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ORIGINAL PAPER

Enhanced alternative oxidase and antioxidant enzymes under Cd^{2+} stress in *Euglena*

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Abstract To identify some of the mechanisms involved in the high resistance to Cd^{2+} in the protist *Euglena gracilis*, we studied the effect of Cd^{2+} exposure on its energy and oxidative stress metabolism as well as on essential heavy metals homeostasis. In *E. gracilis* heterotrophic cells, as in other organisms, $CdCl_2$ (50 μM) induced diminution in cell growth, severe oxidative stress accompanied by increased antioxidant enzyme activity and strong perturbation of the heavy metal homeostasis. However, Cd^{2+} exposure did not substantially modify the cellular respiratory rate or ATP intracellular level, although the activities of respiratory complexes III and IV were strongly decreased. In contrast, an enhanced capacity of the alternative oxidase (AOX) in both intact cells and isolated mitochondria was determined under Cd^{2+} stress; in fact, AOX activity accounted for 69-91% of total respiration. Western blotting also revealed an increased AOX content in mitochondria from Cd^{2+}-exposed cells. Moreover, AOX was more resistant to Cd^{2+} inhibition than cytochrome c oxidase in mitochondria from control and Cd^{2+}-exposed cells. Therefore, an enhanced AOX seems to be a relevant component of the resistance mechanism developed by *E. gracilis* against Cd^{2+}-stress, in addition to the usual increased antioxidant enzyme activity, that enabled cells to maintain a relatively unaltered the energy status.

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Introduction

Heavy metals can induce oxidative stress through different mechanisms, depending on their physical and chemical properties (Schützendübel and Polle 2002; Valko et al. 2005), by directly inducing reactive oxygen species (ROS) production (Fenton reaction), by blocking functional groups of proteins and glutathione, and by displacing essential metals, like zinc or selenium from proteins (Patrick 2003), and zinc from zinc-finger motifs of transcription factors (Hartwig 2001). Thus, the growth of some organisms in heavy-metal polluted environments depends on their ability to synthesize different heavy-metal chelating molecules and to activate efficient antioxidant mechanisms.

Cadmium enters the environment from industrial waste and is one of the most toxic heavy metals for living organisms. The main route of toxicity for this metal is through the depletion of glutathione and the binding to other thiol groups in proteins (Valko et al. 2005); it may also induce the production of lipid peroxides (Patrick 2003; Valko et al. 2005). In mammals, Cd^{2+} is taken up by mitochondria, thus inhibiting cellular respiration and oxidative phosphorylation through ROS-mediated mechanisms (Chavez et al. 1985; Patrick 2003). Other documented Cd^{2+}-sensitive cellular processes are photosynthesis (Pietrini et al. 2003; Faller et al. 2005), membrane lipid composition (Jemal et al. 2000), and the uptake of external nutrients (Sandalio et al. 2001).

Euglena gracilis is a free-living protist that tolerates high concentrations of different heavy-metals (Navarro et al. 1997; Devars et al. 2000; Mendoza-Cozatl et al. 2002).

Increased ROS production and mitochondrial DNA breaks, induced by Cd^{2+} exposure have been described in dark-grown and photosynthetic strains of *E. gracilis* (Watanabe and Suzuki 2002; Watanabe et al. 2003), suggesting that mitochondria are the primary target of Cd^{2+} in this microorganism; unfortunately, mitochondrial function was not evaluated. Interestingly, most of the accumulated Cd^{2+} is localized in the chloroplasts of *E. gracilis* photosynthetic cells (Mendoza-Cozatl et al. 2002, 2006b; Mendoza-Cozatl and Moreno-Sanchez 2005) and in mitochondria of dark-grown cells (Avilés et al. 2003), implying a direct toxic effect on these organelles and their energy metabolism. Mitochondrial Ca^{2+} uptake and release are also directly affected by Cd^{2+} in *E. gracilis* (Uribe et al. 1994).

In most organisms, catalase (CAT) and superoxide dismutase (SOD) participate in the detoxification of H_2O_2 and superoxide, thus avoiding the formation of $OH\cdot$ radicals. To this regard, increased lipid peroxidation and marked changes in antioxidant enzyme activities have been documented in Cd^{2+}-exposed organisms (Sandalio et al. 2001; Dixit et al. 2001; Vitoria et al. 2001; Guelfi et al. 2003; Zhang et al. 2005; Israr et al. 2006). In some studies, Cd^{2+} induced an increase in CAT, SOD or glutathione reductase (GR) whereas, in others, these activities were decreased by Cd^{2+} exposure. For instance, diminution in the glutathione content and of the activity and expression of SOD and glutathione peroxidase (GPx) has been described for soybean plants exposed to Cd^{2+} (Noriega et al. 2007). On the other hand, in *Saccharomyces cerevisiae* Cd^{2+} induces enhancement in the SOD and GPx activities, and accumulation of malondialdehyde (MDA; Liu et al. 2005).

In marked contrast, CAT (Kitaoka et al. 1989), as well as mitochondrial and cytosolic SOD, is absent in *E. gracilis*. These observations suggest a fragile antioxidant system or the presence of alternative mechanisms by which *E. gracilis* maintains its redox balance. To this regard, a chloroplastic SOD (Kanematsu and Asada 1979), two cytosolic GPx (Overbaugh 1985), and a complete ascorbate-glutathione cycle (Shigeoka et al. 1980a,b; 1987) have been characterized as part of the antioxidant mechanism present in *E. gracilis*. Furthermore, a trypanothione reductase has also been described that might be involved in the antioxidant response (Montrichard et al. 1999). Information about other enzymes involved in trypanothione metabolism in this protist is not available as yet.

E. gracilis has a complex respiratory chain constituted by different alternative and classical components, in which the alternative oxidase (AOX) seems essential when cells are subjected to oxidative stress, induced either by cold or respiratory inhibitors (Castro-Guerrero et al. 2004). Cd^{2+} exposure also increases ROS production in *E. gracilis* (Watanabe and Suzuki 2002; Watanabe et al. 2003); hence, AOX might also be involved in the cellular response. Cd^{2+} could also have a potent, direct effect on enzymes, in addition to inducing oxidative stress. To identify and understand better the defense mechanisms of *E. gracilis* under Cd^{2+} stress, a thorough analysis of the oxidative stress induced by Cd^{2+} was undertaken in the present work, including evaluation of antioxidant enzyme and metabolite contents, essential heavy metals homeostasis, and mitochondrial enzyme activities.

Materials and methods

Chemicals

L-Lactate, N, N, N′, *N*′-tetramethyl-*p*-phenylendiamine (TMPD), 1, 6-dichlorophenol indophenol (DCPIP), 5, 5′-dithio-bis(2-nitrobenzoic acid) (DTNB), NADH, glutathione reductase, GSH (reduced glutathione), ascorbic acid, citrate and *tert*-butyl hydroperoxide (TBHP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium cyanide was from J.T. Baker (Phillipsburg, NJ, USA), whereas *n*-propyl gallate, GSSG (oxidized glutathione) and horse heart cytochrome *c* were from ICN Biomedicals Inc. (Costa Mesa, CA, USA). NADPH, hexokinase, glucose-6-phosphate dehydrogenase, glycerol-3-phosphate dehydrogenase and lactate dehydrogenase were from Roche Co. (Basel, Switzerland). Purified recombinant pyruvate phosphate dikinase from *Entamoeba histolytica* was kindly provided by Dr. Emma Saavedra (Instituto Nacional de Cardiología de México).

Culture conditions

Cells of *E. gracilis* Z were grown in the dark in the Hutner's acidic organotrophic medium with glutamate + malate as carbon source (Greenblatt and Schiff 1959) at 25±2 °C under orbital shaking (125 rpm). $CdCl_2$ was added to the culture medium before the cellular inoculum. Mitochondria were isolated as previously described (Castro-Guerrero et al. 2004) from cells reaching the stationary phase (96 h of culture).

Determination of metabolites

The intracellular contents of glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-bisphosphate (F1,6BP), ATP, and trioses phosphate (glyceraldehyde-3-phosphate + dihydroacetonephosphate) were estimated by standard enzymatic assays (Bergmeyer 1974) in cell extracts from suspensions of 1×10^8 cells mixed with 3% (*v*/*v*) perchloric acid and further centrifuged and neutralized with 3 M KOH/ 0.1 M Tris.

Pyrophosphate was determined at 37 °C in a buffer of pH 6 containing 50 mM imidazole plus 10 mM each of

acetate, MES and Tris, 0.15 mM NADH, 5 mM $MgCl_2$, 0.5 mM AMP, 1 mM PEP, 1.5 U/ml LDH, and 0.15 U/ml *E. histolytica* pyruvate phosphate dikinase.

Metals were determined in acid-digested fractions (from 3×10^7 cells harvested after 4 days of culture) by atomic absorption spectrophotometry in a SpectrAA 640 (Varian Australia Pty Ltd.) spectrophotometer as described previously (Navarro et al. 1997; Mendoza-Cozatl et al. 2002).

MDA was determined by measuring the concentration of thiobarbituric acid-reacting substances as described previously (Buege and Aust 1978).

The content of reduced glutathione (GSH) was determined by reverse phase-HPLC (RP-HPLC) coupled to post-column derivatization with DTNB (Ellman's reagent), as described previously (Mendoza-Cozatl et al. 2002), in fresh acid extractions from 3×10^7 cells. Total glutathione (GSH+GSSG) was determined by reducing the samples with 1 mM DTT before acid extraction in the presence of 0.05% (*v*/*v*) Triton X-100.

Enzyme activities

Cellular and mitochondrial respiration in stressed and non-stressed cells was determined at 25 °C by means of a Clark-type oxygen electrode, in air-saturated KME (120 mM KCl, 20 mM MOPS, 1 mM EGTA, pH 7.25) buffer.

Respiration, pyridine nucleotide-independent L-lactate dehydrogenase (L-iLDH), and cytochrome *c* reductase activities in intact fresh mitochondria were determined at 25 °C, as previously described (Castro-Guerrero et al. 2004; 2005). To estimate AOX capacity in respiration experiments, 1 mM cyanide and 3 mM n-propyl gallate (nPG) were added. TMPD (cytochrome c) oxidase activity was evaluated by determining the rate of O_2 uptake after adding 5 mM ascorbate plus 2.5 mM TMPD, which was fully inhibited by 10 mM azide or 1 mM cyanide (Castro-Guerrero et al. 2004). For the Cd^{2+}-sensitivity assays, freshly prepared mitochondria were incubated in the absence of EGTA and with 120 mM KCl, 20 mM MOPS, pH 7.25. Because mitochondria suspended in this last buffer deteriorated rapidly, the assays were carried out for short times. The free Cd^{2+} concentration was calculated by using the software CHELATOR (Schoenmakers et al. 1992), and considering the EGTA added with the mitochondrial sample. Oxygen solubility was determined to be 420 ng atoms/ml (210 μM) at 25 °C and 2,240 m altitude.

Aconitase activity was measured as the formation of *cis*-aconitate (extinction coefficient ε=3.6 mM^{-1} cm^{-1}) from citrate at 240 nm (Hausladen and Fridovich 1994). The reaction mixture contained 10 mM citrate and 0.2 mM $NADP^+$. The reaction was started by adding the mitochondrial protein in the range of 50–150 μg under which the aconitase activity was linear. Control reactions in the absence of citrate or protein indicated no unspecific activity. Mn^{2+} was not added to the reaction mixture since it showed an inhibitory effect in our model (0.6 mM $MnCl_2$ inhibited 50% aconitase activity in *E. gracilis* mitochondrial preparations).

GPx activity was determined in cell extracts by measuring the oxidation of 100 μM NADPH in a reaction coupled to the reduction of 1 mM *ter*-butyl hydroperoxide (TBHP) and in the presence of commercial GR (0.4 U), with 5 mM GSH as electron donor (Overbaugh and Fall 1985). The rate was linear for a protein concentration ranging from 25 to 150 μg. Use of H_2O_2 as substrate instead of TBHP also revealed GPx activity, although the K_m value was higher; the absence of either substrate did not yield activity.

APx activity was determined according to the method of Nakano and Asada (1981) in cell extracts prepared in the presence of 2 mM ascorbate. The reaction was followed by the oxidation of 0.5 mM ascorbate at 290 nm (ε=2.8 mM^{-1} cm^{-1}) starting the reaction with 2 mM H_2O_2 (Shigeoka et al. 1980a,b). The range of protein concentration for rate linearity was from 25 to 100 μg. No activity was registered in the absence of ascorbate. A low activity (<5% of total) was obtained before the addition of H_2O_2; the APx activity was corrected for this basal activity.

GR activity was determined according to Shigeoka et al. (1987) in cell extracts by measuring the oxidation of 0.2 mM NADPH at 340 nm in the presence of 1 mM GSSG at 25 °C. NADPH consumption was not detected in reactions without added GSSG. Rate linearity was achieved from 25 to 150 μg protein.

Triose-phosphate isomerase (TPI) in cell extracts (0.1–0.7 μg protein) was determined through the reverse reaction in 20 mM Tris pH 8.0, 0.15 mM NADH, and 1.7–3.4 U glycerol-3-phosphate dehydrogenase. The reaction was started by adding 1.3 mM glyceraldehyde-3-phosphate.

Western blotting

Immunodetection was achieved by using an antibody raised against AOX from *Chlamydomonas reinhardtii*. For blotting, the mitochondrial extracts (50 μg protein) were first subjected to SDS-PAGE and then the proteins were electrophoretically transferred to a nitrocellulose membrane. The membrane was incubated overnight with the anti-AOX antibody at 4 °C. Then, the membrane was incubated with a second antibody conjugated with horseradish peroxidase for 1 h at room temperature, and the protein was detected by using the ECL detection system kit (Amersham Biosciences, Piscataway, NJ, USA). As control, mitochondrial adenine nucleotide translocator (ANT) was detected in the same membrane with a polyclonal human antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA,

USA). The anti-ANT antibody was used after removing the antibodies of the initial reaction from the membrane using a stripping buffer, composed of 2% SDS, 62.5 mM Tris, and 100 mM β-mercaptoethanol at pH 6.7, and further incubating at 50 °C for 20 min.

Results

Inhibitory concentration of $CdCl_2$ to attain 50% decrease in cellular density, after 4 days culture (IC_{50}), was 30 ± 3 μM (n=3). In 50 μM Cd^{2+}-exposed cells, the rate of cellular respiration was, surprisingly, identical to that attained by control cells. However, the rate of cyanide-resistant respiration accounted for 18 and 69% of the total respiration for control and Cd^{2+}-stressed cells, respectively (Table 1), suggesting an enhanced activity of AOX under Cd^{2+} stress.

Furthermore, the intracellular concentrations of the high-energy related metabolites, ATP, PPi, G6P and F6P, and AMP, were not modified by Cd^{2+} stress (Table 1). In fact, this strong homeostatic control consisted in an *increase* in the metabolites content, as the intracellular water volume in Cd^{2+} stressed *E. gracilis* cells is approximately 3-fold higher than in control cells (Avilés et al. 2003). In turn, the concentrations of ADP, F1,6BP and trioses-phosphate diminished in Cd^{2+}-exposed cells.

Table 1 Effect of Cd^{2+} on growth, respiration, antioxidant enzymes, and high-energy related phosphate metabolites in *E. gracilis* cells

	Z	ZCd_{50}
Cell density at 96 h (×10^6 cell/ml)	7.3±0.2	1.3±0.1*
Cellular respiration	110±7	113±6[a]
+1 mM cyanide	20±8	78±3*
Ascorbate peroxidase (APx)	44±7	86.5±17*
Glutathione peroxidase (GPx)	79±22	122.5±25
Glutathione reductase (GR)	20±7	46±9*
Triose phosphate isomerase	31±3	30±10[a]
ATP	0.96±0.08	0.69±0.27[a]
PPi	0.76±0.12	1.09±0.21[a]
ADP	2.04±0.25 (5)	0.76±0.06 (5)*
AMP	0.99±0.37	1.09±0.2[a]
G6P	4.8±4	3.9±1[a]
F6P	1.46±0.96	1.05±0.36[a]
F1,6BP	0.33±0.16 (5)	0.17±0.03 (9)*
Trioses phosphate	0.83±0.1	0.28±0.07 (7)*

Values are mean±SD of three independent preparations, except where indicated otherwise. Intracellular water volume used for calculations was 2.4 and 6.3 μl (10^7 cell)$^{-1}$ for control (Z) and Cd^{2+} -stressed cells (ZCd_{50}), respectively (Avilés et al. 2003). Units for cellular respiration are ng atoms oxygen (min×10^7 cell)$^{-1}$; for APx, GPx, and GR are nmol (min×mg protein)$^{-1}$, for TPI are μmol (min×mg protein)$^{-1}$; metabolite concentrations are mM

*P<0.005 versus Z (Student t test for non-paired samples)

[a] Not significantly different versus Z

Essential heavy metal homeostasis

Cadmium induces alterations in the composition of trace elements in plants (Sandalio et al. 2001). Perturbation of the Cu and Fe homeostasis can promote oxidative stress through the activation of the Fenton reaction. In order to evaluate a possible impairment in heavy metal homeostasis in *E. gracilis* induced by Cd^{2+}, the intracellular content of several trace nutrient heavy metals was determined. It should be noted that in *Euglena* cultures, the medium is constituted by a variety of heavy metals, such as Zn, Mn, Fe and Cu, which are present at relatively high concentrations (Table 2). Due to the very high affinity of functional groups (carboxylate, sulfhydrylic, imidazole) in biomolecules for heavy metals (Sillen and Martell 1964), their internal concentrations are difficult to determine, although they are expected to be in the micromolar and nanomolar range. Therefore, despite knowing the intracellular water volumes, data in Table 2 were only expressed in terms of content. Nickel is not added to the culture medium but it is a contaminant from other components; although this contaminant was significant, the cell content of this metal remained constant in both types of cells (Table 2).

Except for Zn, all other heavy metals determined (Mn, Fe, Co, Cu) increased when cells were exposed to Cd^{2+}, being Cu and Fe, inorganic catalysts for ROS production, the most affected (Table 2). Likewise, the mitochondrial content of Cu and Fe also increased, respectively, from 0.5±0.1 (n=3) and 2.8±2 (n=7) nmol (mg protein)$^{-1}$ in control mitochondria to 3±0.2 (n=7) and 10.5±2 (n=9) nmol (mg protein)$^{-1}$ in mitochondria from Cd^{2+}-cultured cells.

Variation in the intracellular Zn content correlated with a lowering in the Zn^{2+} concentration in the culture medium from 468±38 μM (concentration at the beginning of the growth curve; n=3) to 44.6±17 μM (n=4) after 96 h in the medium with control cells, and to 178±23 μM (n=5) in the medium with Cd^{2+}-exposed cells. In mitochondria isolated from control and Cd^{2+}-exposed cells, the Zn content was 14±7 (n=6) and 9.3±5 nmol (mg protein)$^{-1}$ (n=8), respectively. The lower Zn content in Cd^{2+}-exposed cells (Table 2) might be related to the well-described inhibitory, competitive effect of Zn on Cd^{2+} transport in photosynthetic *E. gracilis* (Mendoza-Cozatl and Moreno-Sanchez 2005).

The cellular content of Mg^{2+} and Ca^{2+} diminished, and that of K^+ increased under Cd^{2+} stress (Table 2). In turn, the contents of Mg^{2+}, Ca^{2+} and K^+ in control mitochondria were 123±43 (n=8), 19±14 (n=6) and 21.5±12 (n=6) nmol (mg protein)$^{-1}$, respectively, which diminished by 85,

Table 2 Trace elements content in culture medium and in *E. gracilis* cells

	Cd	Mn	Fe	Co	Ni	Cu	Zn	K	Mg	Ca
Culture medium (μM)	52±4	464±9	36±3.6	12.8±5	6.4±1.6	9.6±1.3	468±38	320±20	770±65	248±39
Z cells	0 (29)	46±3 (10)	15±3 (13)	4.6±0.6 (10)	1.2±0.3 (6)	0.6±0.3 (10)	200±9 (7)	156±27 (7)	675±190 (7)	18.6±12 (10)
ZCd_{50} cells	86.3±25* (27)	70±3* (10)	48±9* (12)	9.4±1.5* (13)	1.6±0.6[a] (6)	4.1±0.6* (13)	110±3* (7)	242±60[a] (27)	270±70* (11)	6±3[a] (13)

Values in nanomoles $(10^7 \text{ cells})^{-1}$ are mean±SD of the number of experiments reported in parentheses. Elements were determined in control cells and cells exposed to 50 μM $CdCl_2$. Cells were washed with an EGTA buffer

*$P<0.005$ versus *Z* (Student *t* test for non-paired samples)

[a] Not significantly different versus *Z*

64 and 66% in mitochondria from Cd^{2+}-cultured cells. The Cd^{2+} content in mitochondria isolated from Cd^{2+}-cultured cells was 32±14 nmol $(\text{mg protein})^{-1}$ (n=12). There was a consistent variation pattern for Zn, Mg, Ca, Cu, and Fe between cells and mitochondria (cf. Table 2). However, K^+ increased in cells but it decreased in mitochondria isolated from Cd^{2+}-treated cells. Such an unusual behavior might be related to an enhanced K^+ efflux in these mitochondria, but this has to be studied further.

Oxidative stress metabolites and enzymes

The generation of MDA, a lipoperoxidation product, was increased by sixfold under 50 μM Cd^{2+} stress (0.5±0.1, n=7, in control cells versus 3±0.4 nmol mg protein^{-1}, n=8, in Cd^{2+}-exposed cells). In cells exposed to 25 μM Cd^{2+}, the MDA concentration was 2.2±0.9 (n=5) nmol $(\text{mg protein})^{-1}$, whereas in cells incubated with 10 mM H_2O_2 for 40 min, the MDA concentration was 5.9 and 6.02 nmol $(\text{mg protein})^{-1}$ for control and stressed cells, respectively; this high concentration of H_2O_2 induces cell death (Castro-Guerrero et al. 2004).

Cd^{2+} also induced a moderate diminution in the reduced glutathione level (88±2%, n=3, in control cells versus 69±9%; n=3; in Cd^{2+}-exposed cells), which was accompanied by an increase in the glutathione pool (GSH + GSSG) of approximately three times (6.4±0.7, n=3, in control cells versus 18±0.6 nmol/10^7 cells, n=3; in Cd^{2+}-exposed cells). Thus, Cd^{2+} exposure was associated with a more oxidized glutathione redox state. An increased ubiquinone pool under Cd^{2+} stress was also found (from 0.4 in control mitochondria to 0.8 nmol $(\text{mg protein})^{-1}$ (n=2) in mitochondria isolated from Cd^{2+}-exposed cells); cold and cyanide stress also induce a similar change in the ubiquinone pool (Castro-Guerrero et al. 2004). In turn, cysteine content was similar in control and Cd^{2+}-exposed cells (0.33±0.04, n=3, and 0.43±0.08 nmol/10^7 cells, n=3, respectively), in agreement with previous work (Castro-Guerrero et al. 2004).

Activity of catalase was not detected by either polarography or reaction with hydrogen peroxide in a native gel by using $K_3Fe(CN)_6$ and $FeCl_2$ reaction as contrast stain (data not shown). However, there was a significant increase in the activities of APx, GPx, and GR (48, 35, and 56%, respectively) in Cd^{2+}-stressed cells (Table 1). As internal control, the activity of the cytosolic glycolytic enzyme triose phosphate isomerase (TPI) remained constant in both cell types.

Mitochondrial metabolism

Little is known about the oxidative stress-sensitive aconitase *in E. gracilis* (Kitaoka et al. 1989). A K_m value of 0.8 mM for citrate was determined for both control and Cd^{2+}-stressed mitochondria. Thereafter, to evaluate the content of *active* aconitase, the activity was assayed under maximal rate (V_m) conditions: saturation by substrate (10 mM citrate) and in the absence of products. As expected, in mitochondria isolated from Cd^{2+}-exposed cells, aconitase activity was severely depressed (Table 3).

The activities of respiratory complexes III and IV also decreased, by 50–60%, under Cd^{2+} exposure. This result correlated well with a diminution from 137.6±12 to 52.5±8 in *a*-type cytochrome and from 74.8±5 to 28.7±8 pmol $(\text{mg protein})^{-1}$ in *b*-type cytochrome in control and Cd^{2+}-exposed mitochondria. In contrast, Cd^{2+} stress had no effect on L-iLDH activity (Table 3), whereas SDH and D-iLDH activities decreased by 50 and 40%, respectively.

In good agreement with the observation in intact cells (Table 1), AOX capacity was 4.5 times higher in mitochondria from Cd^{2+}-exposed cells, accounting for 91±3% of total respiration (Table 3). Furthermore, a correlation between the concentration of $CdCl_2$ in the growth medium and the mitochondrial AOX capacity (Fig. 1a) and content (Fig. 1b) was observed.

In addition to inducing oxidative stress, Cd^{2+} may also affect the respiratory chain (Wang et al. 2004). Hence, the

Table 3 Effect of Cd^{2+} on mitochondrial enzyme activities

Enzyme	*Z*	ZCd50
Aconitase	1,066±270 (6)	326±118 (7)*
L-iLDH	274±71 (5)	237±15 (3)[a]
Complex III	95±11 (4)	41±7 (3)*
Complex IV	404±69 (8)	160±62 (4)*
AOX	35±6 (8)	156±37 (8)*

Values are mean±SD of the number of experiments reported in parentheses. Enzyme activities were performed at 25 °C. Activity of aconitase is given as nmol (min mg)$^{-1}$, L-iLDH activity units are nmol DCPIP (min mg)$^{-1}$, complex III activity units are nmol cyt c red (min mg)$^{-1}$ and complex IV and AOX activities units are ng atoms oxygen (min mg)$^{-1}$. Total rates for mitochondrial respiration with 10 mM L-lactate as substrate were: 170±50 (n=8) and 102±23 [n=11; P<0.01 versus *Z* (Student *t* test for non-paired samples)] ng atoms oxygen (min mg protein)$^{-1}$ for control and Cd^{2+}-stressed cells, respectively. Respiratory control values (rate of ADP-stimulated respiration/rate of basal respiration) with L-lactate as substrate for mitochondria isolated from untreated cells were 1.6–2.1; while mitochondria isolated from Cd^{2+}-treated cells seemed to be more prone to suffer mechanical damage during the isolation process, as they were unable to respond to the ADP addition (n=5). Furthermore, the electrical transmembrane potential, as determined by the change in the 0.5 μM rhodamine 123 fluorescent signal, was negligible in mitochondria (0.5 mg protein/2 ml) from Cd^{2+}-treated cells (data not shown). AOX capacity was evaluated in the presence of 10 mM L-lactate and 1 mM cyanide.

*P<0.005 versus *Z*

[a] Not significantly different

direct effect of Cd^{2+} on respiratory enzymes was also evaluated (Fig. 2; Tables 3 and 4). Both in mitochondria isolated from control and Cd^{2+}-treated cells, the most sensitive enzyme to Cd^{2+} was the TMPD (cytochrome c) oxidase, whereas CN-sensitive L-lactate respiration (L-iLDH + cytochrome complex b-c_1 + cytochrome *c* oxidase) showed an intermediate sensitivity. In contrast, the AOX activity was rather resistant to Cd^{2+} (Fig. 2; Tables 3 and 4).

Discussion

E. gracilis is a Cd^{2+}-resistant organism under a variety of conditions (Devars et al. 2000; Mendoza-Cozatl et al. 2002; Avilés et al. 2003). The rate of cellular respiration in Cd^{2+}-cultured cells was similar to that of control cells but showed a lower sensitivity to cyanide (cf. Table 1). This result suggests an increased participation of AOX activity in total respiration. However, electron flux through AOX is not associated to ATP synthesis; as a consequence, the pool of adenine nucleotides decreased, being ADP the most affected (63%) whereas ATP slightly decreased (28%). Such a perturbation in the pool of adenine nucleotides has been profusely documented for a variety of conditions and organisms (Woods and Krebs 1973; Lagunas and Gancedo 1983; Geigenberger et al. 2000; Rolletschek et al. 2004).

The antioxidant defense is certainly one of the cellular processes involved in dealing with heavy-metal induced damage (Sandalio et al. 2001; Vido et al. 2001; Malecka et al. 2001; Guelfi et al. 2003; Casiot et al. 2004). However, the lack of CAT and SOD in *E. gracilis*, two of the most common and relevant defense enzymes, seems a risk factor for this protist when subjected to oxidative stress. Nevertheless, *Euglena* shows high resistance to different stressful conditions such as drastic variations in temperature, pH, and O_2 concentration that usually lead to severe oxidative stress (Castro-Guerrero et al. 2004; 2005; Srivastava et al. 2004). This behavior in *Euglena* suggests the presence of mechanisms that compensate the deficiency of some antioxidant enzymes to contend with oxidative stress.

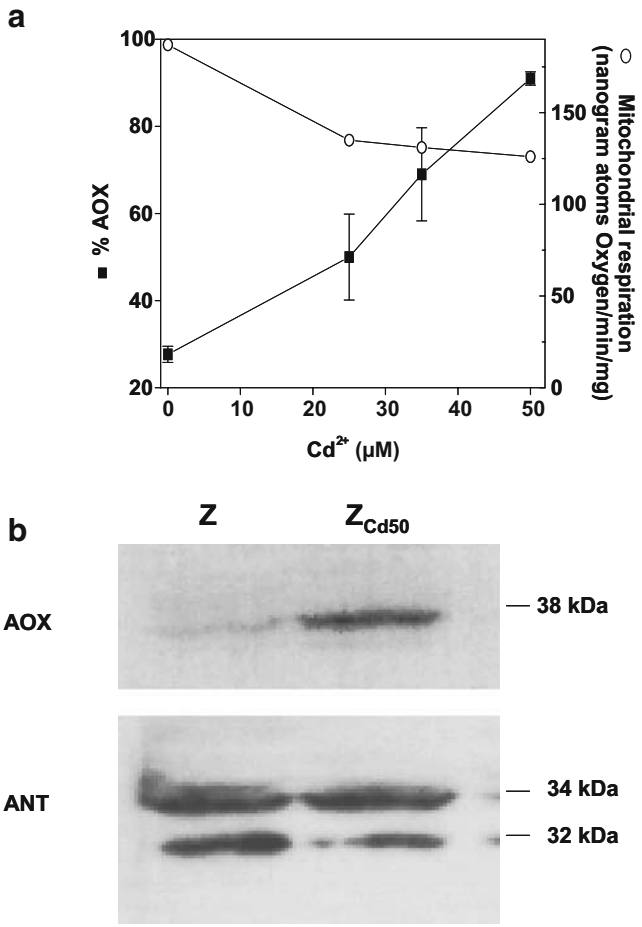

Fig. 1 Effect of Cd^{2+} exposure on mitochondrial respiration and AOX activity and content. **a** Changes in mitochondrial respiratory activity and AOX capacity after $CdCl_2$ exposure. The maximal respiration rate (100%) was obtained with 10 mM L-lactate with no inhibitors added. **b** Western blot of *E. gracilis* mitochondrial preparations (50 μg protein) from control cells and 50 μM $CdCl_2$-exposed cells. Dilutions of antibodies were 1:9,000 and 1:500 for AOX and ANT, respectively

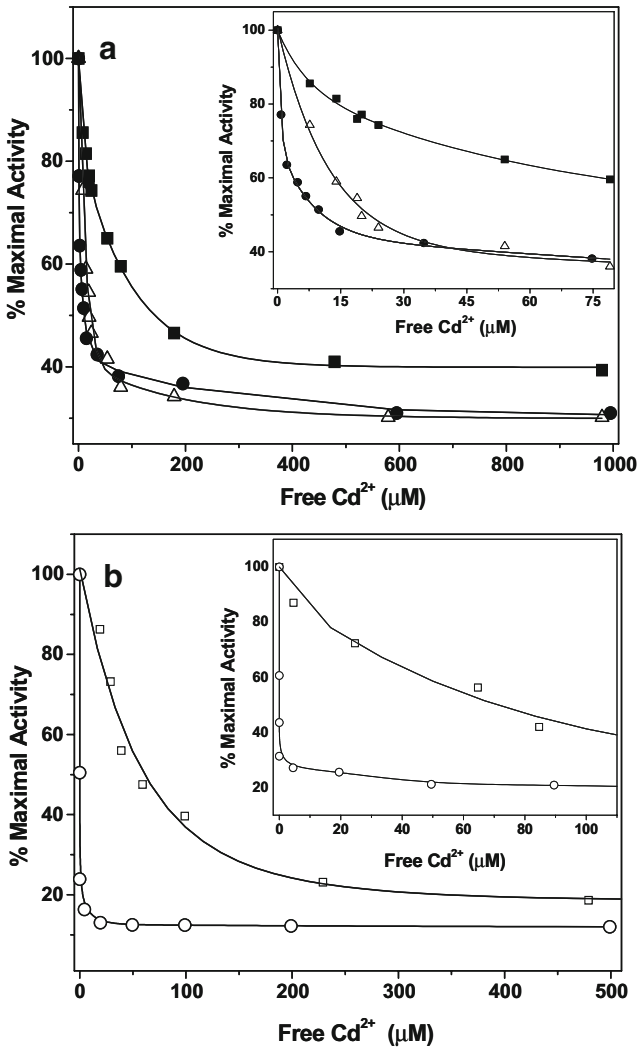

Fig. 2 Inhibition by Cd^{2+} of respiratory enzymes. **a** Mitochondria isolated from control cells. *Filled circle*, TMPD oxidase activity; *open triangle*, L-lactate oxidase; *filled square*, AOX activity. **b** Mitochondria isolated from *Euglena* cells cultivated in the presence of 50 μM Cd^{2+}. *Open circle*, TMPD oxidase activity; *Open square*, AOX activity. Insets show a zoom of the region below 100 μM Cd^{2+}. Enzyme activities and free Cd^{2+} concentrations were determined as indicated under "Materials and methods". The protein concentration used in the activity assays was 1.7–2.5 mg for L-lactate oxidase and AOX (for mitochondria from control cells) and 0.5–0.8 mg for AOX assays (for mitochondria from Cd^{2+}-treated cells), while 0.4–0.8 mg of protein was used for TMPD oxidase assays for both types of mitochondria. Results are representative of four different mitochondrial preparations. Maximal activities and IC_{50} values are shown in Tables 3 and 4

A full assessment of the oxidative stress induced by Cd^{2+} in *E. gracilis* allows for a better understanding of the multitude of effects on enzyme activity, metabolic pathways, and biological functions. For instance, a significant increase in the generation of ROS is usually taken as indicator of oxidative stress, without an appropriate evaluation of the pertinent enzyme activity, metabolite concentration, or cellular function. Moreover, if the antioxidant cellular machinery is concomitantly activated and enhanced, then an increase in ROS might have no biological effect: an increased ROS production is counterbalanced by an increased ROS consumption. Similarly, detection of enhanced gene transcription under oxidative stress (Clifton et al. 2005) has little functional meaning without the parallel assessment of enzyme activities, pathway fluxes, and biological function.

The oxidative stress induced by Cd^{2+} and its appropriate management in *E. gracilis* was apparent by: (a) the increased content of iron and cooper; (b) the enhancement in MDA content; (c) the diminution in the level of reduced glutathione; (d) the diminution in aconitase activity; and (e) the increase in the total content of the antioxidant metabolites, glutathione and ubiquinone, and in the activity of the antioxidant enzymes, APx, GPx, and GR, which could stimulate the ascorbate-glutathione cycle. The lack of CAT and of cytosolic and mitochondrial SOD in *Euglena* was apparently well covered by APx, GPx, and the ascorbate-glutathione cycle for the efficient disposal of superoxide and H_2O_2. In anaerobic parasitic eukaryotes, such as *Trichomonas vaginalis*, *Entamoeba histolytica*, and *Giardia intestinalis*, absence of CAT and GPx can be covered by rubrerythrin, an enzyme with probable peroxide reduction activity (Putz et al. 2005); the presence of this enzyme in *Euglena* has not been explored.

A ramified electron transfer allows organisms to adapt to different environmental conditions (Poole and Cook 2000; Helmerhorst et al. 2002). In *E. gracilis*, the presence of AOX could reduce the generation of oxidative stress by draining electrons from an inhibited respiratory chain. An enhanced AOX has been described for *E. gracilis* and several plants, such as potato leaves, petunia cells, tobacco, soybean, mungo bean, and *Arabidopsis* treated with respiratory inhibitors (antimycin, cyanide) or subjected to cold, conditions under which oxidative stress is also

Table 4 Sensitivity of respiratory enzymes to Cd^{2+} in mitochondria isolated from control and Cd^{2+}-treated cells

	IC_{50} (μM)	
	Control	Cd^{2+} mitochondria
L-Lactate oxidase	14±4 (n=4)	
AOX	38±11 (n=4)	34±10 (n=5)
TMPD oxidase	3.7±1.7 (n=4)	0.21±0.2 (n=4)

For these experiments, mitochondria were incubated in 120 mM KCl, 20 mM MOPS, pH 7.25 (an EGTA-lacking buffer). The free Cd^{2+} concentration was calculated by using the software CHELATOR (Schoenmakers et al. 1992). Enzyme activities were measured as detailed under Materials and Methods. The IC_{50} values were estimated from titration curves, as those shown in Fig. 2. The Cd^{2+} sensitivity of L-lactate oxidase in mitochondria isolated from Cd^{2+}-exposed cells was not determined due to the low activity of this respiratory component and the consequent inaccuracy of the measurements

induced (Vanlerberghe and McIntosh 1992; Wagner and Wagner 1997; Gonzalez-Meler et al. 1999; Castro-Guerrero et al. 2004; Geisler et al. 2004; Sugie et al. 2006).

Indeed, Cd^{2+} exposure inhibited the activity of the respiratory complexes III and IV, whereas AOX activity enhanced and showed resistance to a direct Cd^{2+} exposure. Thus, this inherent resistance to Cd^{2+}, together with the enhanced AOX content and activity induced by Cd^{2+} exposure, indicates a relevant role for AOX in establishing a protective mechanism against Cd^{2+}-induced oxidative stress. The increased AOX capacity under Cd^{2+}-stress promotes an adequate electron flux that limits ROS production and allows for maintaining the ATP level relatively high. The present findings contribute to understand better the cellular and mitochondrial mechanisms used by *E. gracilis* to resist and accumulate heavy metals (Navarro et al. 1997; Devars et al. 2000; Mendoza-Cozatl et al. 2002, 2006a; Avilés et al. 2003; Mendoza-Cozatl and Moreno-Sanchez 2005).

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