Biochimica et Biophysica Acta 1797 (2010) 994–1003

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

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

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ARTICLE INFO

Article history:
Received 13 November 2009
Received in revised form 25 February 2010
Accepted 26 February 2010
Available online 6 March 2010

Keywords:
Alternative oxidase
Nitrogen source
Comparative proteomics
Mitochondria
Bioenergetics
Metabolic flexibility

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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doi:10.1016/j.bbabio.2010.02.034

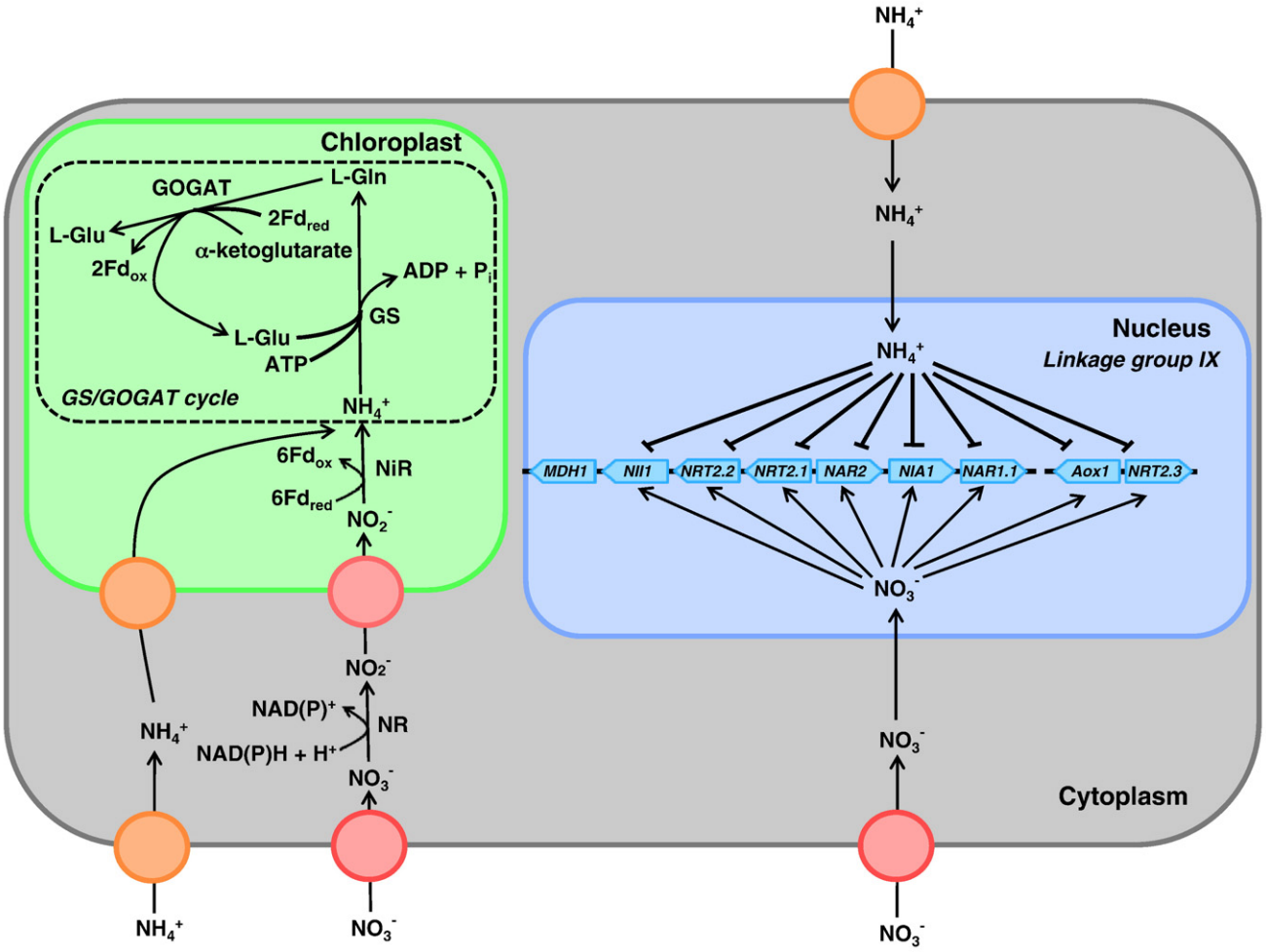

Fig. 1. Inorganic nitrogen assimilation; clustering and regulation by the nitrogen source of nitrate assimilatory genes in *Chlamydomonas*. NR, nitrate reductase; NiR, nitrite reductase; Fd_{red}, reduced ferredoxin; Fd_{ox}, oxidized ferredoxin; GS, glutamine synthase; GOGAT, glutamine 2-oxoglutarate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

source on the mitochondrial energy metabolism, particularly focusing on AOX role in metabolic adaptation as AOX expression level is higher in nitrate-grown cells. Our results provide some information which could rationalize the genetic localization of *Aox1* in a nitrate assimilation gene cluster.

2. Material and methods

2.1. Chlamydomonas strain and culture conditions

The strain used in this set of experiment is a wild-type wall-less *cw15 mt*$^+$ (strain 83). The cells were routinely growth at 25 °C on solidified agar containing 4 mM NH_4Cl or 4 mM KNO_3 under a continuous moderate white light (50 µmol m^{-2} s^{-1}). Cells were transferred at a concentration around 4×10^5 cells ml^{-1} in Erlenmayer Flasks containing TAP (Tris-acetate-phosphate) media supplemented either with 4 mM NH_4Cl or 4 mM KNO_3. The culture were carried out on a rotary shaker at 25 °C under a continuous moderate white light (50 µmol m^{-2} s^{-1}, myxotrophic conditions) and stopped in mid logarithmic phase.

2.2. Extraction and purification of mitochondria

Crude mitochondria preparations were isolated from cell wall-less strains according to Cardol and co-workers [15] and were used for oxygen consumption measurements. The yield of the extraction in both growth conditions is around 2 mg of mitochondrial proteins per liter of culture medium at mid logarithmic phase, corresponding to 5.10^9 cells per liter of culture medium. Crude mitochondria were purified for the proteomic analysis using the procedure of Eriksson and co-workers [16] with some modifications: crude mitochondria were loaded on a three step Percoll gradient (13%, 21% and 45% diluted in a buffer containing 10 mM TRIS, 280 mM Mannitol, 0.1% bovine serum albumin (BSA), 500 µM EDTA, pH 7) and were centrifuged for 50 min at 40,000×*g*. Purified mitochondria were collected at the interface between 21% and 45% Percoll layers, subsequently diluted with 40 ml of buffer without BSA and spun down for 10 min at 10,000×*g*.

2.3. Sample preparation for proteomics

Purified mitochondria were lyzed in a solubilization buffer (7 M urea, 2 M thiourea, 2% ASB-14 w/v, 10 mM dithiothreitol (DTT), anti-protease cocktail Complete EDTA Free (Roche), 0.5 mM EDTA, 50 mM TRIS, pH 7.5), briefly sonicated and intensively vortexed for 30 min at room temperature. Samples were then centrifuged to remove any insoluble material.

In order to improve isoelectrofocusing, the protein samples were precipitated and cleaned using the 2D Clean-Up Kit (GE Healthcare) that enables efficient removal of excess of salts, fatty acids and nucleic acids which may interfere with the first dimension. After the cleaning procedure, the protein pellets were resuspended in the 2D-DIGE label buffer (7 M urea, 2 M thiourea, 2% ASB-14 w/v, Complete EDTA Free (Roche), 0.5 mM EDTA, 50 mM TRIS, pH 8.5) at a protein concentration range of 5–10 mg/ml. Exact protein concentration was measured by using the RC/DC Protein Assay Kit (BioRad Laboratories).

2.4. 2D-DIGE electrophoresis and experimental design

For the comparative proteomic experiment, a set of 3 independent cultures in each experimental condition (nitrate or ammonium) has been carried out. For analytical gels, each biological replicate (25 µg) was either labeled with 0.2 nmol of Cy3 or Cy5 (minimal labeling). At the same time, an internal standard constituted of an equimolar amount of all the biological replicates was labeled with Cy2. For the preparative gel, 700 µg of proteins constituted of an equimolar amount of all the biological replicates were used, out of which 25 µg was labeled with 0.2 nmol Cy2 (minimal labeling). The labeling reactions were stopped after 30 min by adding 5 nmol of lysine. For analytical gels, Cy2-, Cy3- and Cy5-labeled proteins were pooled together before the isoelectrofocusing (IEF). Samples were reduced by adding 10 mM DTT and resuspended in Drystrip rehydration buffer (7 M urea, 2 M thiourea, 2% ASB-14 w/v, 0.6% IPG Buffer (GE Healthcare) v/v), supplemented with Destreak solution (GE Healthcare) to reach a final volume of 450 µl, and laid on a 24 cm regular strip holder. 3-11 NL IPG Drystrips (GE Healthcare) were passively rehydrated in the strip holder for 10 h at 20 °C before IEF. IEFs were run in the following conditions: 200 V for 200 V h (step), 500 V for 500 V h (gradient), 500 V for 500 V h (step), 1000 V for 2000 V h (gradient), 8000 V for 13500 V h (gradient) and 8000 V for 40000 V h (step).

After the first dimension, strips were equilibrated before the second dimension. Strips were reduced in an equilibration buffer (50% glycerol v/v, 2% SDS w/v, 6 M urea, 50 mM Tris, pH 8.8) completed with 300 mM DTT for 15 min. Strips were then alkylated for 15 min in the same equilibration solution in which DTT was substituted by 350 mM of iodoacetamide. After equilibration, strips were laid on the top of a 12.5% acrylamide gel in Laemmli SDS electrophoresis buffer (25 mM Tris, 192 mM glycine, 1% SDS w/v). For the preparative gel, one plate was treated with bind silane for spot picking. Electrophoresis was carried out overnight at 1.5 watts/gel (constant power).

2.5. Protein detection and quantification

After electrophoresis, gels were scanned with a Typhoon 9400 (GE Healthcare) at the specific excitation wavelength of each CyDye. Scanned images were analyzed with the Decyder V6.5 software (GE Healthcare). In brief, the co-detection of the three CyDye-labeled forms of each spot was done by using the DIA (Differential In-gel Analysis) module. The DIA module performs the spot detection, the ratio calculation and the spot abundance normalization via the internal standard. Statistical analysis was carried out in the BVA (Biological Variation Analysis) module after inter-gel matching. Protein spots that showed a statistically significant Student's *t*-test ($p<0.05$, $n=6$) for an increase or decrease in the spot ratio ranging up to +1.2 or down to −1.2 were accepted being differentially expressed between NH_4^+ and NO_3^- experimental conditions.

2.6. In-gel digest and mass spectrometry

Matched spots presenting a statistical difference between NH_4^+ and NO_3^- experimental groups were picked by using the Ettan Dalt Spot Picker (GE Healthcare). Proteins in gel pieces were subsequently in-gel digested according to Shevchenko and colleagues [17], with some modifications. Gel pieces were sequentially washed 3 times with 25 mM NH_4HCO_3 and 100% acetonitrile (ACN) to remove excess of detergent and buffer. After the last dehydration in ACN, pieces of gels were rehydrated with 3 µl of a 5 µg/ml trypsin, proteomic grade solution (Roche) for 2 h at 4 °C to ensure sufficient trypsin diffusion in the gel. Then, the temperature was raised to 37 °C for an overnight digestion.

After the tryptic digestion, resulting peptides were extracted from the piece of gel by adding 5 µl of a 1% trifluoroacetic acid (TFA) v/v, 30% ACN v/v solution and vortexing for 30 min. One microliter of the resulting extract was dropped on a 384-600 MTP Anchorship MALDI target plate (Bruker Daltonic) previously spotted with a 3% w/v HCCA matrix (Sigma) dissolved in acetone. Each drop was then washed with a 10 mM ammonium phosphate solution. Protein identification was carried out by using a MALDI-TOF/TOF instrumentation (Ultraflex II, Bruker Daltonic) in MS and MS/MS modes and the Mascot search engine with a maximal mass error rate fixed at 70 ppm. Peptide modifications were assessed as cysteine carbamidomethylation (as a fixed modification) and methionine oxidation (as a variable modification).

Protein search was performed in NCBI. The functions of the identified proteins were finally purchased using the novel web-portal database ChlamyCyc [18].

2.7. Oxygen consumption measurements

After extraction, crude mitochondria were loaded on a Clark-type oxygen electrode (Hansatech, King's Lynn, UK) in 1 ml of buffer containing 30 mM KCl, 4 mM EDTA, 10 mM $MgCl_2$, 57.5 mM Tris, 2.5 mM KH_2PO_4, 5 µM rotenone, 10 mM succinate and 5 mM NADH, pH 7.4. The cytochrome pathway and AOX were respectively inhibited by 1 mM KCN in aqueous solution and 2.3 mM benzohydroxamic acid (BHAM) in ethanol. State III and state IV respiratory rates were measured at 25 °C. State III respiration was induced by addition of 500 nmol ADP.

3. Results

3.1. Oxygen consumption measurements

Respiration measurements were performed with isolated mitochondria from both growth conditions in state III and state IV. In each state, we measured the total respiration and the respiration after addition of cyanide first and then BHAM, and *vice versa*. The cytochrome pathway respiration corresponds to the respiration after addition of BHAM and which can be inhibited by cyanide, whereas AOX respiration is the respiration after addition of cyanide and which can be inhibited by BHAM. Results presented in Table 1 show that the total respiration and the cytochrome pathway activity are the same between nitrate and ammonium conditions at a given respiratory state, but that AOX activity is 4 to 5 times higher in nitrate-grown cells in comparison with ammonium-grown cells and is independent of the respiratory state. Moreover, in both conditions and in both respiratory states, the cytochrome pathway activity is the same as the total respiration, suggesting that AOX activity is very low. Thus, the measured cytochrome pathway activity cannot be considered as the maximum capacity of this pathway. Contrarily, the measured AOX activity can be considered as its maximum capacity and indicates that AOX is 4 to 5 times more expressed in nitrate condition.

3.2. Comparative proteomic analysis

The DeCyder software detected on average 1160 protein spots on each 2D gel and highlighted a significant statistical variation of expression ($\alpha = 0.05$, ± 1.2) between the mitochondrial proteomes of nitrate and ammonium grown-cells for 51 of them, among which 27 could be identified unambiguously by mass spectrometry: 21 contain proteins which are located in the mitochondrial compartment whereas 6 contain proteins which are located in the cytosol (Table 2). Since cytosolic proteins constitute contaminants of mitochondrial proteins, we will only focus on the differential expression of mitochondrial proteins in this study. Fig. 2 illustrates the pattern of *Chlamydomonas* mitochondrial proteome in a 2D gel and highlights the spots containing mitochondrial proteins which are differentially expressed between nitrate and ammonium conditions. In this gel, proteins from nitrate and ammonium-grown cells are respectively labeled with Cy3 and Cy5, so that they respectively emit green and red fluorescence. Hence, the superimposition of gel images obtained for nitrate and ammonium samples permits to visualize the protein spots which are differentially expressed between nitrate and ammonium conditions in the 2D gel.

Importantly, the percentage of identified spots containing mitochondrial proteins can be considered as an evaluation of mitochondrial purity and of the purification process quality. Since 5 out of the 6 cytosolic protein spots that we identified are cytoskeleton components, that are likely to be attached to mitochondria, they automatically contaminate the mitochondrial protein fraction; as a consequence, the identified spots which contain these proteins must not be taken into account in the calculation of the mitochondrial purity percentage. Excluding cytoskeleton protein spots, we have 21 mitochondrial and 1 cytosolic spots (Table 2); thus we estimate the purity of mitochondria to 95% and we evaluate the contamination by cytosolic proteins to 5%. This percentage is based on the number of identified protein spots, not on the amount of proteins.

3.2.1. Enzymes involved in the TCA cycle

Two TCA cycle enzymes were up-regulated in nitrate-grown cells in comparison with ammonium-grown cells, namely isocitrate dehydrogenase (IDH) NADP-dependent, catalyzing the oxidative decarboxylation of isocitrate into α-ketoglutarate and reducing $NADP^+$ into NADPH, and α-ketoglutarate dehydrogenase (Fig. 3A), catalyzing the oxidative decarboxylation of α-ketoglutarate into succinyl-CoA and reducing NAD^+ into NADH. As both enzymes catalyze an irreversible reaction, they exert a high control on the TCA cycle capacity and their up-regulation probably leads to an increase in the TCA cycle capacity in nitrate-grown cells. Acetyl-CoA synthase, catalyzing the condensation of acetate, the exogeneous carbon substrate provided to the cells, and CoA into acetyl-CoA using energy from ATP, was also up-regulated on nitrate. As acetyl-CoA can notably enter the TCA cycle by acting as a substrate for citrate synthase, the up-regulation of acetyl-CoA synthase appears to follow the increase in the TCA cycle capacity in nitrate-grown cells.

3.2.2. Proteins belonging to the OXPHOS apparatus

The cytochrome c_1 was shown to be down-regulated in nitrate-grown cells in comparison with ammonium-grown cells (Fig. 3B). As it is a catalytic subunit of complex III, which transfers electrons from ubiquinol to cytochrome c, a down-regulation of its expression may constitute a limiting factor for the assembly and the capacity of the whole complex III in nitrate-grown cells. We have also identified two complex I subunits that also displayed a significant variation of expression as a function of the nitrogen source: the 49 kDa ND7 subunit and the 76 kDa subunit. Both proteins are known to bind an iron-sulfur cluster constituting a redox center involved in electrons transfer from NADH to ubiquinone. However, as they display an opposite variation of expression, we could not conclude that complex I capacity is modified according to the nitrogen source. Finally, we also observed the down-regulation of the ATP synthase 60.6 kDa associated protein in nitrate-grown cells (ATP synthase associated protein 1). In *Chlamydomonas*, ATP synthase associated proteins constitute a protein group containing seven members without clear equivalents in mitochondrial ATP synthases from other species. The function of these proteins and their

Table 1
Respiratory rates measured in isolated *Chlamydomonas* mitochondria from nitrate and ammonium conditions. KCN and BHAM were respectively used to inhibit the cytochrome pathway and AOX, and were added in the order indicated by the symbol "+". Values are the result of 3 independent experiments and are expressed in nmol O_2 min^{-1} mg^{-1} of mitochondrial proteins ± SE.

		NO_3^-	NH_4^+
State III	Total respiration	64.2 ± 4.6	63.5 ± 3.8
	+ BHAM	62.9 ± 3.5	65.5 ± 2.6
	+ BHAM + KCN	5.22 ± 0.63	4.15 ± 0.51
	+ KCN	20.3 ± 2.1	8.0 ± 0.7
	+ KCN + BHAM	7.74 ± 1.47	5.56 ± 0.93
State IV	Total respiration	49.1 ± 4.0	48.3 ± 3.9
	+ BHAM	47.7 ± 3.4	49.7 ± 4.0
	+ BHAM + KCN	5.51 ± 0.90	5.22 ± 0.74
	+ KCN	21.7 ± 1.9	10.1 ± 1.6
	+ KCN + BHAM	5.82 ± 0.78	5.80 ± 0.91

Table 2
Protein spots of which the expression level significantly varies in nitrate-grown cells in comparison with ammonium-grown cells ($\alpha = 0.05$, ± 1.2), classified according to their cellular localization (mitochondrion or cytoplasm) and to their general function. These characteristics were purchased in the new *Chlamydomonas* database ChlamyCyc [18].

Master number[a]	Protein name	NO_3^-/NH_4^+ ratio[b]	*t*-test	Accession number in NCBI	pI[c]	MW[d]
Mitochondrial proteins						
Acetate metabolism						
359	Acetyl-CoA synthase	1.25	0.00087	XP_001702039	7.30	74,089
Amino acid metabolism						
715	Agmatine iminohydrolase	1.30	0.008	XP_001700262	6.50	46,082
787	Hydroxypyruvate reductase	1.32	4.60E-05	XP_001691480	9.40	45,116
820	Glutamate dehydrogenase	0.73	5.80E-05	XP_001702270	9.00	49,233
543	Sulfite oxidase	1.27	0.0079	XP_001701253	7.90	64,119
772	Ornithine aminotransferase	1.50	4.20E-06	XP_001702022	6.60	48,655
778	Ornithine aminotransferase	2.03	1.80E-06	XP_001702022	6.60	48,655
83	Prolyl-4 hydroxylase type I	1.37	0.01	XP_001689959	5.10	114,647
Purine nucleotide metabolism						
1003	Urate oxidase II	4.89	2.60E-06	XP_001691000	8.90	23,457
TCA cycle						
792	Dihydrolipoamide succinyltransferase, α-ketoglutarate dehydrogenase E2 component	1.42	5.40E-06	XP_001692539	9.60	47,586
775	Dihydrolipoamide succinyltransferase, α-ketoglutarate dehydrogenase E2 component	1.23	0.002	XP_001692539	9.60	47,586
791	Isocitrate dehydrogenase, NADP-dependent	1.24	0.0071	XP_001698704	9.50	53,886
786	Isocitrate dehydrogenase, NADP-dependent	1.23	0.00061	XP_001698704	9.50	53,886
Mitochondrial respiratory chain						
872	NADH:ubiquinone oxidoreductase 49 kDa ND7 subunit	0.75	0.0029	XP_001697607	8.60	53,030
291	NADH:ubiquinone oxidoreductase 76 kDa subunit	1.27	0.0014	XP_001692885	9.10	79,107
1016	Ubiquinol:cytochrome c oxidoreductase cytochrome c1	0.55	1.80E-09	XP_001690765	6.40	34,040
Mitochondrial ATP synthase						
482	Mitochondrial F1F0 ATP synthase associated 60.6 kDa protein	0.53	0.03	XP_001692395	5.80	63,123
Reactive oxygen species scavenging systems						
513	Hybrid-cluster protein	1.64	0.014	XP_001694671	8.60	70,816
Reactive nitrogen species scavenging systems						
707	Cytochrome P450, nitric oxide reductase	1.38	0.0021	XP_001700272	6.50	44,185
Protein biosynthesis						
573	Non-discriminatory gln-glu-trna synthetase	0.79	0.00023	XP_001700965	6.70	58,456
Other function						
681	Hypothetical protein CHLREDRAFT_98158 (related to phosphate ABC transporter, periplasmic phosphate-binding protein [Vibrio cholerae V51])	1.98	5.00E-07	XP_001696531	6.20	34,303
Cytoplasmic proteins						
Glycolysis						
562	Enolase	0.52	0.048	XP_001702971	5.10	52,026
Cytoskeleton						
609	Alpha tubulin 1	0.27	0.0013	XP_001691876	4.90	50,182
615	Alpha tubulin 1	0.30	0.0052	XP_001691876	4.90	50,182
612	Beta tubulin 2	0.51	0.0087	XP_001693997	4.70	50,157
617	Beta tubulin 2	0.31	0.00025	XP_001693997	4.70	50,157
797	Actin	0.83	0.0082	XP_001699068	5.20	42,094

[a] Master number, position of the protein spot in the master gel.
[b] NO_3^-/NH_4^+ ratio, ratio between the normalized volume of the protein spot in nitrate and ammonium conditions.
[c] pI, isoelectric point.
[d] MW, molecular weight.

involvement in ATP synthase catalytic mechanism remain unclear. Consequently, we could not conclude that the whole ATP synthase apparatus capacity is decreased in nitrate-grown cells. Hence it appears clearly that the sole important modification observed at the level of the OXPHOS apparatus is the decrease in complex III capacity.

3.2.3. Reactive oxygen/nitrogen species (ROS/RNS) scavenging systems

The hybrid-cluster protein (HCP) was shown to be up-regulated in nitrate-grown cells. Previous studies also identified HCP in the mitochondrial compartment [19] and showed that HCP presents a peroxidase activity in *E. coli* [20], highlighting its potential role in ROS scavenging. The cytochrome P450 NO reductase (P450nor) also displayed an up-regulation in nitrate-grown cells. P450nor is responsible for NO detoxification and is important because NO is a complex IV inhibitor [21]: P450nor catalyzes the reduction of NO into N_2O using reducing power from NADH or NADPH [22]. These important proteomics regulations may highlight a potential increase of ROS and RNS production in nitrate-grown cells.

3.2.4. Enzymes involved in amino acid metabolism

The proteomic analysis highlighted the down-regulation of a mitochondrial glutamate dehydrogenase (GDH2) [3] in nitrate-grown cells. This enzyme catalyzes the reversible amination of α-ketoglutarate into L-glutamate using ammonium and reducing power from NADH or NADPH. However, previous studies have shown that the identified isoform mainly catalyzes the reverse reaction: it deaminates L-glutamate into α-ketoglutarate and notably presents an anaplerotic role in the TCA cycle by producing α-ketoglutarate [3,23].

On the contrary, two enzymes involved in L-arginine catabolism were up-regulated in nitrate-grown cells, namely ornithine aminotransferase (Fig. 3C) and agmatine iminohydrolase. On the one hand, in the ornithine aminotransferase pathway, L-arginine catabolism starts with a single cytosolic reaction belonging to the urea cycle which converts L-arginine into urea (an ammonium source) and L-ornithine, which can be used as a substrate by ornithine aminotransferase. This enzyme catalyzes the transamination of L-ornithine and α-ketoglutarate into L-glutamate and L-glutamate-γ-semialdehyde. L-Glutamate-γ-

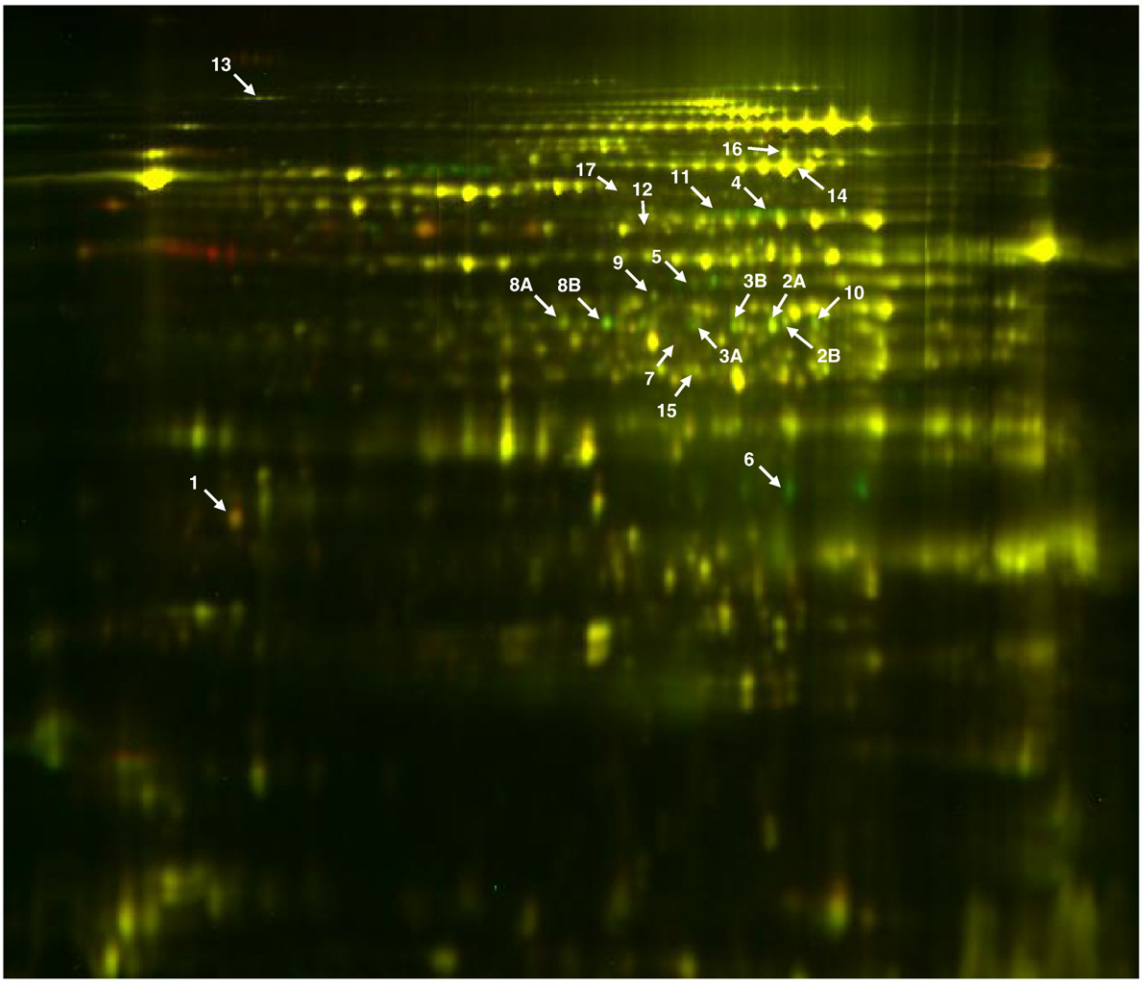

Fig. 2. 2D pattern of the *Chlamydomonas* mitochondrial proteome: superimposition of nitrate and ammonium sample images in a 2D gel. In this gel, proteins from nitrate and ammonium-grown cells are respectively labeled with Cy3 and Cy5, so that they respectively emit green and red fluorescence. The spots containing mitochondrial proteins of which the expression level varies significantly as a function of the nitrogen source ($\alpha = 0.05$, ± 1.2) and which have been identified are pointed out using white arrows. (1) Cytochrome c_1, (2) α-ketoglutarate dehydrogenase E2 component (A, spot 792 in the Master gel; B, spot 775 in the Master gel), (3) isocitrate dehydrogenase NADP dependant (A, spot 786 in the Master gel; B, spot 791 in the Master gel), (4) hybrid-cluster protein (HCP), (5) cytochrome P450 NO reductase (P450nor), (6) urate oxidase II, (7) glutamate dehydrogenase (GDH2), (8) ornithine aminotransferase (A, spot 772 in the Master gel; B, spot 778 in the Master gel), (9) agmatine iminohydrolase, (10) hydroxypyruvate reductase, (11) sulfite oxidase, (12) non-discriminatory gln-glu-tRNA synthetase, (13) prolyl-4-hydroxylase, (14) acetyl-CoA synthase, (15) NADH:ubiquinone oxidoreductase 49 kDa ND7 subunit, (16) NADH:ubiquinone oxidoreductase 76 kDa subunit, (17) ATP synthase associated 60.6 kDa protein.

semialdehyde may be used either for L-glutamate or L-proline biosynthesis. On the other hand, the agmatine iminohydrolase pathway starts with the decarboxylation of L-arginine into agmatine, which can be used as a substrate by agmatine iminohydrolase. This enzyme catalyzes the deamination of agmatine into *N*-carbamoylputrescine and ammonium. *N*-carbamoylputrescine is converted into 4-aminobutyrate by three successive enzymatic reactions notably releasing ammonium, NADH and hydrogen peroxide. At this level, a transamination reaction occurs between 4-aminobutyrate and α-ketoglutarate to give rise to L-glutamate and succinate-γ-semialdehyde, which is finally oxidized into succinate in a reaction releasing NADH or NADPH.

Hydroxypyruvate reductase was also shown to be up-regulated in nitrate-grown cells. In *Chlamydomonas*, this enzyme is located in the mitochondrial compartment [24] and catalyzes the reduction of hydroxypyruvate into glycerate using reducing power from NADH or NADPH. Sulfite oxidase, which catalyzes the oxidation of sulfite into sulfate using H_2O as an electron acceptor and releasing hydrogen peroxide [25], and prolyl-4-hydroxylase, which catalyzes the hydroxylation of L-proline into 4-hydroxy-L-proline notably using α-ketoglutarate, also displayed an up-regulation in nitrate-grown cells. In plants and in *Chlamydomonas*, hydroxyproline-rich glycoproteins (HRGPs) are key components of the cell-wall and, in *Chlamydomonas*, some cell-wall HRGPs are agglutinins involved in sexual recognition between mating-type plus and minus gametes [26].

3.2.5. Enzyme involved in purine nucleotide metabolism

Urate oxidase II, also called uricase, is involved in purine catabolism and was shown to be strongly up-regulated in nitrate-grown cells (Fig. 3D). The guanine and adenine catabolisms start respectively with two and three successive enzymatic reactions that lead to the production of NADH, ammonium and urate. Urate can be oxidized into 5-hydroxyisourate through the urate oxidase II by using molecular oxygen as an electron acceptor and therefore releasing hydrogen peroxide. This reaction is immediately followed by the non-enzymatic decarboxylation of 5-hydroxyisothiourate into allantoin. Then allantoin is converted into urea and glyoxylate by several successive enzymatic reactions. Finally, urea is degraded into ammonium.

3.2.6. Enzyme involved in protein biosynthesis

The non-discriminatory glutamine/glutamate tRNA synthetase displayed a down-regulation in nitrate-grown cells. This enzyme is responsible for loading glutamate or glutamine on a glutamyl-tRNA, which can be used either for protein or heme biosynthesis. However, the down-regulation of the non-discriminatory glutamine/glutamate

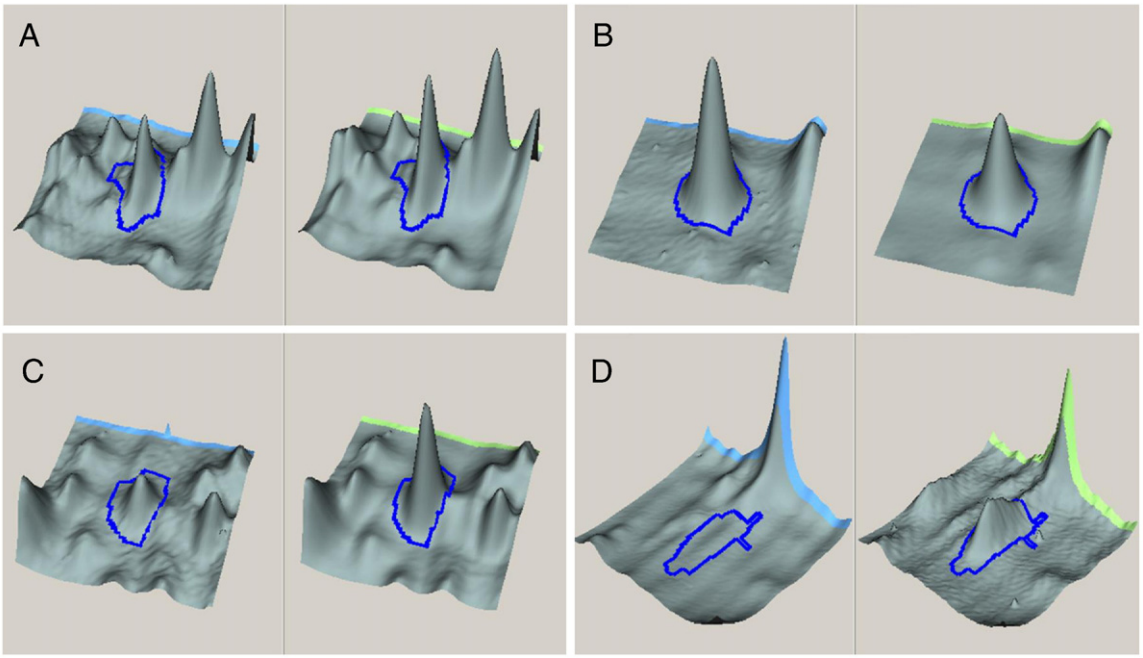

Fig. 3. Examples of some protein spot volume variations between nitrate and ammonium conditions in the Master gel. For each panel (A, B, C and D), the left and the right images illustrate respectively the protein spot for ammonium and nitrate conditions. (A) α-ketoglutarate dehydrogenase E2 component (spot 792), (B) cytochrome c_1, (C) ornithine aminotransferase (spot 778), (D) urate oxidase II.

tRNA synthetase did not allow us to conclude that heme biosynthesis is decreased on nitrate as the reaction catalyzed by this enzyme is not limiting for this pathway.

4. Discussion

In this work, we have studied the effects of the nitrogen source on the soluble mitochondrial proteome of *Chlamydomonas* cells by 2D-DIGE. The major proteomics modifications discussed below are illustrated in Fig. 4. Nitrate (oxidized form) and ammonium (reduced form) are the usual inorganic nitrogen sources used for *de novo* amino acid biosynthesis [1–3]. Photosynthetic eukaryotes preferentially use ammonium as a primary nitrogen source because its assimilation requires less energy than the assimilation of other nitrogen sources, including nitrate, nitrite, urea, purine and amino acids. Previous studies showed that ammonium can either be imported from the extracellular medium or be generated intracellularly from photorespiration, protein turnover or nucleic acid and amino acid catabolisms; then ammonium from both types of sources can enter the GS/GOGAT cycle to generate L-glutamate (Fig. 1) [3]. The genetic localization and the regulation by the nitrogen source of *Aox1* raise an important question: what could be the role(s) of AOX in regard to the assimilation of inorganic nitrogen? Thus, the main objectives of this work were to study the effect of a natural AOX overexpression and to understand its physiological role in such a metabolic context.

4.1. Effect of the nitrogen source on AOX expression and capacity

Baurain and co-workers [9] previously performed western blotting experiments and whole-cell respiration measurements using exactly the same culture conditions and the same *Chlamydomonas* strain (strain 83) as those used in the present study. On the one hand, western blotting experiments showed that the AOX expression level is higher in nitrate-grown cells in comparison with ammonium-grown cells (see Fig. 4 in Baurain et al. [9]). However, AOX was not detected in our proteomic study. This can be explained by the fact that AOX is a highly hydrophobic protein; indeed, the separation method used in 2D-DIGE technology only allows separating soluble proteins. On the other hand, whole-cell respiration measurements demonstrated that AOX capacity is about twice higher in nitrate condition (15 nmol O_2 $min^{-1} \times 10^7$ cells) in comparison with ammonium condition (6.5 nmol O_2 $min^{-1} \times 10^7$ cells; see Table 2 of Baurain et al. [9]). In our work, we performed respiration measurements with isolated mitochondria (Table 1) and we showed that the differential capacity of AOX according to the nitrogen source is also observed at the mitochondrial level (4 to 5 folds higher on nitrate). Altogether, these results demonstrate that AOX is strongly overexpressed on nitrate.

4.2. TCA cycle and primary metabolism: AOX as a trigger factor?

Our results suggest that the TCA cycle capacity is higher in nitrate-grown cells in comparison with ammonium-grown cells. This increase may induce a higher reducing equivalent and organic acid production by this pathway. The increase in the TCA cycle capacity appears to be supported by a higher capacity to convert acetate into acetyl-CoA (Fig. 4). In contrast, we have shown in another study [27] that AOX ablation leads to a decrease in the capacity of the TCA cycle and acetyl-CoA synthase and to an up-regulation of the mitochondrial carbonic anhydrase. This enzyme catalyzes the conversion of carbon dioxide into HCO_3^- and was shown to supply HCO_3^- for the anaplerotic carboxylation of phosphoenolpyruvate into oxaloacetate by phosphoenolpyruvate carboxylase in *Chlamydomonas* [28]. Carbon dioxide is one of the byproduct of the Krebs cycle activity. A previous study carried out in the unicellular green alga *Selenastrum minutum* showed that the TCA cycle carbon dioxide release is higher in nitrate-grown cells in comparison with ammonium-grown cells [29]. Interestingly, it has been evidenced that AOX capacity is importantly decreased in *Chlamydomonas* cells cultivated in a high CO_2 environment [30]. Hence, we suggest that one important role of AOX up-regulation in nitrate-grown cells could be to improve the TCA cycle capacity and to increase carbon dioxide production, which may be converted into HCO_3^- by carbonic anhydrase. In the mitochondrial matrix, HCO_3^- may be used by pyruvate carboxylase and phosphoenolpyruvate carboxylase, leading to a higher production of oxaloacetate in nitrate-grown cells (Fig. 4). Thus we propose that AOX may act as a trigger factor for the TCA cycle in order to

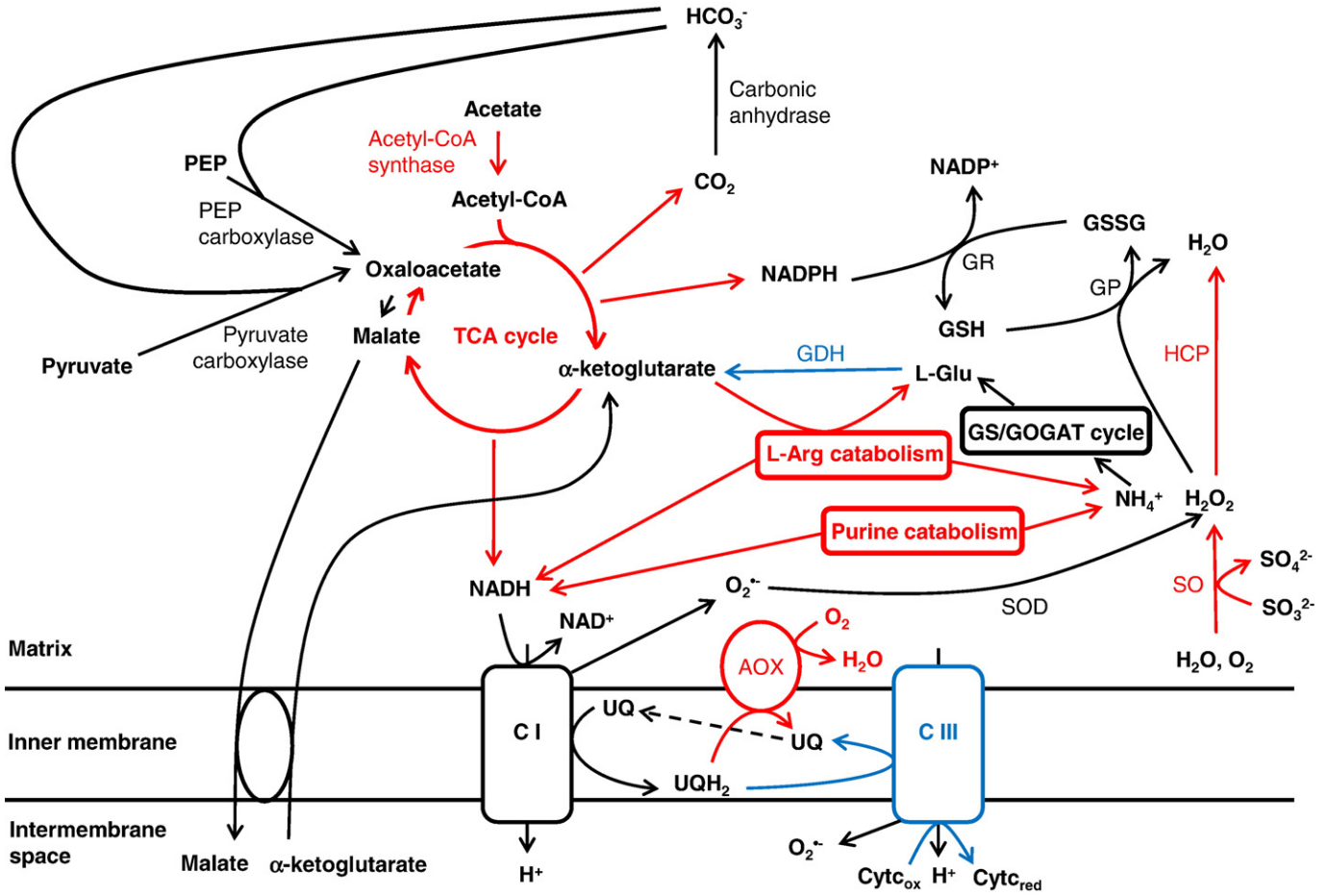

Fig. 4. Scheme illustrating the main adaptations of the mitochondrial proteome in response to the modification of the inorganic nitrogen source. Pathways colored in red and blue indicate respectively that an up-regulation and a down-regulation were highlighted by our proteomic study (except for AOX) in nitrate-grown cells in comparison with ammonium-grown cells. Stoichiometries of the reactions are not showed. GR, glutathione reductase; GP, glutathione peroxidase; HCP, hybrid-cluster protein; SO, sulfite oxidase; SOD, superoxide dismutase; GSSG, oxidized glutathione; GSH, reduced glutathione; GDH, glutamate dehydrogenase; AOX, alternative oxidase; CI, complex I; CIII, complex III; $Cytc_{ox}$, oxidized cytochrome c; $Cytc_{red}$, reduced cytochrome c; UQH_2, ubiquinol; UQ, ubiquinone; PEP, phosphoenolpyruvate.

improve carbon dioxide production and anaplerotic fixation into oxaloacetate.

4.3. Amino-acid and purine nucleotide metabolism: Induction of L-arginine and purine catabolism to promote L-glutamate biosynthesis

Two enzymes involved in two distinct L-arginine catabolic pathways, namely agmatine iminohydrolase and ornithine aminotransferase, are up-regulated in nitrate-grown cells, possibly increasing the capacity of both pathways that are consumers of α-ketoglutarate produced by the TCA cycle and release ammonium, reducing equivalents, hydrogen peroxide and L-glutamate (Fig. 4). We also showed that a mitochondrial isoform of glutamate dehydrogenase (GDH2) is down-regulated, resulting in a possible decrease in the L-glutamate consumption and in the α-ketoglutarate production (Fig. 4). Our results also highlighted a strong up-regulation of urate oxidase II, that was shown to be repressed by ammonium [3], indicating that a strong increase in purine catabolism, which releases ammonium, reducing equivalents and hydrogen peroxide, may occur in nitrate-grown cells. Hence we can hypothesize that the ammonium excess released by L-arginine and purine catabolic pathways in nitrate-grown cells is used by the GS/GOGAT cycle for *de novo* L-glutamate biosynthesis (Fig. 4). In addition, the induction of L-arginine catabolism and the down-regulation of glutamate dehydrogenase could be responsible for an increase in L-glutamate production and for the consumption of α-ketoglutarate produced by the TCA cycle in nitrate-grown cells. Altogether, these results suggest that nitrate-grown *Chlamydomonas* cells tend to induce L-glutamate biosynthetic pathways that do not require nitrate reduction, then lowering the energy cost of inorganic nitrogen assimilation (Fig. 4).

4.4. Respiratory chain: AOX up-regulation as a way to export reducing power?

The increase in TCA cycle, L-arginine and purine catabolism capacities is probably responsible for a higher reducing equivalent production rate in nitrate-grown cells. Moreover, a lower amount of complex III could be responsible for a lower cytochrome pathway capacity (Fig. 4). Altogether, the induction of catabolic pathways and the decrease in the cytochrome pathway capacity could lead to an increase in the ubiquinone pool reduction level and to a higher superoxide anion production by complexes I and III. Interestingly, AOX up-regulation could counteract this effect. However, two possible electron sinks exist in nitrate-grown cells to limit the harmful production of ROS caused by a high ubiquinone pool reduction state: (1) AOX, which is strongly up-regulated, and (2) export of mitochondrial reducing power to the cytoplasm and the chloroplast for nitrate and nitrite reduction, respectively. In *Arabidopsis*, previous studies have also suggested that mitochondrial reducing equivalents can be exported from mitochondria to the cytoplasm, where they could be used to reduce nitrate [14]. In *S. minutum*, it was shown that photogenerated reducing equivalent supply is not sufficient for nitrate and nitrite reduction and that mitochondria could also provide reducing equivalents to allow these reactions to function optimally

[29]. Thus, AOX up-regulation in nitrate-grown cells could contribute to (1) limit the superoxide anion production by the respiratory chain and (2) enhance the TCA cycle capacity, increasing carbon dioxide release and leading to a higher oxaloacetate production and conversion into malate. The latter would allow a net export of mitochondrial reducing power into the cytoplasm for nitrate and nitrite reduction (Fig. 4).

4.5. Induction of ROS and RNS scavenging systems

The hybrid-cluster protein (HCP) and IDH NADP-dependent were up-regulated in nitrate-grown cells (Fig. 4). Both are potential ROS scavenging systems; however, as we did not detect any modification of glutathione reductase (GR) and glutathione peroxidase (GP) expression, the role of IDH NADP-dependent in ROS scavenging remains questionable. Our proteomic analysis highlighted the induction of several ROS sources in nitrate-grown cells: a higher hydrogen peroxide production by L-arginine catabolism, urate oxidase II and sulfite oxidase and possibly a higher superoxide anion production by the respiratory chain (Fig. 4).

Nitrate reductase also catalyzes the reduction of nitrite into NO using an electron from NADH or NADPH in *Chlamydomonas* [31]. Nitrate reductase capacity to produce NO corresponds to about 1% of its capacity to produce nitrite and was shown to be higher than NO synthase capacity in nitrate-grown plants [32]. As nitrate reductase is known to be induced in nitrate-grown *Chlamydomonas* cells [33,34], the production of NO by this enzyme is probably higher. Thus, the observed up-regulation of P450nor in nitrate-grown cells could allow the detoxification of the NO excess synthesized by nitrate reductase.

We also found that sulfite oxidase is up-regulated in nitrate-grown cells (Fig. 4). This enzyme is involved in sulfite detoxification (sulfite is known to break disulfide bridges, link to aldehydes to form hydroxysulfonates, which are metabolic inhibitors, or directly inhibit numerous enzymes [25]). This suggests a higher sulfite/sulfate ratio in nitrate-grown cells, indicating that the mitochondrial reducing status is probably higher in comparison with ammonium-grown cells.

5. Conclusions

AOX up-regulation in nitrate-grown cells could present a dual role: (1) to limit the ubiquinone pool reduction state and thus the ROS production by the respiratory chain and (2) to permit the export of mitochondrial reducing power for nitrate and nitrite reduction. Hence, instead of dissipating the matricial reducing power, AOX could contribute surprisingly to provide redox reactions of nitrate and nitrite reduction with mitochondrial reducing power. Thus, mitochondrial plasticity allows the cell to face the nitrate assimilation energetic cost and the role of AOX in this plasticity makes logical the localization of *Aox1* in a nitrate assimilation gene cluster.

Acknowledgements

This work was supported by a Fond de la Recherche Fondamentale et Collective (FRFC grant 2.4582.05). GM is a recipient of a FRS-FNRS doctoral fellowship, FF is a senior research associate of the FRS-FNRS. We would like to thank Dr. Pierre Cardol for providing us with mitochondria and Dr. Pierre Leprince and Prof. Edwin dePauw for providing us with access to proteomic and mass spectrometry platforms.

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