Deoxyribonucleotide Synthesis in Phycovirus-Infected Green Algae. A New Virus-Induced Ribonucleotide Reductase

Claus Bornemann and Hartmut Follmann

Fachbereich Biologie – Chemie der Universität Heinrich-Plett-Straße 40, D-W-3500 Kassel, Bundesrepublik Deutschland

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Infection of *Chlorella*-like green algae with freshwater phycoviruses is associated with a large and rapid demand for DNA precursors which cannot be met by the algal deoxyribonucleotide-synthesizing enzymes. We have demonstrated in these cells an up to ten-fold increase of the key enzyme, ribonucleotide reductase, 1–2 h post infection. The enzyme activity has been partially enriched from cell extracts. *In vitro*, it differs from that of uninfected algae in three characteristic parameters, *viz.* eight-fold higher resistance to millimolar hydroxyurea concentrations, much higher optimum concentration of an allosteric effector nucleotide, thymidine triphosphate, and an unusually low temperature optimum at 20 °C. We conclude that the large DNA phycoviruses, like Herpes and pox viruses, code for their own specific ribonucleotide reductase.

Introduction

Timely and balanced *de novo* formation of the four 2′-deoxyribonucleotides (dNTPs) permits, but also controls DNA replication and cell proliferation. The key enzymes of deoxyribonucleotide biosynthesis, ribonucleotide reductases (EC 1.17.4) [1, 2] are highly regulated on the level of gene expression, and by allosteric modulation of enzyme activity [3]. Moreover, these enzymes exhibit a unique diversity of protein structure, cofactor and metal requirements in prokaryotic and eukaryotic cells. Four different classes have currently been identified [1, 4, 5], and a number of strict correlations between enzyme properties (*e.g.*, oxygen requirement or sensitivity, inhibition or activation by nucleotides, salt tolerance) and the physiological needs of different organisms were observed [4–6]. It is likely enough that even more types of enzyme and more specific adaptations of dNTP synthesis to individual cellular requirements exist. We are engaged in a continuing search for such systems which has uncovered new enzymes in recent years [5, 23].

Deoxyribonucleotide biosynthesis in plants has found surprisingly little attention, with the exception of unicellular green algae *(Chlorella pyrenoidosa, Scenedesmus obliquus)* in which we identified and characterized an S phase-specific ribonucleotide reductase [7, 8]. The enzyme was isolated from overproducing cells and proved to be of the eukaryotic, tyrosyl radical type [9] but showed an unusual inhibition by ATP as adaptation to the photosynthetic life cycle [6]. The discovery by Van-Etten, Meints, Reisser *et al.* of large, dsDNA-containing viruses which infect endosymbiotic *Chlorella*-like algae and are widely distributed in freshwater environments [10, 11] offers an interesting new case because virus replication requires a rapid and large increase in DNA synthesis after infection [12] which could not be satisfied by the host cells' deoxyribonucleotide metabolism. Animal viruses of the Herpes virus, vaccinia (pox) virus, and African swine fever virus families code for their own ribonucleotide reductases [13–17], but the genetic inventory of the new phycoviruses (genome size, >300 kbp) is far from complete. Preliminary hybridization studies to identify a ribonucleotide reductase gene on *Chlorella* virus DNA have not been successful.

In this note we compare features of ribonucleotide reductase activity in extracts of *Chlorella* Pbi cells not infected or infected by Pbi viruses. Some data have been presented recently [18].

Experimental

Materials and methods

Biochemicals and other reagents were purchased from Merck, Serva, or Boehringer, and radioactive nucleotides from Amersham Buchler. Protein was determined by the method of Bradford [19]. Thin layer chromatography of nucleotides was performed on 20 cm-PEI cellulose sheets, developed in 0.8 M acetic acid for 2 cm and then in 0.8 M ammonium acetate/1.1 M LiCl (pH 4.0). Radioactivity was determined in Aquasafe 300 (from Zinsser) liquid scintillation fluid in a Beckman LS 1801 counter.

Reprint requests to Prof. H. Follmann.

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Algal cultures and extracts

The *Chorella*-like algae Pbi (originally an isolate from *Paramecium bursaria*) were kindly provided by Drs. W. Reisser and B. Becker, University of Göttingen, and were cultured in a mineral medium as described [20]; the medium was supplemented with thiamine hydrochloride (670 $\mu g \times l^{-1}$), vitamin B 12 (70 $\mu g \times l^{-1}$), tetracycline × HCl (670 $\mu g \times l^{-1}$), and ampicillin Na-salt (830 $\mu g \times l^{-1}$). Cultures were maintained in a light thermostat at 21 °C and 6000 lx continuous light. *Chlorella* Pbi viruses were isolated from a local pond (Küchengraben, Kassel). Mass production and plaque titration on agar plates were carried out essentially as described by VanEtten *et al.* [21, 22].

Algae were harvested at a density of $3-8 \times 10^7$ cells $\times ml^{-1}$. For virus infection, cultures of that density were infected with 5 plaque-forming units per cell and were harvested 1–2 h later by low-speed centrifugation.

The algae were suspended in cold 50 mM MOPS buffer, pH 7.0, containing 1 mM dithiothreitol, and were broken in a refrigerated Bühler Vibrogen cell mill under addition of 0.7 mm glass beads. The homogenate was centrifuged for 30 min at 43,000 × *g*. The green supernatant solution was made 1% in streptomycin sulfate, the precipitate removed after 30 min in the cold, and the supernatant brought to 60% ammonium sulfate saturation. After 30 min the greenish protein pellet was dissolved in the above buffer (1 ml per ml packed cells) and dialyzed against a 1000-fold volume of buffer for 2 × 1.5 h. This fraction could be stored at −80 °C for several months without loss in enzyme activity.

Enzyme assays

Ribonucleotide reductase activity was determined in an assay system which involves dephosphorylation of all substrate, effector, and product nucleotides by alkaline phosphatase treatment after the incubation period, and separation of ribonucleosides, deoxyribonucleosides, and liberated bases (if any) by automated HPLC on an Aminex A 9 ion exchange column [23]. Standard assays contained 50 µM guanosine diphosphate, 1 µCi [8-^{3}H]GDP (spec. activity, 12.6 Ci × $mmol^{-1}$), 10 mM dithiothreitol, thymidine triphosphate (0.05–0.5 mM, as specified below), and 1 mg algal/viral protein in a total volume of 0.300 ml 50 mM MOPS buffer pH 7.0. Incubation was for 20 min at the specified temperature and the reaction terminated by boiling.

Results and Discussion

The presently known phycoviruses infect only green algae of endosymbiotic origin. These slow-growing cells cannot be induced to proliferate synchronously in free culture, which impedes determination of cell cycle-dependent ribonucleotide reductase. 5-Fluorodeoxyuridine which leads to overexpression of the enzyme in green algae and other organisms [8] was inhibitory to *Chlorella* Pbi cultures. Nevertheless, ribonucleotide reduction could be reliably measured in cell extracts using a highly sensitive assay with [8-^{3}H]guanosine diphosphate and dithiothreitol as substrates and thymidine triphosphate as positive allosteric effector (Table I). The activity was similar to that observed in comparable *Scenedesmus obliquus* cultures. Virus infection increased the measurable activity (see below). Strong inhibition by hydroxyurea characterizes the formation of deoxyguanylate as radical-linked ribonucleotide reduction and rules out interference from nucleotide salvage pathways. Cytidine diphosphate reduction was also demonstrated but was complicated by high (deoxy)cytidylate deaminase activities present in

Table I. Ribonucleotide reductase activity in cell-free extracts of *Chlorella sp.* Pbi before and after Pbi virus infection.

Assay conditions	% GDP reduction in uninfected	infected algae
Complete[a]	100	100
Thymidine triphosphate omitted	27	7
Dithiothreitol omitted throughout[b]	0	0
Dithiothreitol omitted in extract, present in assay	29	35
+30 mM hydroxyurea	2	5
+1 mM Mg^{2+}	106	152
+1 mM EDTA	33	66

[a] *Cf.* Experimental section. 100% enzyme activity corresponds to 0.23 $nmol \cdot h^{-1} \cdot mg\ protein^{-1}$ in uninfected and 0.64 $nmol \cdot h^{-1} \cdot mg\ protein^{-1}$ in infected algal extracts, respectively.

[b] Work-up and assay in dithiothreitol-free, degassed buffer.

Table II. Fractionation of ribonucleotide reductase. Assay conditions at 30 °C (*cf.* Experimental section) were not corrected for the different optimum requirements.

Fraction	Volume [ml]	Protein [mg·ml^{-1}]	Total activity [pmol·h^{-1}]	Specific activity [pmol·mg^{-1}·h^{-1}]
Uninfected algae (2.1 g packed cells)				
Cell-free extract	25.5	1.73	3,086	66
$(NH_4)_2SO_4$ precipitate	3.0	13.3	3,072	77
Virus-infected algae (2.6 g packed cells)				
Cell-free extract	27.0	1.8	10,719	220
$(NH_4)_2SO_4$ precipitate	2.2	18.3	27,900[a]	693

[a] The increase is probably due to removal of inhibitory material [9].

the extract (not shown); GDP was therefore used as substrate in all subsequent experiments. It has been verified by TLC analysis of the nucleotide phosphorylation levels that reaction mixtures after 30 min still contain about 40% of the initial guanosine diphosphate, 30–40% as the triphosphate, and only 10–20% as monophosphate which would not serve as ribonucleotide reductase substrate.

Partial purification of extracts from uninfected and Pbi virus-infected algae was achieved by removal of nucleic acids with 1% streptomycin sulfate and precipitation of a stable enzyme fraction at 60% ammonium sulfate saturation (Table II), in analogy to our previous purification of *Scenedesmus* ribonucleotide reductase [9].

Algae were infected at a multiplicity of infection of 5 PFU/cell and were analyzed for enzyme activity after 1–2 h, *i.e.* at the onset of viral DNA synthesis and before early release of virus particles at about 3–4 h post infection [10, 12]. We found an up to ten-fold increase in the total and specific activities of ribonucleotide reductase (Table II), accompanied by qualitative changes in several enzyme properties (Fig. 1). Thus, the concentration optimum of the positive effector of GDP reduction, dTTP, shifts from low (10–100 μM) to much higher (500 μM) nucleotide concentration, and the enzyme extracted from virus-infected cells is clearly less sensitive to hydroxyurea (I_{50} = 8 mM) than the one isolated from uninfected algae (I_{50} = 1 mM) (Fig. 1, a, b). Another very pronounced difference

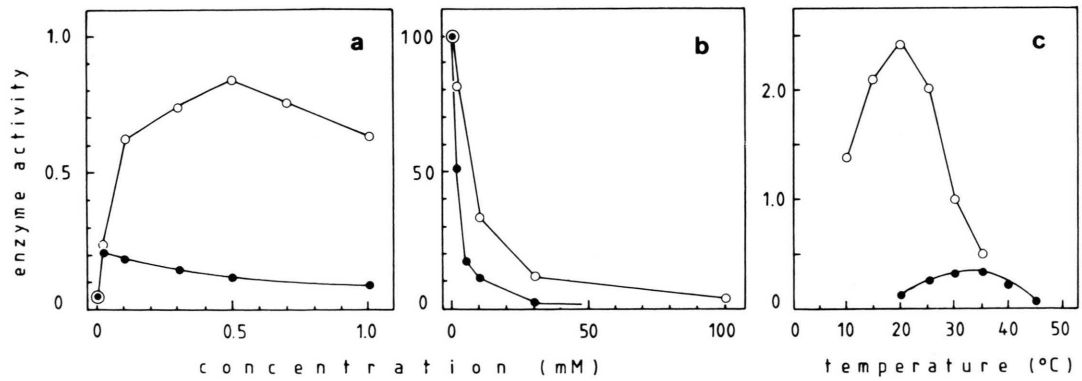

Fig. 1. Ribonucleotide reductase activity in partially purified enzyme extracts prepared from uninfected (●) and Pbi virus-infected (○) *Chlorella sp.* Pbi cultures. a: Dependence of GDP reduction on dTTP as allosteric effector, measured at 30 °C (●) or 20 °C (○). Ordinate: nmol dG formation·mg^{-1}·h^{-1}. b: Inhibition by hydroxyurea, measured at 30 °C. Ordinate: % relative enzyme activity (100% = 0.30 (●) and 0.44 nmol dG·mg^{-1}·h^{-1} (○), respectively). c: Temperature dependence, measured in presence of 0.05 mM (●) or 0.5 mM dTTP concentration (○). Ordinate: enzyme activity in nmol dG·mg^{-1}·h^{-1}.

is seen in the temperature profiles of enzyme activity: The temperature optimum of GDP reduction *in vitro* drops from 30–35 °C to an unusually low value, 20 °C, after virus infection (Fig. 1, c). Magnesium ions had a stimulatory effect only in case of the phycovirus-derived enzyme (Table I) while the pH optimum at pH 7.5 remained unchanged in either type of protein fraction. If enzyme activities in uninfected and infected algae were each determined under their optimum conditions, the virus-induced increase would, in fact, be much higher than reflected by the numbers in Table II.

These results indicate that a new, more active ribonucleotide reductase is rapidly induced in virus-infected algae to enable increased DNA precursor production. The differences observed in several characteristic enzyme parameters rule out mere amplification of the host cell enzyme. A particularly concise correlation of enzyme and physiological conditions is evident in the low temperature optimum as cell growth and virus infection are also optimal at 20 °C. The *Herpes simplex, varicella zoster,* and pseudorabies virus ribonucleotide reductases lack activation and feedback inhibition by deoxyribonucleotides [13, 24, 25], probably to uncouple virus replication from the regulatory function of cellular dNTP pools, and other viral enzymes differ at least quantitatively from their cellular counterparts in allosteric modulation [26, 27]. The large shift observed in the dTTP dependence of GDP reduction (Fig. 1, a) also points to strongly altered allosteric behaviour of the phycovirus-induced enzyme but more detailed characterization of its regulation has to await studies in a more purified enzyme system.

Ribonucleotide reductases depend on transition metals and frequently on additional divalent cations for subunit interaction [1, 2]. These requirements have not yet been completed in our present enzyme preparation which was only moderately affected by Mg^{2+}, Fe^{2+}, Mn^{2+} ions, or EDTA *in vitro*. Such a behaviour is not uncommon with partially purified ribonucleotide reductases in which the essential metal ions remain tightly protein-bound. The iron enzyme of *Scenedesmus obliquus* likewise does not require exogenous metal ions for activity.

Bacteriophages and animal viruses with large dsDNA genomes (>100 kbp; T phages, *Herpes,* pox viruses, ASFV) code not only for their own DNA polymerases but also for specific DNA precursor synthesizing enzymes. Evidence is accumulating that the large *Chlorella* virus also codes for DNA polymerase and probably thymidylate synthase ([10], and personal communication). In view of the results described above we predict that the ribonucleotide reductase induced in the Pbi virus-infected algae represents another virally encoded enzyme. However, since the similarity tree of ribonucleotide reductases is deeply branched (for example, there is little homology between the mammalian *Herpes* virus and vaccinia virus reductase genes [17]), direct identification of the putative *Chlorella* virus reductase genes may be hindered by ignorance of suitable hybridization sequences. We are, therefore, concentrating on further purification and characterization of the virus-induced new enzyme to permit insight into the biochemistry of phycovirus DNA replication, and to provide a basis for comparing viral and host cell ribonucleotide reductases on a more universal scale.

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