationship between increased hydrophobicity and enhanced affinity is also provided by comparing three monoarylated cadaverines in series G; relative affinities increased with alkyl substitutions progressively from the tosyl (VI) to the mesitylenesulfonyl (XXX) and the 2,4,6-triisopropylbenzenesulfonyl derivative (XXXI) so that the latter displayed essentially the same affinity as dansylcadaverine (III). (2,4-Dinitrobenzenesulfonyl)cadaverine (XII) showed a similarly high affinity, but (2,4-dinitrophenyl)cadaverine (XIII) was about five times weaker which, together with other data in series C, suggests that the nature of the connecting segment (i.e., $-SO_2NH-$ vs. $-NHCO-$, $-SNH-$, or $-NH-$) between the aryl group and the alkylamine moiety may be of some importance.

All the simple para-substituted benzenesulfonylcadaverines (series B) had affinities very similar to each other and also to 1-naphthalenesulfonylcadaverine (XIX). Interestingly, however, the affinity of this compound was only about one-third that of dansylcadaverine (III), suggesting that the uncharged, dimethylamino group in the dansyl moiety contributes by reinforcing hydrophobic interactions. This is supported by the observation that isolating the dimethylamino group from the naphthalene ring by interposing methylene groups, as seen in compounds XX and XXI of series E, results in a considerable lowering of affinity. However, it should be pointed out that, while the dimethylamino group in dansylcadaverine is unprotonated ($pK_a \sim 2$) at the pH of experiments, the tertiary amines in the other two (i.e., XX and XXI) will be positively charged, and this alone might be counterproductive to binding.

As a whole, our results suggest that the hydrophobic aryl moieties of the substrates discussed are anchored to a site removed from the active center region of the enzyme. The function of the alkyl side chain is seemingly one of reaching toward the active center and positioning the primary amine nucleophile for attacking the carbonyl group of the thiol ester intermediate. As amply illustrated by the data in Table II, specificity requirements apply with respect to both the nature of the hydrophobic substituent as well as the length of the alkylamine side chain. Among the compounds examined, dansylcadaverine, (2,4,6-triisopropylbenzenesulfonyl)cadaverine, and (2,4-dinitrobenzenesulfonyl)cadaverine represent the most specific structures.

Finally, attention should be drawn to the utility of some of the synthetic compounds in Table II for the covalent modification of transmidase reactive γ-glutamine residues in protein substrates. Dansylcadaverine has already been used for introducing fluorescent labels into specific sites in fibrin (Lorand et al., 1972b; Fretto et al., 1978), casein and β-lactoglobulin (Lorand & Campbell, 1971; Lorand et al., 1971), cold-insoluble globulin (Mosher, 1975), α_2-macroglobulin (Mosher, 1976), proteins in erythrocyte ghost (Dutton & Singer, 1975; Lorand et al., 1975), proteins in sarcoplasmic reticulum (Dutton & Singer, 1975), myosin and actin (Cohen et al., 1979), rhodopsin (Pober et al., 1978), and even guinea pig liver transglutaminase itself (Lockridge, 1971). We are currently exploring the advantages offered by compounds containing haptenic groups (e.g., 2,4-dinitrobenzene; see series C in Table II) against which antibodies are readily available. This type of labeling will obviously facilitate the purification of transamidase specific protein substrates from tissues as well as the rapid isolation of the reactive γ-glutamine containing cross-linking fragments of these proteins.

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2′5′Oligo(A) Polymerase Activity and Inhibition of Viral RNA Synthesis in Interferon-Treated HeLa Cells†

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ABSTRACT: A quantitative assay for 2′5′oligo(A) polymerase, based on the adsorption of cell extract to poly(I)·poly(C)–agarose and incubation with [^{3}H]ATP, has been developed. The [^{3}H]2′5′oligo(A) synthesized is resolved from ATP by chromatography on DEAE-cellulose. The 2′5′oligo(A) polymerase activity has been measured in extracts of control HeLa cells and cells incubated with different concentrations of human fibroblast interferon or for different lengths of time with 100 units/mL of interferon. An increased enzymatic activity can be detected in cells treated with 12.5 units/mL or higher concentrations for 17 h and in cells treated with 100 units/mL longer than 3 h. The synthesis of encephalomyocarditis virus (EMC) RNA is inhibited in these cells in parallel with the increase in polymerase activity. Treatment of HeLa cells with interferon followed 2 h later by actinomycin D prevents the increase in 2′5′oligo(A) polymerase and the inhibition of viral RNA synthesis. After 4-h treatment with interferon, actinomycin D does not show this effect, whereas cycloheximide prevents the increase in polymerase activity. Active RNA synthesis 2 h after the start of interferon treatment and active protein synthesis 4 h afterward is necessary for the increase in 2′5′oligo(A) polymerase activity. Another enzymatic activity, previously reported to be increased in interferon-treated cells, is a double-stranded RNA activated protein kinase, which phosphorylates two polypeptides associated with ribosomes. Assays of this protein kinase have shown an increase in activity in cells treated with 12.5 units/mL or higher interferon concentrations. However, the increase in kinase activity can only be detected in cells treated for at least 10 h. In particular, HeLa cells treated for 7 h with 100 units/mL of interferon do not show increased protein kinase activity; upon such treatment, EMC RNA synthesis is already significantly inhibited. Therefore, an increased protein kinase activity may not be required for an inhibition of EMC RNA synthesis.

Hovanessian et al. (1977) have recently reported that, when double-stranded RNA (dsRNA)[1] is added to extracts of interferon-treated L cells, an enzymatic activity forms an inhibitor of protein synthesis from ATP. The inhibitor has been characterized by Kerr & Brown (1978) as pppA-(2′p5′A)$_{1-4}$2′p5′A$_{OH}$ or 2′5′oligo(A). Cell extracts adsorbed

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[1] Abbreviations used: ds, double stranded; pfu, plaque-forming units.

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