The *Chlamydomonas reinhardtii* Molybdenum Cofactor Enzyme crARC Has a Zn-Dependent Activity and Protein Partners Similar to Those of Its Human Homologue

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The ARC (amidoxime reducing component) proteins are molybdenum cofactor (Moco) enzymes named hmARC1 and hmARC2 (human ARCs [hmARCs]) in humans and YcbX in *Escherichia coli*. They catalyze the reduction of a broad range of N-hydroxylated compounds (NHC) using reducing power supplied by other proteins. Some NHC are prodrugs or toxic compounds. YcbX contains a ferredoxin (Fd) domain and requires the NADPH flavin reductase CysJ to reduce NHC. In contrast, hmARCs lack the Fd domain and require a human cytochrome b5 (hCyt b5) and a human NADH Cyt b5 reductase (hCyt b5-R) to reduce NHC. The ARC proteins in the plant kingdom are uncharacterized. We demonstrate that *Chlamydomonas reinhardtii* mutants defective in Moco biosynthesis genes are sensitive to the NHC *N*°-hydroxylaminopurine (HAP). The *Chlamydomonas reinhardtii* ARC protein crARC has been purified and characterized. The six *Chlamydomonas* Fds were isolated, but none of them are required by crARC to reduce HAP. We have also purified and characterized five *C. reinhardtii* Cyt b5 (crCyt b5) and two flavin reductases, one that is NADPH dependent (crCysJ) and one that is NADH dependent (crCyt b5-R). The data show that crARC uses crCyt b5-1 and crCyt b5-R to reduce HAP. The crARC has a Zn-dependent activity, and the presence of Zn increases its *V*\textsubscript{max} more than 14-fold. In addition, all five cysteines of crARC were substituted by alanine, and we demonstrate that the fully conserved cysteine 252 is essential for both Moco binding and catalysis. Therefore, it is proposed that crARC belongs to the sulfite oxidase family of Moco enzymes.

All eukaryotic molybdenum (Mo)-containing enzymes that have been studied have Mo chelated with an organic motif (molybdopterin [MPT]) forming the so-called Mo cofactor (Moco) (Fig. 1A). Moco is widespread in all kingdoms and synthesized by a conserved pathway, divided in several steps according to the biosynthesis of its intermediates from a guanosine derivative (probably GTP): cyclic pyranopterin monophosphate (cPMP), MPT, and MPT-AMP (adenylated molybdopterin). In *Chlamydomonas reinhardtii*, the CNX2 and CNX3 enzymes catalyze the conversion of GTP into cPMP, CNX5, CNX6, and CNX7 from cPMP into MPT, CNX1G from MPT into MPT-AMP, and CNX1E from MPT-AMP into Moco (21). Two families of Moco-containing enzymes are present in eukaryotes, the sulfite oxidase (SO) family and the xanthine oxidase (XO) family. In Moco proteins, Mo is chelated via two thiol groups of MPT and also with two oxo groups (Fig. 1A). In the SO family, the fifth Mo ligand is a protein-derived cysteine, and in the XO family, this is an inorganic sulfur (28).

A newly identified enzyme called ARC (amidoxime reducing component) is involved in the reduction of a broad range of N-hydroxylated compounds (NHC), present in eukaryotic and prokaryotic organisms (12). An important group of base analogues are for example the NHC of adenine, *N*°-hydroxylaminopurine (HAP), a very powerful mutagen in phages, bacteria, and eukaryotic cells (24). In bacteria, the defect in any enzyme involved in the Moco biosynthesis pathway gives a HAP-hypersensitive phenotype (17), the first evidence of a Moco-dependent enzyme involved in the detoxification of HAP. However, the deletion of known molybdoenzymes in *Escherichia coli* failed to reveal any HAP sensitivity (18), suggesting that a novel type of Moco-dependent activity was involved in HAP detoxification. Nevertheless, a novel Moco-dependent enzymatic activity involved in the reduction of NHC was discovered in humans, hmARCs (human mARCs), operating in conjunction with a human Cyt b5 (hCyt b5) and a human NADH Cyt b5 reductase (hCyt b5-R), capable of converting the prodrug, benzamidoxime, to its active form, benzamidine (12). This conversion is analogous to the reduction of HAP to adenine, as it entails the reduction of an NHC (benzamidoxime) to the corresponding amino form (benzamidine) (Fig. 1). Two highly homologous hmARC proteins are present in humans, hmARC1 and hmARC2 (Fig. 1B). The subcellular localization of ARC proteins is not well defined. The hmARCs were localized in the outer mitochondrial membrane (9), mouse ARC proteins were localized in the inner mitochondrial membrane (5), and rat ARC proteins were localized in peroxisomal membranes (14).

In bacteria, the enzyme involved in the HAP detoxification was identified by analyzing an *E. coli* mutant defective in the YcbX protein. YcbX was capable of avoiding the HAP toxicity by its reduction to adenine (16).

YcbX and hmARCs are hypothetical members of the MOSC protein superfamily. These proteins contain a domain homol-
The cofactor sulfurase C-terminal domain, Cyt domains found in the ARC protein system, MOSC (molybdenum containing proteins) are widely distributed in prokaryotes and eukaryotes and contain a fully conserved cysteine residue substituted were obtained, demonstrating that cysteine 252 is essential for Moco binding and catalysis; therefore, it is proposed that crARC belongs to the SO family of Moco enzymes.

MATERIALS AND METHODS

Chemicals. N^{6}-Hydroxylaminopurine (HAP) was purchased from ICN Biochemicals. The other chemicals were purchased from Sigma-Aldrich.

Chlamydomonas reinhardtii strains and culture conditions. The Chlamydomonas reinhardtii strains used in this work have been described in references 7 and 21. Cells were cultured under continuous light at 23°C in liquid and solid media containing 5 mM ammonium chloride or bubbled (for liquid cultures) with 5% CO₂ in air.

Bacterial strains and culture conditions. The Escherichia coli strains were grown on LB medium or minimal Vogel-Bonner medium (VB) (33) containing 0.2% glucose as the carbon source. The E. coli strains were grown on LB medium or minimal Vogel-Bonner medium (VB) (33) containing 0.2% glucose as the carbon source. The E. coli strain JW5126-1 was used for expression of ARC recombinant proteins because it accumulates eukaryotic molybdenum cofactor (Moco). The E. coli strain BL21(DE3) was used for expression of the remaining recombinant proteins (8).

Tests for inhibition by HAP of E. coli growth. A freshly transformed single E. coli colony of each strain to be tested was inoculated into 1 ml of liquid LB and grown for 2 h at 37°C. Then, it was diluted 50-fold in 0.9% NaCl and 2.5 μl was transferred to minimal VB plates. After the spots had dried, 100 μg of HAP was spotted onto the center of the plate. The plates were incubated for 24 h at 37°C and inspected for growth inhibition zones.

Tests for inhibition by HAP of Chlamydomonas growth. A 5-μl drop from liquid culture of ammonium-grown cells containing about 1,000 cells was laid, in a liquid medium, and found to have a Zn-dependent activity. Finally, crARC mutants with each of its cysteine residues substituted were obtained, demonstrating that cysteine 252 is essential for Moco binding and catalysis; therefore, it is proposed that crARC belongs to the SO family of Moco enzymes.

FIG. 1. Schematic structure of Moco, ARC proteins, their partners, and their reaction catalyzed. (A) Structure of the Moco molecule with the organic motif (MPT) and the Mo atom shown in bold type. The X indicates that the fifth Mo ligand in the ARC protein is unknown. (B to D) Schematic representations of the ARC system in humans (B), E. coli (C), and Chlamydomonas reinhardtii (D). Each of the protein domains found in the ARC protein system, MOSC (molybdenum cofactor sulfurase C-terminal domain), Cyt b5 (Cyt b5 domain), FAD/NADH (FAD- and NADH-binding domain), Fe-S (2Fe-2S-binding domain), and FMN (FMN-binding domain), is shown in boxes (most boxes with a gray background). The MOSC domain is able to bind Moco, which is indicated by Moco above the domain, but it is not known to which amino acids Moco binds. The proteins involved with crARC in the HNC reduction are unknown (question mark). In the table at the bottom of the figure, some reactions carried out by these proteins are shown with some examples of HNC substrates studied and references in the parentheses. The electron donors are NADH in the human system and NADPH in the E. coli system.

Oligo to the C-terminal domain (MOSC) of the eukaryotic Moco sulfatases (MOS). The MOS enzymes are involved in the transfer of a sulfide ligand, yielding sulfated Moco, that is essential for the activity of the XOR family of Moco enzymes. However, except for MOS, all other members of the MOS superfamily are proteins without any confirmed function (28).

MOSC-containing proteins are widely distributed in prokaryotes and eukaryotes and contain a fully conserved cysteine (1).

YcbX contains, in contrast to hmARCs, a ferredoxin (Fd) [Fe2-S2] domain in the C terminus, essential for its activity (16) (Fig. 1C). Recently, the CysJ component of the sulfite reductase complex (8CysJ4CysI) has been identified as one additional component of this system (19) (Fig. 1C). The role of CysJ in HAP reduction is unique and independent of CysJ and sulfite reductase. CysJ provides via its NADPH flavin reducing activity the reducing equivalents needed by YcbX to reduce HAP. Therefore, the role of bacterial CysJ seems to be analogous to hCyt b5-R, that is, to provide the reducing power needed for the reduction of the NHC. In bacteria, the electrons would be funneled from Fd to the MOSC domain of YcbX, while in humans, the electrons would go from the hCyt b5 to the MOSC domain of hmARC proteins. Thus, it appears that the ARC proteins are a widely distributed class of molybdoenzymes existing in organisms from bacteria to mammalian cells with similar biochemical properties.

Although there have been substantial advances in the role of ARC proteins in bacteria and human cells, nothing is known about ARC proteins in the plant kingdom. We have studied this protein in the green alga Chlamydomonas reinhardtii, which has a single gene encoding a protein with similarity to ARC that we call crARC (for Chlamydomonas reinhardtii ARC). In this work, we have shown that crARC is a molybdoenzyme critical for detoxifying HAP, and similar to its human homologue, it requires the NADH-dependent Cyt b5 flavin reductase and Cyt b5-1 but none of the six Chlamydomonas ferredoxins. The enzymatic reduction reaction of HAP by crARC has also been studied in vitro and found to have a Zn-dependent activity. Finally, crARC mutants with each of its cysteine residues substituted were obtained, demonstrating that cysteine 252 is essential for Moco binding and catalysis; therefore, it is proposed that crARC belongs to the SO family of Moco enzymes.
amino acids was cloned in both pQE80 and pASK-IBA5C (Table 1, primer sets 1 and 2) and pASK-IBA8C (Table 1, primer set 3). The resulting cDNAs were cloned in pQE80 (Table 1, primer set 4). The cDNA encoding 368 amino acids was cloned in pQE80 (Table 1, primer set 5). The resulting cDNA encoding 368 amino acids was cloned in E. coli (Table 1, primer set 6). The resulting cDNA encoding 668 amino acids was cloned in pQE80 (Table 1, primer set 7). The resulting cDNA encoding 668 amino acids was cloned in pQE80 (Table 1, primer set 8). The cDNA encoding 668 amino acids was cloned in pQE80 (Table 1, primer set 9). A BLAST search in the Chlamydomonas JGI database using the hCyt b5 sequence (GenPept accession no. NP_058056) yielded 5 sequences with GenPept accession numbers (shown in parentheses): crCyt b5-1 (XP_001697920), crCyt b5-2 (XP_001697853), crCyt b5-3 (XP_001693518), crCyt b5-4 (XP_001693863), and crCyt b5-5 (XP_001697852). Five of these cDNAs were amplified using specific primers, and the resulting cDNAs encoding 111 (crCyt b5-1), 113 (crCyt b5-2), 182 (crCyt b5-3), 100 (crCyt b5-4), and 108 (crCyt b5-5) amino acids were cloned in pQE80 (Table 1, primer set 10). A BLAST search in the Chlamydomonas JGI database using the human hCyt b5-R sequence (GenPept accession no. NP_015586) as a query yielded one sequence with GenPept accession no. crCyt b5-R (XP_001697724). This cDNA was amplified using specific primers, and the resulting cDNA encoding 250 amino acids was cloned in pQE80 (Table 1, primer set 11). A BLAST search in the Chlamydomonas JGI database using the E. coli CysJ sequence (GenPept accession no. NP_417247) as a query resulted in one sequence with GenPept accession no. crCysJ (XP_001697724). This cDNA was amplified using specific primers, and the resulting cDNA encoding 668 amino acids was cloned in pQE80 (Table 1, primer set 12). The full-length ycbX open reading frame was amplified from total genomic E. coli DNA according to GenPept accession no. NP_415467. The resulting cDNA encoding 368 amino acids was cloned in pQE80 (Table 1, primer set 13). The cDNA encoding the N-terminal MOSC domain of YcbX (eMOSC [E. coli MOSC]) resulting in 290 amino acids was cloned in pQE80 (Table 1, primer set 23). The cDNA encoding the C-terminal Fd domain (cFDX) resulting in 110 amino acids was cloned in both pQE80 and pASK-IBA8C (Table 1, primer set 24). Full-length cDNA clones of hmARC1 and hmARC2 were obtained from Source BioScience LifeSciences genomic services (Source BioScience LifeSciences, Nottingham, United Kingdom) IMAGE ID 3847279 for hmARC1 and IMAGE ID 3849257 for hmARC2 according to GenPept accession nos. NP_076583 and NP_060388, respectively. The cDNAs were amplified using specific primers, and the resulting cDNAs encoding 294 (hmARC1) and 292 (hmARC2) amino acids were cloned in pQE80 (Table 1, primer sets 26 and 27). The accuracy of all cDNA sequences was confirmed by DNA sequencing.

Expression and purification of recombinant proteins. Standard expression of the crARC, crARC cysteine-to-alanine variants, hmARC-1, hmARC-2, and YcbX proteins was performed in freshly transformed E. coli TP1000 (moh4) mutant cells (31). The expression of the other proteins was performed in E. coli BL21(DE3). The cells were grown aerobically in LB medium to an A600 of 0.1 before induction. TP1000 cells were induced with 10 μM isopropyl-β-D-thiogalactoside and additionally supplemented with 0.1 mM sodium molybdate to initiate recombinant expression. E. coli BL21 cells were induced with 100 μM IPTG to start expression. Cells expressing proteins with a heme group were supplemented with 1 mM calcium pectinic acid to support heme synthesis. After induction, the cells were grown for an additional 3 h at 22°C. Purification of recombinant proteins expressed by performing Ni-NTA (Ni-NTA) matrix, as recommended by the supplier (Qiagen), under native conditions (4°C) using minimal volumes of washing buffers to wash away dissociation of bound Mo and Zn from the proteins. The protein fractions were analyzed by SDS-PAGE, and only the pure fractions were taken and immediately desalted on a PD10 gel filtration column previously equilibrated with 100 mM Tris-HCl, pH 7.2. The protein concentration was determined by UV absorption measurements using the calculated extinction coefficient (23) of the analyzed polypeptides.

DNA sequencing and sequence analysis. DNA sequencing was performed at the Servicio Central de Apoyo a la Investigación (SCAI) (University of Córdoba, Spain). Sequences were analyzed using the DNAstar software v.4.05 (Lasergene Navigator), the Bioedit Sequence Alignment Editor v. 7.0.9 (Department of Microbiology, North Carolina State University), the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/), and the Chlamydomonas JGI server (http://www.chlamy.org). Enzyme assays. (i) The in vitro HAP reduction by crARC. The HAP reduction by crARC was determined as described previously for hmARCs (9) with minor modifications. Incubations were carried out under aerobic conditions at 37°C in a shaking water bath. Unless stated otherwise, standard incubation mixtures of the constituted system contained 100 pmol crARC, 10 pmol crCyt b5-R (or crCysJ), 100 pmol crCyt b5-1 (or b5-2, b5-3, b5-4, or b5-5), 0.5 mM HAP, and 1.0 mM NADH or NADPH in a total volume of 150 μl of 10 mM potassium phosphate buffer, pH 6.5. After preincubation for 3 min at 37°C, the reaction was started by the addition of NADH or NADPH and terminated after 15 min by adding 150 μl of methanol. The precipitated proteins were sedimented by centrifugation, and the supernatant was analyzed by high-performance liquid chromatography (HPLC). One unit of crARC activity is defined as the amount of enzyme causing the production of 1 μmol of adenine per minute under the described conditions. The apparent kinetic parameters Km and Vmax were estimated using nonlinear regression analysis.

(ii) Determination of the Cyt b5 heme content. For the determination of heme binding to Cyt b5, the absorption at 413 nm was monitored, and the heme/protein ratio was calculated using an extinction coefficient of 117 mM−1 cm−1 (29). (iii) Determination of the flavin reductase FAD content. Binding of FAD to the different reductases used in this work was determined at 450 nm, and the FAD/protein ratio was calculated using an extinction coefficient of 11.3 mM−1 cm−1 (35).

(iv) Determination of the NAD(P)H flavin reductase activity. The rate of NAD(P)H flavin reductase activity was measured by two methods, the reduction of potassium ferricyanide which acts as an artificial electron acceptor or the reduction of crCyt b5.

The activity of crCyt b5-R and crCysJ with potassium ferricyanide was assayed as previously described (30). The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.5), 0.112 mM NADH or NADPH, 0.2 mM potassium ferricyanide, and the appropriate concentration of enzyme in a final volume of 1 ml. The reaction was started by the addition of NADH or NADPH, and ferricyanide reduction was followed by recording the absorbance decrease at 420 nm. The enzyme activity was calculated using the extinction coefficient of 1.02 mM−1 cm−1 (26). One unit of reductase activity is defined as the amount of enzyme causing the reduction of 1 μmol of potassium ferricyanide per minute.

The activity of crCyt b5-R and crCysJ in the presence of crCyt b5 was assayed by the method of Strittmatter and Velick (29). The reaction mixture contained 0.1 mM potassium phosphate buffer (pH 6.8), 0.112 mM NADH or NADPH, 2 mM potassium ferricyanide, and the appropriate amount of crCyt b5-R or crCysJ in a final volume of 1 ml at 25°C. The reaction was initiated by the addition of NADH or NADPH. The reaction was followed by the increase in absorbance at 423 nm. The molar extinction coefficient increase between the reduced and oxidized forms of crCyt b5 was taken as 100 mM−1 cm−1. One unit of reductase is defined as the amount of enzyme catalyzing the reduction of 1 μmol of potassium ferricyanide per minute.

HPLC method for HAP and adenine quantification. HAP and adenine were separated and quantified by HPLC. The HPLC analysis was performed on an Agilent series 1200 from Agilent Technologies. The separation was carried out with a symmetric C18 HPLC method for HAP and adenine quantification. The rate of molybdenum (MPT) bound to the proteins, the analysis of FormA was performed as reported previously (27). ICP-OES. To measure the amounts of Mo and Zn bound to the proteins, inductively coupled plasma optical emission spectrometry (ICP-OES) analysis
| Primer set | Primer | Primer sequence (5’–3’) | DNA cloned | Vector |
|------------|--------|--------------------------|------------|--------|
| crARC5BamHI/ crARC3HindIII | GGATCCATGTCACAATTCGCGCCGTCT | crARC | pQE80 |
| crARC5PstI | CTGCAGCTACGCTGACGCATGGCTCT | crARC | pQE80 |
| crARC5XhoI | CTCGAGCTACTCCTGCAGCTCCGCCCAACCCT | crARC | pQE80 |
| crARC5KpnI | GGTACCGGGCCGATCCGCAGAAGAATCTCGCCTTGGCGGCGGCGGGAAGG |
| crARC5PstI | CTGCAGCTACTCCTGCAGCTCCGCCCAACCCT | crARC | pQE80 |
| crARC5XhoI | CTCGAGCTACTCCTGCAGCTCCGCCCAACCCT | crARC | pQE80 |

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was used. Determinations were carried out on a Yobin-Ivon Ultima 2 ICP-OES. The instrument response was optimized and calibrated with standards prepared from Merck multielement solution VI plus a solution of nitric acid 5% (vol/vol) as a blank. The accuracy of the system was evaluated by running control standards prepared at concentrations lower and higher than the concentration of the samples at the beginning and end of sample runs. Blanks were introduced in sequences before and after sample runs. Recovery was evaluated on samples. The instrument settings were as follows: power, 1,200 W; plasma gas, 12 liters/min; and carrier gas, 0.6 liters/min. Zn was analyzed at a wavelength (λ) of 206.200 nm. Mo was analyzed at a wavelength of 202.030 nm.

RESULTS

The Chlamydomonas reinhardtii Moco mutants are sensitive to HAP. The E. coli mutants defective in molybdenum cofactor (Moco) biosynthesis are hypersensitive to the toxic effect of N6-hydroxylaminopurine (HAP) present in the growth media (18). In order to determine the relationship between N-hydroxylated compound (NHC) toxicity and Moco, we have investigated the phenotypes of different Chlamydomonas mutants in the presence of HAP. Screening the Chlamydomonas mutant library generated in our lab by insertional mutagenesis (7) allowed us to identify five mutations in Moco biosynthesis genes (21). These strains are affected at genes Cnx2 and Cnx3 of the first step of Moco biosynthesis, Cnx5 of the second step, Cnx1G of the third step, and Cnx1E of the fourth step (see introduction). These mutants allowed us to study systematically the effect of inactivated genes in each of the defined Moco biosynthetic steps. As shown in Fig. 2, all the Chlamydomonas Moco mutants tested were hypersensitive to HAP compared to Chlamydomonas parental strain 704. This result indicates that the mutations blocking the synthesis of any Moco intermediate and thus of a functional Moco leads to a Moco-dependent HAP detoxification system. The questions arising now are whether or not this system depends on ARC proteins and if so, whether this is similar to the system found in bacteria or to the one found in humans (Fig. 1). Thus, further studies were performed in order to identify the enzyme complex involved in HAP detoxification.

Cyt b5, but not Fd, is involved in HAP resistance in Chlamydomonas. The protection system against NHC toxicity in a photosynthetic organism like Chlamydomonas has been uncovered. The HAP toxicity found in every Chlamydomonas Moco mutant suggests that an ARC protein could be involved in HAP detoxification in this organism. A search in the Chlamydomonas genome resulted in one sequence that we call crARC. The main difference among YcbX, hmARCs, and crARC is the presence of a Fd domain [Fe2-S2] in the E. coli protein that is absent in the Chlamydomonas and humans proteins. However, the MOSC domain is present in the three proteins (Fig. 1). crARC shows 26.3, 26.1, and 21.3% sequence identity with hmARC1, hmARC2, and YcbX, respectively.

None of the mutants screened from our Chlamydomonas insertional mutant collection (6) were defective in the crARC gene, most probably because the selection medium was not appropriate for this purpose. As the E. coli ycbX mutant is hypersensitive to the toxic effect of HAP, the participation of the different proteins in HAP detoxification could be analyzed from their ability to revert the HAP toxicity found in this mutant (we refer to these experiments as in vivo experiments).

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TABLE 1—Continued

| Primer set | Primer | Primer sequence* (5’–3’) | DNA clonedb | Vector |
|------------|--------|--------------------------|-------------|--------|
| 23 Ycbx5MoscKpnI Ycbx3MoscPstI | GGTACC CGCGCATATTAACCGCGCTTTTATTCATC | ecMOSC | pQE80 |
| 24 Ycbx3FerKpnI Ycbx3FerPstI | GGTACC CGGGTTGAAAAATCTGGCAACGGCTC | ecFDX | pQE80 |
| 25 Ycbx3FerKpnI Ycbx3FerPstI | GGTACC CGGGTTGAAAAATCTGGCAACGGCTC | ecFDX | pASK-IBA5C |
| 26 mARC1SacI mARC KpnI | GAGCTCTGCCCACGCGGCCGC | hmARC1 | pQE80 |
| 27 mARC2SacI mARC2KpnI | GAGCTCTGCCCACGCGGCCGC | hmARC2 | pQE80 |

* The restriction site and mutation introduced are indicated by underlined and italic sequences, respectively. The identity of the restriction enzyme is shown at the end of the primer name.

b crARC-C15A, crARC with the C15A mutation; crARC-C249/252A, crARC with the C249A C252A double mutation.
Therefore, to resolve whether crARC is involved in the detoxification of HAP, this protein was expressed heterologously in the E. coli ycbX mutant.

Figure 3A shows that the HAP toxicity was not reverted in any E. coli ycbX mutant transformed with crARC. The question that now arises is whether crARC needs one ferredoxin (Fd) similar to YcbX or one Cyt b5 similar to hmARCs to be fully functional. We cloned the six ferredoxins present in the Chlamydomonas genome (32). For a control, we also cloned the Fd domain of YcbX (ecFDX [E. coli FDX]). The percentages of identity between the E. coli Fd and Chlamydomonas Fd are 17.3 (crFDX1), 15.4 (crFDX2), 17.1 (crFDX3), 14.5 (crFDX4), 15.7 (crFDX5), and 14.4 (crFDX6). Fig. 3A, spots 1 to 6, shows that the HAP toxicity was not reverted in the E. coli ycbX mutant cotransformed with crARC plus each of the six crFDX or the ecFDX. This result suggests that none of the Chlamydomonas Fds were able to revert the HAP toxicity when coexpressed with ecMOSC (Fig. 3B, spots 1 to 6). These results suggest that the interaction between ecMOSC and ecFDX domains is very specific and that the crFDXs cannot replace the function of the ecFDX domain.

As the crFDXs have failed as crARC partners, we analyzed the Chlamydomonas Cyt b5, since the partner of hmARCs is a hCyt b5. A search in the Chlamydomonas genome using the hCyt b5 sequence as a query resulted in 5 sequences (crCyt b5-1 to crCyt b5-5) that were cloned. The percentages of identity between the hCyt b5 and each crCyt b5 are 28.9 (crCyt b5-1), 26.4 (crCyt b5-2), 15.7 (crCyt b5-3), 28.8 (crCyt b5-4), and 16.3 (crCyt b5-5) (Fig. 4). Interestingly, as shown in Fig. 3C, spots 1, only crCyt b5-1 was able to revert the HAP toxicity when cotransformed with crARC. However, when 100 μM molybdate was also included in the medium, crCyt b5-2 was also able to revert the HAP toxicity (Fig. 3D, spots 2). These results show clearly that crCyt b5 but not crFDX participates in crARC in the HAP detoxification, at least in vivo. These data allowed us to hypothesize that the proteins crARC and crCyt b5-1 or crCyt b5-2 are forming a complex in vivo in the bacterial cell that functions analogously as YcbX. This means that in vivo one or more E. coli proteins should be able to donate electrons to this predicted complex.

In E. coli, the cysJ mutation also causes a HAP hypersensitivity phenotype (19). CysJ provides via its NADPH flavin reductase activity the reducing equivalents needed for the reduction of HAP by YcbX (19). The E. coli cysJ mutant was also cotransformed with crARC plus each of the five crCyt b5. However, none of them were able to revert the HAP toxicity of the cysJ mutant, even in the presence of molybdate in the medium (data not shown). This means that the CysJ protein is able to transfer, at least in vivo, the reducing equivalents to the predicted complex of crARC with crCyt b5-1 or crCyt b5-2.

Purification and characterization of recombinant proteins. To analyze in vitro which Chlamydomonas reductase is involved in the reduction of HAP, we first purify and characterize the potential proteins involved in this reduction.

In contrast to hmARC proteins, crARC does not contain any predicted targeting sequences to the mitochondria or to any other cell compartment. Therefore, we cloned the full-length cDNA, and the recombinant crARC was expressed in a soluble form of 35 kDa. The hmARC1 and hmARC2 proteins were cloned without their predicted signal sequences for mitochondria export. The hmARC1 and hmARC2 proteins were obtained in a soluble form of 33.2 and 33.6 kDa, respectively. YcbX after the recombinant expression was obtained as a soluble protein of 40.6 kDa with a dark red color, corresponding to the bound Fd domain.

Purified crARC, hmARC1, hmARC2, and YcbX were subjected to inductively coupled plasma optical emission spectrometry (ICP-OES) analysis to measure the Mo content which reflects the amount of Moco. As shown in Table 2, these four proteins present similar and almost fully saturated (1:1) Mo/protein ratios.

The visible absorption spectra of crARC in the oxidized state showed an absorption shoulder around 410 nm and a broad shoulder at 465 nm. When crARC was reduced with dithionite, the 410-nm shoulder shifted to 425 nm and the
Heme and FAD contents were determined via extinction coefficient (n two domains, a water-soluble heme-binding domain and a hy-

FIG. 4. Multiple-sequence alignment of Chlamydomonas reinhardtii and human Cyt b5 proteins. The consensus sequences have been calculated with a threshold of 75% with the BioEdit v.7.0.9 program. The sequences and GenPept accession numbers (shown in parentheses) are crCyt b5-1 (XP_001697920), crCyt b5-2 (XP_001697853), crCyt b5-3 (XP_001693518), crCyt b5-4 (XP_001693863), and crCyt b5-5 (XP_001697852) (crCyt b5-1) (the crCyt b5-1 to crCyt b5-5 are from Chlamydomonas reinhardtii), and hCyt b5 (NP_085056) from humans. Highly conserved amino acids are shown on a black background, and moderately conserved amino acids are shown on a gray background. The numbers in the sequence alignment (43, 15, and 13) represent the lengths of poorly conserved inserts that have not been shown in the alignment. The conserved histidines that bind the heme group are indicated by white letters on a black background. The coding sequences for the putative membrane-binding domains are underlined. Gaps introduced to maximize the alignment are indicated by dashes.

TABLE 2. Cofactor content of recombinant proteins

| Protein | Cofactor contenta (mol/mol of protein) |
|---------|---------------------------------------|
| crARC   | 0.83 ± 0.06 Moco                       |
| crCyt b5-1 | 0.17 ± 0.01 heme                     |
| crCyt b5-2 | 0.20 ± 0.03 heme                     |
| crCyt b5-3 | 0.35 ± 0.02 heme                     |
| crCyt b5-4 | 0.19 ± 0.01 heme                     |
| crCyt b5-5 | 0.10 ± 0.01 heme                     |
| crCyt b5-R | 0.34 ± 0.02 FAD                      |
| crCysJ  | 0.55 ± 0.06 FAD                       |
| YcbX    | 0.81 ± 0.07 Moco                      |
| hmARC1  | 0.85 ± 0.11 Moco                      |
| hmARC2  | 0.90 ± 0.09 Moco                      |

a Moco content was determined by quantifying Mo by ICP-OES (n = 3). Heme and FAD contents were determined via extinction coefficient (n = 3).
28.1 kDa and 71.2 kDa, respectively. These proteins were characterized by a light yellow color indicative of bound FAD. When crCyt\textsubscript{b5-R} and crCysJ were subjected to UV-visible absorption spectroscopy in the oxidized state, they showed two distinct absorption peaks at 390 and 460 nm accompanied by a shoulder at around 480 nm (Fig. 5D; data not shown for crCysJ). After dithionite reduction, all these absorption peaks were replaced by broad absorptions between 315 and 500 nm. These spectra are in agreement with the typical signature of flavin reductases. The determination of bound FAD \cite{35} to crCyt\textsubscript{b5-R} and crCysJ revealed that these proteins were saturated at an average of 34% and 55%, respectively (Table 2).

In summary, the spectral properties of the five crCyt\textsubscript{b5}, crCyt\textsubscript{b5-R}, and crCysJ indicate that these recombinant proteins meet the demands of electron carrier proteins.

The flavin reductase activity of crCyt\textsubscript{b5-R} and crCysJ was evaluated by measuring the reduction rate with the artificial electron acceptor ferricyanide and with each of the five crCyt\textsubscript{b5}. As shown in Table 3, crCyt\textsubscript{b5-R} was able to efficiently reduce ferricyanide with NADH but not with NADPH (data not shown). However, crCysJ was able to reduce efficiently ferricyanide with NADPH but not with NADH (data not shown). The crCyt\textsubscript{b5-R} protein was more efficient than crCysJ in the reduction of ferricyanide. These results indicated that

\begin{table}[h]
\centering
\begin{tabular}{lcccc}
\hline
Protein & Flavin reductase activity\textsuperscript{a} measured by: & Reduction of crCyt\textsubscript{b5} protein\textsuperscript{b} & Reduction of ferricyanide\textsuperscript{c} \\
& & crCyt\textsubscript{b5-1} & crCyt\textsubscript{b5-2} & crCyt\textsubscript{b5-3} & crCyt\textsubscript{b5-4} & crCyt\textsubscript{b5-5} \\
\hline
\textit{crCyt} b5-R & 563 ± 8.6 & 107 ± 13 & 0 & 253 ± 33 & 0 & 678 ± 27 \\
crCysJ & 57 ± 11 & 556 ± 125 & 162 ± 19 & 297 ± 36 & 0 & 44.8 ± 5.6 \\
\hline
\end{tabular}
\caption{Determination of flavin reductase activity of crCyt b5-R and crCysJ} \label{table:flavin_reductase_activity}
\end{table}

\textsuperscript{a} The flavin reductase activity was measured by two methods; the reduction of ferricyanide and the reduction of each of the five crCyt\textsubscript{b5} proteins (described in detail in Materials and Methods). NADH was the electron donor for crCyt\textsubscript{b5-R}, and NADPH was the electron donor for crCysJ. Data are means ± standard deviations of 3 independent experiments.

\textsuperscript{b} Measured in milliunits per milligram of protein.

\textsuperscript{c} Measured in units per milligrams of protein.


The in vitro HAP reduction of HAP was performed under the standard conditions: 100 pmol of crARC, 100 pmol of each crCyt b5 and 10 pmol of crCyt b5-R or crCysJ, 0.5 mM HAP, 1 mM NADH (with crCyt b5-R) or 1 mM NADPH (with crCysJ) and 15 min of reaction time. However, crCysJ was able to reduce all the crCyt b5 with NADPH, except for crCyt b5-3. Interestingly, the only combination that was able to significantly affect the folding of the protein, its fluorescence spectrum would be altered. We did not find any significant change in the fluorescence spectra in the mutants (data not shown).

The HAP reduction activity was determined for each cysteine mutant with Zn and without Zn. None of them showed a HAP reduction rate that was different from that of the wild type, and the C252A C252A double mutant that totally abolished HAP reduction activity with and without Zn (Fig. 8A).

The crARC and its variants were subjected to FormA analysis, which allows the quantification of the organic motif of Moco (MPT) bound to the protein and to ICP-OES to measure the Mo content. In all of the crARC variants, the Mo and MPT contents were similar to those of the wild type except in the C252A mutant and the C249A C252A double mutant that had lost 80% and 100% bound Mo and MPT, respectively (Fig. 8B).

These data indicate that the C252 is essential for the binding of Moco to crARC and that the HAP reduction activity depends on the presence of Moco. Interestingly, the neighboring C249 should be also mutated together with the C252 to fully abolish the Moco binding. These data can be explained by assuming that the close residue C249 is able to replace partially C252 to fully abolish the Moco binding. These results indicate that in Chlamydomonas, the HAP reduction occurs by a three-component system consisting of crCyt b5-R, crCysJ and crARC. The crCyt b5-2 was able in vivo to revert HAP toxicity in media with high molybdate, meanwhile in vitro, even with high molybdate (data not shown), crCyt b5-2 was unable to promote HAP reduction. These results are interesting because they suggest that alternative reductases from crCysJ and crCyt b5-R could be involved in the reduction of HAP using crCyt b5-2 and crARC.

The crARC system has a Zn-dependent activity. After determining that crCyt b5-R is the main reductase that with crCyt b5-1 and crARC can reduce HAP, the effects of different metals in the reaction mixture were also studied. The standard reduction of HAP was performed but in the presence of 1 mM concentrations of the different metals listed in Fig. 6A. The results were surprising: Zn caused 10-fold increases in activity in contrast to all other metals tested. The optimal Zn concentration was 1 mM, but even 10 μM increased the reaction rate 3 times; higher Zn concentrations were worse than the optimal but they still caused a positive effect (Fig. 6B).

The kinetic parameters of HAP reduction with Zn or without Zn were determined. The HAP reduction followed a Michaelis-Menten kinetics independent of the presence of Zn (Fig. 6C and D). The kinetic parameters for the reduction of HAP with and without Zn were as follows: with Zn, K_m of 278 μM and V_max of 1.60 μmol·min⁻¹·mg crARC⁻¹; without Zn, K_m of 59 μM and V_max of 0.11 μmol·min⁻¹·mg crARC⁻¹. Regarding the K_m values, the substrate specificity for HAP was higher without Zn than with Zn. However, the V_max was 14.4 times higher with Zn than without Zn, so it seems that Zn increases the crARC capacity to reduce HAP.

We measured the Zn content of crARC, hmARCs, and YcbX by ICP-OES, but we did not detect any Zn joined to these proteins (data not shown). This indicates that these recombinant proteins are expressed and purified without Zn, which might explain the need to add Zn to increase the crARC HAP reduction rate over 14 times.

The fully conserved cysteine is essential for crARC activity. Of all cysteines in ARC orthologous proteins, only one is fully conserved (1), that corresponding to cysteine 252 (C252) in crARC (Fig. 7). The 5 cysteines found in crARC were mutated to alanine. To find out whether the overall tertiary structure of the variants had changed in respect to the wild type, we performed a fluorescence spectroscopy study. As crARC has 10 tryptophan residues distributed along its sequence, if any mutation affected the folding of the protein, its fluorescence spectrum would be altered. We did not find any significant change in the fluorescence spectra in the mutants (data not shown). Therefore, it is unlikely that these cysteine-to-alanine changes significantly affect the folding of the proteins.

The HAP reduction activity was determined for each cysteine mutant with Zn and without Zn. None of them showed a HAP reduction rate that was different from that of the wild type, with the exceptions of the C252A mutant and the C249A C252A double mutant that totally abolished HAP reduction activity with and without Zn (Fig. 8A).
showing that the fully conserved cysteine in an ARC protein is essential for both Moco binding and catalysis.

**DISCUSSION**

The ARC (amidoxime reducing component) protein was discovered in 2006 as a new molybdenum cofactor (Moco)-containing enzyme involved in the reduction of N-hydroxylated compounds (NHC) (12) (Fig. 1). Proteins of this family are distributed throughout the three kingdoms of living organisms and occur either as stand-alone forms or fused to other domains (1). However, the study of this protein in the plant kingdom has not been addressed, and this is the first study of this protein in a plant-like organism, the green alga *Chlamydomonas reinhardtii*.

*Chlamydomonas reinhardtii* mutants defective in several Moco biosynthesis genes and sensitive to N6-hydroxylamino-purine (HAP) (Fig. 2) gave the first clue on the existence of a dependent NHC detoxification system. To investigate whether *Chlamydomonas* has a HAP reduction system related or not with the ARC proteins (Fig. 1D), we have cloned, purified, and studied the *Chlamydomonas reinhardtii* ARC protein, crARC, the six *Chlamydomonas* ferredoxins (Fds), five *Chlamydomonas* Cyt b5, and two *Chlamydomonas reinhardtii* flavin reductases (crCytb5-R and crCysJ), using the human ARCs (hmARCs) and YcbX proteins as controls for comparative experiments.

We have been able to demonstrate by heterologous expression in *E. coli* that *in vivo* the detoxification of HAP by crARC is dependent on crCyt b5-1, crCyt b5-2, and CysJ, but independent of Fd. This signifies that in *Chlamydomonas reinhardtii*, the HAP reduction system is more related to the three-component Cyt b5-dependent human system than to the two-component Fd-dependent bacterial system. The nature of this interaction indicates that CysJ has a broad spectrum of proteins with which it might interact and donate reducing equivalents; apart from its function in the sulfite reductase (10) and YcbX (19), it can even donate reducing equivalents to crCyt b5-1 and crCyt b5-2 proteins at least when they are heterologously expressed in *E. coli*. These facts raise the possibility of discovering other examples of redox carrier proteins functioning with multiple acceptor proteins.

Interestingly, crCysJ, which is homologous to CysJ, also promotes the reduction of HAP with crARC and crCyt b5-1 but in a minor way (Table 4). This explains why *in vivo* CysJ is needed by crARC plus crCyt b5-1 to detoxify HAP, because CysJ replaces the function of its homologous crCysJ.

In *vivo* crCyt b5-2 also reverses the toxicity of HAP if high molybdate is added to the growth medium (Fig. 3D), but this was not confirmed by the *in vitro* HAP reduction (Table 4). However, this result connects the HAP detoxification with the requirement of Moco cofactor for crARC activity. This means that there may be other reductases apart from crCyt b5-R and crCysJ that are able to interact with crCyt b5-2 and crARC in the reduction of HAP, or alternatively, the high molybdate
concentration can alter the structure of one of these proteins, causing a conformational change needed for interactions. Future experiments are required to verify these hypotheses. The results obtained might explain the meaning of a three-component system, so that some of their members can be replaced by other proteins depending on demands of the cell.

The subcellular localization of ARC proteins is not well defined. Mammalian ARC proteins have been localized in the outer (9) and inner (5) mitochondrial membranes but also in the peroxisomal membranes (14). We have studied crARC and its partners with different prediction programs to determine whether they have any peptide signals for subcellular compartmentalization. No predicted signals for organellar compartmentalization could be found in crARC. However, their two partners crCytb5-1 and crCytb5-R contain a hydrophobic membrane-anchoring domain in their C-terminal and N-terminal sequences, respectively. These data suggest that crARC probably exerts its actions in a subcellular compartment. However, additional experiments will be needed to solve its subcellular localization.

Genomes of almost all eukaryotes that use molybdenum have two ARC proteins, with both showing strong similarities at amino acid and nucleotide levels. As there are unsequenced regions in the *Chlamydomonas* genome (22), the possibility of a second crARC protein cannot be excluded. In *Arabidopsis thaliana*, there are two homologous ARC proteins, *Arabidopsis thaliana* ARC1 (atARC1) and atARC2 (Fig. 7) (20). The atARC1 protein has a clear signal for chloroplast export that is absent in the atARC2, so it seems that in *Arabidopsis*, the ARC proteins may exert its functions in different subcellular compartments. Interestingly, we have also found ARC ortholog proteins in cyanobacterial genomes (Fig. 7), and as in *Chlamydomonas*, they lack the ferredoxin domain and there is only one ARC sequence per genome.

We have found that crARC has a Zn-dependent activity that with crCyt b5-1 and crCyt b5-R efficiently reduces HAP. The kinetic parameters for the reduction of HAP by the crARC system showed that Zn increases the crARC capacity to reduce HAP though it also increases its $K_m$. However, the Michaelis-Menten equation fit much better the data points in the absence of Zn than in the presence of Zn. As it is a three-component system, a high number of protein interactions are required to complete the catalytic cycle, therefore a biphasic cooperative behavior cannot be discarded. The $K_m$ of crARC for HAP was similar to those reported for other NHC substrates with heterologously expressed hmARC proteins, but the $V_{max}$ of crARC with Zn was between 10 and 100 times higher (15, 34).

As Zn is not essential for crARC activity but drastically increases its $V_{max}$, we propose that a potential role of Zn could be related to increasing the capacity to eliminate the toxic NHC in the cell.

It is unknown which proteins of the crARC system requires Zn, although it could be related to increasing the proper binding between them to promote a correct electron transfer reaction.

The $V_{max}$ for benzamidoxime when the hmARC proteins were purified from human mitochondria was 12.2 $\mu$mol · min$^{-1}$ · mg protein$^{-1}$ (12). This $V_{max}$ is more than 350 and 40 times higher than those obtained for heterologously expressed hmARC1 and hmARC2, respectively, but only 7 times greater than

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**FIG. 7.** Multiple-sequence alignment of ARC proteins. The sequences and accession numbers (shown in parentheses) (GenPept accession numbers begin with XP and NP; others are deduced from GenBank sequences) are as follows: crARC, *Chlamydomonas reinhardtii* ARC (XP_001694549); atARC1, *Arabidopsis thaliana* ARC1 (NP_174376); atARC2, *Arabidopsis thaliana* ARC2 (NP_199285); hmARC1, human ARC1 (Homo sapiens) (NP_073583); hmARC2, human ARC2 (NP_060368); YcbX, *Escherichia coli* YcbX (NP_415467); ccARC, *Caulobacter crescentus* ARC (AAK22857); scARC, *Streptomyces coelicolor* A3 ARC (CAC04053); and gvARC, *Gloeobacter violaceus* PCC 7421 ARC (NP_926027). The positions of the mutated cysteine to alanine in crARC are indicated by the black arrowheads. For other details, see the legend to Fig. 4.
crARC with Zn ($V_{\text{max}}$ of 1.6 μmol · min$^{-1}$ · mg crARC$^{-1}$). These facts suggest that hmARCs purified from living mitochondria could have retained Zn bound. Therefore, the Zn-dependent activity found with crARC could be a general feature for the other ARC proteins, though this should be confirmed by future experiments.

All ARC proteins contain a fully conserved cysteine that could be considered part of their signature (1) (Fig. 7). We have generated amino acid substitutions of each crARC cysteine to alanine. These substitutions had no effect on the Zn-dependent activity apart from the absolutely conserved cysteine 252 (Fig. 8A) that strongly affected Moco binding (Fig. 8B). Interestingly, to fully abolish Moco binding to crARC, the neighboring C249 also has to be mutated. This means that the C249 in the absence of a functional C252 is able to bind Moco to some extent, but this binding is not appropriate for productive enzyme catalysis. This is the first demonstration in an ARC protein that the fully conserved cysteine is involved in Moco chelation to the protein.

Until now, the Mo coordination spheres of the ARC proteins were unknown (Fig. 1A). In Moco proteins, Mo is chelated to two thiol groups of molybdopterin (MPT) and also with two oxo groups. The fifth Mo ligand is the sulfur of a cysteine in the sulfite oxidase (SO) family or an inorganic sulfur in the xanthine oxidase (XO) family (28), and the exchange of this conserved cysteine to serine or to alanine completely abolishes the enzyme function, as in the case of chicken sulfite oxidase (25). We propose that the fully conserved ARC cysteine is indeed the putative fifth Mo ligand of the ARC proteins (Fig. 9) and therefore, that the ARC proteins belong to the SO family of Moco proteins. Recently, Wahl et al. characterized the hmARCs and found that they do not belong to the XO family, since cyanide treatment neither released sulfur nor significantly affected the activities of hmARCs (34). In addition, by mutating each cysteine to serine, no difference in the activity of the hmARCs was found. It is possible that changing cysteine to serine, another polar amino acid and with a similar electronegative atom (S versus O), could have retained Mo chelation capacity and activity.

Concerning the physiological functions of ARC proteins, it appears likely that one function could be to prevent the accumulation of mutagenic base analogues in the cell as HAP (16) and $N$-hydroxycytosine (34). The hmARC system is able to reduce $N$-hydroxysulfonamides, such as $N$-hydroxy-valdecoxib, which have considerable potential to treat a variety of disorders (13). In addition, hmARC proteins have recently been suggested to act as regulators for the $L$-arginine-dependent biosynthesis of nitric oxide (NO) by catalyzing the controlled elimination of the NO precursor $N^\omega$-hydroxy-$L$-arginine (NOHA) (15). Whether ARC proteins are involved in physiological NOHA reduction and/or are capable of physiologically affecting NO levels has to be investigated in future experiments.

In conclusion, we have characterized for the first time the ARC system in a photosynthetic organism showing that in Chlamydomonas this system is more related to the human

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**FIG. 8.** HAP reduction and Moco contents of crARC and their variants. (A) HAP reduction by crARC and their mutant variants with crCyt b5-I and crCyt b5-R under standard conditions with Zn (+ Zn) or without Zn (− Zn). (B) Mo and MPT contents of crARC and their variants measured by ICP-OES and FormA, respectively. Wt, wild-type crARC; C249/252A, C249A C252A double mutant.

**FIG. 9.** Scheme of the Chlamydomonas ARC system. The figure shows the structure of the Moco molecule. The Mo atom is shown in bold type, illustrating that the fifth Mo ligand in crARC is cysteine 252. The protein domains in the crARC system are shown in boxes (for more details, see Fig. 1 and Discussion).
system than to the bacterial system. The crARC partners are crCyt b5-I and crCyt b5-R. The enzyme has a Zn-dependent activity that does increase its V_{max} more than 10 times. We propose that the fully conserved cysteine 252 of crARC is involved in Moco chelation (Fig. 9). Therefore, the ARC protein would belong to the SO family of Moco enzymes.

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