Inhibition of Protein Synthesis in Chloroplasts from Plant Cells by Virginiamycin

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Z. Naturforsch. **34 c**, 1195–1198 (1979); received July 4, 1979

Chloroplasts, Protein Synthesis, Antibiotics, Photosynthesis

The light-driven incorporation of amino acids by isolated spinach chloroplasts is inhibited by the M component (VM) and not by the S component (VS) of virginiamycin. This inhibitory effect is partially reversible. In chloroplast extracts, poly(U)-directed polyphenylalanine formation is strongly inhibited by VM and not by VS. The *in vivo* synergistic effect of VM and VS observed in bacteria and algae, does not occur in isolated chloroplasts and chloroplast extracts.

Introduction

Cytoplasmic organelles of eucaryotic cells possess autonomous and complete systems for protein synthesis which differ from that of cytosol [1–5], a finding which has fundamental evolutionary implications. As a matter of fact, the initiation and elongation factors and the ribosomes of chloroplasts and mitochondria closely resemble those found in procaryotes, Schizomyces and Schizophyces, but are quite different from the corresponding components present in the cytosol and in the endoplasmic reticulum of eucaryotes [6, 7]. More specifically, the 2 systems for protein synthesis have been distinguished on the basis of their susceptibility to specific inhibitors: chloramphenicol for the 70 S ribosomes of cytoplasmic organelles, and cycloheximide for the 80 S ribosomes of the cytosol [4, 6].

The importance of virginiamycin for this type of study stems from its unique properties. The antibiotic is composed of 2 components, VM and VS, which have a synergistic inhibitory effect [8]. In bacteria, each factor blocks reversibly protein synthesis, whereas their combination possesses an irreversible action [9, 10]. On the other hand, VM produces a reversible bleaching of *Euglena gracilis*, while VS alone has no evident metabolic action but renders permanent the inhibitory effect of its partner on chlorophyll formation and chloroplast multiplication [11–13]. Finally, *Plectonema boryanum*, a photosynthetic procaryote, resembles more closely Euglena and eucaryotic algae than bacteria in its susceptibility to virginiamycin [14].

0341-0382/79/1200-1195 $ 01.00/0

The main purpose of the present work was to ascertain whether virginiamycin inhibits protein synthesis in chloroplasts isolated from plant cells as well as in extracts prepared from these organelles. This study might contribute to clarify: a) the mechanism of action of virginiamycin at the ribosome level, and b) the evolutionary relatedness of the machineries for protein synthesis in bacteria, in cyanophyces and in the cytoplasmic organelles of plant cells.

Materials and Methods

Preparation of chloroplasts from plant cells

Freshly collected spinach leaves were dipped for 15 min in a 10% hypochlorite solution, rinsed with cold water, and suspended in EMST buffer having the following composition: 0.002 M EDTA, 0.001 M $MgCl_2$, 0.004 M β-mercaptoethanol, 0.33 M sorbitol, 0.05 M Tricine-KOH (pH 8.4). Leaf suspensions in melting ice were homogenized for 4 sec (TP-18-10 Ultraturrax Homogenizer, Janke and Kunkel, Ika Werk, Staufen i. Breisgau, Germany) and then passed through 2 layers of muslin filters, one loosely fitted and the other thightly fitted. The homogenates were kept in the dark at 4 °C and used immediately. Chloroplasts were sedimented by centrifugation of plant cell homogenates at 2000 × *g* for 40 sec at 4 °C (angle rotor SS 34, RC 3 B Sorvall centrifuge). Particles were purified by 3 cycles of centrifugation and suspension of the corresponding pellets in EMST buffer.

Protein synthesis in isolated chloroplasts

To chloroplast suspensions in KTM buffer (0.2 M KCl, 0.7 mM $MgCl_2$, 0.05 M Tricine KOH buffer

pH 8.3) kept at 4 °C in the dark, L-[^{35}S]methionine (520 mCi/mmol) was added. Samples were transferred to a shaking water bath at 20 °C, and irradiated for 30 min with a fan-cooled visible light lamp (5000 lux, 500 W Osram, Germany). Polymerization reaction was stopped by addition of 5% TCA and 0.05 M L-methionine. Samples were kept for 15 min in boiling water and 15 min in ice, and then filtered through glass-fiber filters (GFC, Whatman, England). Filters were washed sequentially with 5% TCA, 75% ethanol, and ether, and counted in a scintillation spectrometer.

Preparation of suborganellar extracts from chloroplasts

Suspensions of organelles in SBLA Buffer (Tris-HCl 20 mM pH 2.8, Mg Acetate 50 mM, β-mercaptoethanol 10 mM, KCl 10 mM, Spermine 20 µg/ml, reduced glutathione 0.1 mM) were submitted twice to compression to 6000 psi at 4 °C (French Pressure Cell, Aminco, USA) equipped with a ball-valve. The homogenates were centrifuged at $30{,}000 \times g$ for 30 min at 4 °C (angular rotor A 20, Sorvall RC 3 centrifuge) to remove intact organelles, and supernatants, after addition of 10% glycerol, were frozen at − 70 °C and thawed only once, just before use.

Protein synthesis in suborganellar systems

Reaction mixture had the following composition (for 250 µl): 50 mM NH_4 maleate buffer pH 6.7, 12 mM Mg acetate, 2.5 mM β-mercaptoethanol, 0.5 mM GTP, 5 mM creatin phosphate, 6.25 µg creatin phosphate kinase, 1 mM glutathione, 2.5 mM ATP, 20 µg polyuridylic acid (pU), and 0.125 µCi L-[^{14}C]phenylalanine. Reaction was started by addition of 50 µl of organelle extracts and transfer to a 30 °C water bath. After 30 min incubation, 10% TCA and 0.1 mg bovine serum albumin were added. Samples were boiled for 10 min, and then filtered through glass fiber filters (*v. s.*), which were washed with 5% TCA, 75% ethanol and ether, and counted.

Preparation of virginiamycin components

Crude fermentation product from *Streptomyces virginiae* was fractionated, and VM and VS components crystallized as previously described (*cf.* refs [15] and [16]). Solutions of purified antibiotics were tested for biological activity before use with isolated organelles and suborganellar systems.

Results

Action of virginiamycin on protein synthesis in isolated chloroplasts

It was previously shown that virginiamycin bleaches growing cultures of *Euglena gracilis:* this effect is transient when cells are incubated with VM alone, and permanent in the presence of both virginiamycin components VM and VS [11 − 13]. Since chlorophyll formation is coupled to protein synthesis, and chloroplasts from protists and higher plants share 70 S ribosomes, an inhibitory action of virginiamycin on protein synthesis in isolated plant organelles could reasonably be expected. Indeed, as shown in Table I (section A), light-dependent incorporation of labeled methionine into polypeptides, insensitive to cycloheximide (not shown) but sensitive to chloramphenicol, was strongly inhibited by low concentrations of VM (63 percent inhibition at 1 µg/ml). On the contrary, little or no inhibition by VS, and negligible synergistic effect between the 2 virginiamycin components were observed.

Table I. Action of virginiamycin on light-dependent protein synthesis by isolated chloroplasts.

Experimental conditions	Inhibitors [µg/ml]		Methionine incorporated	
			[cpm]	% of control
A. Inhibitors present				
1. Blank	–		2268	–
2. Dark	–		5042	–
3. Light	–		44112	100
4. Light	CMP	100	8150	18.5
5. Light	VM	1	18666	37.8
6. Light	VS	10	35623	80.1
7. Light	VM + VS	1	17489	39.6
B. Transient contact with inhibitors				
8. Blank	–		4689	–
9. Dark	–		6741	–
10. Light	–		27437	100
11. Light	CMP	100	24687	90
12. Light	VM	1	26819	97.7
13. Light	VS	10	26173	95.4
14. Light	VM + VS	2	23566	85.9

Conditions. A. Chloroplast suspensions were incubated for 30 min at 20 °C in the presence and in the absence of inhibitors. B. After incubation for 5 min with the inhibitors (20 °C, dark), chloroplasts were sedimented and transferred to antibiotic-free medium for protein synthesis. Label: 2.5×10^6 cpm [^{35}S]methionine. Inhibitors: chloramphenicol (CMP) and virginiamycin (VM, VS and VM + VS).

Table II. Action of virginiamycin on polypeptide synthesis by suborganellar preparations of chloroplasts.

Samples	Inhibitors [μg/ml]		Phenylalanine incorporated	
			[cpm]	% of control
1. Blank	–		334	–
2. Sample-pU	–		471	–
3. Complete system	–		6270	100
4. Complete system	CMP	100	510	8.1
5. Complete system	VM	1	565	9.0
6. Complete system	VM	0.1	1229	19.6
7. Complete system	VS	1	5979	95.4
8. Complete system	VM + VS	2	844	13.5

Conditions: as detailed in Materials and Methods.
Inhibitors: chloramphenicol (CMP) and virginiamycin (VM, VS and VM + VS).

Reversibility of virginiamycin produced inhibition in isolated chloroplasts

Previous works with bacteria [9], cyanophyces [14] and eucaryotic algae [12] have shown that the inhibitory action of VM on protein synthesis is reversible in the absence of VS, and irreversible in its presence. Possible reversibility was tested by incubating chloroplasts in the presence of virginiamycin in dark (to prevent photo-inactivation of the antibiotic): the organelles were then centrifuged and transferred to antibiotic-free medium for protein synthesis in light. By comparing sections A and B of Table I it can be concluded that the inhibitory effect of VM on chloroplasts is reversible, although higher concentrations of the drug were less readily removed by washing (not shown).

Inhibition of peptide bond formation in a sub-organellar system derived from chloroplasts

Failure of the virginiamycin component VS in inhibiting protein synthesis in isolated chloroplasts (this antibiotic has a powerful action in bacteria) could be due to lack of penetration. This possibility was checked by evaluating the effect of the 2 virginiamycin components and their combination in a suborganellar system from spinach chloroplasts. Data in Table II show a 91% inhibition of the polymerization reaction by VM at a concentration 10^{-2} M, and 81% inhibition at 10^{-3} M. No inhibition was reproducibly found with VS, nor appreciable synergistic effect was produced by this compound on the inhibitory action by VM.

Discussion

Data presented in Table I indicate that virginiamycin M (group A component) inhibits light-dependent protein synthesis in isolated plant chloroplasts. This result can be accounted for by previous findings that this antibiotic binds to the 50 S subunits of 70 S ribosomes [16–18], the type of particles present in both procaryote cytoplasm and eucaryote organelles. It is tempting to extrapolate all these observations and to infer that sensitivity to group A components *in vivo* as well as *in vitro* is general property of 50 S ribosomal subunits, irrespective of their origin and preparation.

The unique property of synergimycins is that they contain 2 components (to which either one of 2 basic structures A and B were assigned) displaying a synergistic inhibitory effect on peptide bond formation *in vivo* [8]. Two patterns of inhibition were observed in microorganisms. In bacteria, single synergimycin components inhibit reversibly protein synthesis, while their association produces an irreversible block [9, 10]. In algae, on the other hand, only group A components produce a transient halt of protein and chlorophyll formation, whereas group B components, though apparently inactive *per se*, render irreversible the inhibitory effect of their partners [12]. From data in Tables I and II it cannot be decided whether the inhibition pattern in plant chloroplasts mimics that in Euglena chloroplasts, as expected. As a matter of fact, in the experiments herewith related no activity of virginiamycin S (group B compounds) were observed.

Although the binding of VS to ribosomes has been conclusively shown [19, 20], a lack of activity *in vitro* has been repeatedly observed with group B synergimycins [15, 16, 21]. In the case of bacteria, for which these antibiotics are powerful growth inhibitors, the claim was made that the *in vitro* activity is linked to the nature of the template used for directing cell-free protein formation [22]. Obviously, such explanation does not hold for experiments in Table I, where endogeneous messengers were involved. A more likely explanation is that no biochemical reaction for *in vitro* activity of group B synergimycins is presently available. However, the introduction of a new biophysical test (group A compounds modify the spectrofluorimetric shift accompanying fixation of group B compounds to 50 S subunits) [22] should make it

possible to compare the *in vitro* systems from Schizophyces and eucaryotic organelles, with respect to their sensitivity to antibiotics of the virginiamycin family.

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