DIGUANOSINE NUCLEOTIDES OF FUNGI THAT REGULATE RNA POLYMERASES ISOLATED AND PARTIALLY CHARACTERISED.[1]

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SUMMARY

Three unusual phosphorylated diguanosine compounds called 'hot spots' HS-1, HS-2 and HS-3 (ref. LéJohn, H.B. Proc. Can. Fed. Biol. Soc. 18, 159, 1975) have been isolated as acid-soluble materials from several fungi, Achlya, Blastocladiella emersonii, Aspergillus niger and Rhizopus stolonifer in their vegetative phase. The nucleotides were purified from acid extracts of Achlya and Blastocladiella. The tentative structures of HS-3 and HS-2 determined are GppppG and GppppGp. HS-1 structure is still in doubt but it is related to HS-2. The structures were deduced from enzymatic digestion and UV analyses of the products, molar ratios of guanosine and phosphate, and chromatographic behaviour on PEI-cellulose. All three compounds accumulated in an inverse manner with rates of RNA synthesis and directly with rates of protein synthesis. The acid-soluble pools of the three compounds fluctuated during the life cycle of Achlya, and just prior to sporulation, were excreted into the medium. HS-2 was convertible to HS-3 by acid hydrolysis.

INTRODUCTION

Finamore and Warner (1) reported in 1963 that brine shrimp, Artemia salina contain an unusual symmetrical pyrophosphate anhydride, P^1, P^4-diguanosine 5′-tetraphosphate in large amounts as acid-soluble nucleotide. Two years later, Warner and Finamore (2) isolated a homologue P^1, P^4-diguanosine 5′-triphosphate from the same organism. These compounds have since been found in Daphnia magnum (3). These molecules appear to be important in regulating DNA synthesis in embryogenesis (4). More recently, the 5′-termini of eukaryotic and viral mRNAs have been shown to be 'capped' with unusual methylated oligonucleotide fragments with the generalised structure, 7mG(5′)ppp(5′)NmpNp (5-9). Their biological role remains obscure. This communication provides evidence that fungi produce large quantities of three highly phosphorylated diguanosine compounds that are similar to the di-

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guanosine tetraphosphates of brine shrimp. These compounds are synthesised during active protein synthesis and influence in some way the mode of transcription of these organisms. Evidence for their regulation of the cellular RNA polymerases is presented in a separate communication (10). We also show that their pool sizes expand and shrink in concert with DNA synthesis. The tentative structures of the three compounds called HS-1, 2 and 3 (hot spots) were determined.

MATERIALS & METHODS

Organisms. Achlya sp. (1969), Blastocladiella emersonii, Aspergillus niger and Rhizopus stolonifer were used but this report concerns material isolated from Achlya only. Conditions for producing synchronous cell populations have been described (11).

Enzyme Digestion. Snake venom phosphodiesterase (SVPDE) (Sigma Chemical Co.,), bovine spleen phosphodiesterase (SPDE) (Sigma and Worthington products), nucleotide pyrophosphatase (NP) (Sigma), and bacterial alkaline phosphatase (BAP)[3] from Sigma were used to partially and completely digest the HS compounds.

Chromatography. HS compounds, their digested products were separated by the one and two dimensional chromatographic system of Cashel and Kalbacher (12) using PEI-cellulose plates (Brinkmann). A slight modification was the use of pH 3.65 instead of pH 3.4, 1.5 M KH_2PO_4 buffer suggested by Cashel and Kalbacher (12).

Purine Staining. Products of HS compounds after acid, alkali and enzyme hydrolyses were stained with $AgNO_3$-Na dichromate as described by Reguera & Asimov (13). This procedure was taken as the HS compounds display poor UV absorptivity on PEI-cellulose plastic sheets.

Miscellaneous. Periodate oxidation, acid and alkali labilities, ionisation constants, and molar ratios of guanosine to phosphate of HS compounds were studied as discussed by Cashel and Kalbacher (12).

RESULTS

Isolation of GTP and HS Compounds. Achlya cells grown to about mid-log phase (14 hr) as aerated 10-litre cultures at 28°C were collected by filtration, washed, and extracted by 1 M formic acid. For every g wet weight of cells, 1 ml acid was used. The cells were soaked in acid for 1 hr at 0°C and the liquid expressed by vacuum filtration. The acid solution was concentrated ten-fold under vacuum at 37°C and 100 µl of the concentrate streaked onto PEI-cellulose plates (20 cm x 20 cm) and the site of application overlaid with 20 µl of acid extract obtained from cells grown in ^{32}P-orthophosphate. The plates were dried, then moistened with methanol at the site of application before chromatographing in 1.5 M KH_2PO_4. The solvent was allowed to migrate to 15 cm from the origin and HS compounds were located by autoradiography. The relative positions of the HS compounds and GTP are, HS-1, 1 cm;

[3]Abbreviation: BAP=bacterial alkaline phosphatase; NP=nucleotide pyrophosphatase; SPDE-bonine spleen phosphodiesterase; SVPDE=snake venom phosphodiesterase.

HS-2, 2 cm; HS-3, 5 cm; and GTP, 6.5 cm from the origin. Cellulose from the pertinent areas were scrapped off and packed into small columns. Free P_i was removed by washing the columns with 0.1 M triethylamine-HCO_3 (TEA-HCO_3), pH 9 buffer until no more radioactivity could be detected in the eluate, then HS compounds were eluted with 2 M TEA-HCO_3, pH 9. The eluate was evaporated to dryness at 37°C in vacuo and the residue taken up in a small volume of H_2O. Repeated drying and resuspension of the residue removed all traces of TEA-HCO_3. They are stable as powder at -20°C but breakdown in solution even at low temperatures.

UV Spectral Analysis. The HS compounds and GTP were spectrally analysed between A_{320} and A_{220} at acid (0.1 N HCl), alkali (0.01 N NaOH) and neutral pH values. Estimates were also made of their respective pK values. These data are summarised in Table 1 and compared with data reported (14, 15) for GTP, UTP, ATP and CTP. Spectral properties of the HS compounds were consistent with those of GTP only. The pK values, however, suggested that the nucleotides may be modified with methyl groups as shown by the literature data for 7mG (see Table 1) but no methyl groups have been detected in them (unpublished).

Enzymatic Digestion. Intact molecules of HS-3 and HS-1 were resistant to BAP hydrolysis, but HS-2 was converted to HS-3 with release of P_i. However, with

Table 1. Spectral data of HS compounds and comparison with literature values for GTP, ATP, UTP, CTP and 7-mGuo. (see ref. 14 & 15).

Spectral ratios	HS-3	HS-2	HS-1	7mGuo	GTP	ATP	UTP	CTP
$A_{250/260}$	0.95	0.94	0.93	-	0.96	0.85	0.75	0.45
$A_{280/260}$	0.69	0.67	0.72	0.79	0.67	0.22	0.38	2.12
$A_{290/260}$	0.54	0.55	0.56	-	0.49	0.027	0.04	1.67
λmax (pH 1)	257	258	258	256-258	256	257	262	280
λmax (pH 12)	255	256	256	-	-	-	-	-
λmin (pH 1)	240	242	242	230	228	230	230	242
λmin (pH 12)	237	235	236	-	-		-	-
pK	7.2*	7.4*	-	7.1	3.3,9.3	4.0	9.5	4.8

*Because of the instability of these compounds, this may represent a composite of pK values for the intact molecule and breakdown products.

prolonged incubation of all HS compounds in buffer under alkaline conditions (pH 9 or greater), they became progressively susceptible to BAP digestion with release of ppG, pG, Guo and P_i. Spleen phosphodiesterase (SPDE) released guanosine and ppppG from HS-3. Guanosine, ppppGp and pppGpp were formed by SPDE action on HS-2 and 1 respectively. These phosphorylated products were unstable and were broken down into a variety of guanosine polyphosphates. Products identified were pppG and ppG from HS-3, pppGp and ppGp from HS-2 and pppGpp, ppGpp, ppGp and unidentified substances from HS-1. The unique reaction of SPDE on these compounds is puzzling since the enzyme requires a free 5′-hydroxyl end for catalysis. Contamination by snake venom phosphodiesterase (SVPDE) was eliminated because SVPDE cleaved HS-3 into equimolar amounts of pG and pppG in a limited digestion. HS-2 was resistant to SVPDE suggesting that the free 3′-hydroxyl groups present in HS-3 must be blocked in this compound. Preliminary treatment with BAP made it susceptible to SVPDE digestion with the same products as HS-3. This suggested that HS-2 is a higher phosphorylated homologue of HS-3.

HS-3 was cleaved by nucleotide pyrophosphatase to give the same products as SVPDE. Taken together, these results indicated that HS-3 is a diguanosine tetraphosphate and HS-2 a diguanosine pentaphosphate. The structure of HS-1 is still in question since yields of this compound are always very low. Because the nucleotide products of enzyme digestion are 5′-linked polyphosphates, the HS compounds must have the guanosine residues connected by 5′-5′ pyrophosphates. All nucleotide products were detected by Ag-chromate staining (13) as well as UV absorption.

Table 2. Molar ratios of major constituents of HS compounds and their sensitivities to acid, alkali and periodate treatment.

Compound	Total P/guanine	Ribose/guanine	Periodate oxidation	Lability pH 2	Lability pH 10
HS-3	4.12	1.0 ± 0.05	+	+	+
HS-2	5.31	0.9 ± 0.11	+	+	+
HS-1	ND	ND	+	+	+
GTP(cellular)			+	-	+

+ signifies that property is a characteristic of the compound.
ND, not determined as yet.

General Properties. Sensitivity of the HS compounds to oxidation by periodate, lability to alkali and acid conditions and the chemical constitution of HS-3, 2 and 1 were studied and the data are summarised in Table 2. All three were oxidised by Na periodate, labile to acid (pH 2 or lower) and alkali (pH 9 or higher). The molar ratios of guanine, ribose and P_i complement the enzyme data indicating that HS-3 is diguanosine tetraphosphate and HS-2 pentaphosphate. HS-1 composition is yet to be unequivocally determined.

Table 3. ^{32}P-orthophosphate entry into HS pools during the developmental cycle of Achlya as a function of cellular protein content. Cells were grown at 22°C to reduce growth rate which leads to a clearer exposition of the metabolic changes.

Development time (hr)	Cellular protein (mg)	Acid-soluble nucleotides: * ^{32}P entry (CPM x 10^{-5}) HS-3	HS-2	HS-1
6	0.1	2.0	3.09	1.2
9	0.13	5.3	12.9	3.1
11	0.40	39.1	29.8	3.97
12	1.10	62.2	41.2	5.19
13	1.38	62.8	36.2	6.17
15	1.52	94.1	77.9	11.33
16	1.73	68.3	55.1	7.78
17	1.94	87.2	75.6	7.38
18	2.31	72.5	60.1	5.7
19	2.38	20.7	27.7	7.1
20	2.52	78.5	55.3	4.4
21	2.40	38.5	28.6	3.8
22	2.41	31.2	13.9	3.6
23	2.43	25.09	9.1	3.5
24**	2.38	-	-	-

*Samples were taken in triplicate and the pool sizes represent an average of the three. Standard deviation of less than 15%.

**Inception of sporulation.

Kinetics of HS Accumulation. Table 3 shows the results of a study in which the cellular HS pools were labelled with ^{32}P-orthophosphate. During exponential growth, the compounds not only increased with increase in cell volume as expected, but they periodically decreased to very low levels implying that they might be active in cellular metabolism. Towards the end of the exponential growth period, the compounds were excreted into the medium (Table 4) marking the onset of hyphal differentiation into sporangia. Cellular GTP did not shrink correspondingly (not shown).

Rates of DNA, RNA, Protein and HS Syntheses. To determine whether these unusual nucleotides had any relationship with macromolecular metabolism, Achlya cells were grown under synchronous conditions and pulsed for 10 min at hourly intervals with labelled uridine, thymidine, amino acid mixture and P_i. Specific activities of RNA, DNA and protein were determined and ^{32}P label in HS compounds estimated from the acid soluble pool fraction after chromatography and PEI-cellulose separation. The results are shown in Table 5. The data for HS-3 only is recorded here.

Whereas HS synthesis occurred when rates of protein synthesis were high,

Table 4. Efflux of HS compounds into the growth medium at late stages of cellular development.

Growth medium was collected at the specified intervals, dried in vacuo, residue dissolved in a fixed volume of 1 M HCOOH and an aliquot applied on PEI-cellulose sheets and chromatographed. The areas corresponding to HS-3, 2 and 1 were cut out and the radioactivity determined. At the same times, samples of cells were collected and the cellular content of HS compounds similarly determined.

Growth time (hr)	(CPM x 10^{-5}) HS compounds in cells			HS compounds in medium		
	HS-3	HS-2	HS-1	HS-3	HS-2	HS-1
15	92.12	46.16	26.83	2.74	0.78	2.26
16	92.03	39.01	25.99	6.12	2.67	3.98
17	58.22	34.01	12.92	7.68	4.12	6.61
18	50.42	27.98	15.73	20.14	8.89	11.49
19	33.65	29.49	7.69	-	-	-
20	31.99	25.12	4.83	33.2	20.5	17.8
21	———		Sporulation	———		

Table 5. Relative rates of RNA, DNA, protein and HS syntheses as a function of stage of development.

Growth time (hr)	CPM x 10^{-3}/10 min in HS-3 only	Specific activities (arbitrary units) RNA	DNA	protein
9	2.1	3.65	0.45	0.51
10	6.2	5.11	1.05	0.98
11	7.1	3.45	1.21	1.41
12	9.8	2.78	1.45	1.47
13	16.1	1.27	1.55	1.63
14	24.9	1.39	2.13	1.98
15	33.9	0.35	1.00	2.88
16	22.0	0.19	1.10	3.35
17	17.0	0.15	0.45	3.31
18	11.9	0.13	0.26	4.29
19	7.3	0.16	0.28	4.50
20-21	——— sporulation ———			

the inverse was true for RNA synthesis. At the late stages when protein synthesis was still active, decline of HS compounds from the cellular pool was accounted for by their excretion into the medium (Table 4). Rate of DNA synthesis, on the other hand, was altered correspondingly as the HS pools changed. The conclusion of Finamore and Clegg (4) about GppppG relation to DNA synthesis may well be important here.

DISCUSSION

A plethora of highly phosphorylated nucleosides have now been reported to exist in various cells (5-9, 16-18). Sometimes, they are modified by methylation (5-9). A variety of roles have been ascribed them. The nucleoside polyphosphates we have isolated and described here may be yet another class of these bizzare molecules. They resemble the diguanosine nucleotides found in brine shrimp (1, 2) but not the oligonucleotides found at the 5′ termini of mRNAs (5-9) since we have no evidence that they are methylated (unpublished data of H.B.L.). Because of their close correlation with protein biosynthesis, and their regulatory effects

on the activities of RNA polymerases (10), they may play a vital role in differentiation of these fungal cells. This conjecture is supported by the observation that they are excreted into the growth medium only when the cells begin to differentiate and produce asexual spores. The excretion patterns and, correspondingly, sporangial maturation can be altered by application of dibutyryl cyclic AMP, Zn^{++}, Fe^{+++}, Ca^{++}, cytokinins and cytochalasins A and B (manuscript in preparation). We also have evidence that HS-3, at least, can be synthesised in a cell free system with ribosomes, phage MS2 RNA, uncharged tRNA and GTP (Piamenta and LéJohn, manuscript in preparation). Their connection with protein synthesis may therefore be realistic. What roles other than regulation of transcription they have await elucidation in the future.

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