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Inhibition of nitrate reductase by azide in microalgae results in triglycerides accumulation

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ABSTRACT

Sodium azide (Az) induces accumulation of triglycerides (TAG) in the green alga *Chlorella desiccata* with minimal growth retardation (Rachutin-Zalogin & Pick, joint manuscript). To clarify the mechanism of this effect, the involvement of respiration, production of reactive oxygen species (ROS) and suppression of nitrate reductase (NR) was investigated. Different respiratory inhibitors induced minor or no TAG accumulation, ruling out respiration as the primary Az target. ROS generators failed to induce massive TAG accumulation, but the singlet oxygen quencher DABCO inhibited Az-induced TAG biosynthesis and this inhibition was suppressed in 30% D_2O. Az-induced TAG accumulation was observed in nitrate, but not in ammonium medium, in which growth is not dependent on NR. Effects of cyanide, cyanate, a singlet oxygen quencher, D_2O and of CO_2 limitation on TAG accumulation are consistent with inhibition of NR. *C. desiccata* NR activity is Az-sensitive. The results suggest that Az-induced TAG accumulation results primarily from photoinactivation of NR.

1. Introduction

We recently found that sodium azide (Az) induces massive accumulation of TAG in different species of microalgae (Rachutin-Zalogin & Pick, joint manuscript). The purpose of the present study was to try to clarify the mechanism of this effect.

1.1. Azide

Az is a well-known metabolic inhibitor in plants and mammals with many potential targets. First, it is an effective respiratory inhibitor [1], which binds irreversibly to the heme cofactor in cytochrome C oxidase (complex IV) in a process similar to carbon monoxide, in the mitochondrial respiratory chain [2]. Az also inhibits catalase (CAT) and superoxide dismutase (SOD), which scavenge ROS, thus enhancing oxidative stress in plant and animal cells [1,3]. In plants, Az was reported to inhibit oxygen evolution of PSII by occupying the chloride binding site in the immediate vicinity of the Mn cluster [4–7]. A number of ATPases have been shown to be inhibited by Az, including F-ATPases (ATP synthases) [8,9], ABC transporters [10], translocase SecA [11], DNA topoisomerase IIα [12], and ecto-ATPases [13]. Another known target of Az in plants and algae is the enzyme NAD(P)H:nitrate reductase (NR). In addition, azide is a strong physical quencher of singlet oxygen ($^1O_2$) and is frequently employed to show involvement of $^1O_2$ in oxidation processes [14,15].

1.2. Nitrate reductase

The enzyme NAD(P)H:nitrate reductase (NR) is the major enzyme controlling nitrogen assimilation in plants and algae. It catalyzes the first step in the assimilation of nitrate, namely reduction of nitrate to nitrite, by electron transfer from NAD(P)H. A second function of NR is the generation of nitrous oxide (NO), an important second messenger in green algae and in plants [16,17]. In structure, NR of plant and fungi is a homodimer. Each monomeric unit contains a two-electron transport chain, composed of flavine adenine dinucleotide (FAD), cytochrome b_{557} (Cyt) and a pterin molybdenum cofactor and a nitrate binding site [18,19]. The expression, stability and activity of NR are subject to complex regulations. Light stimulates both the synthesis of NR at the expression level and its post-translational activation, whereas darkness stimulates NR degradation and post-translational inactivation. The expression level of NR is transcriptionally regulated by nitrate availability and by light, and is suppressed by ammonium [20]. In higher plants, the post-translational regulation of NR is controlled by reversible phosphorylation and by binding of 14-3-3 protein to the phosphorylated enzyme resulting in inactivation. Two types of protein kinases, two Ca-dependent protein kinases (CDPKs) and one SNF1-related protein kinase phosphorylate Ser_{534} (in *Arabidopsis thaliana* NR) at the "hinge 1" domain of the protein, and binding of 14-3-3 protein to the phosphorylated enzyme (P-NR/14-3-3) leads to inactivation. Activation is promoted by dephosphorylation by a light-activated type 2A protein

Abbreviations: Az, sodium azide; NR, nitrate reductase; DABCO, 1,4-Diazabicyclo[2.2.2]octane; DHR, dihydrorhodamine 123; Tiron, 1,2-Dihydroxy-benzene-3,5-disulfonic acid.

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phosphatase. The activation level of NR is influenced by environmental factors, such as hypoxia, leading to NR activation, and by CO_2 limitation which deactivates NR (reviewed in [21,22]). The activation of NO production from nitrite, by the NR isoform NIA2 in *A. thaliana*, is regulated by phosphorylation of a different site, Ser_{627} in the "hinge 2" domain of the protein, by MAP kinase 6 [23]. In contrast to higher plants, NR in green algae is not regulated by phosphorylation and by 14-3-3 proteins [24].

NR is an unstable protein with a half-life of a few hours and its degradation is influenced by environmental conditions. Interestingly, it has been shown that the enzyme is most stable in its active (dephosphorylated) state and most unstable in its deactivated state (P-NR/14-3-3 complex), suggesting that phosphorylation coordinately controls both the activation level and the overall NR protein level (reviewed in [21]).

Az and cyanide are potent inhibitors of NR in both plants and in green algae, but their mechanisms of inhibition are not clear [25–27]. Earlier studies have shown that cyanide enhances inactivation of NR in the presence of NAD(P)H [28,29]. Inactive NR can be reactivated either by an oxidant such as ferricyanide, or by blue light photoirradiation [30]. Cyanide was proposed to stabilize the inactive state by binding to the pterin molybdenum center at the active site [31]. The mechanism of inhibition of NR by Az is also not entirely clear. It has been demonstrated that Az acts as a competitive inhibitor with respect to nitrate [25,28,32] and that Az inhibits the photoreactivation of NR [33]. Az also inhibited photoreduction of Cyt b by flavin in endoplasmic reticulum NR preparations from *Neurospora crassa* leading to inactivation of the enzyme. [34].

2. Materials and methods

2.1. Chemicals

Sodium azide (Az) was purchased from Merck. CM-H_2DCFDA (5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate) (C6827), and DHR (dihydrorhodamine 123) were purchased from Invitrogen Molecular Probes. Atpenin A5 was purchased from Enzo Life Sciences. All other chemicals were purchased from Sigma-Aldrich Chemicals.

2.2. Algal strains and cultivation conditions

Chlorella desiccatta strain and culturing conditions are as described in the joint manuscript (Rachutin-Zalogin & Pick, joint manuscript).

2.3. Lipid content

Estimation of neutral lipid contents by Nile Red fluorescence, by thin-layer chromatography and by HPLC was performed as described in the joint manuscript (Rachutin-Zalogin & Pick, joint manuscript). It should be noted that in *C. desiccata*, the increase in Nile Red fluorescence is linearly correlated with TAG level as determined in cell lipid extracts.

2.4. Measurement of reactive oxygen species

Stock solution of 5 mM dihydrorhodamine 123 (DHR) in DMSO was prepared and stored at −20 °C in the dark. Washed cells (2×10^7 cells/ml) were incubated for 0.5 h in the dark with 5 μM DHR. Then, the cells were washed, diluted to 1×10^7 cells/ml and exposed to different induction conditions: complete medium (+N), nitrogen deficient medium (−N) or +N with different chemicals: H_2O_2, NaN_3, Tiron (1,2-dihydroxy-benzene-3,5-disulfonic acid, TIR) and DABCO (1,4-diazabicyclo[2.2.2]octane) for 15 h in the light. Finally, the cells were centrifuged, resuspended in PBS (×2) and their DHR (Ex: 488 nm, Em: 529 nm) and Chl (Ex: 435, Em: 685 nm) fluorescence was measured by a plate reader Tecan infinite M200. DHR fluorescence was normalized to Chl fluorescence.

2.5. Nitrate reductase assay

NR activity was measured in lyzed cell extracts of *C. desiccata* and of *Dunaliella tretiolecta*. Freshly washed cells containing 4×10^8–1×10^9 cells were disrupted by glass beads with a DC-Stirrer. Nitrate reductase enzymatic assay was performed as previously described [35,36]. Nitrite production was measured by absorbance at 543 nm.

3. Results

In order to identify the target of Az, leading to TAG accumulation in *C. desiccate* (Rachutin-Zalogin and Pick, accompanying manuscript), we tested the inhibitory effect of Az on three enzymatic activities which are considered the major cellular components that are sensitive to micromolar concentrations of Az in plants and in algae: respiration, neutralization of reactive oxygen species production (ROS) and nitrate reductase (NR). Other potential azide-sensitive activities were ignored because under our growth conditions, they are not expected to be inhibited by Az: inhibition of PS-II activity is expected only at 1–10 mM Az and the inhibition is competitive with respect to chloride anions (5), likewise, protein translocation into chloroplasts is inhibited by mM Az concentration and seems to be competitive with ATP (11), whereas inhibition of F1-ATPases is not relevant, because Az inhibits just the ATP hydrolysis activity and not ATP synthesis (8), which are the relevant activities for photosynthesis and respiration.

3.1. Inhibition of respiration

To test if inhibition of respiration is responsible for TAG induction, we compared the effects of respiratory inhibitors directed against different complexes in the respiratory chain, at different concentration ranges, on cell growth and on TAG level: Rotenone (Complex I, 0.1–20 μM), Atpenin (Complex II, 0.1–10 μM), Antimycin A (Complex III, 5–100 μM) and Cyanide (Complex IV, 20–1000 μM). Of the tested chemicals, only Antimycin A induced at best 40% of the TAG level compared to Az at similar growth-inhibitory concentrations (Fig. 1C). Rotenone and Atpenin induced less than 20% of TAG (not shown). Surprisingly, even KCN, which resembles Az in that it inhibits cytochrome oxidase (Complex IV) at similar concentrations, led to a minor TAG accumulation and to higher growth inhibition compared to Az at 50-fold higher concentrations (Fig. 1B). These results suggest that inhibition of respiration is not the major cause for induction of TAG biosynthesis.

3.2. ROS production

Inhibition of catalase (CAT), or of superoxide dismutase (SOD) by Az is expected to induce enhanced levels of reactive oxygen species (ROS). Since ROS are known to be involved in triggering various stress responses in plants and algae, they might be involved also in triggering TAG biosynthesis.

In order to test if Az-treatment indeed involves enhanced ROS production in *C. desiccata*, we utilized two fluorescent ROS (and of reactive nitrogen species) sensors, the carboxy fluorescein derivative CM-H_2DCFDA (not shown) and dihydrorhodamine 123 (DHR, Fig. 2). Both probes showed that Az induces enhanced ROS generation, and that the ROS level can be decreased by the ROS quencher Tiron (1,2-dihydroxy-benzene-3,5-disulfonic acid, TIR) [37,38] but not by the singlet oxygen ($^1O_2$) quencher 1,4-diazabicyclo[2.2.2]octane (DABCO) [39]. We next tested if H_2O_2, superoxide or $^1O_2$ generators (Benzyl viologen (BV) and Rose bengal (RB), respectively), trigger TAG accumulation. None of these chemicals induced more than 20% TAG induction in comparison to Az at growth inhibitory concentrations (not shown). Another test for the possible involvement of ROS is the effect of ROS