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THE BIOSYNTHESIS OF MULTI-L-ARGINYL-POLY(L-ASPARTIC ACID) IN THE FILAMENTOUS CYANOBACTERIUM *ANABAENA CYLINDRICA*

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Summary

The cyanobacteria produce multi-L-arginyl-poly(aspartic acid), a high molecular weight (M_r = 25 000—125 000) branched polypeptide consisting of a poly(aspartic acid) core with L-arginyl residues peptide bonded to each free carboxyl group of the poly(aspartic acid) An enzyme which will elongate Arg-poly(Asp) has been isolated and purified 92-fold from the filamentous cyanobacterium *Anabaena cylindrica* The enzyme incorporates arginine and aspartic acid into Arg-poly(Asp) in a reaction which requires ATP, KCl, $MgCl_2$, and a sulfhydryl reagent The enzymatic incorporation of arginine is dependent upon the presence of L-aspartic acid but not visa versa, a finding which suggests the order of amino acid addition to the branched polypeptide-aspartic acid is added to the core followed by the attachment of an arginine branch The elongation of Arg-poly(Asp) in-vitro is insensitive to the addition of protein synthesis inhibitors and to the addition of nucleases. These findings support the notion previously suggested from in-vivo studies that Arg-poly(Asp) is synthesized via a non-ribosomal route and also demonstrate that amino-acetylated transfer-RNAs play no part in at least one step of the biosynthetic mechanism

Introduction

The cyanobacteria produce a characteristic subcellular inclusion known as the cyanophycin or structured granule [1,2] This inclusion contains a high molecular weight branched polypeptide (M_r = 25 000—125 000) whose structure consists of a poly(L-aspartic acid) core with L-arginyl residues peptide bonded to each free carboxyl group of the poly(aspartic acid) core [3,4] Since

Abbreviations Arg-poly(Asp), multi-L-arginyl-poly(L-aspartic acid), ATP, adenosine-5'-triphosphate, EDTA, ethylenediaminetetraacetic acid, POP, 2,5-diphenyloxazole, dimethyl-POPOP, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene, Tris, tris(hydroxymethyl)aminomethane

the molar ratio of arginine to aspartic acid in the polypeptide is 1 1, each arginine branch is only one residue long Because of the similarity in structure to synthetically produced multichain poly(amino acids) [5,6], the branched polypeptide from the cyanobacteria has been called multi-L-arginyl-poly(L-aspartic acid) (Arg-poly(Asp)) [4].

In vivo studies suggest that the pathway of Arg-poly(Asp) biosynthesis differs from the normal route of protein synthesis [7]. The addition of the protein synthesis inhibitor chloramphenicol to an exponentially growing culture of the filamentous cyanobacterium *Anabaena cylindrica* results in the intracellular accumulation of Arg-poly(Asp) This observation indicates that the synthesis of Arg-poly(Asp) is non-ribosomal, although the involvement of parts of the normal protein synthetic machinery (e g transfer-RNA) in the biosynthesis of Arg-poly(Asp) cannot be excluded.

This paper reports the identification and isolation of a soluble enzyme which will elongate Arg-poly(Asp) Although the number of enzymes involved in the biosynthesis of Arg-poly(Asp) is not yet known, the enzyme reported here will be called multi-L-arginyl-poly(L-aspartic acid) synthetase (EC 6 3 2 -)

Materials and Methods

Materials and culture

[^{3}H] Arginine, [^{3}H]aspartic acid, Protosol, POP, and dimethyl POPOP were purchased from New England Nuclear (Boston, Mass). All biochemicals including ATP, nucleases, chloramphenicol, and trypsin were purchased from Sigma Chemical Company (St Louis, Mo) All other chemicals were J T Baker (Phillipsburg, N J) reagent grade or equivalent. Arg-poly(Asp) was isolated from *Anabaena cylindrica* and purified using a dilute acid wash procedure [4]. *Anabaena cylindrica* was grown as previously described [7]

Assay of Arg-poly(Asp) synthetase activity

The activity of Arg-poly(Asp) synthetase was measured by following its ability to incorporate radioactive arginine into a trichloroacetic acid insoluble product Enzyme reactions were carried out in 16 × 100 mm pyrex tubes and the standard reaction mixture contained the following in a total volume of 125 µl 50 mM Tris, pH 8 2, 10 mM β-mercaptoethanol, 20 mM KCl, 20 mM $MgCl_2$, 4 mM ATP, 34 µm chloramphenicol, 100 µm aspartic acid, 10 µm [^{3}H]arginine-HCl (400 mCi/mM), 0 87 mg/ml purified Arg-poly(Asp), 50 µl cell-free extract Assays were incubated in a shaking waterbath at 28°C. At timed intervals 50 µl-aliquots are taken from each reaction mixture and placed on 2 3 cm diameter discs of Whatman number 3 MM filter paper. After briefly allowing the paper to soak up the added reaction mixture, the reaction was terminated by dropping the discs into 5% trichloroacetic acid containing 1 0 mM arginine HCl The paper discs were washed two times with cold 5% trichloroacetic acid followed by a brief wash in 0.1 M potassium phosphate buffer (pH 7 0) Since Arg-poly(Asp) is soluble in dilute acid [8] but not soluble in 5% trichloroacetic acid [9], a buffer wash prior to the first dehydration step was necessary to neutralize the trichloroacetic acid remaining on the filter paper. If the buffer wash step was deleted, a significant fraction of the Arg-poly(Asp) was solubi-

lized in the first dehydration step The paper discs were dehydrated by washing them consecutively with 70% ethanol, 95% ethanol, absolute ethanol, ethanol/ethyl ether (1 : 1, v/v), and anhydrous ether. After the final ether wash, the discs were dried under a heat lamp, put in scintillation vials, and 2 ml of a toluene scintillation counting fluid consisting of 4 0 gm POP and 0.25 gm dimethyl-POPOP in 1 liter of scintillation grade toluene was added The counting efficiency of the paper disc method is approximately 10%, but the speed and number of assays which can be carried out make this procedure the method of choice Enzyme activity is expressed as units where 1 unit is taken as that amount of enzyme which will incorporate 1.0 nM of arginine into trichloroacetic acid-insoluble material in 1 h at 28° C using the standard reaction mixture described above. Specific activity is given as units of enzyme activity per mg of protein

Isolation of multi-L-arginyl-poly(L-aspartic acid) synthetase

Arg-poly(Asp) synthetase was isolated from chloramphenicol treated cells When growing *Anabaena cylindrica* reached a cell density of approximately 0 1 mg dry weight per ml, chloramphenicol was added to the culture at a final concentration of 5 μg/ml Cells were harvested 48 h after the addition of the protein synthesis inhibitor using a Szent-Gyorgyi and Blum continuous flow attachment on a Sorvall RC-2B centrifuge The fresh weight of the cell pellet was determined, and cells were resuspended (4 ml/g fresh weight) in a buffer containing 0.05 M Tris, 0.005 M β-mercaptoethanol, 0 001 M $MgCl_2$, 0 001 M EDTA (pH 8 2) The cell suspension was cavitated using the flat-tip probe on a Bronson Sonifier (W-185-C) for two 15-s periods of cavitation (setting 1), each period separated by 1 min to allow cooling. The broken cell suspension was centrifuged for 60 min at 37 000 $\times$ *g* in the SS-34 rotor of a Sorvall RC-2B centrifuge The supernatant was removed and centrifuged 4 h at 40 000 rev /min in a Beckman 42.1 rotor (124 000 $\times$ *g*) using a Spinco Model L The supernatant from the 124 000 $\times$ *g* 4 h centrifugation was then fractionated by ammonium sulfate precipitation A 100% saturated solution of ammonium sulfate (pH 8 8) was added and the fraction insoluble between 27% and 37% saturation was collected The 27%—37% fraction was redissolved in a mercaptoethanol buffer consisting of 0 02 M Tris, 0.005 M β-mercaptoethanol, 0 001 M EDTA (pH 8 0) and dialyzed overnight against a large volume of the same buffer. The dialyzed material was clarified by centrifugation and applied to a DEAE-cellulose column (20 0 cm $\times$ 2 50 cm) which had been pre-equilibrated with the mercaptoethanol buffer The column was washed with 100 ml of buffer and the enzyme eluted with a 1000 ml linear gradient of buffer and buffer containing 0.40 M KCl Column fractions of 13 ml each were collected and assayed for enzyme activity. The enzyme eluted just after the blue chromoprotein *c*-phycocyanin Fractions containing enzyme were pooled and the protein precipitated by the addition of solid ammonium sulfate to a final concentration of 50% saturation The light blue precipitate was collected by centrifugation, dissolved in a minimum volume of mercaptoethanol buffer and dialyzed against a large volume of this same solution overnight Following dialysis, aliquots were frozen in solid CO_2/ethanol and stored at —70° C The enzyme activity is stable under these storage conditions for at least 6 months

Analysis of the reaction product

The isoelectric point of the reaction product was determined by polyacrylamide gel isoelectric focusing A 125 μl reaction mixture was incubated 1 h at 28°C and then stopped by the addition of 5.0 ml cold 5% trichloroacetic acid containing 1 mM arginine HCl The resulting precipitate was washed two times with cold 5% trichloroacetic acid and once each with 95% ethanol, absolute ethanol and diethyl ether Following the ether wash, the precipitate was air dried and then dissolved in a solution containing 6 0 M urea and 10% sucrose The reaction product was isoelectrically focused on 10 0 cm rods of polymerized acrylamide (7 50% T, 2 6% C) containing 2.4% pH 3—10 ampholine (LKB Instruments Inc , Rockville, Md.) and 6 0 M urea [10] Following focusing, the gels were stained with 0.1% coomassie blue, and the pH profile of parallel gels was measured [4] The positions of the stained material in the gels were determined by scanning the gels at 550 nm using the linear transporter attached to a Gilford spectrophotometer The distribution of radioactivity in the stained gels was determined by cutting the gel into 1 mm slices and counting each slice in 5.0 ml of toluene based scintillation fluid (see above) to which 0 2 ml of Protosol/water (9 1, v/v) was added

The size distribution of the reaction product was measured by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate The trichloroacetic acid insoluble product was prepared as described above and dissolved in a solution containing 0 04 M Tris (pH 7 0), 1 65% sodium dodecyl sulfate, and 8 3% glycerol Following electrophoresis in rods of polymerized acrylamide containing sodium dodecyl sulfate [11], the gels were stained overnight with 0.5% fast green in methanol/acetic acid/water (50 20 30, v/v/v) [12] and then destained in methanol/acetic acid/water (10 7 5 82 5, v/v/v)

TABLE I

PURIFICATION OF MULTI-L-ARGINYL-POLY(L-ASPARTIC ACID) SYNTHETASE

Details of the purification procedure are given in Materials and Methods One unit of enzyme activity is defined as the amount of enzyme which will incorporate 1 nmol of arginine in 1 h at 28°C using the standard reaction mixture described in Materials and Methods

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification (-fold)
1 Supernatant fluid 37 000 × *g* (60 min)	109	1362	1927	1 41	100	1 0
2 Supernatant fluid 124 000 × *g* (240 min)	86	886	1260	1 42	65 4	1 0
3 Ammonium sulfate precipitation (27-34% saturation)	19	50 6	800	15 8	41 5	11 2
4 DEAE-cellulose	10 5	6 0	779	130	40 4	92 1

The location of stained material and the measurement of the radioactivity in the gels was carried out as described for the isoelectric focusing gels except that the gels were scanned for absorbance at 630 nm. For comparison, proteins with known molecular weights were electrophoresed on parallel polyacrylamide gels

The solubility properties of the reaction product were determined by incubating a 125 μl reaction mixture for 1 h at 28°C, at which time 1 0 ml distilled water was added and the mixture placed on ice to stop the reaction Following centrifugation at 10 000 × *g* for 5 min, the supernatant was removed and mixed with 1 0 ml of 20% trichloroacetic acid, while 1 0 ml of 10% trichloroacetic acid was added to the pellet The precipitates were washed once in 10% trichloroacetic acid and twice with 95% ethanol After drying at 40°C, the precipitates were taken up in 0 1 ml Protosol/water (90 10, v/v) and counted in toluene based scintillation fluid The solubility in dilute acid of the material sedimenting at 10 000 × *g* for 5 min was determined by centrifuging a second diluted reaction mixture as above, but the pellet was resuspended in 1 0 ml of 0 1 M HCl. After gentle agitation for 15 min, the acid extract was centrifuged at 10 000 × *g* for 5 min and the radioactivity of the trichloroacetic acid insoluble material in both the pellet and acid supernatant was measured

The effect of the trypic digestion upon the reaction product was determined by adding trypsin (EC 3.4.4 4) to one of two parallel reaction mixtures at a final concentration of 250 μg/ml Aliquots were taken prior to and 10, 20, and 40 min following trypsin addition, and the [^{3}H]arginine incorporated into trichloroacetic acid-insoluble material was measured

Miscellaneous assays

Algal dry weight was determined as previously described [8] Measurement of α-amino groups was by the method of Rosen [13], and protein was determined by the method of Lowry et al [14]

Results

Purification of Arg-poly(Asp) synthetase

The enzyme purification is described in Materials and Methods and a summary of a typical purification procedure is presented in Table I The enzyme activity was purified 92-fold with a recovery of 40.4% of the activity initially in the 37 000 × *g* (60 min) supernatant While the second isolation step (124 000 × *g*, 240 min) gives no net purification, it is routinely carried out since removal of membrane fragments and ribosomes facilitates the further steps of the purification. Enzyme activity in Table I is measured as the ability to incorporate [^{3}H]arginine into trichloroacetic acid-insoluble material, although the preparations will also incorporate [^{3}H]aspartic acid into trichloroacetic acid insoluble material The ratio of arginine incorporation to aspartic acid incorporation is constant throughout the various steps of the purification procedure, an observation which suggests that one enzyme may be responsible for both activities

Properties of the enzyme reaction

The enzyme isolated from *Anabaena cylindrica* incorporates [^{3}H]arginine

TABLE II

RATE OF INCORPORATION OF ARGININE INTO TRICHLOROACETIC ACID-INSOLUBLE MATERIAL BY MULTI-L-ARGINYL-POLY(L-ASPARTIC ACID) SYNTHETASE AS A FUNCTION OF THE COMPOSITION OF THE REACTION MIXTURE

The composition of the complete reaction mixture is given in Materials and Methods In the boiled control, the cell free extract was boiled for 10 s prior to its addition to the reaction mixture Specific activity is given as the nmol arginine incorporated for 1 h per mg protein

Reaction mixture	Specific activity	Percent complete reaction mixture
Complete	125	100
Boiled	0 0	0
—β-Mercaptoethanol	51	40
—Arg-poly(Asp)	5	4
—Chloramphenicol	122	98
—KCl	17 5	14
—$MgCl_2$	1 2	1
—ATP	1 0	1

into trichloroacetic acid-insoluble material (Table II) Under the standard assay conditions the rate of amino acid incorporation is linear for at least 40 min and is directly proportional to the amount of enzyme added to the reaction mixture Table II lists the effects of deleting components from the standard reaction mixture Little activity is measured if the enzyme preparation is boiled or if Arg-poly(Asp), KCl, $MgCl_2$, or ATP are left out of the assay mixture The removal of chloramphenicol from the reaction mixture does not affect the rate of incorporation while the removal of β-mercaptoethanol inhibits incorporation by 60% No activity is recovered if a sulfhydryl reagent such as β-mercaptoethanol or dithiothreitol is absent from the enzyme isolation buffer. The pH optimum of the reaction is 8 2 and the optimum temperature for incorporation is 28°C (Fig 1)

Further characterization of the requirement for Arg-poly(Asp) is shown in Fig 2 The rate of amino acid incorporation is proportional to the Arg-poly-(Asp) concentration from 0 to 0 87 mg/ml, and at higher concentrations the enzyme is inhibited The requirement for Arg-poly(Asp) cannot be met by other peptides or proteins even though they may have an N- or C-terminal aspartic acid or arginine The materials tested include the dipeptides, L-arginine-L-aspartic acid, L-aspartyglycine, and glycyl-L-aspartic acid, the synthetic polypeptides, poly-L-arginine and poly-L-aspartic acid, and the proteins, bovine serum albumin, protamine, soybean trypsin inhibitor, bovine hemoglobin, pancreatic ribonuclease, and lysozyme Also, the addition at a final concentration of 1 mg/ml of each of the above peptides or proteins to a reaction mixture containing Arg-poly(Asp) had no effect on the rate of arginine incorporation

The reaction is ATP-dependent and there is no incorporation if the following compounds are used to replace ATP β,γ-methylene adenosine-5′-triphosphate, adenosine-5′-diphosphate, adenosine-5′-monophosphate, cyclic adenosine-3′ 5′-monophosphate, cyclic adenosine-2′ 3′-monophosphate, cytidine-5′-triphosphate, guanosine-5′-triphosphate, inosine-5′-triphosphate, phosphoenolpyruvate, carbamyl phosphate, D-glucose 6-phosphate, 6-phosphogluconate, or D-ribulose-1,5-diphosphate

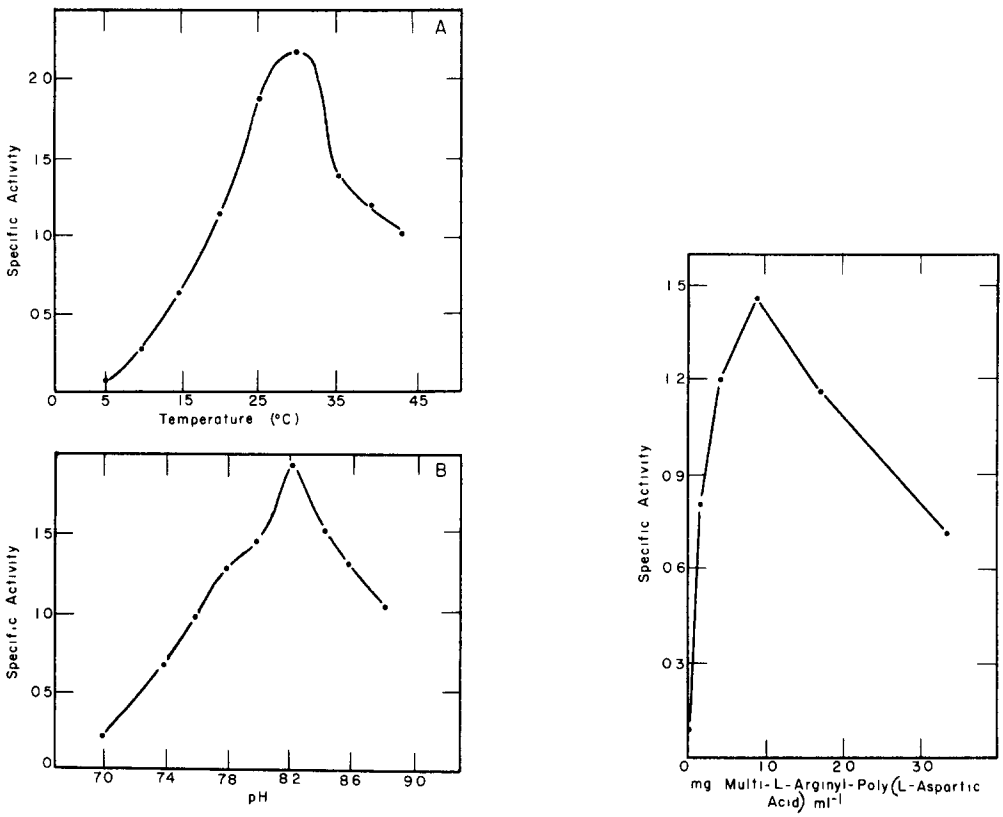

Fig 1 (A and B) The activity of multi-L-arginyl-poly(L-aspartic acid) synthetase as a function of the assay temperature and the pH of the reaction mixture The composition of the enzyme assay mixture is the same as given in Materials and Methods Specific activity is given as the nmoles arginine incorporated for a 1 h incubation per mg protein Graph A shows the specific activity as a function of the assay temperature In Graph B, the pH of the Tris buffer in the reaction mixture was adjusted to the pH values indicated, and the specific activity of the enzyme measured at 28°C

Fig 2 The effect of varying the concentration of multi-L-arginyl-poly(L-aspartic acid) in the reaction mixture on the activity of multi-L-arginyl-poly(L-aspartic acid) synthetase The assay conditions are similar to that given in Materials and Methods except that multi-L-arginyl-poly(L-aspartic acid) has been added at various final concentrations Specific activity is given as the nmol arginine incorporated for a 1 h incubation per mg protein

The incorporation of [^{3}H]arginine is dependent upon the presence of L-aspartic acid. If aspartic acid is either deleted from the reaction mixture or replaced by D-aspartic acid, arginine incorporation is inhibited 98% (Table III). However, L-aspartic acid incorporation is only moderately reduced (44% inhibition) by the absence of L-arginine or the replacement of D-arginine for L-arginine

The incorporation of [^{3}H]arginine by multi-L-arginyl-poly(aspartic acid) synthetase is insensitive to the addition of a variety of antibiotics including chloramphenicol (100 μg/ml), erythromycin (25 μg/ml), streptomycin sulfate (50 μg/ml), tetracycline (25 μg/ml), rifampin (25 μg/ml), 7-methyltryptophan (10^{-4} M); and 7-azatryptophan (10^{-4} M). The addition of deoxyribonuclease (EC 3 1 4.5) or pancreatic ribonuclease (EC 2 7 7 16) to the reaction mixture

TABLE III

THE DEPENDENCE OF THE AMINO ACID INCORPORATING ACTIVITY OF MULTI-L-ARGINYL-POLY(L-ASPARTIC ACID) SYNTHETASE ON THE PRESENCE OF AMINO ACIDS

The reaction assay mixture is similar to that given in Materials and Methods Radioactive amino acids (400 Ci/mol) were used at a final concentration of 10 μM, and unlabeled amino acids were added at a final concentration of 100 μM Specific activity is given as nmol amino acid incorporated for 1 h per milligram protein

Radioactive amino acid	Unlabeled amino acid	Specific activity
L-[^{3}H] Arginine (—)	none	2 6
L-[^{3}H] Arginine (—)	L-Aspartic acid	95 0
L-[^{3}H] Arginine (—)	D-Aspartic acid	2 2
L-[^{3}H] Aspartic acid (—)	none	7 9
L-[^{3}H] Aspartic acid (—)	L-Arginine	14 0
L-[^{3}H] Aspartic acid (—)	D-Arginine	8 0

at a final concentration of 100 μg/ml had no effect on the rate of incorporation, even though these enzymes were able to digest radioactive DNA and RNA added to a control reaction mixture. However, the addition of the arginine analogue canavanine (10^{-4} M) inhibited the incorporation of [^{3}H]arginine 72%, an inhibition which could be overcome by increasing the concentration of L-arginine in the reaction mixture

Characterization of the reaction product

Arg-poly(Asp) dissolved in 6 0 M urea and electrofocused on a polyacryl-

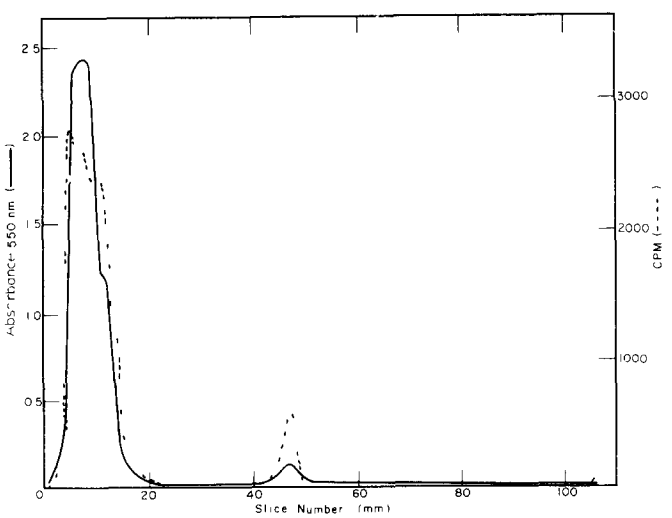

Fig 3 Polyacrylamide gel isoelectric focusing of the trichloroacetic acid-insoluble enzyme product produced by multi-L-arginyl-poly(L-aspartic acid) synthetase The 7 5% gels containing 6 0 M urea and 2 4% pH 3 10 ampholine were run as described in Materials and Methods The absorbance at 550 nm of the coomassie blue stained gels is given as a function of the distance from the top of the gel (———) The profile of radioactivity in the gel (- - - - - -) was determined by cutting the gel into 1 mm slices and counting each slice

TABLE IV

THE SOLUBILITY PROPERTIES OF THE TRICHLOROACETIC ACID-INSOLUBLE PRODUCT FORMED BY MULTI-L-ARGINYL-POLY(L-ASPARTIC ACID) SYNTHETASE

A reaction mixture as prepared and fractionated as described in Materials and Methods Total nmol of arginine incorporated are for a 1 h period at 28°C

Fraction	nmol arginine incorporated	% of complete	% in pellet
Complete reaction mixture	2 93	100	—
1 10 000 × *g* 15 min supernatant	0 05	2	—
2 10 000 × *g* 15 min pellet	2 66	91	100
a 0 1 M HCl soluble	2 30	78	90
b 0 1 M HCl insoluble	0 27	9	10

amide gel containing a pH gradient from 3 0 to 10.0 produces two bands a major band containing greater than 97% of the coomassie staining material at pH 4.75 and a minor band at pH 6.10 [4]. Arg-poly(Asp) synthetase incorporates radioactive arginine into a product with isoelectric points similar to that for Arg-poly(Asp) (Fig. 3). In the figure shown 91.2% of the total radioactivity is incorporated into the major band and 8 8% into the minor band.

Table IV lists the solubility properties of the reaction product. Over 90% of the amino acids incorporated in a 1 h period are in a particulate fraction which sediments during centrifugation at 10 000 × *g* for 5 min The Arg-poly-(Asp) which was added to the reaction mixture is particulate under the conditions of the assay and is also sedimented by this treatment. 90 percent of the counts which sediment at 10 000 × *g* are soluble in 0 1 M HCl, a concentration of dilute acid which is known to solubilize Arg-poly(Asp) [8]

The reaction product is resistant to trypic digestion. The addition of

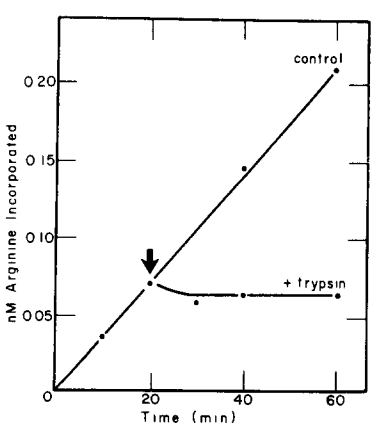

Fig 4 The effect of trypsin on the incorporation of arginine into trichloroacetic acid insoluble material by multi-L-arginyl-poly(L-aspartic acid) synthetase The composition of the reaction mixture is given in Materials and Methods At the time indicated by the arrow, trypsin at a final concentration of 250 μg/ml was added to one of two duplicate reaction mixtures Aliquots were taken at the times indicated, and the nmol arginine incorporated into trichloroacetic acid insoluble material was measured

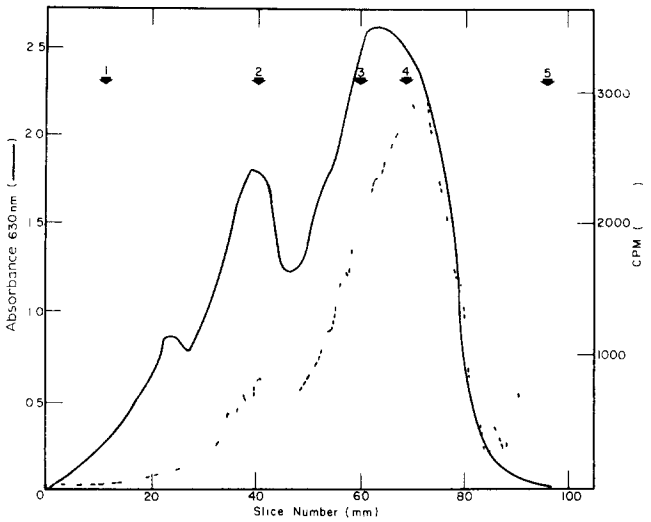

Fig 5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the trichloroacetic acid insoluble reaction product produced by multi-L-arginyl-poly(L-aspartic acid) synthetase The preparation of reaction product and the acrylamide gel electrophoresis procedures are run as described in Materials and Methods The absorbance at 630 nm of a fast green stained gel is given as a function of the distance from the top of the gel (———) The profile of radioactivity in the gel (- - - - -) was determined by cutting the gel into 1 mm slices and counting each slice The numbered arrows indicate the positions of marker proteins of known molecular weight which were run on parallel gels M_r values 5, β-lactoglobulin 18 400, 4,glyceraldehyde-3-phosphate dehydrogenase 37 000, 3, ovalbumin 43 500, 2, bovine serum albumin 68 000, 1, gamma globulin 150 000

trypsin to the reaction mixture at a final concentration of 250 μg/ml inhibits further incorporation of arginine into trichloroacetic acid insoluble material, but does not solubilize the counts already incorporated (Fig. 4) Measurements of the free α-amino groups in the reaction mixture before and after trypsin addition showed that trypsin was active under the conditions of the assay

The size distribution of the enzyme product was determined by electrophoresis in the presence of the detergent sodium dodecyl sulfate (Fig 5) Arg-poly(Asp) dissolved in sodium dodecyl sulfate migrates as a broad band with a molecular weight range estimated by comparison to known markers of M_r = 25 000 to 125 000, in agreement with previous findings [3] The distribution of fast green staining material within this size range is not random and three weight classes can be distinguished with molecular weights centering at 39 000, 67 000 and 100 000 The significance of this distribution of material is not known, although the enzyme incorporates radioactivity into all molecular weight classes

Discussion

An enzyme which can incorporate radioactive arginine or aspartic acid into multi-L-arginyl-poly(aspartic acid) has been isolated and purified 92-fold from the filamentous cyanobacterium *Anabaena cylindrica* Evidence that the enzymatic product is Arg-poly(Asp) includes the following

(1) The isoelectric points of the enzyme product as determined by polyacryl-

amide gel isoelectric focusing is the same as that for Arg-poly(Asp) (Fig. 3)

(ii) The enzyme product is particulate at neutral pH and low ionic strength but soluble in dilute acid (Table IV), properties similar to that reported for Arg-poly(Asp) [3,9].

(iii) The addition of trypsin to a reaction mixture inhibits further incorporation but does not result in the solubilization of the radioactive amino acids already incorporated (Fig. 4), indicating that the enzyme product is resistant to tryptic hydrolysis. Arg-poly(Asp) is known to be insensitive to trypsin even though arginine constitutes one half of the residues in the molecule [4]

(iv) The size distribution of the product as measured by sodium dodecyl sulfate-acrylamide gel electrophoresis is the same as isolated Arg-poly(Asp)

The incorporation reaction represents the elongation of pre-existing chains of Arg-poly(Asp) and not the de-novo synthesis of the polypeptide because incorporation will not occur if Arg-poly(Asp) is deleted from the reaction mixture (Table II, Fig. 2) and because the total amount of arginine or aspartate which is incorporated is not enough to account for the high molecular weight material produced Estimates of the amount of amino acids incorporated suggest that each chain of primer Arg-poly(Asp) is extended by the addition of several new residues. Whether additional enzymes or factors are involved in biosynthesis, or whether different reaction conditions are needed for de-novo synthesis is not known

The enzymatic reaction is dependent upon the presence of $MgCl_2$, KCl, ATP, and a sulfhydryl reagent No high energy phosphate compound yet tried can replace ATP (see above) and the reaction cannot be driven by the analogue of ATP, β,γ-methylene adenosine-5'-triphosphate The requirement for a sulfhydryl reagent indicates that the enzyme may contain an active sulfhydryl group as is the case for the enzymes which synthesize the cyclic peptide antibiotics tyrocidine and gramicidine [15]

While the enzymatic incorporation of [^{3}H]arginine into Arg-poly(Asp) is dependent upon the presence of L-aspartic acid (Table III), the reverse is not the case. [^{3}H] Aspartic acid incorporation into Arg-poly(Asp) is only inhibited 44% if L-arginine is removed from the reaction mixture or if D-arginine replaces L-arginine Since Arg-poly(Asp) contains arginyl residues peptide bonded to the carboxyl-groups of a polyaspartic acid backbone, these observations suggest that the mechanism of chain elongation consists of the addition of an aspartic acid residue to the backbone and then the addition of an arginyl residue to the newly added aspartate Thus aspartic acid could be incorporated in the absence or arginine but no visa-versa Since the enzyme will not add arginine to poly-(aspartic acid) or to any other peptide or protein containing aspartic acid, the sequence of addition must be quite specific.

None of the inhibitors of protein synthesis used effects the incorporation reaction except for the arginine analogue canavinine, and this in a manner competitive with arginine In vitro, the enzyme is not associated with ribosomes, although its intracellular location is unknown Because the enzymatic incorporation of amino acids is insensitive to the presence of nucleases in the reaction mixture, transfer RNAs are not involved in the reaction mechanism

Protein modification enzymes are known which will transfer arginine to a polypeptide chain containing an aspartic acid N-terminal [16] However, Arg-

poly(Asp) synthetase will only transfer arginine to Arg-poly(Asp), and the modification mechanism is known to proceed via a charged transfer RNA, an intermediate excluded in the enzyme mechanism

The non-ribosomal synthesis of peptide bonds is usually associated with the production of small peptides as in the formation of glutathione [17], peptide antibiotics [15], and the peptide bridges in the peptidoglycan of bacterial cell walls [18,19] Thus the production of high molecular weight (25 000—125 000) branched polypeptides by a soluble enzyme presents a unique opportunity to study the enzymology of peptide bond synthesis

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