novan et al., 1981; Kayser et al., 1981; Michaels, 1979). It is noteworthy that studies in progress in this laboratory on the primary structure of *Stoichactis* toxin[3] have revealed no homology with toxin A-III; 142 of (approximately) 150 residues of the former protein have been definitely placed to date. It is therefore probable that factors beyond primary structure must be responsible for the ability of these two proteins to effect membrane penetration and disruption.

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Activity of Synthetic Thymosin α_1 C-Terminal Peptides in the Azathioprine E-Rosette Inhibition Assay†

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ABSTRACT: The helical C-terminal portion of the thymic hormone thymosin α_1 exhibits immunological activities in several in vitro assays. The C-terminal region spanning positions 17–28 was subdivided into 11 overlapping peptide segments to collect further information on the molecular signal hypothesis for T lymphocyte differentiation by thymosin α_1 derived peptides. All peptides were synthesized by classical means and tested in the azathioprine E-rosette inhibition assay. The results provided additional evidence that a basic–acidic–lipophilic sequence character is a possibly essential feature of a molecular signal for T cell differentiation. Five to seven structures beginning N terminally with lysine fitted this functional key. They showed immunological in vitro activities similar to and even better than the parent hormone thymosin α_1 in the ability to express in immature spleen cells from adult thymectomized mice the E-receptor sensitive to azathioprine inhibition.

During the past decade, elucidation of the function of the thymus gland in the immune system has been the focus of numerous investigations. Several polypeptide factors have been isolated, their structures have been determined, and for some, specific hormonal functions in the development of the immune system have been postulated (Low et al., 1979a,b; Bach et al., 1979; Ahmed et al., 1979; Low et al., 1981). Thymosin α_1, an octaeicosapeptide component of the thymus-derived peptide mixture thymosin fraction 5, has been reported to exhibit activity in several in vitro immunological assays (Low et al., 1979a,b; Birr et al., 1981a,b; Abiko et al., 1980a–c). Evidence suggests that it may play a regulatory role in latter stages of T lymphocyte differentiation (Low et al., 1979a,b; Ahmed et al., 1979). The synthesis of thymosin α_1 has been reported by several groups (Wang et al., 1979; Birr & Stollenwerk, 1979a,b; Wong & Merrifield, 1980; Abiko et al., 1980a–c) including a biological synthesis of the desacetyl derivative (Wetzel et al., 1980).

Although thymosin α_1 has exhibited activity in numerous assay systems, due to their nonuniformity and the probable multifunctional role of the peptide in lymphocyte differentiation, a relationship confining a specific biological function to a partial sequence or active site has not been established as it has been for the related thymic peptides thymopoietins I and II (Goldstein et al., 1979) and facteur thymique sérique (FTS) (Pleau et al., 1979; Imaizumi et al., 1981). Recent

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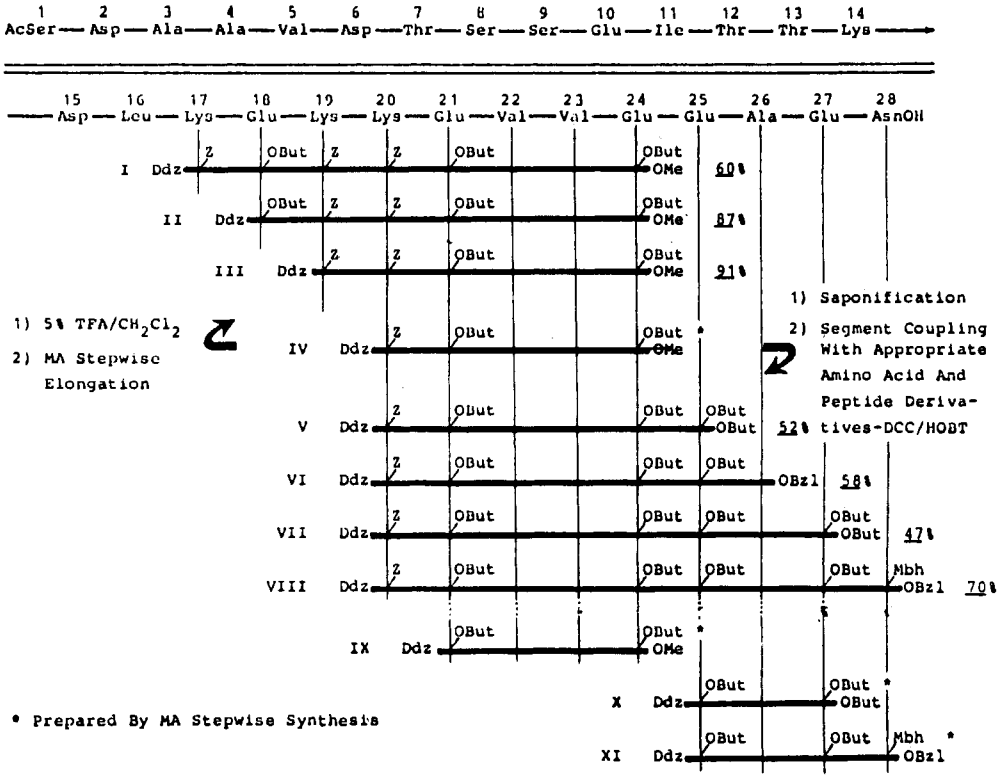

FIGURE 1: Synthesis of C-terminal thymosin α_1 peptides.

studies, however, have indicated that the C-terminal region of thymosin α_1 is important and perhaps essential for the exhibition of biologic activity in certain in vitro assay systems. Abiko and co-workers (Abiko et al., 1980a–c, 1979a,b) have demonstrated that longer C-terminal sequences (regions 14–28 and 19–28) exhibit partial activity when compared to the complete sequence in the restoration of E-rosette forming ability of human peripheral T lymphocytes from patients suffering from impaired immunological function due to minimal change nephrotic syndrome or chronic renal failure. We have reported that several shorter C-terminal sequences in the 17–28 region (Birr et al., 1981a,b; Ciardelli et al., 1981) and also some modified sequences of this region (Birr et al., 1981a,b) display varying degrees of activity in the allogenic one-way mixed lymphocyte reaction (MLR) and α-amanitine inhibited E-rosette assay. Both of these assays utilize peripheral T lymphocytes isolated from healthy human donors and are therefore dependent upon the present immune status of the individual and composition of the lymphocyte subpopulations. These differences often result in large variations in absolute activity values, making comparison of results obtained from assays performed with T lymphocytes from different donors difficult.

In the absence of a single definitive assay system for the determination of biologic activity of thymic factors and related peptides, it is important to consider the results obtained in several available assay systems before any specific biologic function is proposed. We report here the results of several peptides having sequences contained in the C-terminal region of thymosin α_1 evaluated in the azathioprine E-rosette inhibition assay (Dardenne & Bach, 1975). This bioassay has been utilized extensively for the study of FTS and of several of its analogues (Bach et al., 1979; Pleau et al., 1979). It has been applied to the quantitation of circulating thymic hormone activity in serum of normal individuals and a wide variety of patients with primary and secondary immunodeficiencies (Incefy et al., 1977; Iwata et al., 1981). The assay was employed in earlier studies with thymosin fraction 7 (Goldstein et al., 1972) and fraction 5 (Burton et al., 1978) as well as in a recent evaluation of the biologic activity of synthetic thymosin α_1 (Wong & Merrifield, 1980). Although, as is the case with other T cell surface markers, the function of the E-receptor is unknown, recent evidence indicates an involvement in the modulation of T cell immune responses (Van Wauwe et al., 1981).

Materials and Methods

Preparation of Thymosin α_1 C-Terminal Peptides. The synthesis of the C-terminal overlapping segments was performed by using classical solution peptide synthesis procedures as illustrated in Figure 1. The protected pentapeptide IV, previously prepared for the total synthesis of thymosin α_1 using the mixed anhydride (MA) method in combination with the [2-(3,5-dimethoxyphenyl)prop-2-yl]oxycarbonyl (Ddz) protecting group (Birr & Stollenwerk, 1979a,b), was used as a key intermediate for the synthesis of several other segment peptides. The protected peptides I–III were obtained from the protected pentapeptide IV by simple N-terminal stepwise elongation using the MA method as previously described (Birr et al., 1979a,b). Peptides V–VIII were obtained from IV by saponification of the C-terminal methyl ester and condensation of the resulting pentapeptide free carboxylic acid with the appropriate amino acid or peptide derivatives using the *N,N'*-dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBt) technique (König & Geiger, 1970). The small protected peptide derivatives required for the segment condensations as well as compound IX were also prepared by using the MA method. The strategy of maximum protection was employed throughout. C-Terminal carboxylic acid moieties were protected as either methyl (OMe), *tert*-butyl (OBu^t) or benzyl (Bzl) esters as illustrated. Side-chain carboxylic functions of the glutamic acid residues were protected as their *tert*-butyl esters and side-chain amino functions of the lysine residues as their benzyloxycarbonyl (Z) derivatives. The amide

function of asparagine was protected by the 4,4′-dimethoxybenzhydryl (Mbh) residue.

Preparation of Ddz-Lys(Z)-Lys(Z)-Glu(OBu^t)-Val-Val-Glu(OBu^t)OMe (III). (*a*) *Procedure A.* According to Birr et al. (1979a,b), 1 g of segment IV, Ddz-20–24-OMe, was dissolved in 14 mL of dichloromethane to which 0.7 mL of trifluoroacetic acid was added for N-terminal deprotection. After 20 min at ambient temperature, the mixture was neutralized with 1.03 mL of *N*-methylmorpholine.

(*b*) *Procedure B.* A 693-mg sample of Ddz-Lys (Z) was dissolved in 15 mL of dry dichloromethane. Protected from moisture the stirred solution was cooled to −15 °C, and 152 μL of *N*-methylmorpholine was added, followed by 162 μL of isobutyl chloroformate. The reaction was stirred for 8 min at −15 °C. Then the preparation from procedure A was added at once, and the mixture was agitated for a further 3 h without cooling. For workup the mixture was diluted with 100 mL of chloroform and extracted with 0.5 M $KHSO_4$ at 0 °C (!) followed by washings with 5% $KHCO_3$ and water. The organic layer was separated and evaporated at 30 °C to dryness. The residue was crystallized from ethyl acetate/ether; yield 1.125 g (91%); mp 216–218 °C dec.

This segment 19–24 (III) of thymosin α_1 by the same procedure was N-terminally elongated up to position 17 (segments I, II).

Preparation of Ddz-Lys(Z)-Gly(OBu^t)-Val-Val-Glu(OBu^t)-Glu(OBu^t)OBu^t (V). A 250-mg sample of Ddz-20–24-OH (from IV) and 73.5 mg of 1-hydroxybenzotriazole were dissolved in 20 mL of dry tetrahydrofuran and activated at 0 °C by addition of 50 mg of *N,N′*-dicyclohexylcarbodiimide. This mixture was allowed to react with 65 mg of Glu(OBu^t)OBu^t for 2 h at 0 °C and a further 4 h at ambient temperature.

After evaporation of the solvent, the residue was redissolved in ethyl acetate, the precipitated dicyclohexylurea was separated, and the filtrate was washed as in procedure B. For purification, the product in ethyl acetate was passed through basic alumina, which was further eluted with the same solvent. From evaporation of the eluate the hexapeptide was obtained as a colorless glass: yield 160 mg (52%); TLC (0.25-mm thin-layer chromatograms on Merck 60 F_{254} silica gel) R_f 0.91 [$CHCl_3$/CH_3OH/CH_3COOH, 85:10:5 (v/v)] and 0.58 [$CHCl_3$/C_2H_5OH, 9:1 (v/v)]. Amino acid analysis gave Glu 2.90 (3), Val 2.00 (2), and Lys 0.99 (1).

Preparation of Ddz-Lys(Z)-Glu(OBu^t)-Val-Val-Glu(OBu^t)-Glu(OBu^t)-AlaOBzl (VI). According to procedure B, 2.1 g of Ddz-Glu(OBu^t)·CHA, after liberation from the cyclohexylamine (CHA) salt, was activated via mixed anhydride formation with 468 μL of isobutyl chloroformate and allowed to react with 500 mg of AlaOBzl·HCl and 443 μL of *N*-methylmorpholine. The oily residue from evaporation was dissolved in ethyl acetate and filtered over basic alumina. From evaporation of the eluate, the dipeptide Ddz-Glu(OBu^t)-AlaOBzl was obtained as a colorless glass: yield 1.31 g (96%); TLC (conditions as above) R_f 0.51 [$CHCl_3$/CH_3COCH_3, 85:15 (v/v)] and 0.85 [$CHCl_3$/CH_3OH/CH_3COOH, 85:10:5 (v/v)].

According to procedure A, 176 mg of Ddz-Glu(OBu^t)-AlaOBzl was N-terminally deprotected, neutralized with 330 μL of *N*-methylmorpholine, and evaporated. The residue was redissolved in 2 mL of dimethylformamide and added to the solution of 250 mg of Ddz-20–24-OH (from IV) and 73.5 mg of 1-hydroxybenzotriazole in 3 mL of dimethylformamide, which had been activated at 0 °C for 10 min with 100 mg of dicyclohexylcarbodiimide. The segment condensation was stirred for 1 h at 0 °C and a further 3 days at ambient temperature. For workup and purification the preparation was processed as described for V. Heptapeptide VI was obtained as a colorless glass: yield 190 mg (58%); TLC (conditions as above) R_f 0.78 [($CHCl_3$/C_2H_5OH, 9:1 (v/v)]. Amino acid analysis gave Glu 3.06 (3), Ala 1.00 (1), Val 1.77 (2), and Lys 1.05 (1).

Segments VII and VIII were prepared from IV by the same procedure. Deprotection of compounds I–IX (Figure 1) was accomplished by conventional procedures. Treatment with anhydrous trifluoroacetic acid in the presence of anisole (Ddz, OBu^t, and Mbh removal) with prior saponification (OMe removal) and/or hydrogenolysis (Z and OBzl removal) where necessary provided the desired free peptides. After purification by ion-exchange chromatography using DEAE-Sephadex A-25 (OAc^- form) and aqueous acetic acid gradients, the peptides were homogeneous on thin-layer chromatography in three solvent systems and provided satisfactory amino acid analyses (see Results and Table I).

Azathioprine E-Rosette Inhibition Assay. The bioassay was employed as described by Dardenne & Bach (1975) and Iwata et al. (1979a,b). It is based upon differing sensitivities to azathioprine (AZ) demonstrated by spleen cells of adult thymectomized mice and normal mice. Certain thymic factors such as purified and synthetic FTS (Bach et al., 1978; Iwata et al., 1979b), thymosin α_1 (Wong & Merrifield, 1980), thymosin fraction 7 (Goldstein et al., 1972), and serum from young mice or normal young individuals (Bach et al., 1979; Iwata et al., 1981) have the ability to restore to normal the sensitivity to AZ that is lacking in spleen cells of adult thymectomized mice. Details of the assay were the same as previously described (Dardenne & Bach, 1975; Iwata et al., 1979a,b) with the following minor modifications. The lyophilized peptides were dissolved in enough sterile distilled water to reach a concentration of 10^{-3} M. Series of 10-fold dilutions to 10^{-11} M in Hanks balanced salt solution (Gibco, Grand Island, NY) were prepared and tested for each peptide. Each test dilution (60 μL) was mixed with AZ (60 μL of a 20 μg/mL solution in Hanks balanced salt solution) and a suspension of spleen cells (50 μL) from adult thymectomized C57B1/6 mice and incubated for 75 min at 37 °C. E-rosette formation and counting were performed as described (Dardenne & Bach, 1975). Each determination was performed in duplicate. A control sample consisting of the test peptide and spleen cell suspension but without AZ was carried out for each dilution.

Results

The analytical data obtained for the peptides prepared and tested in this study are exhibited in Table I.

In the assay, of the 11 segment peptides examined, 6 were able to induce azathioprine sensitivity under the conditions tested (Table II). Three of the peptides (17–24, 20–25, and 20–27) exhibited activity approximately equal to that of the parent peptide, thymosin α_1, which was typically active in the range 10^{-6}–10^{-7} M in this assay (Wong & Merrifield, 1980), and a synthetic sample, which was active at 10^{-6} M in this study. Three sequences (18–24, 20–24, and 20–28) exhibited somewhat lower activity at 10^{-5} M while the remaining five segments (19–24, 21–24, 20–26, 25–27, and 25–28) were unable to induce azathioprine sensitivity at concentrations as high as 10^{-3} M.

Discussion

In this bioassay azathioprine, an immunosuppressant, is utilized in vitro to inhibit the E-rosette forming ability of mouse

Table I: Analytical Data for Synthetic C-Terminal Thymosin α_1 Segment Peptides

peptide	$[\alpha]^{25}_D$ [a]	TLC: $R_f \times 100$ [b]			amino acid analysis [c]				
		1	2	3	Ala	Asp	Glu	Lys	Val
17-24	−92.0	6	9	0			3.00 (3)	3.06 (3)	1.38 (2)
							3.00	2.75	1.78
18-24	−75.4	14	18	2			3.00 (3)	2.00 (2)	1.80 (2)
							3.00	2.10	1.69
19-24	−63.4	14	17	3			2.00 (2)	2.07 (2)	1.83 (2)
							2.00	1.87	2.07
20-24	−56.0	23	38	6			2.00 (2)	0.96 (1)	1.74 (2)
							2.00	0.92	1.89
21-24	−49.4	40	61	28			2.00 (2)		1.76 (2)
							2.00		2.07
20-25	−73.0	19	29	5			3.00 (3)	1.03 (1)	1.85 (2)
							3.00	0.90	1.71
20-26	−60.0	20	31	5	0.69 (1)		3.00 (3)	0.98 (1)	1.72 (2)
							3.00	1.02	1.60
20-27	−75.4	18	25	3	0.95 (1)		4.00 (4)	1.07 (1)	1.97 (2)
					1.41		4.00	0.93	1.71
20-28	−72.0	14	20	2	1.06 (1)	1.06 (1)	4.00 (4)	1.03 (1)	1.84 (2)
25-27	−18	21	29	1	0.98 (1)		2.00 (2)		
					1.26		2.00		
25-28	−31.2	18	22	2	1.05 (1)	1.25 (1)	2.00 (2)		
					1.03	0.98	2.00		

[a] Specific rotation values expressed in degrees and determined at a concentration of 0.5% in 2 N NH_4OH. The value for segment 21-24 was determined at a concentration of 0.25% in the same solvent. [b] Solvent systems: (1) 1-butanol/pyridine/acetic acid/water, 5:5:1:4; (2) ethyl acetate/pyridine/acetic acid/water, 15:20:6:11; (3) 1-pentanol/pyridine/2-butanone/formic acid/water, 40:28:11:5:15. [c] Hydrolysis conditions: upper values, 12 N HCl/propanoic acid, 1:1, 30–50 min, 160 °C; lower values, 6 N HCl, 48 h, 110 °C.

Table II: Activity of Thymosin α_1 C-Terminal Peptides in the Azathioprine E-Rosette Inhibition Assay

position	sequence [a]	act. [b] (M)
17-24	KEKKEVVE	10^{-7}
18-24	EKKEVVE	10^{-5}
19-24	KKEVVE	IA
20-24	KEVVE	10^{-5}
21-24	EVVE	IA
20-25	KEVVEE	10^{-6}
20-26	KEVVEEA	IA
20-27	KEVVEEAE	10^{-6}
20-28	KEVVEEAEN	10^{-5}
25-27	EAE	IA
25-28	EAEN	IA
thymosin α_1		10^{-6}

[a] Sequence key: K = Lys, E = Glu, V = Val, A = Ala, and N = Asn. [b] Activity is expressed as the lowest concentration of peptide inducing in immature mouse spleen cells the E-rosetting ability sensitive to azathioprine inhibition. IA, inactive.

spleen cells. Cells from adult thymectomized mice are much less sensitive to this inhibition, and this is interpreted as a reflection of their lower level of differentiation due to the lack of thymic influence. The mechanism of azathioprine inhibition has not been entirely elucidated. As an immunosuppressant, the drug primarily affects cell-mediated immunity, although humoral immunity may also be significantly affected (Maibach, 1965; Svorcova et al., 1980). Azathioprine probably inhibits de novo purine biosynthesis in a manner similar to the related immunosuppressant 6-mercaptopurine. Any inhibition of the purine recycling enzymes adenosine deaminase and purine-nucleoside phosphorylase, deficiencies of which are known to result in immune defects (Osborne, 1981), apparently has yet to be reported. It has been previously suggested that inhibition of E-rosette formation by azathioprine is due to its antimetabolic properties (Verhagen et al., 1980). The reduction of θ antigenicity in some lymphocytes in vivo (Poulter et al., 1974) may result from a similar mechanism.

Although activity was detected in only some of the segment peptides tested, it was not possible from these results to locate a specific sequence or active site responsible for induction of azathioprine sensitivity in this assay. The three most active peptides did contain a common pentapeptide sequence (20–24) which was itself active at a higher concentration; however, two other segments (19–24 and 20–26) also contain this common sequence but showed no activity in this assay. Nevertheless, it is probable that this region of the molecule is important, though not alone sufficient for the activity of thymosin α_1 in this assay.

It may be of significance that five of seven segments beginning N terminally with lysine exhibited activity while of the remaining four peptides beginning N terminally with glutamic acid only one was active. The occurrence of a basic–acidic–lipophilic sequence of amino acids in thymus-derived peptides and its relationship (Birr et al., 1981a,b) to activity in E-rosette assay systems have been discussed by us and subsequently by R. Geiger.[1] The active pentapeptide of the thymopoietins (region 32–36, TP 5) (Goldstein et al., 1979) Arg-Lys-Asp-Val-Tyr incorporates such a relationship and does show activity in certain E-rosette assay systems (Nash et al., 1981; Abiko et al., 1980a–c) including one involving azathioprine inhibition (Verhagen et al., 1980). Longer segments containing the entire pentapeptide showed activity while those containing only a part of the pentapeptide sequence possessed no activity (Abiko et al., 1979a,b). Removal of one or more of the N-terminal basic residues resulted in inactive peptides while substitution of residues afforded varying results depending on the assays employed (R. Geiger;[1] Abiko et al., 1980a–c). However, in no case was a peptide found to be active that did not contain a basic–basic–acidic–lipophilic–lipophilic amino acid sequence.

In this study, all of the seven peptides beginning N terminally with lysine contain at least one basic–acidic–lipophilic sequence. As stated, five of these peptides were active in the assay. Of the four segments beginning N terminally with

[1] Personal communication and a lecture, 1980, Struktur von Peptiden mit Wirkung auf Lymphozyten, Zürich, Switzerland.

glutamic acid, three were inactive, and albeit the shortest segments tested, they did not possess a basic–acidic–lipophilic sequence. The single active N-terminal glutamic acid segment (18–24) did include such a sequence. The failure of the two lysine N-terminal fragments (19–24 and 20–26) to show activity is difficult to interpret. The requirements for activity in this assay system are certainly more subtle than this simple sequence relationship. A correct spatial electronic presentation, essential for binding to a receptor site, is dependent upon both sequence and conformation, the latter being subject to other allosteric influences.

At its first appearance, FTS does not seem to contain such a sequence relationship at all, yet it is highly active in this assay. Recently, however, a pentapeptide sequence essential for activity in this assay has been reported for FTS (Imaizumi et al., 1981). This peptide segment 3–7 of the FTS sequence, Lys-Ser-Gln^5-Gly-Gly, does begin N terminally with lysine followed by hydrophilic and then more lipophilic amino acid residues. It is of additional significance that a FTS analogue containing glutamic acid substituted for glutamine in position 5 retains full biologic activity while substitution of the lipophilic amino acid norvaline at the 5 position results in an inactive analogue (Blanot et al., 1979). Therefore, FTS and particularly the Glu-5 analogue may, in fact, be related to the previously mentioned thymus-derived peptides through sequence similarities.

The results obtained in this study did confirm that the C-terminal region of thymosin α_1 contains molecular signal functions important in T lymphocyte differentiation. They provided additional evidence in support of the suggestion (Birr et al., 1981a,b) that a basic–acidic–lipophilic sequence of amino acids is of significance in the interaction of small thymus-related peptides with T cells, as established in vitro on immature spleen cells by the restoration of their E-rosetting ability sensitive to azathioprine inhibition.

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