Free, Conjugated and Bound Polyamines during the Cell Cycle in Synchronized Cultures of *Scenedesmus obliquus*

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The levels of free, conjugated and bound polyamines (PA) were analyzed during the cell cycle of the synchronized unicellular green alga *Scenedesmus obliquus*. The polyamines putrescine (PUT) and spermidine (SPD) in their free and conjugated forms accumulated per cell to a maximum in the cell cycle at about the 16th hour after onset of illumination. The polyamines bound to macromolecules and membrane systems showed an additional peak around the 8–10th hour of the cell cycle. The possible role of the different forms of polyamines in DNA replication, mitosis, cell division and development of the photosynthetic apparatus is discussed.

Introduction

The main polyamines (PAs), putrescine (PUT), spermidine (SPD) and spermine (SPM) are ubiquitous in cells of higher plants. They occur in three different forms: In the free form as cations (S), conjugated to small molecules (SH), such as phenolic acids and bound to various macromolecules (PH), such as nucleic acids (Flink and Pettijohn, 1975), phospholipids (Chapel *et al.*, 1984) and proteins (Serafini-Fracassini and Mossetti, 1985; Park *et al.*, 1980; Kauss and Jeblick, 1986; Apelbaum *et al.*, 1988). The binding of polyamines to macromolecules is considered to constitute a mechanism of regulation of basic processes in plants, such as cell division, morphogenesis, senescence and responses to environmental and stress conditions (Slocum *et al.*, 1984). The enzyme transglutaminase is capable of inserting polyamines covalently between two carboxyl groups of two glutamate residues in a protein (Icekson and Apelbaum, 1987). Such binding of exogenous polyamines to proteins retards protein degradation, inhibits chlorophyll loss and stabilizes chloroplast thylakoid membranes (Besford *et al.* 1990, 1991, 1993). Furthermore, endogenous polyamines were determined in photosynthetic pigment-protein complexes, including the reaction center of PS II (Kotzabasis *et al.*, 1993 a).

In experiments employing aphidicolin-synchronized cell cultures of *Nicotiana tabacum*, Pfosser *et al.* (1990) reported that free putrescine levels were higher at the end of G 1-phase and at the S/G 2 transient phase, but declined rapidly at the beginning of DNA replication. Maki *et al.* (1991) reported an increase of the polyamine level prior to the DNA synthesis during the S-phase and prior to cytokinesis of *Catharanthus roseus* synchronized by specific inhibitors of arginine and ornithine decarboxylase. In synchronized cultures of photoautotrophically grown *Euglena* (Adlakha *et al.*, 1980) the arrest of cell division blocked the utilization of polyamines.

In the present communication we investigated the changes of the endogenous polyamine levels during the natural cell cycle in suspension cultures of the unicellular green alga *Scenedesmus obliquus*, synchronized by a light-dark regime, without the addition of any chemical factors influencing the cell cycle.

Abbreviations: HPLC, high performance liquid chromatography; PA(s), polyamine(s); PH, form bound to macromolecules; PUT, putrescine; S, free cationic form; SH, bound form conjugated to small molecules; SPD, spermidine.

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Materials and Methods

Synchronization of a Scenedesmus culture

Synchronized cultures of the unicellular green alga *Scenedesmus obliquus,* wild type strain D 3 (Gaffron, 1939) were used. The culture tubes (40 mm diameter, 400 mm height) were kept in a temperature controlled water bath (30 °C) in front of a panel of fluorescent lamps with an irradiation intensity of 20 W m^{-2} of white light. The cultures were continuously percolated with air enriched with 3% carbon dioxide (Senger *et al.,* 1972). Synchronization of the culture was achieved by a periodical light-dark change with 14 h light and 10 h darkness. The density of the culture was photoelectrically controlled at the onset of the light period (Pfau *et al.,* 1971). Under these conditions the cells were completely synchronized. Separation of cells into daughter cells (autospores) occurs between the 16th and 20th hour after onset of illumination.

Determination of polyamines

Samples of algae suspension were drawn every 2 h from the synchronized culture, adjusted to 32 µl PCV and centrifuged at 3000 × *g* for 10 min. The pellet was extracted with 5% (v/v) perchloric acid (32 µl PCV in 1 ml perchloric acid). The extract was kept on ice for 30 min and centrifuged at 3000 × *g* for 20 min (Ecospin, Sorvall-Instruments, DUPONT). Aliquots of the supernatant and pellet (redissolved in 1 N NaOH) were hydrolyzed according to the procedure of Tiburcio *et al.* (1985). 200 µl of the supernatant and the resuspended pellet were mixed with 12 N HCl in a proportion 1:1 (v/v), put in flame-sealed ampoules and hydrolyzed at 110 °C for 18 h. The hydrolyzed product was centrifuged at 3000 × *g* for 10 min in order to remove carbonized material and evaporated at 70–80 °C. Dried samples of the supernatants (SH-PAs) and from pellets (PH-PAs) were redissolved in 200 µl of 5% (v/v) perchloric acid. In order to characterize and estimate all forms of polyamines by HPLC, their derivatization is necessary, especially the benzoylation of 200 µl of each polyamine fraction (S-, SH-, PH-PAs) according to a modified method of Flores and Galston (1982). Immediately, 1 ml 2 N NaOH and 10 µl benzoylchloride were added to 200 µl of the polyamine aliquots and suspended by vigorous shaking for 30 sec. After a 20 min incubation time at 25 °C, 2 ml saturated NaCl were added to stop the reaction. Benzoylpolyamines were extracted into 3 ml of diethylether. The ether phases were then evaporated to dryness. The benzoylpolyamine-residues were redissolved in 200 µl 63% (v/v) aqueous methanol and 20 µl of this extract were injected in a HPLC system according to an improved method by Kotzabasis *et al.* (Kotzabasis *et al.,* 1993 b). This analysis was performed with a Hewlett-Packard 1090 HPLC equipped with a DPU multichannel integrator, a diode array system (Hewlett Packard, Frankfurt, F.R.G.) and an 85 B personal computer. A narrow bore column C 18, 2.1 × 200 mm, 5 µm particle size (Hypersyl, Hewlett Packard) was used for the separation of polyamines. The above method (Kotzabasis *et al.,* 1993 b) allows the direct estimation of specific polyamines in each fraction of total polyamines.

Cell number

The cell number was determined in a hemacytometer chamber (Thoma neu) under the microscope.

Results

Changes in the polyamine pattern of the unicellular green alga *Scenedesmus obliquus* were investigated during the cell cycle in a synchronized culture. The entire cell cycle lasts 24 h and is directly controlled by light-dark periods (14 h light – 10 h dark). The HPLC profiles of polyamines suggested that in *Scenedesmus* cells only putrescine (PUT) and spermidine (SPD) prevail, whereas the tetramine spermine (SPM) does not exist or only in traces.

Analysis of the total polyamine level (free, conjugated and bound polyamines together) demonstrated dramatic changes during the cell cycle (Fig. 1). The concentration of total polyamines at the 16th hour is about 10 times higher than the corresponding value in the beginning of the cell cycle. The total putrescine (PUT) and spermidine (SPD) followed the same pattern (Fig. 1) with an 11-fold increase of their initial values at the 16th hour of the cell cycle.

Analyzing the polyamines separately with respect to their three forms of binding the free cationic forms of putrescine (S-PUT) and spermidine (S-SPD) showed similar curves to the corresponding ones for total putrescine and total spermidine with only a single peak at the 16th hour (Fig. 2).

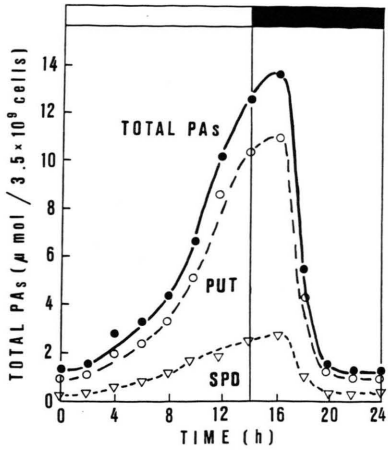

Fig. 1. Changes in the amount of total polyamines (PAs), putrescine (PUT) and spermidine (SPD) per cells during the cell cycle of synchronized *Scenedesmus obliquus* cells. Cells were synchronized in a 14:10 light-dark regime. For determination of polyamines see **Methods.**

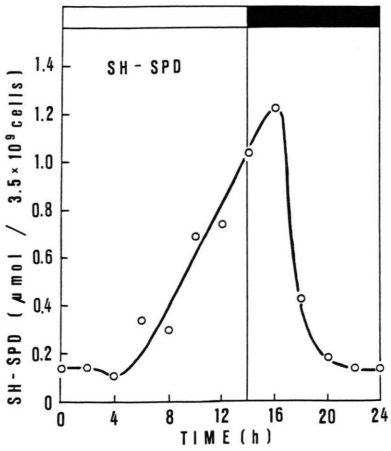

Fig. 3. The amount of conjugated spermidine (SH-SPD) per cells during the life cycle of synchronized *Scenedesmus obliquus.* For experimental details see Fig. 1.

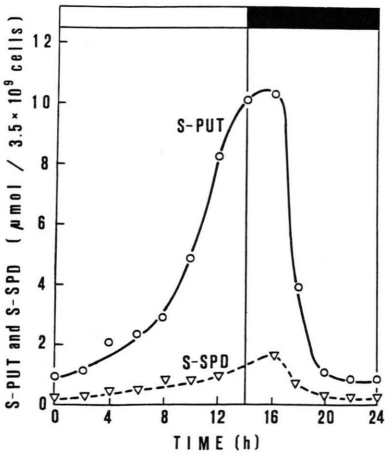

Fig. 2. The amounts of free putrescine (S-PUT) and free spermidine (S-SPD) during the cell cycle of synchronized *Scenedesmus obliquus.* For experimental details see Fig. 1.

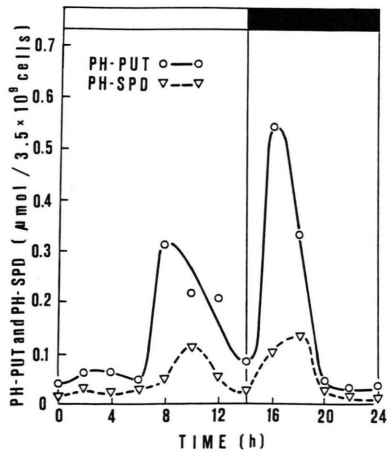

Fig. 4. Changes in the amount of bound putrescine (PH-PUT) and bound spermidine (PH-SPD) per cells during the cell cycle of synchronized *Scenedesmus obliquus.* For experimental details see Fig. 1.

Remarkable is a delay of about 4 h in the appearance of spermidine bound to small molecules (SH-SPD) during the cell cycle (Fig. 3) in comparison to the free cationic form of spermidine (S-SPD) (Fig. 2). The fraction conjugated to small molecules (SH) did not contain putrescine but only spermidine (SH-SPD). The SH-SPD follows the same pattern as the S-SPD with a single maximum at the 16 th hour (Fig. 3). The concentrations of spermidine in its free cationic form (S-SPD) and bound to small molecules (SH-SPD) add up to about 95% of total spermidine.

Only the polyamines bound to the macromolecules and membrane systems of *Scenedesmus* revealed a different profile during the cell cycle as compared to the free cationic forms (S-PAs) and the fraction of polyamines bound to small molecules (SH-PAs). The putrescine bound to macromolecules (PH-PUT) peaks at the 8th and 16th hour, whereas the corresponding spermidine fraction

(PH-SPD) peaks at the 10 th and 18 th hour (Fig. 4). The formation of macromolecules bound putrescine (PH-PUT) and spermidine (PH-SPD) starts with a delay of about 6 h in comparison to the synthesis of polyamines in their free cationic forms (S-PAs) in the cell cycle of *Scenedesmus.*

Discussion

The increase in total putrescine and spermidine (Fig. 1) as well as their soluble free cationic forms (S-PUT and S-SPD) (Fig. 2) follows in general the growth pattern of a synchronous culture, like dry weight or chlorophyll formation (Senger, 1970 a, b) up to the 16 th hour of the cell cycle. From this time on the cells separate quickly into 8 daughter cells. This explains the sharp decline of all polyamine concentrations when calculated on the base of cell number. A calculation based upon the culture volume would result in steady values from the 16 th to the 24 th hour.

The maximum amount of the polyamine fractions as above mentioned is reached at the 16 th hour. Up to this stage cells have grown, performed a series of subsequent mitosis steps and reached the M-phase of the cell cycle. It has been assumed that polyamines play an important role in mitosis. According to Adlakha *et al.* (1980) and Flores and Galston (1982) polyamines induce and stabilize the polymerization of actin and convert G-actin to F-actin. Likewise in *Scenedesmus,* a peak value of polyamines was found in the M-phase of synchronized *Euglena* (Galston, 1983).

At the current stage of investigation we cannot exclude the possibility that the curves presented here are composed of several curves with different peaks for polyamines of various compartments or binding to different molecules. For example, from the different slopes of the putrescine (PUT) and spermidine (SPD) curves (Fig. 1) one can calculate a PUT/SPD ratio, which has a peak at the 12 th hour. This would coincide with the maximum of DNA replication in the S-phase of the cell cycle of *Scenedesmus* (Feller *et al.,* 1980). A publication by Maki *et al.* (1991), reporting two peaks of polyamines at the S- and the M-phase of synchronous cell cultures of *Catharanthus roseus* is in agreement with this interpretation.

Only the fraction of the polyamines bound to macromolecules and membranes (PH-PAs) produces distinct peaks during the light period (Fig. 4). Since it is reported that polyamines retard the degradation of pigment-protein complexes and also stabilize photosynthetic membranes (Besford *et al.,* 1990, 1991, 1993) it seems logical to correlate the peaks of PH-PAs with the maximum in photosynthetic capacity around the 8 th hour of the life cycle (Senger, 1970 b). Furthermore, the fact that polyamines exist in intact chloroplasts, thylakoids, photosystem II, light harvesting complex, in the cyt b 559 complex and in the reaction center of PS II (Kotzabasis *et al.,* 1993a) might account for the appearance of the PH-PAs peak at the 8 th hour. The rapid decline of photosynthetic capacity after its maximum around the 8 th hour thus correlates with the decrease in PH-PAs. However, at the current stage of knowledge we cannot rule out that the peaks of the macromolecules bound forms of putrescine (PH-PUT) and spermidine (PH-SPD) at the 8 th and 16 th hour of the life cycle might represent the requirement for polyamines prior to DNA replication (Torrigiani *et al.,* 1987, 1989; Follmann *et al.,* 1979) which takes place in the life cycle of *Scenedesmus* around the 12 th hour (Follmann *et al.,* 1979).

The second peak in the macromolecules bound forms of polyamines (PH-PAs) around the 16 th hour (Fig. 4) is most probably linked to some other process, like cell wall formation prior to separation of daughter cells.

The observation that the onset of the biosynthesis of soluble free cationic forms of polyamines (S-PAs) coincides with the beginning of the life cycle, followed in 2 h steps by the forms of polyamines conjugated with small molecules (SH-PAs) and the forms bound to macromolecules or membranes (PH-PAs) is in agreement with the structural development of the cells. However, the high ratio of putrescine to spermidine and the absence of spermine cannot be explained at the current state of knowledge.

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