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Scientific Research

Cyclic Bis(3'-5')diadenylic Acid (c-di-AMP) Analogs Promote the Activities of Photosynthesis and Respiration of *Chlamydomonas reinhardtii*

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ABSTRACT

Physiological changes in the photosynthesis, respiration and cell division of *Chlamydomonas reinhardtii*, a freshwater green alga, in response to adenine nucleotides were investigated. In advance of this investigation, two adenine nucleotides, di(2'-*O*-methyl)-cyclic bis(3'-5')diadenylic acid (1) and its *N*-benzoyl-protected analog 2 were synthesized from the commercially available adenosine phosphoramidite. The respective analogs significantly promoted the cell division (cell number) of *C. reinhardtii* strains 137c mt$^+$ and BR mt$^+$. Moreover, they significantly enhanced the O_2 evolution (photosynthesis) and O_2 uptake (respiration) of both strains. c-di-AMP analogs seem to play an effective role as a physiological activator in planta.

KEYWORDS

c-di-AMP; O_2 Evolution (Photosynthesis); O_2 Uptake (Respiration); *Chlamydomonas reinhardtii*

1. Introduction

Prokaryotes and eukaryotes have been suggested to utilize c-di-AMP (**Figure 1**) for the regulation of cell cycle progression [1]. c-di-AMP performs an important function as a novel second messenger in addition to acting as a signaling molecule for DNA integrity in *Bacillus subtilis* during sporulation [2,3]. Moreover, an examination of the role of *B. subtilis* in *β*-lactam resistance revealed that c-di-AMP is essential for peptidoglycan homeostasis [4]. c-di-AMP functions as a new second messenger in *Staphylococcus aureus* with a role in controlling cell size and envelope stress [5]. According to Woodward *et al*. [6], *Listeria monocytogenes*, an intracellular bacterial pathogen, is detected in the cytosol of host immune cells and the host response is often dependent on microbial secretion systems. Moreover, c-di-AMP secreted by intracellular *L. monocytogenes* has been shown to activate a host type I interferon (INF) response [6]. Thus, the evidence of the existence and biological action of c-di-AMP in bacteria has been confirmed. To our knowledge, however, the existence of c-di-AMP in animals has not been disclosed. Nevertheless, a biological response to mucosally administered c-di-AMP, including strong adjuvant activities was observed in mice by Ebensen *et al*. [7].

Moreover, as far as we know, the existence of c-di-AMP in planta has not been reported. However, we have disclosed that c-di-AMP promotes the cell division of *Chlamydomonas reinhardtii*, a freshwater green alga and that di(2'-*O*-methyl)-*c*-di-AMP (**1**) exhibits activity comparable to c-di-AMP (**Figure 1**) [8,9]. While developing a route for the analog synthesis, it was also revealed that the new lipophilic analog **2** possessing benzoyl-protected adenines would be readily accessible (**Figures 1** and **2**).

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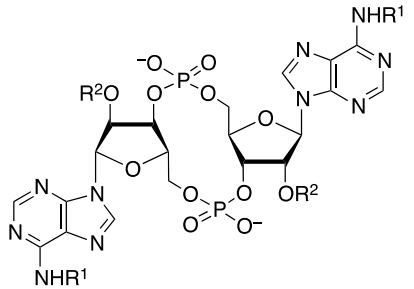

Figure 1. c-di-AMP and its 2'-modified analogs.

The purpose of the present study was to confirm that the 2'-modified c-di-AMPs, including the new lipophilic analog **2**, promote physiological activities such as photosynthesis and respiration in *C. reinhardtii*.

2. Materials and Methods

2.1. Synthesis of 2'-Modified c-di-AMPs as Physiological Effectors

Di(2'-*O*-methyl)-c-di-AMP (**1**) (triethylammonium salt) was synthesized by our method [8]. The *N*-benzoyl-protected analog **2** was obtained as follows (**Figure 2**). A mixture of the fully protected analog **3** (7.5 mg, 7.5 µmol) and $N(C_2H_5)_3$ (35 µl) in CH_3CN (0.5 ml) was stirred for 22 h at room temperature (rt). The mixture was evaporated to afford a white oily material, which was purified by reversed-phase medium-pressure liquid chromatography (eluent: A = H_2O, B = a 20:80 mixture of H_2O and CH_3CN; gradient: 0 - 5.5 min with a linear gradient from A 100% to A 90%/B10%, 5.5 - 20 min A90%/B10%) using a Purif-pack ODS-30 column (size 20) on a Purif-compact (Moritex, Japan) to give **2** (7.3 mg, 89%) as triethylammonium salt: ^{1}H NMR (500MHz, CD_3OD) δ 1.29 (t, J = 7.3 Hz, 18H), 3.18 (q, J = 7.3 Hz, 12H), 3.78 (s, 6H), 4.10 (m, 2H), 4.34 (d, J = 4.0 Hz, 2H), 4.38 (d, J = 10 Hz, 2H), 4.45 (d, J = 12 Hz, 2H), 4.58 (s, 1H), 6.32 (s, 2H), 7.51 (dd, J = 8.0 and 7.5 Hz, 4H), 7.61 (t, J = 7.5 Hz, 2H), 8.02 (d, J = 8.0 Hz, 2H), 8.70 (s, 2H), 8.76 (s, 2H); ^{31}P NMR (202 MHz, CD_3OD) δ –2.56; HRMS (ESI) calcd for $C_{48}H_{65}N_{12}O_{14}P_2$ $[M - H]^-$ 893.1815, found 893.1813.

The 2'-modified c-di-AMPs **1** and **2** thus obtained were dissolved in a solution of Tris-acetate-phosphate (TAP) [10,11] containing various elements, which is used as a culture medium for the cells of *C. reinhardtii*.

2.2. Culture Condition of *C. reinhardtii* Cells

A small quantity of *C. reinhardtii* strain 137c mt$^+$ cells maintained on Tris-acetate-phosphate (TAP) agar plates [10,11] was transferred to a 50 ml-conical beaker containing 5 ml TAP medium and pre-cultured by shaking back and forth (40 strokes min^{-1}) under a light condition of 14 µmol m^{-2} s^{-1} at 26˚C for 3 days [8]. A small quantity of the *C. reinhardtii* strain BR mt$^+$ [12] was also transferred to a beaker and pre-cultured at 26˚C for 3 days, but without shaking, because the cells of the *C. reinhardtii* strain BR mt$^+$ show more active swimming in culture medium compared to the cells of the *C. reinhardtii* strain 137c mt$^+$. Following the 3 days of pre-culture, the cell culture was started using 1.4 × 10^3 (strain 137c mt$^+$) and 1.3 × 10^3 (strain BR mt$^+$) cells/ml (at time zero) in plastic dishes (35 mm in interior diameter, 10 mm in height) containing 2 ml TAP medium in the absence and presence of the respective 10 µM c-di-AMP analogs **1** and **2** under the same conditions as used for the preculture, according to a modified version of the method of Tezuka *et al*. [8]

2.3. Measurement of Photosynthesis and Respiration

To estimate the photosynthesis and respiration of the cells of the *C. reinhardtii* strains 137c mt$^+$ and BR mt$^+$ in the plastic dishes cultured for 3 days, the cell suspension (2 ml) in the dishes was transferred to our own specially designed glass cuvette (17 mm in interior diameter, 60 mm in height) and placed on a mixer. To estimate the O_2 evolution and the O_2 uptake of the cells, a Mettler-Toledo AG electrode (Switzerland) was used as an O_2 gas analyzer to measure the quantity of dissolved oxygen in the cultured cell suspension. The cell suspension in the cuvette on the mixer was stirred with a stirring bar during the measurement of photosynthesis and respiration. The photosynthesis was estimated by measuring the increase in dissolved oxygen in the suspension under a light condition of 14 µmol m^{-2} s^{-1} at 26˚C for 3 min and then the respiration was continued to estimate the decrease in dissolved oxygen in the suspension under a dark condition using a black cloth at 26˚C for 3 min. The activities of photosynthesis and respiration were expressed as nmol O_2 (cells per ml)$^{-1}$ min^{-1}.

2.4. Estimation of Cell Numbers

After quantification of the O_2 in the suspension under light and dark conditions, the cell suspension in the cuvette was used to count the cell numbers as an index of cell division. Previous to the estimation of cell numbers, glutaraldehyde (final concentration of 0.5%) was added to an aliquot (0.5 ml) of the suspension as described above. Glutaraldehyde was used to stop the movement of *C. reinhardtii* cells with 2 flagella and to obtain a favorable estimation of cells under a cell counter. The cell

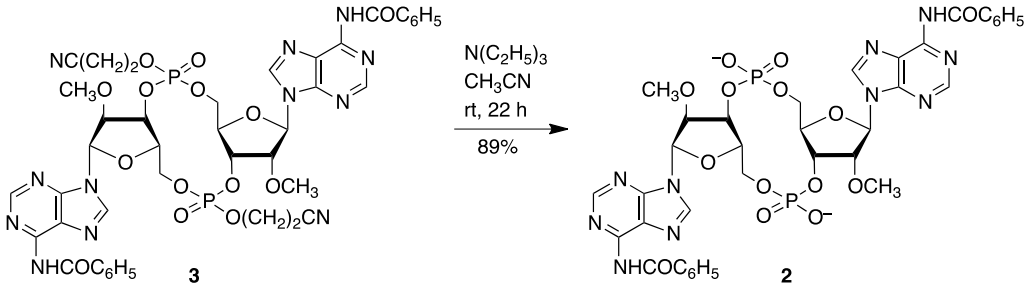

Figure 2. Synthesis of *N*-benzoyl-protected di(2'-*O*-methyl)-c-di-AMP 2.

numbers in the suspension containing 0.5% glutaraldehyde were estimated automatically using a cell counter (TC10 Automated Cell Counter: Bio-Rad. Laboratories, CA). The cell quantity of *C. reinhardtii* was expressed as the number of cells per ml. Experiments to measure the effects of the c-di-AMP analogs on the activities of photosynthesis and respiration were repeated four times, as were the experiments to determine the numbers of *C. reinhardtii* cells. Similar results were obtained each time and representative results are shown.

2.5. Statistical Analysis

A one-way ANOVA ($p < 0.05$) was used to assess differences in photosynthesis, respiration and cell division among control, di(2'-*O*-methyl)-c-di-AMP (**1**) and the *N*-benzoyl-protected analog **2** for *C. reinhardtii* strain 137c mt^+ and strain BR^+. The calculation of significant differences between *C. reinhardtii* strains was performed applying the multiple comparison test of Tukey-HSD ($p < 0.05$). For these analyses, we used the free statistical software R version 2.15.1 (R core team 2012) [13].

3. Results

The *N*-benzoyl-protected analog **2** was newly synthesized from the fully protected cyclic dinucleotide **3** (Figure 2). Thus, treatment of **3** with triethylamine removed the cyanoethyl groups on the phosphates to afford **2** in 89% yield. The activities of O_2 evolution (photosynthesis) and O_2 uptake (respiration) of the *C. reinhardtii* strains 137c mt^+ and BR mt^+ on the basis of the number of cells in the suspension were significantly promoted by di(2'-*O*-methyl)-c-di-AMP (**1**) and its *N*-benzoyl-protected analog **2** (Figure 3). The degree of promotion of the activities of photosynthesis and respiration of strain 137c mt^+ showed a tendency similar to that of strain BR mt^+.

The numbers of cells of the *C. reinhardtii* strains 137c mt^+ and BR mt^+ in the cell suspension were significantly promoted by the respective analogs of c-di-AMPs as described above (Figure 4). The percentage increase in the number of cells of strain 137c mt^+ also showed a tendency similar to that of strain BR mt^+.

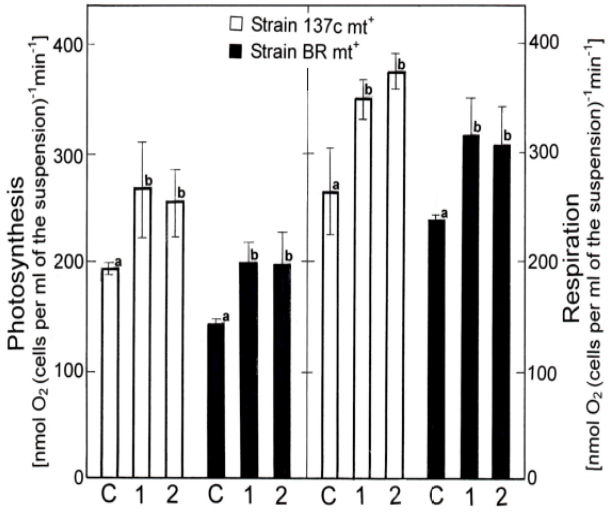

Figure 3. Effects of the 2'-modified c-di-AMPs on photosynthesis (O_2 evolution) and respiration (O_2 uptake) of *Chlamydomonas reinhardtii*. Cells of the *C. reinhardtii* strains 137c mt^+ and BR mt^+ were cultured in the presence and absence of 10 µM di(2'-*O*-methyl)-c-di-AMP (1) and its *N*-benzoyl-protected di(2'-*O*-methyl)-c-di-AMP 2 under a light condition of 14 µmol m^{-2} s^{-1} at 26˚C for 3 days [8]. Cells of strain 137c mt^+ were cultured by shaking and those of strain BR mt^+ were cultured without shaking. The activities of photosynthesis and respiration were measured under a light condition of 14 µmol m^{-2} s^{-1} and a dark condition at 26˚C, as described in the Materials and Methods. The activities were expressed on the basis of one ml of the cell suspension. Vertical bars represent the means ± SD calculated from the results of three replicates. According to a one-way ANOVA, different letters (a, b) indicate statistical significance ($p < 0.05$) among control, di(2'-*O*-methyl)-c-di-AMP (1) and *N*-benzoyl-protected analog 2. C = control, 1 = di(2'-*O*-methyl)-c-di-AMP (1), 2 = *N*-benzoyl-protected di(2'-*O*-methyl)-c-di-AMP 2.

Based on the results in Figures 3 and 4, the activities per cell in the suspension were expressed (Table 1). The degree of promotion of the activities of photosynthesis and respiration (Figure 3) showed a tendency different from that of the number of cells in the suspension (Figure 4).

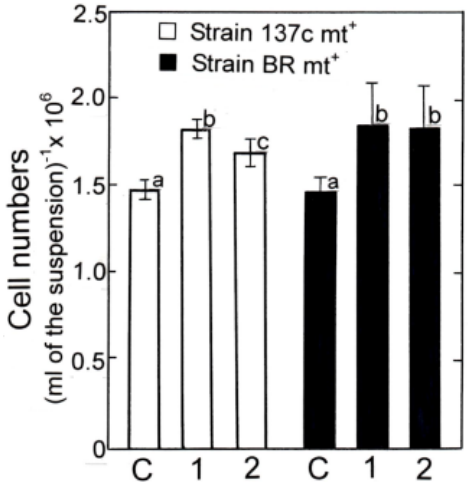

Figure 4. Effects of the 2'-modified c-di-AMPs on the cell numbers of *Chlamydomonas reinhardtii*. After measuring the activities of photosynthesis and respiration as described in Figure 3, the number of cells in suspension was estimated automatically for each of the strains using a cell counter as described in the Materials and Methods. Vertical bars represent the means ± SD calculated from the results of three replicates. According to a one-way ANOVA, different letters (a, b, c) indicate statistical significance ($p < 0.05$) among control, di(2'-*O*-methyl)-c-di-AMP (1) and the *N*-benzoyl-protected analog 2. C = control, 1 = di(2'-*O*-methyl)-c-di-AMP (1), 2 = *N*-benzoyl-protected di(2'-*O*-methyl)-c-di-AMP 2.

4. Discussion

We established a new route to the lipophilic c-di-AMP analog **2** with protected adenines (Figure 2). Because the fully protected 2'-*O*-methyl-c-di-AMP **3** is readily available by our method [8], removal of the cyanoethyl groups of **3** just led to the analog **2**. Considering that di(2'-*O*-methyl)-c-di-AMP (**1**) exhibits activity comparable to that of the natural c-di-AMP [8], the new analog **2** could be equivalent to the *N*-benzoyl-protected c-di-AMP.

Di(2'-*O*-methyl)-c-di-AMP (**1**) and its *N*-benzoyl-protected analog **2** promoted the activities of photosynthesis and respiration of *C. reinhardtii* as shown in Figure 3. These results are the first example of c-di-AMP analogs promoting a physiological action such as photosynthesis or respiration in planta. Moreover, these phenomena appeared to be partially dependent on the promotion of cell numbers by c-di-AMPs as shown in Figure 4.

Namely, *C. reinhardtii* strain 137c mt^+ and strain BR^+ with the respective adenine nucleotides such as di(2'-*O*-methyl)-c-di-AMP (**1**) and its *N*-benzoyl-protected analog **2** showed significantly higher activities of photosynthesis, respiration and cell division (cell numbers) than those of control ($p < 0.05$).

In our previous study [8], the promotion of the number of cells by c-di-AMPs was approximately twofold higher than that in the present study (Figure 4). This was probably related to the fluence rate of irradiance and the temperature in the growth chamber, since the rate and the temperature in the present study were ca. 20% and 1˚C, respectively, both lower than those in the previous study. The difference in these values between the present study and previous study may have resulted in the lower cell numbers (*i.e.*, lower cell division). *C. reinhardtii* cells attempted to escape from the relatively high irradiation in our additional experiments (data no shown). Therefore, *C. reinhardtii* was cultured under a light condition of relatively low irradiation in the present study.

Moreover, the promotion of photosynthesis and respiration may be partially caused by the promotion of metabolic activities of chloroplasts and mitochondria by c-di-AMPs, although we did not analyze the metabolic activities using chloroplasts and mitochondria as intact cell organelles isolated by a biochemical approach in the present study. In other words, the promotion may have been caused by the metabolic alteration in chloroplasts and mitochondria activated due to c-di-AMPs. This is because the degree of promotion of the activities of photosynthesis and respiration (Figure 3) did not necessarily show the same tendency as the degree of the promotion of cells (Figure 4), as shown in Table 1. Furthermore, in our additional experiments, the respiration of *Escherichia coli* was also promoted by c-di-AMPs (in preparation). These promotional effects of c-di-AMPs on the physiological activities and cell division of *C. reinhardtii* (eukaryote) seem to mirror the physiological and biochemical responses of prokaryotic cells such as *E. coli* to c-di-AMPs.

The promotions of photosynthesis and respiration induced by analog **2** were similar to those induced by the analog **1** (Table 1). This means that the benzoyl-protecting groups on analog **2** do not affect the promotions and thus lipophilicity is not the prominent factor in these effects. Therefore, for the design of the c-di-AMP analogs that can be utilized as physiological/biochemical modulators in planta, the deprotection of benzoyl groups on the adenine bases is actually unnecessary.

In conclusion, it was confirmed that the c-di-AMP analogs do in fact promote the activities of photosynthesis and respiration in *C. reinhardtii*. In other words, the c-di-AMP analogs probably play an important role as physiological/biochemical modulators in planta.

Acknowledgements

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Table 1. Relationship between the activities of photosynthesis (O_2 evolution) and respiration (O_2 uptake), and the number of cells of *Chlamydomonas reinhardtii*. The data were calculated from the results in Figures 3 and 4. The activities per cell in the suspension were expressed.

C. reinhardtii	Strain 137c mt^+	Strain BR mt^+
Photosynthesis [fmol O_2 $cell^{-1}$ min^{-1}]		
Control	129.80 (100%)	96.78 (100%)
di(2'-*O*-methyl)-c-di-AMP (**1**)	143.44 (110.5%)	106.90 (110.5%)
N-benzoyl-protected di(2'-*O*-methyl)-c-di-AMP **2**	148.29 (114.2%)	106.99 (110.5%)
Respiration [fmol O_2 $cell^{-1}min^{-1}$]		
Control	176.55 (100%)	162.26 (100%)
di(2'-*O*-methyl)-c-di-AMP (**1**)	189.45 (107.3%)	162.26 (100%)
N-benzoyl-protected di(2'-*O*-methyl)-c-di-AMP **2**	218.88 (124.0%)	167.10 (103.0%)

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