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Azide improves triglyceride yield in microalgae

Tatyana Rachutin Zalogin, Uri Pick *

Department of Biological Chemistry, Weizmann Institute of Science, 234 Herzl Street, Rehovot 76100, Israel

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ABSTRACT

Many species of microalgae accumulate under growth-limiting conditions, such as nitrogen deprivation, large amounts of triglycerides (TAG). The regulation of this process is not clear. Here we demonstrate that sodium azide (Az) induces synthesis of high levels of TAG in the lipid-accumulating marine species *Chlorella desiccata*. In comparison to N deprivation, Az leads to only minor growth retardation and to smaller inhibition of photosynthesis and respiration, resulting in a 60–80% increase in TAG yield. Maximal TAG induction level by Az is strictly dependent on light intensity and requires high CO_2. The cell morphology, TAG level and composition are similar in both treatments. From 17 tested microalgae species, 15 were responsive to Az under different culturing conditions. The results suggest that the higher TAG yield in Az-treated compared to N-deprived cultures, results from the better metabolic state and higher photosynthetic activity of the culture. The potential of Az to improve TAG yield production from microalgae is discussed.

1. Introduction

Many species of microalgae, particularly green algae and diatoms, can accumulate large amounts of triglycerides (TAG), amounting to over 50% of their biomass.

The growth-limiting conditions that induce TAG accumulation include nutrient deprivation, extreme temperature, high light intensity, high pH and high salinity [1]. Limiting the availability of nitrogen (N) is the most common way to induce TAG accumulation in microalgae [2]. Typically, N limitation increases the cellular lipid level in green microalgae by 2–10 fold in parallel with inhibition of cell division and biomass [3,4].

The regulation of massive TAG biosynthesis in green algae is not clear. It is not well understood why under nutrient limitation most green algae accumulate carbohydrates, primarily starch, whereas others accumulate TAG, and what triggers TAG massive accumulation. The recent finding that blocking starch biosynthesis in *Chlamydomonas reinhardtii* by suppression of ADP-glucose pyrophosphorylase converts this species from a starch to a TAG accumulator under N-deprivation [5], suggests that there is no specific trigger for TAG accumulation.

In an attempt to artificially induce TAG accumulation in microalgae, we tried to interfere in energy and carbon metabolism by addition of different inhibitors. Our rationale was that inhibition of alternative metabolic pathways for carbon utilization and/or energy production, such as respiration, may shift the metabolism towards TAG accumulation.

Here we show that the respiratory inhibitor sodium azide (Az) induces massive TAG accumulation in the oleaginous green alga *Chlorella desiccata* and in other microalgae. In contrast to N-deprivation, Az induces maximal TAG accumulation in *C. desiccata* with minimal growth retardation, resulting in a larger TAG yield.

2. Materials and methods

2.1. Chemicals and strains

Sodium azide (NaN_3) was purchased from Merck. All other chemicals were purchased from Sigma-Aldrich Chemicals.

2.2. Algal strains and cultivation conditions

Dunaliella tertiolecta was obtained from the culture collection of Dr. W. H. Thomas (La Jolla, CA). *Dunaliella bardawil* Ben-Amotz and Avron is a local isolated species, American Type Culture Collection, Rockville, Md. #30861. *Dunaliella parva* is a local isolate, obtained from Prof. B. Ginzburg at the Hebrew University, Jerusalem. *Phaeodactylum tricornutum* (strain CCMP632) was a gift from Dr. A. Vardi, Department of Plant Sciences at the Weizmann Institute. *C. reinhardtii* strains CW15 and cc-1009 were obtained from Prof. A. Danon, Department of Plant Sciences at the Weizmann Institute. *C. desiccata* (UTEXID LB2437) was obtained from The Culture Collection of algae at the University of Texas at Austin. All other microalgae species were obtained from the Culture Collection of Algae and Protozoa (CCAP), SAMS Research Services Ltd., Scottish Marine Institute, Scotland, UK.

D. tertiolecta, *Dunaliella salina* Teodoresco, *D. parva* and *D. bardawil* were cultured in *Dunaliella* medium containing 5 mM KNO_3, 5 mM $MgSO_4$, 0.2 mM $CaCl_2$, 0.2 mM KH_2PO_4, 1.5 μM + 6 μM $FeCl_3$ + Na_2EDTA, 7 μM $MnCl_2$, 1 μM $CuCl_2$, 1 μM $ZnCl_2$, 1 μM $CoCl_2$, 1 μM

Abbreviation: Az, sodium azide.

* Corresponding author. Tel.: +972 8 934 2732.
E-mail address: uri.pick@weizmann.ac.il (U. Pick).

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$(NH_4)_6Mo_7O_{24}$, 50 mM $NaHCO_3$, 50 mM Na-Tricine pH 8 and 0.6–2.0 M NaCl.

Pseudochlorococcum polymorphum, *Scenedesmus dimorphus*, *Chlorella sorokiniana* and *Chlorella zofingiensis* were cultured in fresh water medium containing 14.6 mM $NaNO_3$, 9.2 mM KH_2PO_4, 4 mM $MgSO_4$, 75 μM $CaCl_2$, 10 μM $FeCl_3$ + 20 μM Na_2EDTA, 0.77 μM $ZnSO_4$, 0.31 μM $CuSO_4$, 1.61 μM Na_2MoO_4, 46.3 μM H_3BO_3, 9.15 μM $MnCl_2$, 0.172 μM $Co(NO_3)_2$, 2 mM $NaHCO_3$ and 50 mM Na-tricine pH 8.0 (the two last components are not used for *C. zofingiensis*).

P. tricornutum was cultured in F/2 medium [6] supplemented with 2 mM $NaHCO_3$ and 50 mM Na-tricine pH 8.0.

C. desiccata, *Chlorella vulgaris*, *Nannochloris atomus*, *Nannochloropsis salina*, *Dunaliella primolecta* and *Chaetoceros muelleri* were cultured in artificial sea water medium (ASW) containing: 461 mM NaCl, 28.5 mM $MgSO_4$, 29 mM $MgCl_2$, 10.2 mM $CaCl_2$, 10 mM KNO_3, 0.4 mM KH_2PO_4, 0.5 mM $NaHCO_3$, 20 mM Tris–Cl pH 7.6, 113 μM Na_2SiO_3, 3 μM $FeCl_3$ + 12 μM Na_2EDTA, 39 μM $CuSO_4$, 26 μM Na_2MoO_4, 72 μM $ZnSO_4$, 42 μM $CoCl_2$, 9 μM $MnCl_2$, Vitamin B_{12}—1 mg/L, Biotin—1 mg/L, Thiamin-HCl—200 mg/L.

Microalgae were cultured under two conditions: low CO_2 medium—most species were grown in culture flasks on an orbital shaker at 100 rpm, illuminated continuously with fluorescent lamps at 70 μmol m^{-2} s^{-1} at 24 °C (continuous illumination) or under 16/8 h photoperiod at 18 °C (L/D cycles, *P. tricornutum*). High CO_2 medium—*C. desiccata*, *N. atomus*, *D. primolecta* and *Isochrysis galbana* were also grown in vertical glass tubes (3.7 or 2.5 cm diameter, 32 cm height) submerged in a Perspex water bath, illuminated continuously with fluorescent lamps at 110 μmol m^{-2} s^{-1}, and supplied with 5% CO_2/air mixture bubbled through a capillary from the bottom of the tube. The temperature of the culture was maintained at 24 °C and pH was not controlled.

2.3. Cell harvest and transfer to nitrogen deprivation

Cells were harvested by centrifuging at 3000 rpm for 5 min at 4 °C. For nitrogen starvation experiments, cultures were first cultured for 2–3 days in complete growth media. Next, cells were washed once and then resuspended in nitrogen deficient medium. Cell concentration was determined by Z2 Coulter Particle Analyzer (Beckman Coulter, Hialeah, Florida) or by Cellometer Auto M10 (Nexcelom Bioscience, Lawrence, MA).

2.4. Determination of lipid content

2.4.1. Nile red staining and fluorescence spectrometry

Nile red (NR, 9-diethylamino-5H-benzo[α]phenoxazine-5-one) was prepared as a stock solution in dimethylformamide (1 mM) and stored in single-use aliquots at −20 °C in the dark. Cells were washed in fresh growth medium and resuspended at final concentrations of 5×10^5–1×10^7 cells/mL, depending on the species. NR was added 3–10 min before the measurement, to a final concentration of 1 μM. Emission spectra at excitation and emission wavelengths of 488 nm and 520–750 nm, respectively, were measured in a Cary Eclipse Spectrophotometer (Varian, Australia Pty Ltd.) Intracellular neutral lipid content was estimated in equal number cell suspensions from the fluorescence emission intensity at 580–585 nm of Nile red-stained cells.

2.4.2. Thin-layer chromatography (TLC)

Lipids were extracted as previously described [7]. In brief, fixed number of cell pellets (1×10^7–1×10^8) were heated for 5 min to 70 °C in 200 μL DMSO and mixed on vortex with 3 mL methanol. After 15 h at 4 °C, the pellet was removed by centrifugation, and the extract was supplemented with 3 mL diethylether, 3 mL N-hexane and 3 mL distilled water. After mixing and phase separation, the hexane phase was evaporated and the dried lipids were dissolved in 200 μL chloroform, and stored in Teflon-sealed vials at −20 °C. Half to ten μL of the extract was applied to TLC silica-gel plates (5 × 7.5 cm, 60 F_{254}, Merck, Darmstadt, Germany), and developed in a closed jar in mixture of N-hexane:diethylether:acetic acid (85:15:1, v/v/v). Lipid spots were visualized by 5 min incubation in iodine vapor. The plate was scanned by Image Scanner III (Epson Expression™ 10000XL), using scanning software LabScan™ 6.0 (Powered by Melanie, Swiss Institute of Bioinformatics). TAGs were quantified by densitometry software ImageQuant™ TL relative to triolein standards.

2.4.3. High-performance liquid chromatography (HPLC)

Lipids were extracted from 2×10^8 cells as described above. The lipid pellet was dissolved in acetonitrile: *iso*-propanol:hexane, 2:2:1, v/v/v; 10 μL samples were filtered through 0.2 μm filter and injected at 10 °C for HPLC analysis. The analysis was carried out at 40 °C on a

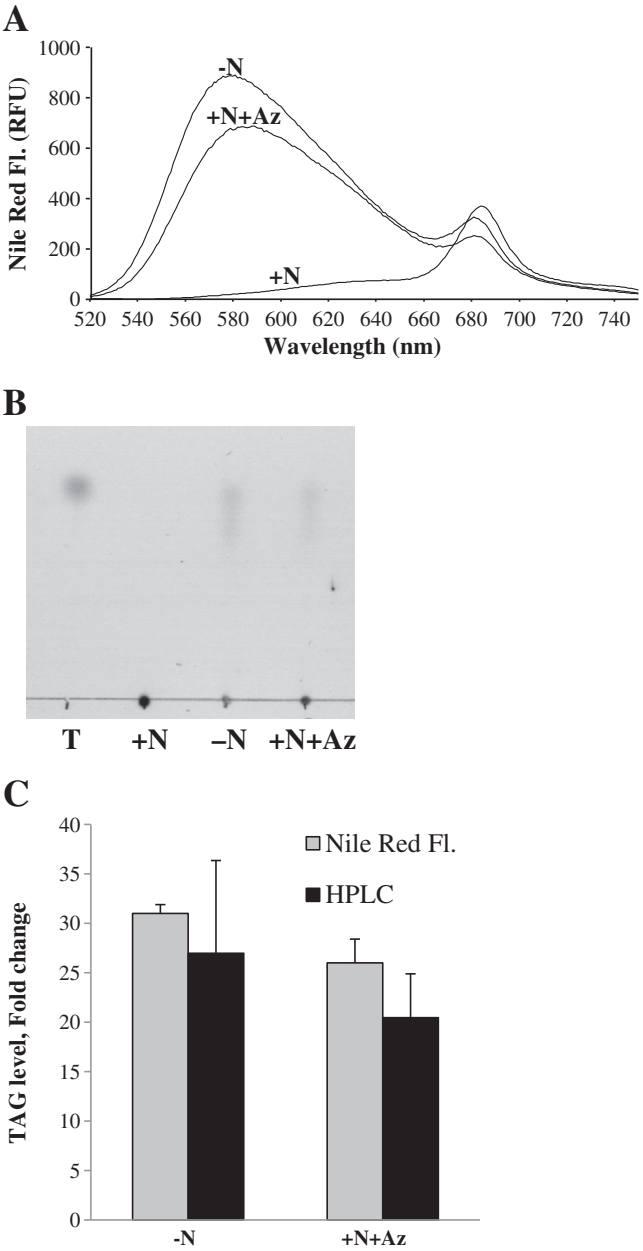

Fig. 1. Az-induced TAG accumulation in *C. desiccata*. Cells were cultured at high CO_2 for 3 d. A, NR fluorescence emission spectra in intact cells. +N, complete growth medium; −N, N-deprived medium; +N + Az, complete medium with 20 μM NaN_3. B, TAG quantification by TLC. T, 1 μg triolein. C, Comparison of neutral lipid (TAG) contents by NR fluorescence in intact cells and by HPLC analysis of cell extracts. Results are expressed in fold increase relative to control (+N) cells. Means ± SD of 3 independent experiments.