Regulation of the Alternative Oxidase *Aox1* Gene in *Chlamydomonas reinhardtii*. Role of the Nitrogen Source on the Expression of a Reporter Gene under the Control of the *Aox1* Promoter[1]

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In higher plants, various developmental and environmental conditions enhance expression of the alternative oxidase (AOX), whereas its induction in fungi is mainly dependent on cytochrome pathway restriction and triggering by reactive oxygen species. The AOX of the unicellular green alga *Chlamydomonas reinhardtii* is encoded by two different genes, the *Aox1* gene being much more transcribed than *Aox2*. To analyze the transcriptional regulation of *Aox1*, we have fused its 1.4-kb promoter region to the promoterless arylsulfatase (*Ars*) reporter gene and measured ARS enzyme activities in transformants carrying the chimeric construct. We show that the *Aox1* promoter is generally unresponsive to a number of known AOX inducers, including stress agents, respiratory inhibitors, and metabolites, possibly because the AOX activity is constitutively high in the alga. In contrast, the *Aox1* expression is strongly dependent on the nitrogen source, being down-regulated by ammonium and stimulated by nitrate. Inactivation of nitrate reductase leads to a further increase of expression. The stimulation by nitrate also occurs at the AOX protein and respiratory levels. A deletion analysis of the *Aox1* promoter region demonstrates that a short upstream segment (−253 to +59 with respect to the transcription start site) is sufficient to ensure gene expression and regulation, but that distal elements are required for full gene expression. The observed pattern of AOX regulation points to the possible interaction between chloroplast and mitochondria in relation to a potential increase of photogenerated ATP when nitrate is used as a nitrogen source.

Besides the cyanide-sensitive cytochrome pathway, mitochondria from higher plants, some protists, and many fungi possess an alternative pathway that is resistant to cyanide but sensitive to salicylhydroxamic acid and *n*-propyl gallate. Cyanide-resistant respiration is mediated by a single non-phosphorylating enzyme, the alternative oxidase (AOX), which bypasses proton-translocating complexes III and IV of the cytochrome pathway to directly transfer electrons from reduced ubiquinone to molecular oxygen. In the thermogenic spadix of *Sauromatum guttatum* and other Araceae, the free energy of the alternative pathway is involved in heat production during anthesis (Moore and Siedow, 1991). Although its precise function in other tissues is still not fully understood, the AOX is often considered to be a regulatory enzyme balancing carbon metabolism and electron transport. According to the energy overflow hypothesis (Lambers, 1982), shunting electrons to the alternative pathway would allow continued operation of glycolysis and tricarboxylic acid cycle when the cytochrome pathway is impaired or restricted by a high adenylate charge (for review, see Wagner and Krab, 1995; Vanlerberghe and McIntosh, 1997). The alternative pathway is also thought to prevent over-reduction of respiratory chain components that might otherwise result in the generation of harmful reactive oxygen species (for review, see Moller, 2001).

Enhanced alternative respiration is observed following various developmental or environmental stimuli, and especially in stress conditions. Regulation of AOX activity is complex and occurs at both transcriptional and posttranslational levels. In isolated mitochondria from higher plants, AOX activity strongly increases upon reduction of an intersubunit disulphide bridge, yielding a non-covalently linked dimeric protein. Under its reduced form, the enzyme is activated by α-keto acids, including pyruvate, through the formation of a thiohemiacetal. Both regulatory mechanisms occur at the same highly conserved Cys residue of the enzyme (for review, see Affourtit et al., 2002). In fungi and protozoa, most studies so far point to a monomeric AOX that is only stimulated by ADP, AMP, and GMP, whereas the AOX from higher plants is unaffected by these purine nucleotides (for review, see Joseph-Horne et al., 2001). Comparisons of AOX protein sequences reveal a domain of about 40 amino acids surrounding the regulatory Cys in the plant sequences that is not found in the fungal sequences (Umbach and Siedow,

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2000). This conserved domain may be involved in dimerization and posttranslational regulation of the plant enzyme. These discrepancies in structural and regulatory properties between AOXs from both groups suggest potential differences in their physiological roles (for review, see Joseph-Horne et al., 2001).

In higher plants, where AOX is generally encoded by small multigene families, many conditions are known to enhance *Aox* gene transcription and/or AOX protein accumulation. Some of these conditions include saline (Hilal et al., 1998) and oxidative stresses (Wagner, 1995; Vanlerberghe and McIntosh, 1996), exposure to heavy metals (Padua et al., 1999), inhibition of mitochondrial protein synthesis (Zhang et al., 1996), cytochrome pathway restriction (Vanlerberghe and McIntosh, 1992, 1994; Saisho et al., 1997; Wagner and Wagner, 1997; Tanudji et al., 1999; Ducos et al., 2001), and incubation with metabolites such as acetate, Cys, or salicylic and citric acids (Elthon et al., 1989; Rhoads and McIntosh, 1992; Vanlerberghe and McIntosh, 1996; Lennon et al., 1997; Potter et al., 2000). In fungi, AOX induction is especially dependent on cytochrome pathway restriction (Lambowitz et al., 1989; Sakajo et al., 1991; Minagawa et al., 1992; Li et al., 1996; Yukioka et al., 1998; Huh and Kang, 1999, 2001) and triggering by reactive oxygen species (Yukioka et al., 1998; Huh and Kang, 2001).

The alternative pathway of the unicellular green alga *Chlamydomonas reinhardtii* has been mainly investigated in terms of cell respiratory capacities. Under photoautotrophic conditions, the addition of cyanide only weakly reduces (about 20%) the cell respiratory rate (Husic and Tolbert, 1987; Goyal and Tolbert, 1989), indicating that the alternative pathway capacity is constitutively high in *C. reinhardtii*. This capacity considerably decreases during the log growth of cells cultivated at high CO_2, but returns as the culture enters the stationary phase. At high CO_2, when the capacity of the alternative pathway is low, addition of cyanide or salicylic acid induces its reappearance (Goyal and Tolbert, 1989). The presence of acetate in the medium determines a 2- to 3-fold stimulation of respiration in air-grown cells (Fett and Coleman, 1994; Endo and Asada, 1996), due to an increase of the cytochrome pathway capacity without significant modification of the alternative pathway capacity (R.F. Matagne, D. Bauzain, and M. Dinant, unpublished data). The AOX enzyme from *C. reinhardtii* is recognized by a monoclonal antibody raised against the AOX of *S. guttatum* (Derzaph and Weger, 1996).

The algal protein is encoded by two different genes, the *Aox1* gene being much more transcribed than *Aox2*. Both genes lack the 40-amino acid conserved domain and the regulatory Cys typical of the plant sequences and are thus more similar to the *Aox* genes described in fungi (Dinant et al., 1998, 2001). In the genome of *C. reinhardtii*, the *Aox1* gene, previously named *Nar5* (Quesada et al., 1998), lies in opposite orientation by the side of *Nrt2;3*, a gene coding for a nitrite/nitrate transporter (Quesada et al., 1998; Rexach et al., 1999; Navarro et al., 2000). Both *Aox1* and *Nrt2;3* genes are under control of the nitrogen source. Their expression is down-regulated by ammonium and is considerably stimulated by nitrate and nitrite in a deletion mutant that lacks several nitrate-related assimilation genes, including the *Nia1* gene encoding nitrate reductase (Quesada et al., 1998, 2000).

In this work, we have fused the *Aox1* promoter region to the promoterless arylsulfatase (*Ars*) reporter gene to study the transcriptional regulation of the *Aox1* gene in *C. reinhardtii*. We show that the expression of the *Aox1/Ars* chimeric construct does not respond to the classical inducers for AOX. The expression is primarily regulated by the nitrogen source, being down-regulated by ammonium and stimulated by nitrate. Moreover, in nitrate-grown cells, inactivation of nitrate reductase by azide, tungstate, or mutation of the *Nia1* gene increases the expression of the chimeric construct. This kind of regulation also occurs at the AOX protein and respiratory levels. A deletion analysis of the promoter region allowed us to define segments required for basal expression and regulation of the *Aox1* gene.

RESULTS

The *Aox1* Promoter Efficiently Drives the Expression of the *Ars* Reporter Gene

To investigate the transcriptional regulation of the *Aox1* gene, we fused its 1.4-kb promoter region to the Ars-encoding *Ars* reporter gene deprived of its own promoter. The *Aox1* promoter segment extends from −1364 to +60 with respect to the transcription start site and encompasses the ATG start codon of *Nrt2;3* (see "Materials and Methods" and Fig. 5A). This chimeric construct called *Aox1/Ars* was introduced into the wall-less Arg-requiring 325 strain of *C. reinhardtii* by cotransformation with the pASL plasmid bearing the wild-type *Arg7* gene as the selectable marker. The pJD54 plasmid harboring the promoterless *Ars* gene was used as a control. The percentage of transformants expressing ARS was determined by in situ staining of approximately 500 Arg-independent clones. No colony transformed with pJD54 displayed ARS activity, whereas 39% of the colonies transformed with *Aox1/Ars* produced the enzyme. Sixteen transformants exhibiting various levels of ARS activity and two negative controls were selected for further analyses.

To verify the status of the chimeric construct in the genome of the 16 transformants, their DNA was purified and submitted to PCR amplification. By using two different sense oligonucleotides in the *Aox1* promoter region and an antisense primer in the *Ars* coding sequence, we concluded that 10 transformants

bore at least one copy of the 1.4-kb promoter region, whereas five of the six others carried one or more 5′-truncated fragments containing at least approximately 650 bp of the 1.4-kb segment. Southern-blot hybridizations with a 1.2-kb probe located in the promoter region showed that most transformants had received several copies of the *Aox1/Ars* construct (data not shown). As expected, control and untransformed strains only displayed the signal corresponding to the endogenous *Aox1*. Clone 6, bearing a unique full copy of the chimeric construct, as well as clone 2, carrying two truncated copies, and clone 3, having at least one full copy among about four integration events, were selected for further study.

In Contrast to a Number of Known AOX Inducers, the Nitrogen Source Strongly Modulates the Expression of the Chimeric Construct

The expression of AOX at transcriptional and/or protein level was reported to increase upon exposure to a number of stress or inhibitory conditions (see the introduction). To analyze the expression of the *Aox1* gene from *C. reinhardtii* under different experimental conditions, cells from clone 6 were cultivated for 8 h in Tris-acetate-phosphate $(TAP)_{NH_4}$ liquid medium containing various agents susceptible to stimulate the alternative pathway. As shown in Table I, most treatments did not markedly modify the expression of the *Aox1/Ars* construct. Only paraquat and the three respiratory inhibitors, rotenone, antimycin A, and myxothiazol, very moderately stimulated the expression of the chimeric construct. Similar results were obtained with clone 3, as well as in 24-h cultivations of clones 3 and 6 (data not shown).

Because the expression of the chimeric construct was much higher in medium containing nitrate as nitrogen source (see below), ARS activities were reexamined in TAP_{NO_3} medium, except for saline and osmotic stresses (Table I). Only copper was able to slightly enhance expression of the chimeric construct. Such an effect of copper on *Aox* transcription was previously reported for sycamore (*Acer pseudoplatanus*) suspension cells and interpreted as a part of a general stress response, as the consequence of a copper-induced inhibition of the cytochrome pathway, or as the result of some interaction between iron and copper pools in cells (Padua et al., 1999). Otherwise, none of the tested agents was able to significantly increase the expression of the *Aox1/Ars* construct, hinting that the transcriptional regulation of *Aox1* in *C. reinhardtii* is different from that of many other organisms.

To study the impact of mitochondrial mutations on the expression of the chimeric construct, we crossed clone 6 with several *dum* mitochondrial mutant

Table I. *ARS activities of clone 6 grown for 8 h in TAP_{NH4} or TAP_{NO3} liquid medium supplemented with various stress agents, inhibitors, or metabolites susceptible to stimulate the alternative pathway*

nd, Not determined; ns, ARS activity not significantly different from controls. Growth reduction varied from 25% to 50% in comparison with controls except otherwise specified: (1) growth reduction larger than 75%; (2) no growth reduction.

Agent, Inhibitor, or Metabolite	Concentration	Mean ARS Activity	
		TAP_{NH4}	TAP_{NO3}
		nmol naphthol h^{-1} mg^{-1} protein	
None	/	500	2500
Saline stress			
Lithium chloride	10 mM (1)	200	nd
Sodium chloride	100 mM	300	nd
Potassium chloride	100 mM	400	nd
Osmotic stress			
Mannitol	400 mM (1)	ns	nd
Oxidative stress			
Paraquat	200 nM	650	ns
Tert-butyl hydroperoxide	100 μM	ns	ns
Hydrogen peroxide	1 mM	350	ns
Heavy metals			
Copper	100 μM	ns	3500
Cadmium	100 μM	ns	ns
Respiratory inhibitors			
Rotenone	40 μM (2)	600	1750
Antimycin A	2 μM	600	1750
Myxothiazol	6 μM	650	2000
Metabolites			
Cys	1 mM (2)	ns	ns
Salicylic acid	1 mM	400	ns
Citric acid	5 mM	350	ns

strains isolated in our laboratory (for review, see Remacle et al., 2001b). The mutants used were deprived of complex I (*dum20* and *dum25*), complex III (*dum2*), complex IV (*dum19*), or complex I + III (*dum24*) activities. Meiotic products carrying both the *dum* mutation and the *Aox1/Ars* construct were selected on nitrate-containing TAP agar medium. None of these progeny clones expressed an ARS activity significantly higher in TAP_{NH_4} or TAP_{NO_3} medium than clone 6 (data not shown). *Aox1* transcript level similarly was not significantly modified in mutant cells (data not shown).

To examine the influence of the nitrogen status on the expression of the chimeric construct, ARS activities of transformants 2, 3, and 6 were analyzed after transfer for 8 h from TAP_{NH_4} into TAP liquid medium deprived of nitrogen or containing different nitrogen sources. As shown in Figure 1A, ammonium removal (N-free medium) led to a nearly 2-fold stimulation in the expression of the *Aox1/Ars* construct, whereas substitution of 4 mM nitrate for equimolar ammonium resulted in a 4- to 5-fold increase of expression. Moreover, cultivation in the presence of both ammonium and nitrate gave rise to an intermediate expression level. Taken together, these results suggest that ammonium and nitrate mediate competing effects on the expression of the *Aox1* promoter.

To analyze the expression of the chimeric construct in response to the nitrogen-source concentration, cells from clone 6 were transferred into TAP liquid media containing increasing molarities of ammonium or nitrate, and ARS activities were determined after 8 h (Fig. 1B). Whereas expression in ammonium slowly decreased with concentration down to one-third of the N-free level, ARS activity steeply increased and peaked (5-fold stimulation) around 1 mM nitrate. Beyond that point, ARS activity grew much more slowly to finally reach a 6-fold increase. Such a biphasic dose response curve to nitrate was also observed with cells from clone 3 (data not shown).

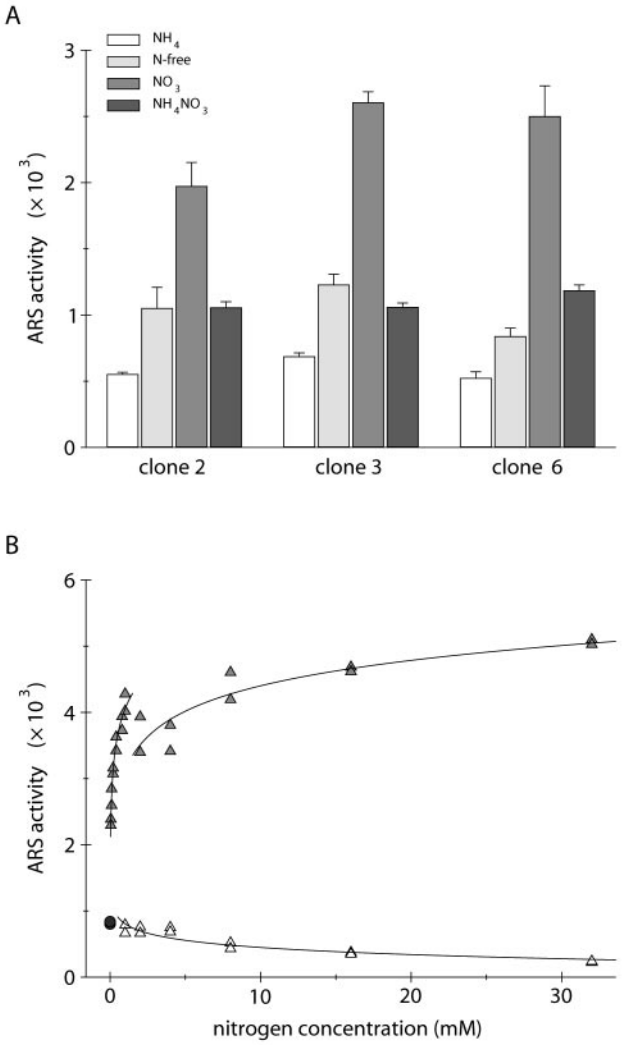

Figure 1. Effect of different nitrogen sources and concentrations on the expression of the *Aox1/Ars* chimeric construct. A, Ammonium-grown cells from clones 2, 3, and 6 were transferred for 8 h into TAP liquid media deprived of nitrogen (N-free) or containing 4 mM ammonium chloride (NH_4), 4 mM sodium nitrate (NO_3), or 4 mM ammonium nitrate (NH_4NO_3). ARS activities (nanomoles of naphthol per hour per milligram of protein) are the means of independent experiments ± SE ($n = 4$ for clones 2 and 3; $n = 6$–12 for clone 6). B, Ammonium-grown cells from clone 6 were transferred for 8 h into TAP liquid media deprived of nitrogen (black circles) or containing 1 to 32 mM ammonium chloride (white triangles) or 0.05 to 32 mM sodium nitrate (gray triangles). Each symbol is the ARS activity determined in an independent culture.

Azide Enhances the Expression of the Chimeric Construct without Blocking the Cytochrome c Oxidase

Searching for treatments able to stimulate the expression of the *Aox1/Ars* construct, we also examined the effect of the metalloprotein inhibitor sodium azide. Given its extensive coordination chemistry with metals, it is a competitive inhibitor of many heme- or copper-containing enzymes, including the mitochondrial cytochrome *c* oxidase (Smith and Wilcox, 1994; Saisho et al., 2001). As shown in Figure 2A, ARS activity was notably higher when cells from clone 6 were cultivated in the presence of 200 μM sodium azide. In absolute value, the increase of enzyme activity was much higher in nitrate-grown than in ammonium-grown cells. Because the addition of azide during the enzymatic incubation did not modify ARS activity, the observed stimulation actually resulted from a higher accumulation of the ARS enzyme. The same treatment applied to cells carrying both the *dum19* mutation (lack of a functional cytochrome *c* oxidase) and the chimeric construct also induced a 2-fold increase of ARS activity in cells grown in the presence of nitrate (Fig. 2B). These results thus indicate that the higher expression of the *Aox1/Ars* construct following azide addition is particularly prominent for cells grown in nitrate-

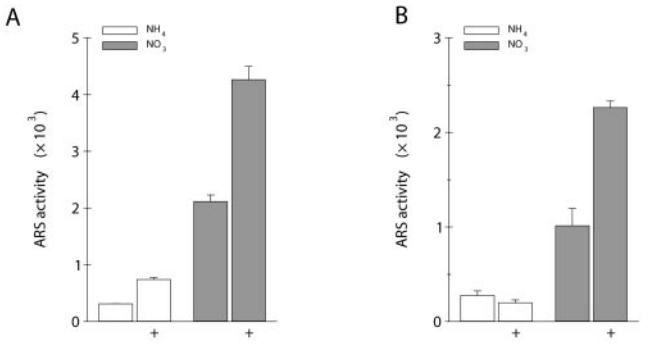

Figure 2. Effect of sodium azide on the expression of the *Aox1/Ars* chimeric construct. Ammonium-grown cells from clone 6 (A) or from a *dum19* progeny clone lacking a functional cytochrome *c* oxidase (B) were transferred for 8 h into TAP liquid media containing 4 mM ammonium chloride (NH_4) or 4 mM sodium nitrate (NO_3), each in the absence (−) or in the presence (+) of 200 μM sodium azide. ARS activities (nanomoles of naphthol per hour per milligram of protein) are the means of four or five independent experiments ± SE.

containing medium and is unrelated to the inhibition of cytochrome *c* oxidase.

Inactivation of Nitrate Reductase Further Enhances the Expression of the Chimeric Construct in Nitrate-Containing Medium

Nitrate reductase, the first enzyme of the nitrate assimilation pathway, is a heme-containing protein that is also inhibited by sodium azide (Guerrero and Gutierrez, 1977). In *C. reinhardtii*, this key enzymatic component of nitrogen metabolism is only present when cells are cultivated in a medium deprived of ammonium and containing nitrate (for review, see Fernandez et al., 1998). Because the positive azide effect on the expression of the *Aox1/Ars* construct was markedly high for cells grown in nitrate-containing medium, we wondered whether part of this stimulation was related to the inhibition of nitrate reductase. As shown in Figure 3A, the in vitro nitrate reductase activity was dramatically reduced by 200 μM sodium azide and was completely abolished by about 300 μM. Interestingly, these kinetic data are in accordance with the important azide stimulation of the chimeric construct expression in clone 6 cultivated for 16 h in TAP_{NO_3} liquid medium (Fig. 3B). Similar observations were made on clone 3 and in 8-h cultivations of both clones (data not shown). Because growth of clone 6 in these culture conditions was reduced by 85%, it can be thought that 200 μM sodium azide also markedly reduces the in vivo activity of nitrate reductase. Taken together, these results suggest that a large part of the positive azide effect in the presence of nitrate is related to the inhibition of nitrate reductase.

The nitrate reductase enzyme uses molybdenum as a metal cofactor and is inactivated by substituting tungstate for molybdate in the growth medium (Vega et al., 1971; Galvan et al., 1992; Loppes et al., 1999). To analyze by another approach the effect of nitrate reductase inactivation on the expression of the *Aox1/Ars* construct, cells from clone 6 were maintained for 10 generations on TAP agar medium supplemented with 1 μM molybdate or 1 μM tungstate. They were then transferred for 16 h into TAP liquid media containing 4 mM ammonium, 4 mM nitrate, or 4 mM nitrate with 200 μM sodium azide, each supplemented with molybdate or tungstate. As shown in Figure 3C, tungstate did not affect ARS activity in TAP_{NH_4} medium nor in azide-supplemented TAP_{NO_3} medium. In contrast, expression in TAP_{NO_3} medium was increased more than twice by tungstate to reach

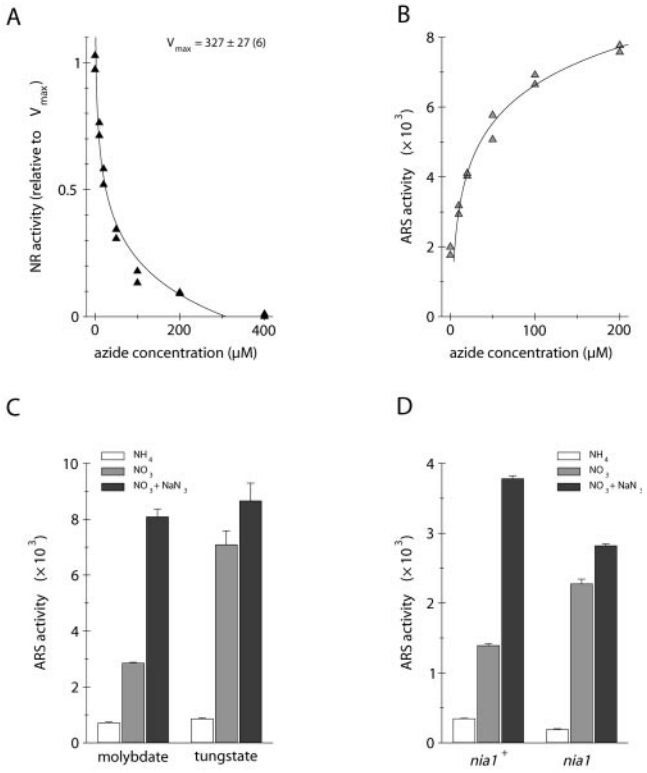

Figure 3. Expression of the *Aox1/Ars* chimeric construct in relation to nitrate reductase inactivation. A, Nitrate reductase (NR) activity in crude extracts from strain 83, measured in the presence of increasing concentrations of sodium azide. The activity is expressed relative to the uninhibited activity V_{max} (nanomoles of NO_2 per hour per milligram of protein). B, Ammonium-grown cells from clone 6 were transferred for 16 h into TAP_{NO_3} liquid medium containing increasing concentrations of sodium azide. ARS activity is expressed in nanomoles of naphthol per hour per milligram of protein. C, Cells from clone 6 pregrown in the presence of 1 μM molybdate or 1 μM tungstate were transferred for 16 h into TAP liquid media containing 4 mM ammonium chloride, 4 mM sodium nitrate, or 4 mM sodium nitrate with 200 μM sodium azide, each supplemented with 1 μM molybdate or 1 μM tungstate. ARS activities are the means of six independent experiments ± SE. D, Ammonium-grown cells from *nia1*$^+$ or *nia1* (lacking a functional nitrate reductase) strains derived from clone 6 were transferred for 8 h into TAP liquid media containing 4 mM ammonium chloride, 4 mM sodium nitrate, or 4 mM sodium nitrate with 200 μM sodium azide. ARS activities are the means of three independent experiments ± SE.

the sodium azide level, whereas both nitrate reductase activity and cell growth were reduced by about 90% (data not shown). These results were repeated on clone 3, as well as in 8-h cultivations. Here again, the data strongly suggest that in nitrate-grown cells, the inactivation of nitrate reductase markedly enhances the expression of the chimeric construct.

To further test this hypothesis, clone 6 was crossed with a *nia1* mutant strain lacking a functional nitrate reductase, and a meiotic product carrying both the *nia1* mutation and the *Aox1/Ars* construct was isolated. ARS activities were measured in cells cultivated for 8 h in TAP liquid media containing ammonium, nitrate, or nitrate with sodium azide. As shown in Figure 3D, ARS activity of *nia1* cells was about 12-fold higher in TAP_{NO_3} than in TAP_{NH_4} medium. Moreover, the ARS activity recorded in TAP_{NO_3} medium was nearly twice higher with the *nia1* mutant than with a *nia1*$^+$ wild-type clone isolated from the same cross. Finally, azide barely increased the expression of the chimeric construct in the *nia1* mutant, whereas the *nia1*$^+$ clone showed the familiar stimulation pattern. These results were repeated in 24-h cultivations and following direct cotransformation of a *nia1* strain with the *Aox1/Ars* construct (data not shown). Taken with the observations reported above, these data indicate that any kind of nitrate reductase inactivation enhances the nitrate-mediated stimulation of the chimeric construct expression. Moreover, because sodium azide also enhances the expression of the *Aox1/Ars* construct in ammonium-grown wild-type cells (Fig. 2A), it might marginally act on another enzymatic system unrelated to nitrate reductase.

The AOX Protein and Alternative Respiration Levels Parallel the Expression of the Chimeric Construct

Having demonstrated that the chimeric construct is transcriptionally down-regulated by ammonium and stimulated by nitrate, it was of interest to examine the AOX protein levels of the wild-type strain cultivated in the same growth conditions. A polyclonal antibody raised against the AOX of *C. reinhardtii* was tested for this purpose and was found to be 100-fold more sensitive than the monoclonal antibody raised against the AOX of *Sauzomatun gutattum*. This specific antibody preparation could thus be used to study the AOX abundance in membrane fractions from strain 83 cultivated for 16 h in TAP liquid media deprived of nitrogen or containing different nitrogen sources. A band of approximately 40 kD was detected in all culture conditions (Fig. 4A). This band most probably corresponds to the product of *Aox1* because the *Aox2* gene is only poorly transcribed (Dinant et al., 2001) and because the *Aox2* promoter (GenBank accession no. AF537324) is unable to drive significant expression of the *Ars* reporter gene under a broad range of growth conditions (data not shown). However, the mass of this protein is higher than the predicted mass (33.4 kD) of the mature AOX1 enzyme (Dinant et al., 2001). A similar discrepancy between the molecular masses calculated from the deduced amino acid sequence and determined by SDS-PAGE was observed for AOX proteins from soybean (*Glycine max*; Finnegan et al., 1997). A second, less intense band of approximately 35 kD was sometimes detected on the immunoblots (Fig. 4A). Reyes-Prieto et al. (2002) also detected the 40-kD polypeptide and a minor band around 36 kD using the same antibody. Considering that both bands disappear in an antisense strain deprived of alternative respiration (M. Dinant and R.F. Matagne, unpublished data), we presume that the lower band should correspond to an isoform (maybe a degradation product) of the AOX1 protein.

The data presented in Figure 4A show that AOX accumulation was low in TAP_{NH_4} medium, higher in N-free medium, and still increased in nitrate-containing media. In the same loading conditions, we

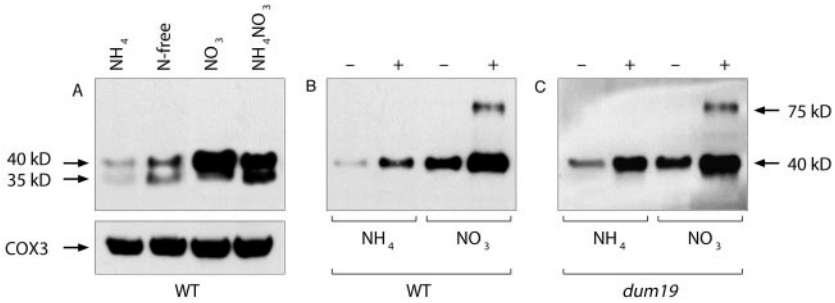

Figure 4. Effect of nitrogen source and sodium azide on the AOX protein accumulation (western blots). Except in A, each lane was loaded with 30 μg of membrane proteins. A, Ammonium-grown wild-type cells (strain 83) were transferred for 16 h into TAP liquid media deprived of nitrogen (N-free) or containing 4 mM ammonium chloride (NH_4), 4 mM sodium nitrate (NO_3), or 4 mM ammonium nitrate (NH_4NO_3). Membrane proteins (15 μg for cells maintained in N-free medium; 30 μg for other conditions) were loaded on the gel to get similar signals for subunit III of cytochrome *c* oxidase. Ammonium-grown cells from wild-type strain (B) or from a *dum19* mutant (strain 193) lacking a functional cytochrome *c* oxidase (C) were transferred for 16 h into TAP liquid media containing 4 mM ammonium chloride (NH_4) or 4 mM sodium nitrate (NO_3) in the absence (−) or in the presence (+) of 200 μM sodium azide. Time exposure of the screen was longer for A than for B and C.

did not detect any change in the amount of subunit III of cytochrome *c* oxidase. The stimulation by 200 μM sodium azide was also investigated at the protein level on cells grown for 16 h in TAP_{NH_4} or TAP_{NO_3} liquid medium (Fig. 4B). AOX accumulation in the presence of azide was higher on both nitrogen sources, the abundance of the protein being particularly high in nitrate-containing medium. Moreover, another band with an apparent mass of 75 kD was systematically detected in the latter condition. This additional band could correspond to a homodimeric form of AOX associated with its overexpression. Similar responses to azide in both ammonium and nitrate media were obtained with membrane fractions from a *dum19* mutant (strain 193) deprived of cytochrome *c* oxidase (Fig. 4C), thus confirming that the positive azide effect is unrelated to the cytochrome *c* oxidase inhibition. Because in ammonium-grown *dum19* cells, azide treatment increased the amount of AOX protein but not ARS expression (compare Figs. 2B and 4C), one could suggest that, at least in the mutant, AOX protein accumulation does not require transcriptional activation of the *Aox1* gene.

To further assess the physiological relevance of the AOX regulation by the nitrogen source, we examined the respiratory rates of wild-type cells from strain 83 grown for 16 h in TAP liquid media containing 4 mM ammonium or nitrate. Capacities of the alternative and cytochrome pathways of respiration were determined by the addition of potassium cyanide (inhibitor of cytochrome *c* oxidase) and *n*-propyl gallate (inhibitor of AOX), respectively. As shown in Table II, total respiratory rates were not affected by the nitrogen source. However, the alternative pathway capacity (i.e. the oxygen uptake inhibited by *n*-propyl gallate in the presence of cyanide) was at least twice higher in nitrate-containing medium than in ammonium-containing medium (15.0 versus 6.5), whereas the cytochrome pathway capacity (oxygen uptake inhibited by cyanide in the presence of *n*-propyl gallate) was barely increased (21.5 versus 15.7). Together, these results show that there is a close correlation among the transcriptional regulation of the *Aox1/Ars* construct, the AOX protein accumulation, and the capacity of the alternative pathway.

Table II. *Dark respiratory rates of whole cells from strain 83 grown for 16 h in TAP_{NH4} or TAP_{NO3} liquid medium*

Respiratory O_2 consumption was measured in the absence (Total) or in the presence of KCN or *n*-propyl gallate. Both inhibitors were added to a final concentration of 1 mM. Additions were made in the order indicated. Values are the mean of three to six independent experiments ± SE.

	TAP_{NH4}	TAP_{NO3}
	nmol O_2 min^{-1} 10^{-7} cells	
Total	28.7 ± 0.3	28.6 ± 0.5
+KCN	6.8 ± 0.3	15.7 ± 1.5
+PG	0.3 ± 0.2	0.7 ± 0.3
Total	27.1 ± 0.9	30.0 ± 1.1
+PG	15.7 ± 1.2	21.8 ± 2.0
+KCN	0.0 ± 0.0	0.3 ± 0.2

A 0.3-kb Proximal Upstream Segment Is Sufficient for Regulated Expression of the Chimeric Construct, But Distant Elements Are Required for Full Expression

To define a minimal promoter still able to mediate *Aox1* transcriptional regulation, a series of 5′-deleted fragments was produced by PCR and fused to the *Ars* reporter gene. Six new chimeric constructs were generated, named *Aox1*Δ1/*Ars*, *Aox1*Δ2/*Ars*, *Aox1*Δ3/*Ars*, *Aox1*Δ4/*Ars*, *Aox1*Δ5/*Ars*, and *Aox1*Δ6/*Ars*, that contained portions of the *Aox1* upstream region up to positions −1129, −935, −694, −466, −253, and −59, respectively (Fig. 5B). After linearization, these constructs were introduced with pASL into *C. reinhardtii*, and Arg-independent colonies were selected on TAP agar medium. In a first step, 25 clones from each cotransformation were picked up at random and submitted to in situ staining to determine the percentage of transformants expressing ARS. With constructs *Aox1*Δ1/*Ars* to *Aox1*Δ5/*Ars*, the co-expression rate was high (56%–80%), whereas it fell to 8% with *Aox1*Δ6/*Ars* (Fig. 5B). This indicates that the −253 to +59 segment of the *Aox1* promoter is sufficient to drive the expression of the *Ars* reporter gene. For three different constructs, the total DNA from the 25 clones was purified and used in PCR amplification of the *Aox1-Ars* junction to determine the percentage of cotransformed cells. The rates obtained (79%–87%; Fig. 5B) were high and homogenous enough to examine ARS activities in pools of transformants. As shown by Ohresser et al. (1997), all of the Arg-independent colonies from one cotransformation experiment can be harvested and assayed for ARS activity as a single population without prior selection of the transformants harboring the chimeric construct. This procedure bypasses the so-called "position effects" and obviates the need for molecular characterization of integration events in dozens of individual transformants. About 300 colonies from each cotransformation were thus harvested as a pool and plated onto fresh TAP agar medium. After 3 d, these cells were transferred for 16 h into TAP liquid media containing 4 mM ammonium, 4 mM nitrate, or 4 mM nitrate with 200 μM sodium azide, before determination of ARS activities. The results of two independent cotransformation experiments are presented in Figure 6A. Except for *Aox1*Δ6/*Ars* that gave rise to very low ARS activities, not significantly different from that of a pool bearing either pJD54 (promoterless reporter gene) or no construct, ARS expression could be analyzed with all of the constructs. The general trend in TAP_{NO_3} and azide-supplemented TAP_{NO_3} media was a decrease of expression from *Aox1*Δ1/*Ars* to *Aox1*Δ5/*Ars*. However, ARS activities of these five constructs remained higher in TAP_{NO_3}

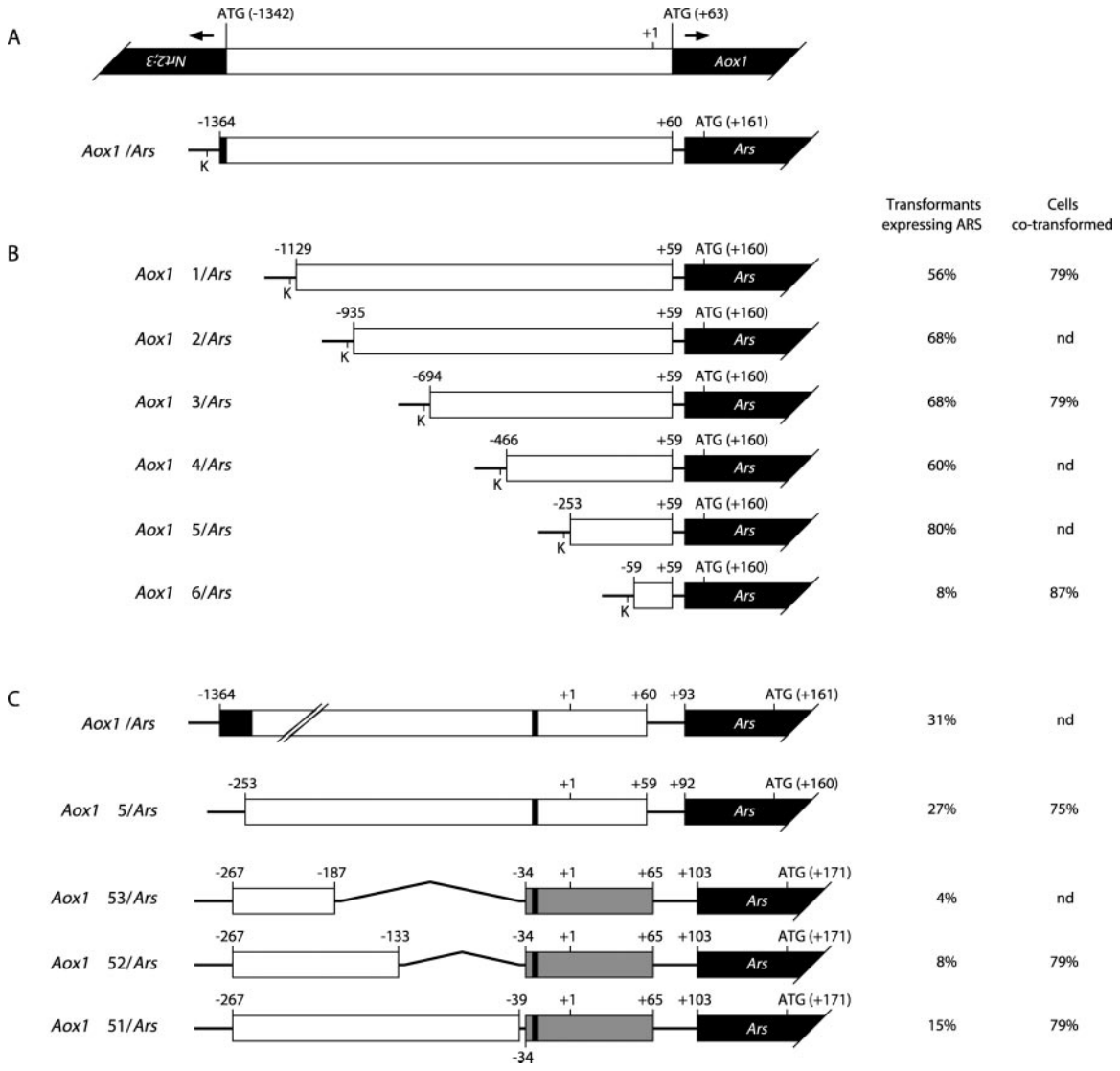

Figure 5. A, Schematic drawings of the *Aox1* and *Nrt2;3* genes arranged in opposite orientation and of the *Aox1/Ars* chimeric construct. *Nrt2;3*, *Aox1*, and *Ars* coding sequences are shown as black boxes. B, Constructs *Aox1*Δ1/*Ars* to *Aox1*Δ6/*Ars* are derivatives of pJD54; K, unique *Kpn*I restriction site used for linearization. C, Constructs *Aox1*Δ51/*Ars* to *Aox1*Δ53/*Ars* are derivatives of pJD100. Gray boxes represent the *TubB2* minimal promoter of constructs *Aox1*Δ51/*Ars* to *Aox1*Δ53/*Ars*. Numbering is relative to the transcription start site, which is preceded by a thin black area symbolizing the putative TATA box. Whenever determined, the co-expression rate on TAP agar medium and the cotransformation percentage are given in B and C ($n = 25$; see text for details). nd, Not determined.

than in TAP_{NH_4} medium and were still subject to azide stimulation. This indicates that proximal elements ensuring basal transcription and regulation lie in the short promoter segment present in *Aox1*Δ5/*Ars* and not in *Aox1*Δ6/*Ars* (−253 to −59), but that distant elements are required for full expression.

To further demonstrate that critical elements are present within the −253 to −59 promoter region, three 3′ deletions of the *Aox1*Δ5 fragment were cloned into pJD100. This plasmid encodes the *Ars* reporter gene driven by a 35-bp minimal promoter derived from the *C. reinhardtii TubB2* gene (Davies and Grossman, 1994). The new chimeric constructs, named *Aox1*Δ51/*Ars*, *Aox1*Δ52/*Ars*, and *Aox1*Δ53/*Ars*, contained portions of the *Aox1* upstream region from position −267 to positions −39, −133, and −187, respectively (Fig. 5C). Because these constructs could not be linearized, they were introduced under circular form into *C. reinhardtii* by cotransformation with pASL. Circular *Aox1/Ars* and *Aox1*Δ5/*Ars* were used as controls. After selection of Arg-independent colonies, the percentage of transformants expressing ARS was determined as above. Though lower than in the previous experiment, probably because circular plasmids are more subject to disruption during integration (Davies and Grossman, 1994; Loppes and Radoux, 2001), the co-expression rates were maximal with controls (27% and 31%) and minimal with

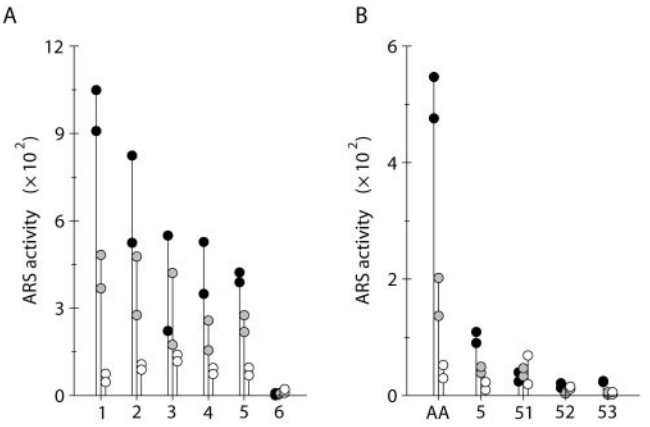

Figure 6. Deletion analysis of the *Aox1* promoter. Ammonium-grown cells from pools of transformants obtained with six linear constructs (A) or with five circular constructs (B) were transferred for 16 h into TAP liquid media containing 4 mM ammonium chloride (white circles), 4 mM sodium nitrate (gray circles), or 4 mM sodium nitrate with 200 μM sodium azide (black circles). Each symbol is the average ARS activity (nanomoles of naphthol per hour per milligram of protein) determined from a single cotransformation experiment. AA, *Aox1*/*Ars* construct; 1 to 5, constructs *Aox1*Δ1/*Ars* to *Aox1*Δ5/*Ars*; 51 to 53, constructs *Aox1*Δ51/*Ars* to *Aox1*Δ53/*Ars*.

*Aox1*Δ52/*Ars* and *Aox1*Δ53/*Ars* (4% and 8%), suggesting that these two constructs were unable to promote significant *Ars* transcription (Fig. 5C). The cotransformation rate for three different constructs were still highly homogenous (75%–79%; Fig. 5C), thus allowing the recording of ARS activities in pools of transformants as previously done.

In two independent cotransformation experiments, about 200 colonies from each cotransformation were pooled, plated onto fresh TAP agar medium, and then transferred for 16 h into TAP liquid media containing ammonium, nitrate, or nitrate with sodium azide. ARS activities with *Aox1*Δ52/*Ars* and *Aox1*Δ53/*Ars* were negligible and not significantly different from those of a pool bearing either pJD100 or no construct (Fig. 6B). In contrast, the *Aox1*Δ51/*Ars* construct lacking the −39 to +59 upstream segment of *Aox1*Δ5/*Ars* was still expressed but no more regulated. As previously, the deleted *Aox1*Δ5/*Ars* construct showed a lower expression level than the full *Aox1*/*Ars* construct, but basically responded to both nitrate and azide stimulations.

Together, the results obtained with the different chimeric constructs indicate that (a) elements between −133 and −39 are needed for sufficient transcription of *Aox1*; (b) elements conferring a response to nitrate and sodium azide lie in the −39 to +59 segment; and (c) elements in the distal part of the *Aox1* promoter are required to ensure full gene expression and regulation.

DISCUSSION

To study the transcriptional regulation of the main AOX gene from *C. reinhardtii*, we fused the full 1.4-kb upstream region of *Aox1* to the *Ars* reporter gene. We showed that expression of the chimeric construct was generally not enhanced by a number of known AOX inducers, including stress agents, respiratory inhibitors, and metabolites. A slight stimulation of the ARS expression was mostly observed when cells with less abundant AOX content (i.e. ammonium-grown cells) were exposed to respiratory inhibitors and paraquat (Table I). This contrasts with the situation described in many higher plants and fungi where the tested agents strongly stimulate the expression of the *Aox* gene and/or the accumulation of the AOX protein (see the introduction). However, it is worth noting that in the absence of inducers, the capacity of the alternative pathway is constitutively high in *C. reinhardtii* (see Table II). In several fungi, the AOX activity conversely is negligible under normal growth conditions and becomes detectable only when the cytochrome pathway of respiration is blocked by chemical inhibition or by mutation (Lambowitz et al., 1989; Sakajo et al., 1991; Minagawa et al., 1992; Li et al., 1996; Yukioka et al., 1998; Huh and Kang, 1999, 2001).

It has also to be pointed out that AOX isoforms unresponsive to cytochrome pathway inhibition and chemical induction have been described in several organisms (Saisho et al., 1997; Tanudji et al., 1999; Huh and Kang, 2001). In eudicot plant species in particular, the AOX is encoded in two discrete gene subfamilies: The *Aox1* subfamily is responsive to stress agents and respiratory inhibitors, whereas the *Aox2* subfamily shows tissue and developmental stage specificity but does not respond to *Aox1*-inducing agents (Considine et al., 2002).

Apparently peculiar to *C. reinhardtii* is the regulation of the amount of *Aox1* transcript and AOX protein by the nitrogen source (Quesada et al., 1998, 2000; this work). Simple removal of ammonium from the culture medium resulted in a significant increase of expression at both transcriptional and protein levels (Figs. 1A and 4A). Addition of nitrate further enhanced the expression, whereas the combination of ammonium and nitrate led to an intermediate expression level. In this alga, four high-affinity nitrate/nitrite transport systems are responsible for the entry of nitrate and nitrite into the cell. These transporters are differentially regulated by the nitrogen source and exhibit variable affinity for their substrates (for review, see Galvan and Fernandez, 2001). As shown in Figure 1B, the expression of the *Aox1*/*Ars* construct in relation to the nitrate concentration displays a biphasic curve. The change between the two response curves might reflect a switch between two of these transport systems. Moreover, one transporter (system IV) is known to be resistant to the ammonium inhibition affecting the other transport systems (Rexach et al., 1999; Navarro et al., 2000), which could account for the competing effects of ammonium and nitrate on the expression of *Aox1* transcript and AOX

protein accumulation. The same hypothesis has been proposed by Llamas et al. (2002) to explain the transcriptional regulation of the *Nia1* gene by nitrate and ammonium.

In nitrate-grown cells, the inactivation of nitrate reductase by sodium azide, tungstate, or mutation of the *Nia1* gene further enhances the expression of the chimeric construct and the amount of AOX protein. Hence, intracellular accumulation of nitrate would provide a permanent positive signal, as observed for the expression of nitrate-regulated genes (Rexach et al., 2002). Such a model is in accordance with the up-regulation of the *C. reinhardtii Aox1* transcript in the *G1* mutant lacking *Nia1* and five other nitrate-related assimilation genes but retaining a functional system IV transporter (Quesada et al., 1998; Navarro et al., 2000).

The 75-kD polypeptide visualized after azide treatment of nitrate-grown cells could correspond to a dimeric form of the AOX enzyme. However, the dimerization domain surrounding the regulatory Cys typical of the plant sequences is missing in both AOX isozymes from *C. reinhardtii* as well as in all fungal sequences (Umbach and Siedow, 2000; Dinant et al., 2001). Besides, the algal AOX enzyme is not stimulated by pyruvate (M. Dinant, personal observation). Because treatment with dithiothreitol did not eliminate the band at 75 kD in the SDS gel (data not shown), the detected signal could represent an artifact, possibly created occasionally during heating of the sample, as observed for the AOX of *Acanthamoeba castellanii* (Jarmuszkiewicz et al., 1997).

Although the major effect of sodium azide occurs through nitrate reductase inhibition in nitrate-containing medium, stimulation was also observed in the presence of ammonium, mainly at protein level (Fig. 4, B and C). This stimulation, independent from cytochrome *c* oxidase inhibition, must result from the inactivation of another unidentified enzymatic system.

In *C. reinhardtii*, regulatory sequences appear in most cases to be located within 200 to 300 bp from transcription start sites (for review, see Kindle, 1998). This rule is in agreement with our 5′-deletion analysis, demonstrating that a shortened promoter corresponding to the −253 to +59 segment is sufficient to ensure basal transcription and regulation of the *Aox1* gene. More specifically, elements lying between −133 and −39 are required for gene expression, whereas elements conferring response to the nitrogen source and azide should be found in the −39 to +59 region. However, the most expressed and regulated constructs extend to the *Nrt2;3* promoter range, which suggests that distant elements are probably also involved in the expression of the *Aox1* gene.

The 1.4-kb segment lying between *Aox1* and *Nrt2;3* was examined for conserved elements susceptible to mediate transcriptional regulation of the *Aox1* gene. Various 4- to 6-bp motifs previously identified in other *C. reinhardtii* promoters were found, but none concerned a nitrogen-related element described in the *Nia1* gene promoter from either *C. reinhardtii* (Loppes and Radoux, 2001, 2002) or *Chlorella vulgaris* (Cannons and Shiflett, 2001). A linker-scanning mutagenesis is necessary to identify responsive elements in the *Aox1* promoter (Quinn et al., 2000; Loppes and Radoux, 2002).

Our data raise the question of the physiological signification of the AOX regulation by the nitrogen source. The photosynthetic reduction of nitrate into nitrite requires the oxidation of NAD(P)H. Light reactions inside the chloroplast drive the coupled production of NADPH and ATP. In illuminated green tissues and microalgae, oxygen evolution is stimulated when nitrogen is supplied as nitrate rather than ammonium (Bloom et al., 1989; de la Torre et al., 1991), which suggests that nitrate assimilation enhances the noncyclic electron flow by acting as an alternative electron acceptor in the chloroplast. As hypothesized by Quesada et al. (2000), the nitrate reduction might result in an increased production of photogenerated ATP. In this context, the specific stimulation of AOX production by nitrate and not by ammonium might indicate the requirement for the cell to modify the partitioning of electron flow to adapt mitochondrial ATP synthesis during assimilation of these different nitrogen sources. Alternatively, the regulation observed at transcriptional, protein, and alternative pathway capacity levels might simply be due to the side effect of a nitrogen-related *Nrt2;3* enhancer on the *Aox1* gene expression. However, it is worth mentioning that the two genes partly differ in their regulation pattern: Although both are down-regulated by ammonium and better expressed in nitrate-containing medium, *Nrt2;3* is only transcribed when the regulatory *Nit2* gene is functional, which is not the case for *Aox1* (Quesada et al., 1998; R.F. Matagne and M. Dinant, unpublished data). Further investigations on other microalgae and higher plants would clear up this intriguing question.

MATERIALS AND METHODS

Chlamydomonas reinhardtii Strains and Culture Conditions

The host strain used in cotransformation experiments was the cell wall deficient, Arg-requiring *cw15 arg7-8 mt*$^+$ mutant (strain 325, lacking argininosuccinate lyase). Two other strains were used in additional experiments: *cw15 mt*$^+$ (strain 83) and *cw15 dum19 mt*$^-$ (strain 193, lacking cytochrome *c* oxidase). These three strains were able to assimilate nitrate as sole nitrogen source. Mutant strains used in genetic crosses were the following: *dum20 mt*$^-$ and *dum25 mt*$^-$ (strains 235 and 228, lacking NADH-ubiquinone oxidoreductase), *dum2 mt*$^-$ (strain 166, lacking ubiquinone-cytochrome *c* oxidoreductase), *dum19 mt*$^-$ (strain 239, lacking cytochrome *c* oxidase), *dum24 mt*$^-$ (strain 283, lacking both NADH-ubiquinone and ubiquinone-cytochrome *c* oxidoreductases), and *cw15 nia1 mt*$^-$ (strain 69, lacking nitrate reductase). The *dum* strains inactivated in mitochondrial genes and the *nia1* strain (also known as *nit1*) are reviewed by Remacle et al. (2001b) and Fernandez et al. (1998), respectively.

Cells were grown on solidified agar medium at 25°C under continuous illumination (70 μE m^{-2} s^{-1}). The routinely used medium was TAP con-

taining 7.5 mM NH_4Cl as nitrogen source (Harris, 1989) and 100 mg L^{-1} Arg when required. For liquid cultures, we used TAP medium containing 4 mM NH_4Cl, 4 mM $NaNO_3$, or 4 mM NH_4NO_3, or TAP deprived of any nitrogen source. Stress agents, inhibitors, and metabolites were sterilely added to the TAP liquid media after autoclaving. In the experiments devoted to the effects of tungstate, media were prepared according to Loppes et al. (1999).

General Methods

Crosses and isolation of meiotic products were performed as described by Harris (1989). Manipulation of nucleic acids was achieved by standard procedures (Sambrook et al., 1989; Ausubel et al., 1993). Plasmid DNA for sequencing and cotransformation was purified using Concert Rapid Plasmid Miniprep System (Invitrogen, Bethesda, MD) and Nucleobond AX (Macherey-Nagel, Düren, Germany). Total *C. reinhardtii* DNA for Southern blotting was obtained by the procedure of Newman et al. (1990). A faster cell lysis protocol communicated by L. Ellis was used in the preparation of total DNA for PCR amplification. In brief, 0.5 cm^2 of cells resuspended in 100 μL of PCR buffer containing 4 mg mL^{-1} proteinase K was incubated for 1 h at 58°C and then transferred for 1 h at 95°C. Dimethyl sulfoxide (6%, v/v) was required for optimal PCR amplification. Oligonucleotide synthesis and automated sequencing were ordered from GENOME Express (Paris). Restriction enzymes were purchased from Roche Diagnostics (Mannheim, Germany), whereas calf intestinal alkaline phosphatase, T4 DNA polymerase, T4 DNA ligase, and RadPrime DNA labeling system were ordered from Invitrogen. Amplification of DNA was carried out using either a standard *Taq* DNA polymerase from Amersham Biosciences (Piscataway, NJ) or a Super *Taq* Plus DNA polymerase from HT Biotechnology Ltd. (Cambridge, UK), an enzyme mixture displaying both high processing and proofreading activities. The buffers were those recommended by the suppliers, and the thermocycler was a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis).

Chimeric Constructs

A 1,522-bp DNA fragment extending from *Aox1* to *Nrt2;3* coding sequences was amplified by PCR using forward primer 5 from Dinant et al. (2001) with the MDRP4 reverse oligonucleotide (5′-TATGGAGCCCAGAAGACGAGAAGC-3′), which is complementary to amino acids 32 to 25 of the *Aox1* coding sequence (Dinant et al., 2001), and cloned into the pGEM-T Easy vector (Promega, Madison, WI) for sequencing (GenBank accession no. AF537323). Taking advantage of a unique *Nru*I site located 3 bp before the *Aox1* initiation codon, a 1,424-bp *Eco*RI-*Nru*I fragment (from positions −1364 to +60 relative to the transcription initiation site) was gel-purified, blunt-ended, and inserted into the *Sal*I restriction site of the pJD54 plasmid harboring a promoterless *Ars* gene (Davies et al., 1992). This construct named *Aox1/Ars* would produce a transcript with a 160-nt leader sequence (including 60 nt from *Aox1* and 69 nt from *Ars*; see Fig. 5A).

The *Xho*I-1423-R reverse oligonucleotide (5′-AAGCCTCGAGATATCGAACGGTGAC-3′), which is complementary to the *Aox1* 5′-untranslated region sequence from positions +68 to +44 relative to the transcription initiation site, was combined with forward primers *Xho*I-235-F (5′-GTTACTCGAGGTGCACGGAAAAGCG-3′), *Xho*I-429-F (5′-AACCCTCGAGGCGCTGCCAGAATGA-3′), *Xho*I-670-F (5′-TTGGCTCGAGGTTTTCGGCATCCCC-3′), *Xho*I-898-F (5′-TGTCCTCGAGATGACAGCGACCTAG-3′), *Xho*I-1111-F (5′-CGGCCTCGAGGTGCAACCCGATGCG-3′), and *Xho*I-1305-F (5′-CCTGCTCGAGTCGTCTACACTGGAA-3′) to amplify upstream regions between positions +59 and −1129, −935, −694, −466, −253, and −59, respectively. These PCR products were *Xho*I-digested and inserted into the *Sal*I restriction site of the dephosphorylated pJD54 plasmid to generate chimeric constructs *Aox1*Δ1/*Ars* to *Aox1*Δ6/*Ars*. These deleted constructs would produce a transcript with a 159-nt leader sequence (lacking one more base from the *Aox1* 5′-untranslated region than original *Aox1/Ars*; see Fig. 5B).

Similarly, the *Kpn*I-1097-F forward primer (5′-GCTCGGTACCCAGGGGCTCGGCCTC-3′), which is complementary to the *Aox1* upstream sequence from positions −276 to −252, was used with the reverse oligonucleotides *Kpn*I-1326-R (5′-TCGAGGTACCAGTGTAGACGATTCG-3′), *Kpn*I-1232-R (5′-CACTGGTACCGACCGCCGCCGCAAC-3′), and *Kpn*I-1178-R (5′-TATGGGTACCAACTTCAAGCACAGA-3′) to amplify upstream regions between positions −267 and −39, −133, and −187, respectively. Likewise, the PCR products were *Kpn*I-digested and inserted into the *Kpn*I restriction site of the dephosphorylated pJD100 plasmid harboring a 35-bp β-tubulin (*TubB2*) minimal promoter fused to the *Ars* gene (Davies and Grossman, 1994). These constructs, named *Aox1*Δ51/*Ars* to *Aox1*Δ53/*Ars*, would produce a transcript with a 170-nt leader sequence (including 65 nt from *TubB2* and 69 nt from *Ars*; see Fig. 5C).

All junctions were checked by automated sequencing. Computer analyses were performed with the aid of the GCG software package (Genetics Computer Group, Madison, WI; available at the Belgian EMBL Node, ULB/VUB).

C. reinhardtii Cotransformation

Nuclear cotransformation of *C. reinhardtii* strain 325 was performed using the glass bead method (Kindle, 1990) with minor modifications (Loppes and Radoux, 2001) using 1 μg of pASL (Adam and Loppes, 1998) bearing the wild-type *Arg7* gene as the selectable marker (Debuchy et al., 1989) and 5 μg of cotransforming DNA. Except otherwise stated, plasmids were linearized by *Bam*HI (pASL) or *Kpn*I (chimeric constructs) before cotransformation. Cells were then spread onto TAP agar plates for selection of Arg-independent transformants.

To determine the percentage of transformants expressing ARS, a number of Arg-independent clones were transferred and maintained for 3 d on TAP agar medium and then in situ stained for ARS activity (Ohresser et al., 1997). Total DNA from these clones was also purified to estimate the percentage of cotransformed cells by PCR amplification of the *Aox1-Ars* junction using the reverse oligonucleotide *Aox1-Ars*-R (5′-TTCTGAATGGCGTCCTGGTC-3′), which is complementary to amino acids 42 to 36 of the *Ars* coding sequence (de Hostos et al., 1989), either with forward primers *Xho*I-1305-F (*Aox1/Ars* and *Aox1*Δ1/*Ars* to *Aox1*Δ6/*Ars*) or with *Kpn*I-1097-F (*Aox1*Δ51/*Ars* to *Aox1*Δ53/*Ars*). To examine the status of the chimeric construct in the genome of the original *Aox1/Ars* transformants, the *Aox1-Ars*-R reverse oligonucleotide was combined either with forward primer 5 from Dinant et al. (2001) or with forward primer in-p*Aox1*-F (5′-TACGTACCGTGCTGCGAGGAGC-3′), which is complementary to the *Aox1* upstream region from positions −607 to −586 relative to the transcription initiation site. The copy number of the chimeric construct was also investigated in these clones through Southern-blot hybridizations following restriction of total DNA by *Hin*fI. The radiolabeled DNA probe was a 1.2-kb *Sal*I fragment of the *Aox1* upstream region covering positions −1359 to −140 with respect to the transcription initiation site. In deletion studies, several hundreds transformants from each experiment were pooled and maintained on TAP agar medium for further analyses.

Isolation of Membrane Fractions

Crude fractions containing mitochondrial and chloroplast membranes were prepared by cell sonication (Vibra Cell, Sonics and Materials, Newtown, CT) in MOPS buffer (280 mM mannitol, 10 mM MOPS-KOH, pH 7.4, and 0.1 M bovine serum albumin) as described by Remacle et al. (2001a).

Enzymatic Assays

Cells from isolated clones or pools of transformants were transferred from fresh TAP agar plates into glass tubes containing 2 mL of liquid medium at a density of 6×10^6 cells mL^{-1} and maintained for 8, 16, or 24 h under continuous light with agitation. After overnight freezing, ARS activity was determined on 50 to 400 μL of the thawed cell suspension using α-naphthyl sulfate as a substrate and tetrazotized-*o*-dianisidine as a post-coupling agent (Ohresser et al., 1997).

Cytochrome *c* oxidase activity was monitored by recording reoxidation of ferrocytochrome *c* in membrane fractions (2 μg proteins) as described by Remacle et al. (2001a). NADH-nitrate reductase activity was assayed in crude cell extracts according to Hageman and Reed (1980) by measuring the amount of nitrite formed after 15 min in the presence of potassium nitrate and NADH. Crude cell extracts were prepared by cell sonication in Tris-HCl buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 20 μM FAD) as described by Loppes et al. (1999).

Total protein content was determined according to Bradford (1976). The spectrophotometer apparatus used in all assays was a computer-controlled

UV/Vis Lambda 20 running UV WinLab software (PerkinElmer Life Sciences, Wellesley, MA).

Whole-Cell Respiration

Cells from cultures in liquid medium were harvested by centrifugation and resuspended into 1 mL of fresh medium at a density of 5×10^6 cells mL^{-1}. Respiratory rates were measured as oxygen uptake in the dark at 25°C using a Clark-type oxygen electrode (Hansatech, King's Lynn, UK). Capacities of the alternative and cytochrome pathways were estimated by addition of 1 mM potassium cyanide (in aqueous solution) and 1 mM *n*-propyl gallate (in ethanol), respectively.

Western-Blot Analysis

Membrane fractions were solubilized in an equal volume of denaturation buffer (125 mM Tris-HCl, pH 6.8, 10% [w/v] SDS, 20% [v/v] glycerol, and 0.1% [w/v] bromophenol blue) and boiled for 5 min. The proteins were separated by SDS-PAGE according to Laemmli (1970) using a 12% (w/v) polyacrylamide resolving gel and subsequently electrotransferred onto a Hybond C Super membrane (Amersham Biosciences) in transfer buffer (25 mM Tris, 192 mM Gly, and 20% [v/v] methanol). Prestained standard (Invitrogen) was used to estimate molecular mass and the efficiency of electrotransfer. Immunodetection of the AOX protein was carried out using the BM chemiluminescence western-blot kit (Roche Diagnostics) according to the manufacturer's instructions. Polyclonal antibodies raised against the AOX1 protein of *C. reinhardtii* (a kind gift from Dr. S. Merchant, University of California, Los Angeles) was used at dilution of 1:54,000. Antibodies against the subunit III of yeast cytochrome *c* oxidase were obtained from Molecular Probes (Eugene, OR) and used at concentration of 1 μg mL^{-1}.

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