REGULATION OF FUNGAL RIBONUCLEOTIDE REDUCTASE BY UNUSUAL DINUCLEOTIDES[1]

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SUMMARY: Three phosphorylated dinucleosides designated HS1, HS2, and HS3, isolated from the water-mould Achlya, were shown to significantly inhibit ribonucleotide reductase activity from Achlya. All three compounds decreased CDP reduction in fungal extracts by 50% at concentrations of 0.1mM. At the same concentration HS3 also inhibited partially purified CDP reductase from Chinese hamster ovary cells by at least 80% but showed only 10% inhibition with enzyme from E. coli. ADP reductase activity from Achlya was inhibited 50% by both HS1 and HS3 at 0.1mM. HS2 however, showed no inhibitory effect on purine reduction. The levels of ribonucleotide reductase during the asexual growth cycle of Achlya correlated with thymidine uptake into DNA and with the synthesis of HS compounds.

Recently, LéJohn et al (1) reported the isolation of three highly phosphorylated dinucleoside compounds† synthesized during active protein synthesis in the water-mould Achlya. These compounds have been shown to interact with the various fungal RNA polymerases in a complex manner (2). We wished to determine whether the same compounds may also play a role in the regulation of DNA synthesis. Little is known of the process by which eucaryotic organisms regulate the replication of their DNA. DNA synthesis is often confined to specific stages of development and specific periods of the cell cycle. It has been suggested that ribonucleotide reductase may be a major point of control in this process (3). In other eucaryotic systems this enzyme is responsible for the production of a balanced supply of the four deoxyribonucleotides and is known to be subject to complex allosteric regulation by a variety of ribonucleoside and deoxyribonucleoside triphosphates (4). Furthermore, ribonucleotide reduction may be the rate-limiting step in DNA synthesis and cell division (5). Thus this enzyme seemed a likely candidate for HS regulation.

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†A revised report being prepared (McNaughton, D.R., Klassen, G.K. and LéJohn, H.B.) will show that contrary to the structures proposed in a preliminary communication (1), HS3 dinucleoside polyphosphate contains uridine and adenosine that are highly modified. Both HS2 and HS1 dinucleoside phosphates contain only uridine that is also modified. The modifying components on the uridine moities absorb UV.

Ribonucleotide reductase has been isolated from a variety of eucaryotic organisms but has not been significantly purified from any. To our knowledge, there have been only two reports demonstrating pyrimidine ribonucleotide reduction in fungal extracts. The CDP reductase activity found in Saccharomyces (6) resembles the E. coli and mammalian type enzyme in general properties, while that from Pythomyces (7) appears to require coenzyme B12 and thus resembles the second class of ribonucleotide reductase found in Lactobacillus (8).

We report here the demonstration in Achlya of both purine and pyrimidine ribonucleotide reductase activites resembling those found in mammalian systems. Furthermore, CDP reduction is significantly inhibited by low concentrations of all three unusual dinucleotide compounds, while ADP reduction is similarly inhibited by two of the three compounds. HS3, the most potent inhibitor of the three compounds, also inhibited CDP reduction by mammalian ribonucleotide reductase but showed only marginal effect on enzyme from E. coli.

MATERIALS & METHODS

Organisms. The conditions for growing Achlya sp. (1969) and harvesting of cells have been described (9,10). E. coli KL161, a thymine requiring strain, was obtained from the E. Coli Genetic Stock Centre. Chinese hamster ovary cells (11) were routinely maintained in α-minimal essential medium (Flow Laboratories Inc.) as previously described (12).

Preparation of cell extracts. Routinely, Achlya cells were harvested by filtration 10 hours after spore germination. The cells were ground to a fine powder in liquid nitrogen and 0.5 ml of buffer (0.1M Hepes* pH=8, 5.0mM dithiothreitol) added for every gram of cell powder. The extract was centrifuged at 50,000g for 20 min and the supernatant was then passed over a column of Sephadex G25 (1 X 16 cm, equilibrated with 50mM Hepes pH=7.5, 5.0mM dithiothreitol). The extract was then frozen in liquid nitrogen and stored at -76°C. Protein content of the extracts was measured by the method of Lowry et al (13).

E. coli was grown and crude extract prepared as described (14). The extract was dialysed 4 hours against 50mM Hepes pH=7.5, 5.0mM dithiothreitol.

Chinese hamster ovary cell crude extract was prepared as described (15). The mammalian enzyme was partially purified by precipitation at pH=5.2, followed by affinity chromotography on ATP-agarose (Lewis and Wright, manuscript in preparation).

Enzyme assay. CDP reductase activity was routinely measured in a reaction mixture of 50mM Hepes pH=7.5, 10mM $MgCl_2$, 10mM dithiothreitol, 10mM ATP and 0.4mM [^{14}C]CDP (4000 cpm/nmole) in a total volume of 150 µl. After incubation at 22°C for 60 minutes the reaction was terminated by heating in boiling water. The deoxycytidine formed was measured as previously described (15).

ADP reductase was routinely measured in a reaction mixture of 50mM Hepes pH=7.5, 10mM dithiothreitol, 1.0mM dGTP and 0.4mM [^{14}C]ADP (4300 cpm/nmole) in a total volume of 150 µl. The deoxyadenosine formed was measured as described by Cory (16).

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* Abbreviations: Hepes = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, TEAB = triethylammonium bicarbonate

Thymidine incorporation. Thymidine uptake into acid precipitable material was measured by adding (^{3}H)thymidine (0.4 µCi/ml, 20nM) to 25 ml of Achlya culture at various times after spore germination. After 10 minutes incubation, cells were filtered, washed with phosphate buffered saline and incubated in ice-cold 10% trichloroacetic acid for 10 minutes. The cells were then filtered onto a glass fibre filter (Gelman #61630) and treated as previously described (15).

^{32}P-orthophosphate pulsing. Twenty-five ml samples of cells were removed at the intervals indicated and pulsed with 200 µCi (^{32}P)orthophosphate for 10 min. The labelled cells were washed with water by vacuum filtration and extracted for 30 min with 0.2 ml of 1.0M formic acid at 0°C. HS compounds in the acid extracts were separated on PEI-cellulose plates (Brinkman) and the area corresponding to HS3 cut and radioactivity determined.

Isolation and purification of HS compounds. Mid-log phase cells of Achlya (14 hr. old) grown and harvested as described (2) were used for extraction of HS compounds. Typically, 50-60g wet weight of cells were extracted with 50 ml of 1M formic acid, pH=3.5, at 0°C for 1 hr. The acid extract collected by filtration was carefully adjusted to pH=7 with NaOH and then diluted 10 fold. All subsequent purification steps were performed at 4°C. After dilution, 2 ml of a formic acid extract from cells grown in medium supplemented with (^{32}P)orthophosphate was added and the entire volume was then flushed through a column (3X45 cm) of DEAE-Sephadex A-25 at a flow rate of 50 ml/hr. The column was pre-equilibrated with 0.2M TEAB buffer at pH=7. The loaded column was then washed with 500 ml of 0.2M TEAB before nucleotides were eluted with a linear gradient (0.2M - 1.2M) of TEAB at pH=8. The absorbance of each fraction was determined and fractions corresponding to peaks of UV absorbance assayed by chromatography on PEI-cellulose for HS content as described (1). The three HS compounds eluted at the same molarity of 1.1M TEAB. The pertinent fractions were pooled, diluted 10 fold and loaded onto another DEAE-Sephadex A-25 column (1X30 cm) pre-equilibrated with 0.1M NaCl:0.05M Na formate, pH=3.6 buffer. After loading, the column was washed with the NaCl-formate buffer and nucleotides eluted with a linear gradient of NaCl (0.1M - 0.6M) in 0.05M Na formate, pH=3.6 buffer. The three HS compounds eluted at NaCl concentrations as follows: HS3, 0.28M; HS2, 0.35M; and HS1, 0.40M. ATP marker eluted at 0.22M. The HS fractions were pooled according to species, concentrated to 1 ml by evapomix and desalted on Sephadex G10 columns using 0.04M TEAB, pH=7 as elution buffer. The desalted HS solutions were brought to dryness by evapomix, washed six times with successive additions of 1 ml water. The final residue was dissolved in 1 ml of water and stored at -20°C until use.

Chemicals. Radioactive thymidine, CDP and ADP were purchased from Amersham/Searle. ATP-agarose was prepared by PL Biochemicals. Sephadex G10, G25 and DEAE-Sephadex A-25 were obtained from Pharmacia Ltd. All remaining chemicals were obtained from Sigma Chemical Co.

RESULTS

Ribonucleotide reductase in Achlya. When prepared as described, crude extracts of Achlya cells harvested 10 hours after spore germination contained at least 20 mg of protein per ml. After passage through Sephadex G25 to remove endogenous inhibitors of low molecular weight, both purine and pyrimidine ribonucleotide reductions were demonstrated. The activity increased linearly with protein concentrations above 0.75 mg protein per assay. The reaction had an optimum temperature of 22°C and proceeded linearly for at least 60 min.

Both CDP and ADP reductase activities resembled those found in mammalian systems (17). Table 1 shows that CDP reduction in Achlya is dependent upon

TABLE 1 PROPERTIES OF RIBONUCLEOTIDE REDUCTASE FROM ACHLYA

OMISSION OR ADDITION	CDP REDUCTASE UNITS*	ADP REDUCTASE UNITS
complete	1.92	0.77
- ATP	0.28	-
- dGTP	-	0.01
+ dATP (1.0 mM)	0.21	0.08
+ Hydroxyurea (1.0 mM)	0.24	0.36
+ $Fe(NH_4)_2(SO_4)_2$ (0.1 mM)	1.95	0.63

* 1 unit of ribonucleotide reductase = 1 nmole deoxynucleoside formed per hour per mg extract protein.

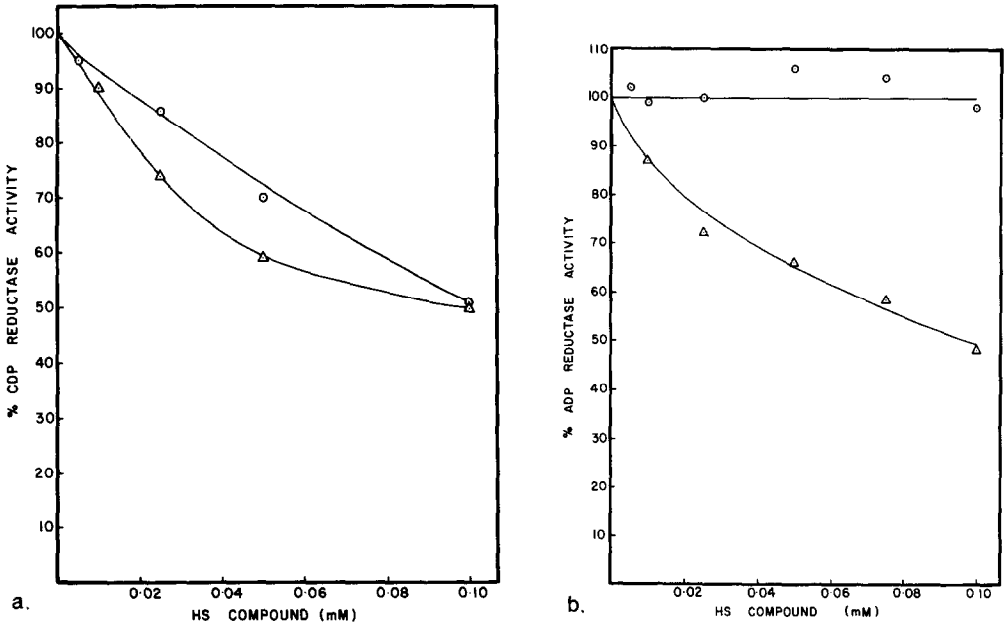

Figure 1. The inhibition of Achlya ribonucleotide reductase activity by HS compounds.
CDP reductase (Fig. 1a) and ADP reductase (Fig. 1b) were assayed as described in Materials and Methods, with 0.80 mg extract protein per assay. Each point for HS3 (△——△) represents the average of duplicate points from three separate experiments utilizing two separate preparations of HS3 compound and cell extract. The points for HS2 (⊙——⊙) represent the average of duplicate assays from a single HS2 and enzyme preparation.

the presence of ATP. Similarly ADP reduction requires dGTP. As expected dATP, a general feedback inhibitor of this enzyme, inhibited both reactions. Also hydroxyurea, a specific inhibitor of ribonucleotide reductase (15), inhibited both CDP and ADP reduction to the same degree as mammalian enzyme. Unlike many mammalian systems (18), ferrous ion did not stimulate the reaction.

Inhibition of ribonucleotide reductase with dinucleotide compounds. The unusual dinucleotides isolated from Achlya were tested for their effects on purine and pyrimidine ribonucleotide reductase activities in vitro. Both HS2 and HS3 significantly inhibited pyrimidine reduction at concentrations less than 0.1mM. Figure 1a shows that both compounds decrease CDP reduction by 50% at 0.1mM. Purine ribonucleotide reduction responded in a similar manner to HS3, being inhibited 50% by a concentration of 0.1mM (Figure 1b). Figure 1b also shows that HS2 has no effect on ADP reduction in the concentration range tested.

The low levels of HS1 found in Achlya prevented a detailed study of its interaction with ribonucleotide reductase. However, single point assays at 0.1mM demonstrated that HS1 reduced both CDP and ADP reduction by approximately 50% (data not shown).

To determine whether the inhibitory effects of the HS compounds were specific for fungal ribonucleotide reductase, the effect of HS3 on enzyme from E. coli and Chinese hamster ovary cells was tested. Table 2 shows that HS3 inhibited enzyme from eucaryotic sources to a much greater extent than from E. coli. Mammalian enzyme was the most sensitive, being inhibited 80% at 0.1mM.

TABLE 2. HS3 INHIBITION OF CDP REDUCTASE FROM DIFFERENT SOURCES

SOURCE	% INHIBITION BY HS3	
	0.05 mM *	0.10 mM *
Escherichia coli	3%	10%
Achlya sp.	40%	50%
Chinese hamster ovary	65%	80%

* HS3 concentration was determined based on a lower limit extinction coefficient of 25,000. The extinction coefficients of uridine and adenosine were taken as 12,500 and 15,000 respectively. HS3 contains uridine and adenosine in a 1:1 ratio. Both bases are highly modified in an unelucidated way. Because HS3 shows a hyperchromic shift of about 20%, these concentrations are likely to be within 20% of the actual values.

TABLE 3. SPECIFICITY OF HS INHIBITION OF ACHLYA CDP REDUCTASE

ADDITION	% ACTIVITY	ADDITION	% ACTIVITY
none	100% (2.0)*	7. ADP	96%
1. PO_4 (1.0 mM)	101	8. GDP	96
2. P_2O_7 (5.0 mM)	102	9. UDP	101
3. Adenosine†	99	10. ATP	104
4. Cytidine	92	11. CTP	91
5. Guanosine	98	12. GTP	98
6. Uridine	98	13. UTP	96

* Figure in parenthesis is the rate of the control assay in nanomoles of deoxycytidine formed per hour.

† All ribonucleosides and ribonucleotides were assayed at a concentration of 0.10 mM.

In control experiments, a number of ribonucleosides and their di- and triphosphate derivatives as well as phosphate and pyrophosphate were tested for their effect on ribonucleotide reduction. Table 3 shows that as expected, none of the compounds tested significantly inhibited CDP reduction at concentrations equivalent to, or greater than, those tested for the dinucleotide inhibitors.

Ribonucleotide reductase levels and HS pool sizes in vivo. The specific activity of ribonucleotide reductase was determined during the period from 8 to 15 hours after Achlya spore germination. As can be seen in Figure 2, CDP reductase activity peaked at 9 hours and then declined over 10 fold within the next 4 hours. The rate of thymidine incorporation into acid precipitable material also peaked at 9 hours and subsequently declined along with ribonucleotide reductase levels. Also shown in Figure 2, the rate of HS3 synthesis began to increase at 9 hours and showed a dramatic peak at 11 hours. Cellular pool sizes of HS compounds are known to rapidly increase and are maintained after this time (2). Thus, there is a very good correlation between the fall in both ribonucleotide reductase activity and thymidine incorporation into DNA and the increase in HS synthesis. Whether the drop in enzyme activity is due to inactivation and/or decreased synthesis is under investigation.

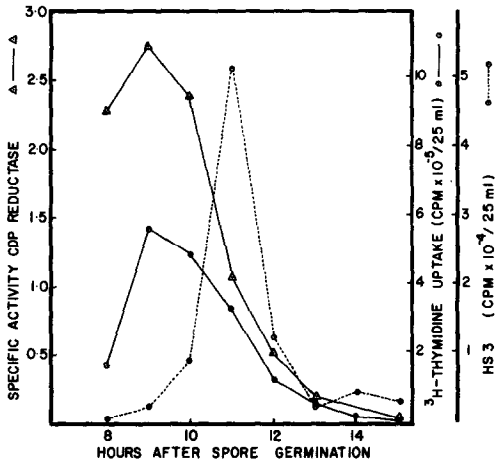

Figure 2. Levels of ribonucleotide reductase, thymidine uptake and HS3 synthesis during the growth cycle of Achlya.
15 litre carboys of glucose (5 g/l), yeast extract (0.5 g/l) medium were inoculated with 6 X 10^7 Achlya spores. At hourly intervals samples were filtered and cell extracts prepared as described. Also, 25 ml volumes of the fungal culture were pulsed for 10 minutes with (^{3}H)thymidine or (^{32}P)orthophosphate as described in Materials and Methods. Specific activity of CDP reductase (△——△), thymidine uptake (⊙——⊙), and HS3 synthesis (⊙·····⊙).

DISCUSSION

The three unusual dinucleotides isolated from Achlya have been shown to reduce purine and pyrimidine ribonucleotide reduction by 50% at concentrations well below their estimated intracellular levels of 0.2 to 0.5 mM (2). Thus the inhibition demonstrated is probably physiologically significant. Ribonucleotide reductase is known to be responsive to a complex pattern of allosteric control by a variety of ribonucleoside triphosphates. Therefore, it is not entirely surprising that highly phosphorylated dinucleosides should influence ribonucleotide reduction. HS2 inhibition of CDP reduction but not of ADP reduction resembles the effect of many known regulators of this enzyme which often preferentially affect either purine or pyrimidine reduction. Whether in fact, the effects of HS compounds are mediated through specific allosteric nucleotide binding sites remains to be determined. The observation that ribonucleotide reductase levels *in vivo* fall off as HS synthesis increases would again support the physiological significance of these compounds. However, further work is in progress to determine the exact connection between ribonucleotide reductase, DNA synthesis and the levels of HS compounds.

The inhibition of CDP reductase with HS3 is much greater with enzyme from

Chinese hamster ovary and Achlya cells than with enzyme from E. coli and suggests that the HS compounds may be specific regulators of eucaryotic ribonucleotide reductase. The higher levels of inhibition seen with enzyme from ovary cells as compared to Achlya may be due to the increased purity of the mammalian preparation. Similar, if not identical, HS compounds exist in mammalian cells (Goh, Lewis, Goodridge, Wright and LéJohn, manuscript in preparation), and we are presently studying their interaction with mammalian ribonucleotide reductase.

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