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# Plasticity of the mitoproteome to nitrogen sources (nitrate and ammonium) in *Chlamydomonas reinhardtii*: The logic of *Aox1* gene localization

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ABSTRACT

Nitrate and ammonium constitute primary inorganic nitrogen sources that can be incorporated into carbon skeletons in photosynthetic eukaryotes. In *Chlamydomonas*, previous studies and the present one showed that the mitochondrial AOX is up-regulated in nitrate-grown cells in comparison with ammonium-grown cells. In this work, we have performed a comparative proteomic analysis of the soluble mitochondrial proteome of *Chlamydomonas* cells growth either on nitrate or ammonium. Our results highlight important proteomics modifications mostly related to primary metabolism in cells grown on nitrate. We could note an up-regulation of some TCA cycle enzymes and a down-regulation of cytochrome $c_1$ together with an up-regulation of L-arginine and purine catabolism enzymes and of ROS scavenging systems. Hence, in nitrate-grown cells, AOX may play a dual role: (1) lowering the ubiquinone pool reduction level and (2) permitting the export of mitochondrial reducing power under the form of malate for nitrate and nitrite reduction. This role of AOX in the mitochondrial plasticity makes logical the localization of *Aox1* in a nitrate assimilation gene cluster.



## 1. Introduction

Nitrate and ammonium are primary nitrogen sources for many organisms, including bacteria, protozoa, algae, plants and fungi. These organisms preferentially assimilate ammonium (reduced form) since its assimilation into organic form requires less energy than nitrate. However, microorganisms essentially exploit nitrate because it is much more abundant in natural soils (from 10 to 10.000 folds) [1–3].

The general scheme of inorganic nitrogen assimilation in photosynthetic eukaryotes is illustrated in Fig. 1. In these organisms, nitrate assimilation requires successively two transport and two reduction steps that consume eight electrons [1]. First, the extracellular nitrate is transported to the cytoplasm, where it is reduced into nitrite by the nitrate reductase (NR) using reducing power that comes from NADH or NADPH. Then, cytoplasmic nitrite is transported to the chloroplast stroma, where it is reduced into ammonium by the nitrite reductase (NiR), using reducing power coming from reduced ferredoxin [2,4]. Finally, ammonium is incorporated into carbon skeletons mainly by the glutamine synthase/glutamate 2-oxoglutarate aminotransferase (GS/GOGAT) cycle [1]. In these cyclic reactions, GS catalyzes the amination of an L-glutamate molecule into L-glutamine using ammonium and energy from ATP. Then, GOGAT catalyzes the transamination of L-glutamine and α-ketoglutarate using reduced ferredoxin. This reaction leads to the generation of two molecules of L-glutamate, one of them being used by GS to permit a continuous operation of the cycle whereas the second will enter the primary metabolism for anabolic purposes [2,3]. In the same organisms, ammonium assimilation only requires ammonium transport from the extracellular medium to the cytoplasm and then to the chloroplast stroma, where it is incorporated into L-glutamate by the GS/GOGAT cycle [1]. In *Chlamydomonas*, the genome sequencing has revealed the existence of 13 putative nitrate/nitrite transporters and 8 putative ammonium transporters [1]. Nitrate transporters can be grouped in three different protein families: NRT1 (one member), NRT2 (putative nitrate and nitrite transporters, six members) and NAR1 (putative nitrite and bicarbonate transporters, six members) [1,2,5]. Ammonium transporters form a protein family, AMT1, some of its members being located in the plasma membrane and the others in the chloroplast membrane. The presence of AMT1 transporters in both membranes allows ammonium to reach the chloroplast, where it is used in the GS/GOGAT cycle [1,6].

*Abbreviations:* NR, nitrate reductase; NiR, nitrite reductase; GS, glutamine synthase; GOGAT, glutamate/2-oxoglutarate aminotransferase; AOX, alternative oxidase; ROS, reactive oxygen species; RNS, reactive nitrogen species; 2D-DIGE, two-dimensional differential in-gel electrophoresis; TCA cycle, tricarboxylic acid cycle; IDH, isocitrate dehydrogenase; CoA, coenzyme a; OXPHOS apparatus, oxidative phosphorylation apparatus; HCP, hybrid-cluster protein; P450nor, P450 NO reductase; GDH, glutamate dehydrogenase; HRGP, hydroxyproline-rich glycoprotein; GP, glutathione peroxidase; GR, glutathione reductase

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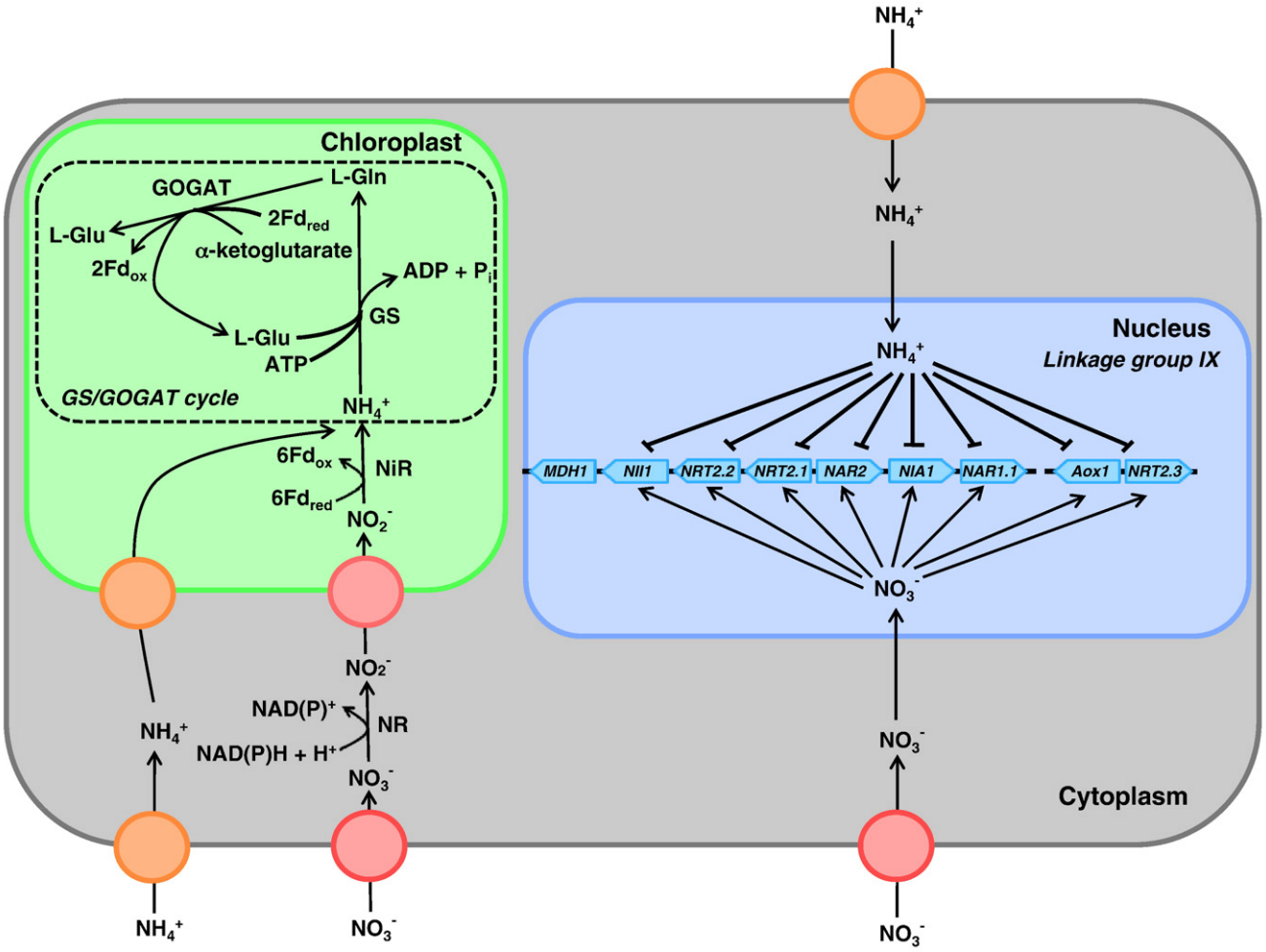


**Fig. 1.** Inorganic nitrogen assimilation; clustering and regulation by the nitrogen source of nitrate assimilatory genes in *Chlamydomonas*. NR, nitrate reductase; NiR, nitrite reductase; $Fd_{red}$, reduced ferredoxin; $Fd_{ox}$, oxidized ferredoxin; GS, glutamine synthase; GOGAT, glutamine 2-oxoglutarate aminotransferase.

In protozoa, plants and fungi, a cyanide-insensitive alternative respiratory pathway is also found in addition to the cyanide-sensitive cytochrome respiratory pathway, consisting of complexes III and IV (Fig. 4). The alternative pathway consists of a single protein located at the level of the mitochondrial inner membrane: the alternative oxidase (AOX). AOX is a terminal oxidase which catalyzes the reduction of molecular oxygen into water using electrons from ubiquinol. This reaction is not coupled to proton translocation across the inner membrane, so that the alternative pathway is energy-dissipating [7,8]. On the basis of structural and regulatory differences, two types of AOXs can be distinguished: homodimeric "plant-type" and monomeric "fungi-type" AOXs [8]. It has been evidenced that the *Chlamydomonas* AOX presents all the fundamental characteristics of the fungi-type enzyme [9]. In this organism, two genes encode an alternative oxidase : *Aox1* and *Aox2*, the transcription level of *Aox1* being much more important than that of *Aox2* [10].

Very interestingly, in *Chlamydomonas*, the *Aox1* gene is found in a gene cluster group involved in the nitrate assimilation pathway (Fig. 1). The genes coding for nitrate assimilation pathway form two clusters in linkage group IX [2]. One of these clusters contains *NII1*, encoding nitrite reductase, *NIA1*, encoding nitrate reductase, *MDH1*, encoding chloroplastic malate dehydrogenase, and *NAR1.1*, *NAR2*, *NRT2.1* and *NRT2.2* [2,11,12]. The second cluster is made of *NRT2.3* and *Aox1*, previously named *NAR5* [2,12,13]. *NRT2* and *NAR2* genes of these two clusters encode components of three out of the four high-affinity nitrate/nitrite transport systems that have been identified in *Chlamydomonas* plasma membrane, whereas *NAR1* genes encode two nitrite/bicarbonate transporters that have been identified in *Chlamydomonas* chloroplast membrane [1,2,5]. Except for *MDH1*, all the genes of these two clusters are coregulated by the nitrogen source: they are activated on nitrate and repressed on ammonium (Fig. 1) [5,12]. It was previously reported that *Aox1* displays a higher expression at both transcriptional and protein levels in *Chlamydomonas* cells grown on nitrogen-free medium in comparison with ammonium-grown cells [8,9]. Interestingly, the expression levels are even much more important in nitrate-grown cells. The regulation by the nitrogen source has shown to be concentration-dependent at the transcriptional level. In addition, the AOX capacity appears to be at least twice higher in nitrate-grown cells in comparison with ammonium-grown cells while the total respiratory rate does not vary significantly [9]. Importantly, the AOX regulation pattern in response to the nitrogen source which is found in *Chlamydomonas* appears to be specific to this unicellular alga, since it is the reverse to that found in higher plants. Indeed, in *Arabidopsis thaliana*, five genes encode an alternative oxidase, out of which three (*aox1a*, *aox1d* and *aox2*) are transcriptionally induced by ammonium and two (*aox1a* and *aox1d*) are transcriptionally repressed by nitrate in both root and shoot. This regulation by the nitrogen source also occurs at the protein and at the functional levels since AOX capacity was found to be three folds higher on ammonium [14].

In this work, we compared the soluble mitochondrial proteomes of *Chlamydomonas* cells grown on nitrate and on ammonium using the technique of two-dimensional differential in-gel electrophoresis (2D-DIGE). Our purpose was to characterize the impact of the nitrogen