Structure and Action of Heteronemertine Polypeptide Toxins. Membrane Penetration by *Cerebratulus lacteus* Toxin A-III†

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ABSTRACT: The heteronemertine worm *Cerebratulus lacteus* produces a family of three structurally homologous proteins that function as direct lytic factors for a variety of cells [Kem, W. R., & Blumenthal, K. M. (1978) *J. Biol. Chem. 253*, 5752–5757]. It is demonstrated herein that the hemolytic activity of the most abundant variant, designated toxin A-III, is unaffected by either extensive iodination or complete blockage of carboxylate groups by tyramine or glycine ethyl ester. Iodination of A-III with lactoperoxidase produced a derivative that is preferentially labeled at His-67 and to a lesser extent at Tyr-6. The ratio of labeling at these two positions is approximately 3 to 1. Iodinated A-III is completely insoluble in 10% Cl_3CCOOH. However, following treatment with trypsin-containing liposomes, 15% of the input counts are converted to a Cl_3CCOOH-soluble form. Incubation with free trypsin in the presence of liposomes containing N^{α}-tosyl-L-lysine chloromethyl ketone results in approximately 60% of the input counts becoming Cl_3CCOOH soluble. Free trypsin renders toxin A-III 90% soluble in 10% ClCCOOH. Electrophoretic analysis of the labeled tryptic peptides generated in the presence of liposomes shows that internal trypsin hydrolyzed the Arg-13–Ser-14 bond, generating exclusively peptide T-1 (residues 1–13) while external trypsin produces peptide T-11 (residues 60–71) as the major radioactive product. These data are consistent with insertion of at least the amino-terminal 13 residues of A-III into the liposome and imply that membrane penetration by this protein may be important for its cytolytic activity.

Extracts of mucus secreted from the skin of the Atlantic coast heteronemertine *Cerebratulus lacteus* contain a number of small proteins of biological interest. Two classes of toxins, the cytolytic A toxins (Kem & Blumenthal, 1978) and the neurotoxic B toxins (Kem, 1976), have been purified from these secretions in recent years.

The *Cerebratulus* A toxins have molecular weights of about 10 000 and are highly basic and cross-linked proteins (Kem & Blumenthal, 1978). The complete covalent structure of toxin A-III, the most abundant homologue, has recently been determined (Blumenthal & Kem, 1980; Blumenthal, 1980). The toxin is a single polypeptide chain of 95 amino acid residues cross-linked by three disulfide bonds, Cys-17–Cys-38, Cys-23–Cys-34, and Cys-48–Cys-61. However, there is as yet little or no information on structure–function relationships either in toxin A-III or in other cytolytic proteins.

Functionally, it has been shown that low concentrations of A-III are capable of causing membrane disruption in a variety of cells. Human erythrocytes are 50% lysed at an A-III concentration of 3 μg/mL while Ehrlich ascites cells are approximately 3-fold more sensitive. Posner and Kem have shown that sublytic concentrations of A-III (0.25–1.0 μg/mL) cause reversible depolarization of Cardiac Purkinje fiber membranes (Posner & Kem, 1978); higher concentrations cause irreversible depolarization and loss of intracellular components. Toxin A-III has been shown to be devoid of phospholipase A activity (Kem & Blumenthal, 1978).

The mechanism by which A-III causes cytolysis remains unclear, although the low concentration required for effect would seem to argue against a detergent-type action. Specific interaction of A-III with a cell surface molecule has yet to be demonstrated. Marker-release studies in progress in this laboratory have shown that A-III, in the concentration range of 10–100 μg/mL, causes release of $^{22}Na^+$ and [^{3}H]sucrose from liposomes containing sphingomyelin, phosphatidylcholines, or phosphatidylserine, in addition to cholesterol and dicetyl phosphate. Activity studies with this toxin have shown that, of these lipids, sphingomyelin alone is an effective inhibitor of A-III-induced hemolysis (Kem et al., 1980).

We have been interested in the mechanism of A-III-induced lysis in the hope that the toxin could provide a well-characterized and easily manipulated model for the interaction of polypeptides with membranes. In the present study we show that A-III is capable of interaction with preformed sphingomyelin containing membranes and that a portion of the toxin's sequence can become exposed on the interior of sphingomyelin liposomes. A preliminary report of these data has appeared (Blumenthal & Kreider, 1982).

Experimental Procedures

Materials. C. lacteus toxin A-III, purified as described previously (Kem & Blumenthal, 1978), was repurified by gel filtration on Sephadex G-50 prior to use in these studies. Trypsin, treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone, was purchased from Worthington. Cholesterol, dicetyl phosphate, bovine brain sphingomyelin, tyramine, and lacteroperoxidase were obtained from Sigma. [1-^{14}C]Glycine ethyl ester (40–60 mCi/mmol) and $Na^{125}I$ (carrier free) were products of New England Nuclear. Cellulose TLC[1] sheets were purchased from Schleicher and Schuell, and N^{α}-tosyl-L-lysine chloromethyl ketone was from Calbiochem. Fresh human erythrocytes, obtained by venipuncture, were washed as described by Kem & Blumenthal (1978) prior to use for hemolysis assay. All other reagents were of the purest grade commercially available.

Preparation of Liposomes. Liposomes were prepared essentially as described by Huang & Thompson (1974). Lipids were dissolved in either chloroform or chloroform–methanol

† From the Department of Biological Chemistry, College of Medicine, University of Cincinnati, Cincinnati, Ohio 45267. *Received February 22, 1982.* These studies were aided by a grant from the National Science Foundation (PCM80-20330). K.M.B. is a Research Career Development awardee of the National Institute of Neurological and Communicative Disorders and Stroke (5-K04-00304).

[1] Abbreviations: TLCK, N^{α}-tosyl-L-lysine chloromethyl ketone; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; BSA, bovine serum albumin; BAPA, benzoylarginine-*p*-nitroanilide; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; PTH, phenylthiohydantoin.

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(1:1) and stored at −20 °C. For liposome preparation, a mixture of sphingomyelin, cholesterol, and dicetyl phosphate (20:15:2 molar ratio) was dried under a nitrogen stream and then resuspended at a phospholipid concentration of 4 mg/mL in 0.5–1.0 mL of 10 mM Tris-HCl, pH 7.2, containing 0.145 M NaCl (Tris–saline) and 1 mg/mL either trypsin or TLCK. The suspension was then vortexed to homogeneity at room temperature and subjected to gel filtration on columns of Sephadex G-50 (TLCK–liposomes) or Sephacryl S-200 (trypsin–liposomes). Columns were pretreated with blank liposomes to minimize irreversible adsorption. The material eluting at the void volume of either column was used without further purification. The trapping efficiency in this procedure was 0.5–1.0% as assessed by entrapment of $^{22}Na^+$, [^{3}H]sucrose, or ^{86}Rb.

Chemical Modification of Toxin A-III. Coupling of toxin carboxylate groups to either tyramine or glycine ethyl ester was carried out at pH 4.8 in the presence of 0.1 M 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide as described by Carraway & Koshland (1972) and desalted by gel filtration on Sephadex G-25. Incorporation of either blocking group was quantitated by amino acid analysis, tyramine eluting from the amino acid analyzer between ammonia and arginine (66.9 min).

Native toxin A-III, or tyramine coupled A-III, was iodinated enzymically with lactoperoxidase as described elsewhere (Bolton, 1977). Iodinated proteins were desalted either by gel filtration in the presence of 0.1 mg/mL bovine serum albumin or by dialysis against Tris–saline. In one experiment, aimed at assessing the effect of more exhaustive iodination of A-III on biological activity, iodination was done with iodine monochloride as described by Roholt & Pressman (1972).

Analytical Methods. The extent of A-III modification by tyramine was assessed by amino acid analysis, following acid hydrolysis at 108 °C for 22 h in vacuo. The relative degree of iodination at Tyr-6 and His-67 was determined by subjecting samples of the iodinated, reduced, and carboxymethylated protein to automated Edman degradation following complete hydrolysis with trypsin. Sequence runs employed a Beckman 890C sequencer equipped with a Sequemat autoconverter and used dimethylallylamine program 102974 supplied by the manufacturer. The PTH derivatives obtained were then subjected to liquid scintillation counting.

The activity of modified A-III samples was compared to that of native toxin by using the hemolysis assay described by Kem & Blumenthal (1978); in these studies, the A-III concentration was determined spectrophotometrically (E_{280} = 13 500) while that of tyramine A-III was done by amino acid analysis.

Trypsinization Studies. Hydrolysis was carried out for 2 h at 37 °C in Tris–saline buffer, pH 7.4, and was initiated by addition of labeled toxin. Liposomes containing trypsin were suspended in Tris–saline containing 1 mg/mL TLCK prior to addition of toxin. For TLCK–liposomes, the external trypsin concentration was adjusted to 2 μg/mL (after addition of toxin), the same trypsin concentration used for solution digestion of A-III. At the end of hydrolysis, each reaction mixture was made 0.5 mg/mL in BSA, mixed with an equal volume of 20% trichloroacetic acid, filtered on Millipore HAWP filters, and washed twice with 1 mL of 10% Cl_3CCOOH. The washed filters were then subjected to liquid scintillation counting.

Results

Activity of Modified A-III. Toxin A-III was iodinated with either lactoperoxidase–H_2O_2 or ICl as described under Experimental Procedures. With ICl, the specific radioactivity of the desalted protein indicated an incorporation of about 0.9 g-atom of iodine/mol of toxin, whereas with lactoperoxidase the level of incorporation varied from 5 to 10% of this value. Hemolysis assays performed on ICl treated toxin (Figure 1) show its activity to be indistinguishable from that of native A-III assayed in parallel. Thus, trace iodinated A-III almost certainly retains full hemolytic potency. All subsequent studies employed lactoperoxidase-labeled toxin.

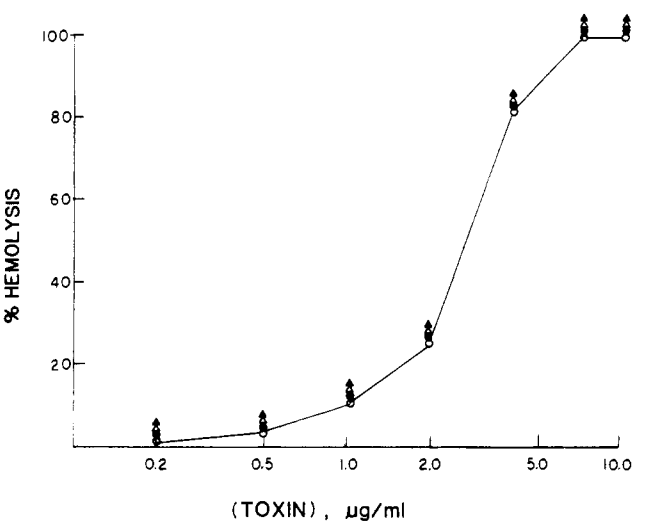

FIGURE 1: Lysis of human erythrocytes by native (○), ICl-treated (●), tyramine-treated (△), or glycine ethyl ester treated (▲) toxin A-III. Assays were carried out as described by Kem & Blumenthal (1978).

Table I: Amino Acid Composition of Native and Modified A-III

amino acid	residues per mol		
	native[a]	glycine ethyl ester	tyramine
aspartic acid	6	6.1	6.1
threonine	3	2.8	2.7
serine	8	7.6	7.4
glutamic acid	4	4.1	3.9
proline	2	2.2	2.2
glycine	*12*	*15.7*	*12.0*
alanine	13	13.0	12.8
cysteine[b]	6	5.6	5.1
valine	7	6.0	6.0
isoleucine	6	5.0	5.3
leucine	5	5.1	5.1
tyrosine	1	0.9	1.1
phenylalanine	2	2.0	2.0
histidine	1	1.0	0.9
lysine	14	13.8	13.7
arginine	3	2.9	2.9
tryptophan	2	nd	nd
tyramine			*4.1*

[a] Calculated from the amino acid sequence (Blumenthal & Kem, 1980). [b] Determined as *S*-(carboxymethyl)cysteine.

Toxin samples treated with either glycine ethyl ester or tyramine in the presence of EDC were prepared as described under Experimental Procedures. After desalting and acid hydrolysis, their amino acid compositions (Table I) clearly indicate incorporation of 4 mol of either blocking group/mol of toxin. Thus, the four carboxylates known to exist in A-III (Blumenthal & Kem, 1980) have been quantitatively blocked. Hemolysis assay of these samples (Figure 1) reveals that they retain full biological activity. Thus, none of the toxin carboxylate groups are essential for cytolysis.

Characterization of Iodinated A-III. Lactoperoxidase is known to cause iodination of both tyrosine and histidine res-

Table II: Sequence Analysis of Iodinated A-III Tryptic Digest[a]

Edman cycle	cpm released $\times 10^{-3}$	Edman cycle	cpm released $\times 10^{-3}$
1	2.5	6	46.1
2	2.7	7	13.5
3	2.7	8	149.8
4	2.6	9	43.1
5	5.7	10	6.0

[a] Iodinated A-III was reduced and alkylated, mixed with 1.0 mg of BSA, and hydrolyzed with trypsin prior to application to the sequencer. Approximately 50% of the input counts are released in cycles 1-10.

idues, of which toxin A-III contains one each, widely separated in the linear sequence of the molecule (Tyr-6 and His-67). Since the position of labeling was of critical importance for interpretation of the trypsinization data, the trace-iodinated A-III was analyzed further.

Iodinated A-III was reduced and alkylated and hydrolyzed with trypsin, and the resulting peptides were analyzed by thin-layer chromatography and electrophoresis. Autoradiography of the resulting fingerprint showed the presence of one major and one minor spot (data not shown), consistent with iodination of both potential sites in the toxin.

Since the fingerprints indicated iodination at two sites, it became important to determine the degree of labeling at each. A sample of iodinated, reduced, and alkylated A-III, containing 550 000 cpm, was mixed with 1.0 of mg BSA and hydrolyzed for 4 h at 37 °C with 25 μg of trypsin. The hydrolysate was then subjected to 10 cycles of automated Edman degradation and the radioactivity released in each cycle quantitated (Table II). Approximately 50% of the total radioactivity applied to the cup was released in the first 10 cycles. Of this, 46 140 cpm (16.8%) was found at step 6 while 149 772 cpm (54.5%) was found at step 8.

Examination of the sequence of toxin A-III (Blumenthal & Kem, 1980) shows the single toxin tyrosine to be located at position 6 of peptide T-1 while His-67 is found at position 8 of peptide T-11. Thus, the radioactive PTH-amino acids released at cycles 6 and 8 must represent Tyr-6 and His-67, respectively. Unexpectedly, the preponderance of the label was found associated with His-67. The ratio of [^{125}I]iodo-His-67 to [^{125}I]iodo-Tyr-6 was calculated as approximately 3.25 to 1, on the assumption that the counts found at positions 7 and 9 represent carry over from steps 6 and 8, respectively.

Fingerprint analysis of the tryptic peptides derived from tyramine-treated, iodinated A-III revealed the presence of four labeled spots, as expected. No attempt was made to assess the level of iodination at each site.

Trypsinization Studies. It is now recognized that a number of proteins are capable of posttranslational penetration of membranes. If any portion of the A-III sequence is capable of crossing a liposomal membrane, then tryptic sites in the toxin may become accessible to encapsulated trypsin. Trypsin-containing liposomes were prepared as described under Experimental Procedures and separated from free trypsin on Sephacryl S-200 (Figure 2). Assay of column fractions with the chromogenic trypsin substrate benzoylarginine-*p*-nitroanilide showed no detectable hydrolysis by the liposome peak after 2 h at 37 °C, at which time hydrolysis by peak 2 was complete. After 16 h, a small amount of hydrolysis was observed in the liposome region (approximately 5% of maximum). Since this hydrolysis was not blocked by the presence of 0.1 mg/mL TLCK, we ascribe it to leakage of substrate into the liposome rather than to external trypsin adsorbed to

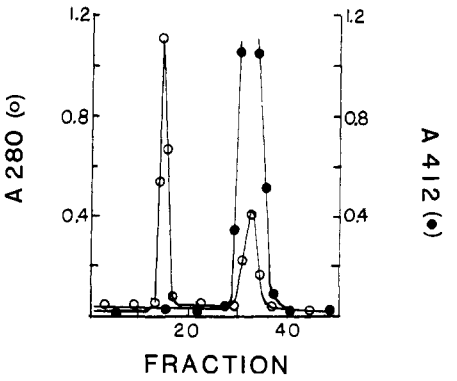

FIGURE 2: Purification of trypsin-containing liposomes. Liposomes, prepared as described under Experimental Procedures, were separated from free trypsin on a column (0.9 × 50 cm) of Sephacryl S-200 equilibrated with Tris–saline buffer. Fractions were monitored for absorbance at 280 nm (○) and for trypsin activity (●) with the chromogenic model substrate BAPA.

Table III: Hydrolysis of A-III by Free and Liposomal Trypsin[a]

expt	conditions	% Cl_3CCOOH soluble
1	A-III alone	0
2	A-III + trypsin (2 μg/mL)	90
3	A-III + trypsin-liposomes + TLCK (1 mg/mL)	15
4	A-III + trypsin (2 μg/mL) + TLCK-liposomes	55–60

[a] Digestions were initiated by addition of iodinated A-III in experiments 1-3 and by addition of trypsin in experiment 4. All reactions were carried out for 2 h at 37 °C.

the membrane. These results indicate both that entrapment of trypsin has taken place and that removal of free trypsin by the column step is complete.

Hydrolysis of iodinated A-III by trypsin-containing liposomes was initiated by addition of toxin to a liposome suspension in Tris–saline containing 1 mg/mL TLCK. For TLCK–liposomes, toxin was added 5 min prior to the addition of trypsin to allow time for toxin–liposome interaction to occur. Marker release studies with sphingomyelin liposomes have shown that this is ample time for toxin–liposome interaction to occur.[2] Table III shows that free trypsin converts 90% of labeled A-III to a Cl_3CCOOH-soluble form; in the absence of trypsin or in the presence of trypsin and TLCK essentially all of the label remains Cl_3CCOOH insoluble. Trypsin-containing liposomes, in the presence of external TLCK, convert 15% of the label to Cl_3CCOOH-soluble material, and this value is highly consistent from experiment to experiment. Conversely, external trypsin, in the presence of TLCK–liposomes, solubilizes 60–65% of the input counts.

Sequencer data has shown that a maximum of 23% of the ^{125}I is at Tyr-6 while a maximum of 77% is found at His-67. Solubilization of counts by internal trypsin indicates that some portion of the A-III sequence must span the liposomal membrane. The fact that only 15% of the input counts are solubilized by internal trypsin strongly suggests that the peptide released in this experiment is T-1 while generation of peptide T-11 by external trypsin is responsible for its solubilization of 60–65% of the input label. Thus, the amino-terminal end of toxin A-III must span the membrane with at least residues 1–13 being located inside the liposome, Arg-13 being the first trypsin-sensitive site in the protein.

[2] K. M. Blumenthal, unpublished experiments.

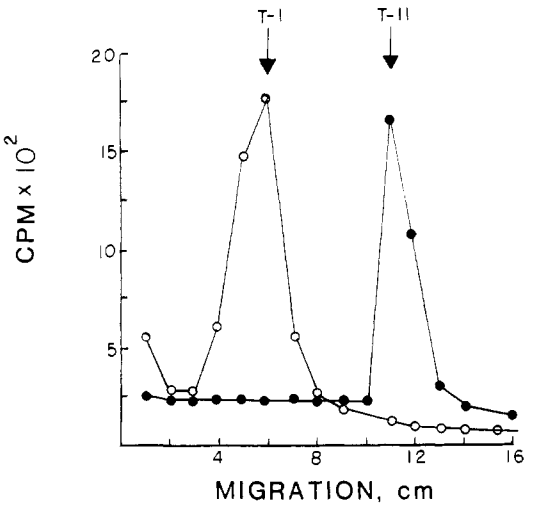

FIGURE 3: Analysis of labeled peptides. Iodinated A-III was subjected to trypsinization by trypsin–liposomes (○) or by free trypsin in the presence of TLCK–liposomes (●), and the digests were separated into liposomal and supernatant fractions by centrifugation. These were analyzed by paper electrophoresis at pH 1.9. The electrophoretic mobilities of iodinated peptides T-1 and T-11 of toxin A-III are indicated by vertical arrows.

Identification of Tryptic Peptides. To verify that the peptides generated in the trypsinization studies were indeed T-1 and T-11, we first centrifuged digests to remove liposomes. Pellets and supernatants were then analyzed by paper electrophoresis at pH 1.9, following reduction with β-mercaptoethanol, and compared to iodinated samples of authentic T-1 and T-11 obtained during sequence analysis of toxin A-III (Blumenthal & Kem, 1980). Figure 3 shows that the pellet from the trypsin–liposomes contains a single radioactive peak having a mobility identical with that of iodinated T-1 while the supernatant from the external trypsin digest contains largely iodinated T-11. It should be noted that only a small aliquot of the supernatant was spotted to obviate the electrophoretic streaking caused by spotting large amounts of salt. This accounts for the apparently small amount of T-11 observed; in fact, this supernatant contains 3–4 times as many counts as does the trypsin–liposome pellet.

The trypsin–liposome supernatant has a large number of counts in a diffuse streak reaching back to the origin, as would be expected for high molecular weight material (data not shown). The remaining pellet (TLCK–liposomes) lacks T-1 but has a relatively small number of counts migrating in the position of T-11. It is likely that these counts represent T-11 that is adsorbed to the liposomes, since the number of counts found varies with the input counts in the experiment.

Discussion

The results presented herein offer conclusive evidence that a portion of the N-terminal sequence of *C. lacteus* toxin A-III is capable of being inserted into and spanning the membrane of a sphingomyelin liposome. Sphingomyelin was chosen because liposomes containing this phospholipid have been found to be sensitive to the effects of A-III in marker release studies.[2] In addition, the nonessentiality of toxin carboxylate groups for cytolysis has been demonstrated, and we have shown that iodination of the single tyrosine and histidine residues does not diminish toxicity.

The most interesting result described herein is the partial digestion of A-III by trypsin-containing liposomes. It should thus be emphasized that nontrapped trypsin has been completely eliminated from the liposome preparation by Sephacryl S-200 gel filtration. No hydrolysis of the chromogenic trypsin substrate BAPA by these liposomes is detectable after a 2-h incubation at 37 °C in either the presence or absence of TLCK. After 16–18 h at 37 °C, a small degree of hydrolysis is observed that is insensitive to TLCK, suggesting that this hydrolysis results from an inward leakage of the substrate but not the inhibitor. Transmembrane movement of these solutes has not been directly assayed, since BAPA hydrolysis is undetectable over the time course of A-III hydrolysis by these liposomes.

Treatment of iodinated A-III with trypsin–liposomes for 2 h at 37 °C results in conversion of about 15% of the input label to a Cl_3CCOOH-soluble form. Analysis of this digest by pH 1.9 paper electrophoresis (Figure 3) shows that a single radioactive spot having electrophoretic mobility identical with that of iodinated peptide T-1 (residues 1–13) remains associated with the liposomes after centrifugation. The supernatant from this digest contains only high molecular weight material as judged by its nonmigration at pH 1.9. Since 16–23% of the iodine incorporated into A-III is at Tyr-6, these data show that at least the first 13 residues of A-III can cross the membrane of a preformed sphingomyelin liposome, rendering the Arg-13–Ser-14 bond accessible to internal trypsin.

Treatment of A-III with 2 μg/mL free trypsin in the presence of liposomes containing TLCK causes solubilization of 60–65% of the input label after 2 h at 37 °C. Electrophoretic analysis of the peptides generated in this digest shows that most of the counts are present as peptide T-11 (residues 60–71), the only histidine-containing peptide in A-III, and that most of the counts remain in the supernatant after removal of liposomes by centrifugation. Since most of the iodination of A-III occurs at His-67, these data indicate that a significant portion of the C-terminal end of the molecule remains outside the liposome while part of the N-terminal end is internalized.

Model studies with iodinated insulin A indicate that a peptide of M_r of about 2300 would be partially (about 50%) soluble in 10% Cl_3CCOOH. Thus, the fact that more extensive solubilization of input label does not occur in the trypsin-out experiments suggests that Lys-19 and Lys-20 are not available to external trypsin. Whether this indicates that they are inside the liposome or protected by close association with the membrane cannot be determined from our data.

In our studies of A-III structure, it was pointed out that the carboxy-terminal portion of the chain had a significantly higher degree of hydrophobicity than did the amino end. This raised the question of whether the C-terminal end might have a functional role in membrane interaction, particularly since Chou–Fasman (Chou & Fasman, 1978) analysis suggested it to have a helical or hairpin helix conformation of the type recently proposed to be important for penetration of membranes by proteins (Engelman & Steitz, 1981). The experiments described herein indicate that, in contradistinction to the earlier hypothesis, it is the N-terminal end of A-III that is important for membrane penetration.

It is now well-known from both chemical and electrophysiologic studies that a number of protein toxins are capable of penentrating and/or aggregating within preformed membranes. Studies with membranes containing a photoreactive glycolipid have shown membrane penetration by the A_1 subunit of cholera toxin (Wisnieski & Bramhall, 1981). Bhakdi et al. (1981) have shown that hydrophilic monomers of staphylococcal α-toxin are induced to form amphiphilic hexamers upon association with detergent micelles. Electrophysiologic studies have shown that diphtheria toxin and *Stoichactis helianthus* cytotoxin cause formation of transmembrane channels in lipid membranes containing lecithin and cholesterol (Do-

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.

Acknowledgments

The expert technical assistance of Nancy Kreider was invaluable in the performance of these experiments. I am also indebted to Dale Blankenship for his excellent work on amino acid analysis and sequence determinations and to Marcia McLachlan for preparation of the manuscript.

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[3] K. M. Blumenthal and W. R. Kem, unpublished experiments.

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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0006-2960/82/0421-4233$01.25/0