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Top-down control analysis of the cadmium effects on molluscan mitochondria and the mechanisms of cadmium-induced mitochondrial dysfunction

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Submitted 22 April 2010; accepted in final form 13 September 2010

Kurochkin IO, Etzkorn M, Buchwalter D, Leamy L, Sokolova IM. Top-down control analysis of the cadmium effects on molluscan mitochondria and the mechanisms of cadmium-induced mitochondrial dysfunction. Am J Physiol Regul Integr Comp Physiol 300: R21–R31, 2011. First published September 15, 2010; doi:10.1152/ajpregu.00279.2010.—Cadmium (Cd) is a toxic metal and an important environmental pollutant that can strongly affect mitochondrial function and bioenergetics in animals. We investigated the mechanisms of Cd action on mitochondrial function of a marine mollusk (the eastern oyster Crassostrea virginica) by performing a top-down control analysis of the three major mitochondrial subsystems (substrate oxidation, proton leak, and phosphorylation). Our results showed that the substrate oxidation and proton leak subsystems are the main targets for Cd toxicity in oyster mitochondria. Exposure to 12.5 μM Cd strongly inhibited the substrate oxidation subsystem and stimulated the proton conductance across the inner mitochondrial membrane. Proton conductance was also elevated and substrate oxidation inhibited by Cd in the presence of a mitochondrially targeted antioxidant, MitoVite, indicating that Cd effects on these subsystems were to a large extent ROS independent. Cd did not affect the kinetics of the phosphorylation system, indicating that it has negligible effects on F1,F0 ATP synthase and/or the adenine nucleotide transporter in oyster mitochondria. Cd exposure altered the patterns of control over mitochondrial respiration, increasing the degree of control conferred by the substrate oxidation subsystem, especially in resting (state 4) mitochondria. Taken together, these data suggest that Cd-induced decrease of mitochondrial efficiency and ATP production are predominantly driven by the high sensitivity of substrate oxidation and proton leak subsystems to this metal.

Cadmium; reactive oxygen species; proton leak; substrate oxidation; mitovite

TOXIC METALS SUCH AS CADMIUM (Cd) are important contaminants in marine ecosystems. Most (~97%) Cd in the biosphere is derived from human activities, although in some coastal areas Cd levels are naturally elevated due to the leaching of Cd-enriched soils or deposition of Cd-hyperaccumulating diatoms (3, 28, 54). Cd is an extremely toxic metal with no known biological function in animals. Because of its rapid bioaccumulation and long residence time in the organisms, Cd represents an important health concern for humans and wildlife (54–55).

Because of its affinity to thiol groups and nitrogen-containing ligands, Cd can bind to a broad range of biological macromolecules (such as proteins and DNA), damaging their structure and interfering with their functions (5). Mitochondria are key intracellular targets for Cd due to their ability to accumulate Cd and because of the sensitivity of mitochondrial enzymes to Cd-induced damage (14, 48, 56, 73). Because of the central role of mitochondria in critical cellular processes such as bioenergetics, redox signaling, and cell death, Cd-induced mitochondrial damage has long-ranging consequences for cellular function, energy homeostasis, and whole-organism performance and survival. Previous studies have shown that Cd suppresses respiration and leads to the partial uncoupling, as well as elevated rates of reactive oxygen species (ROS) production in animal and plant mitochondria (11, 13, 14, 18, 23, 35, 65, and references therein). Several mechanisms have been proposed to explain effects of Cd on mitochondrial bioenergetics, including inhibition of electron transport chain (ETC), oxidative damage to mitochondrial enzymes and opening of the mitochondrial permeability pore (1, 23, 52, 73). However, the sites of toxic action of Cd in mitochondria and the relative sensitivity of different mitochondrial processes to Cd are not well understood.

Top-down control analysis is a useful approach for the study of the control of oxidative phosphorylation and for the identification of mitochondrial processes that are modulated by external stressors such as Cd (9, 15, 25, 33). The top-down control analysis allows partitioning the complex system involved in the control of mitochondrial oxidative phosphorylation to three interconnected blocks of reactions: phosphorylation, proton leak, and substrate oxidation subsystems (Fig. 1). The substrate oxidation subsystem encompasses all processes that create proton motive force (Δp) and includes the activities of electron transport chain, tricarboxylic acid cycle, and metabolite transport. The phosphorylation subsystem (including activity of F0, F1-ATPase and transport of adenylates and inorganic phosphate) uses Δp to synthesize ATP, whereas the proton leak subsystem involves all cation (including proton) cycles that dissipate Δp without ATP production. All three subsystems are linked by a common intermediate, Δp. The top-down control analysis involves determining the kinetic response (measured as O2 consumption) of each subsystem to experimental perturbation of Δp. This, in turn, provides a way to quantify the control that each subsystem confers over mitochondrial oxidative phosphorylation under different physiological conditions. With an elasticity analysis (7, 16, 30), it also
allows identifying which subsystems are modulated by stressors such as Cd. Although the top-down control analysis gives a relatively low-resolution picture of mitochondrial function, it provides a quantitative insight into the overall control structure of oxidative phosphorylation, pinpoints critical functions of mitochondrial bioenergetics that are most sensitive to Cd and focuses future searches for molecular targets of Cd. The top-down control analysis has been previously used to identify mitochondrial sites of Cd action in plants (37, 41–43); however, to our knowledge, it has never been used to investigate Cd effects in animal mitochondria.

In this study, we used a marine mollusk, the eastern oyster *Crassostrea virginica*, as a model to analyze the mitochondrial targets of Cd effects. Oysters are an ecologically and economically important bivalve species that serves as an ecosystem engineer in western Atlantic estuaries (31, 61). Like all marine bivalves, oysters can strongly concentrate Cd in soft tissues to levels that exceed environmental concentrations by orders of magnitude (28, 58, 68), making these mollusks susceptible to the toxic effects of Cd, as well as important vectors of Cd transfer to higher levels of the food chain. Our earlier studies showed that Cd exposure suppressed ADP-stimulated respiration and the rate of ATP synthesis, stimulated production of ROS and resulted in a partial uncoupling in oyster mitochondria (18–19, 65, 67). However, the mechanisms underlying these effects are not fully understood. The aims of the present study, therefore, were to determine 1) which of the three mitochondrial subsystems (substrate oxidation, phosphorylation, or proton leak) is most impacted by Cd in oyster mitochondria, 2) whether Cd exposure affects the control over oxidative phosphorylation exerted by different subsystems, and 3) whether ROS are implicated in Cd-induced modulation of mitochondrial control and function. To achieve these aims, we used top-down control and elasticity analyses to determine the sites of action of Cd in oyster mitochondria and tested whether Cd-induced effects could be reversed by a mitochondrially targeted antioxidant, MitoVitE.

**MATERIALS AND METHODS**

**Animal collection and maintenance.** Adult *C. virginica* (7- to 12-cm shell length) were obtained from local oyster suppliers (Cuttyhunk Shellfish Farms, Cuttyhank, MA, for MitoVitE studies and from Taylor Shellfish Farms, Shelton, WA, for all other experiments). Oysters were shipped within 48 h of collection to the University of North Carolina at Charlotte and placed in tanks with recirculated aerated artificial seawater (ASW) (Instant Ocean, Kent Marine, Aircworth) at 20°C and 30% salinity for 2 wk prior to experimentation. Temperature and salinity were maintained within 1°C and 1‰ of their respective target values. Our previous studies have shown that this temperature and salinity are close to the optima for oysters (I. M. Sokolova, unpublished data).

**MitoVitE synthesis.** The synthesis of Mito-VitE, an α-tocopherol analog followed the previously reported procedures (20, 21, 30, 62, 64) optimized as described in the supplemental material in the online version of this article. All commercially available starting materials and reagents were used as purchased. Solvents were purified using standard procedures (2), and reactions were carried out under inert argon (Ar) atmosphere.

**Measurements of mitochondrial respiration and membrane potential.** Mitochondrial respiration and membrane potential were measured simultaneously in a water-jacketed, temperature-stabilized four-port chamber (World Precision Instruments, Sarasota, FL) in the presence or absence of Cd (34 μM total Cd added as CdCl₂ or 12.5 μM free Cd²⁺; see below) and in the presence or absence of a mitochondrially targeted ROS scavenger, MitoVitE (10 μM). We used 12.5 μM free Cd, which is within the range of the intracellular Cd levels found in Cd-exposed oysters (65, 68, and references therein) and is similar to experimental Cd concentrations used in earlier studies (18, 19, 65).

Because BSA is known to bind Cd (24, 57), we determined the coefficient of Cd binding to BSA by measuring the amount of total and free Cd in the assay media at different Cd concentrations. Briefly, 50 μl of 109CdCl₂ (154 ng Cd/ml with a specific activity of 15.53 nCi/ng Cd) was added to 0.95 ml of assay media containing 10 mg/ml BSA and different concentrations of nonradioactive Cd (as CdCl₂) ranging from 15.6 μM to 2 mM. The mixture was incubated at 20°C for 1 h and subjected to ultrafiltration through Amicon Ultra-4 filters with the cut-off size of 10 kDa (Millipore, Billerica, MA) at 2,500 g for 20 min. Prior to the filtration, Ultra-4 filters were exposed overnight to a 5% (vol/vol) solution of cetrimethylammonium bromide (CTAB; Aldrich, Milwaukee, WI) at 36°C. CTAB eliminates negative charges on the filter surface so that the filter cannot bind cations and acts simply as a size sieve (24). The ultrafiltrate-containing free Cd and the remaining nonfiltered media (containing BSA and bound Cd) were collected, and their volumes were adjusted to 1 ml each with the assay media. Radioactivity was measured using a Wallac Wizard 1480 gamma counter (Perkin Elmer, Waltham, MA).

The relationship between the free and total Cd in the assay media is described by the following second order polynomial (R² = 0.998):

$$[\text{Free Cd, } \mu M] = -0.002x^2 + 0.07624x - 12.955,$$

where $x$ is the total...
µM Cd concentration in the assay media. This equation was used to calculate the total concentration of Cd (34 µM of total Cd) that was needed to achieve the desired concentration of free Cd (12.5 µM of total Cd) in our assay.

Oxygen concentrations in the respirometry chamber were determined using a fiber optic oxygen sensor connected to the Microx TX3 oxygen monitor (Precision Sensing, Düsseldorf, Germany) and OxyMicro ver. 2.00 software (World Precision Instruments, Sarasota, FL). A two-point calibration (0% and 100% of air saturation) was performed prior to each measurement with saturated Na₂SO₃ solution and air-saturated assay media serving as 0% and 100% calibration points, respectively. For each mitochondrial preparation, we determined states 3 (ADP-stimulated respiration), 4 (resting respiration after ADP was depleted), and 4oL (resting respiration in the presence of oligomycin), and we calculated respiratory control ratios (RCR) as a ratio of state 3 to state 4 respiration. RCR values obtained in this study ranged between 2 and 3 (see also results) typical for molluscan mitochondria respiring on succinate (34, 47, and references therein). In a pilot study, we also tested for the presence of alternative oxidase (AOX) in oyster mitochondria using an AOX inhibitor, salicylhydroxamic acid (SHAM, 200 µM) in potassium cyanide-inhibited mitochondria; no measurable AOX activity was detected in mitochondria from oyster gills (data not shown).

Mitochondrial membrane potentials (ΔΨ) were determined by ΔΨ-dependent mitochondrial accumulation of tetracyanophosphonium (TPP⁺) ions using a TPP⁺-selective electrode (KWIKTPP-2) and a Super Dri-Ref reference electrode (World Precision Instruments, Sarasota, FL) connected to a pH meter (model 1671; Jenco Instruments, San Diego, CA). Oxygen and TPP⁺ concentrations in the assay media were monitored using Logger Pro 3.2 with a Vernier LabPro interface (Vernier Software and Technology, Beaverton, OR). An H⁺/K⁺ exchanger nigericin (123 nM) was added to the assay media to collapse the pH gradient (ΔpH) and convert it into the electrical gradient (ΔΨ). Our pilot studies showed that this concentration of nigericin does not affect respiration of oyster mitochondria (data not shown). The TPP⁺ electrode was filled with a solution containing 10 mM TPP⁺ and 10 mM NaCl, pH 7.2 and calibrated before each measurement using stepwise additions of TPP⁺ (2–10 µM). In the experiments, testing the effects of MitoVitE on mitochondrial subsystems, calibration was achieved by the stepwise addition of TPP⁺ instead of MitoVitE. Our pilot experiments showed that 10 µM of TPP⁺ or MitoVitE are nontoxic for oyster mitochondria and sufficient for determination of the entire range of membrane potentials at the protein concentrations (2 mg/ml) used in this study (data not shown). Corrections for the nonspecific binding of TPP and MitoVitE were conducted after fully collapsing the mitochondrial membrane potential with 0.4 mM of KCN and 0.5 mM 2, 4-dimotephenol similar to the methods described by Lötscher et al. (49) and Chamberlin (17). TPP⁺ or MitoVitE binding was not affected by Cd (data not shown). Nonspecific binding was 0.67 ± 0.11 µmol·µL⁻¹·mg⁻¹ protein and 1.75 ± 0.21 µmol·µL⁻¹·mg⁻¹ protein for TPP⁺ and MitoVitE, respectively.

A mitochondrial matrix volume of 1 µl/mg protein was used based on results from a previous study with invertebrate mitochondria (17); similar values have been reported for other animals, including mollusks (6–7). It is worth noting that the potential effects of variation in mitochondrial volume on ΔΨ are small because even a twofold error in the mitochondrial volume causes only a 17-mV error in the calculated ΔΨ (7). Further, any variation should not affect the overall pattern of ΔΨ change in response to experimental titrations (see Kinetic responses of the mitochondrial subsystems) or Cd exposure. It has been shown that Cd has no effect on mitochondrial volume in oysters (67) or other organisms (38–40, 67).

The mitochondrial membrane potential was calculated on the basis of the extramitochondrial and intramitochondrial TPP⁺ or MitoVitE concentrations using the Nernst equation as described elsewhere (7). It is worth noting that the binding corrections for the two phosphonium probes used to calculate the mitochondrial membrane potential in this study were determined at a low membrane potentials in fully depolarized mitochondria, and the ΔΨ-dependent binding behavior of the two probes was not measured. Theoretically, if nonspecific binding of TPP⁺ and MitoVitE is differentially affected by ΔΨ, this could lead to systematic differences between ΔΨ values estimated with TPP⁺ and MitoVitE. To avoid this bias, we only compared kinetic responses between control and Cd-exposed mitochondria measured with the same type of probe. The absolute difference of ΔΨ values between control and Cd-exposed mitochondria measured with the same probe is unaffected by the value of the nonspecific binding correction. It is also worth noting that the calculation of ΔΨ is insensitive to potential errors in the nonspecific probe binding correction, so that even a twofold error in the binding coefficient only alters ΔΨ value by ~17 mV.

Kinetic responses of the mitochondrial subsystems. The kinetic responses of all subsystems (proton leak, substrate oxidation, and phosphorylation) were measured in the presence of 0.5 mM of ADP (a concentration that was sufficient to achieve maximum oxygen uptake), 20 µM rotenone, 10 mM succinate, and 1% BSA (fatty acid free) in 1 ml of assay media containing 2 mg of mitochondrial protein. Our pilot studies have shown that 10 mM succinate is a saturating concentration for all three studied subsystems under the assay conditions used in this study (data not shown). The kinetic response of the oxidation subsystem was determined by a stepwise addition of small amounts of mitochondrial uncoupler [3-chlorophenyl)hydrazonolmononitride (0.2–1.8 µM) to respiring mitochondria in the presence of 5 µg/ml oligomycin to inhibit ATP synthesis. This titration resulted in depolarization of mitochondria and stimulation of their oxygen consumption. The kinetic response of the mitochondrial proton leak subsystem was determined in the presence of oligomycin (5 µg/ml) by sequential addition of small amounts of malonate (2–18 mM) to the respiring mitochondria. Malonate titration resulted in a progressive depolarization of the mitochondria and a decrease in respiration. The phosphorylation subsystem was also titrated by malonate (2–18 mM) but without oligomycin to achieve the maximum respiration rate. We did not use hexokinase in this assay because our earlier studies showed that oyster mitochondria become partially uncoupled in the presence of Mg²⁺ needed for hexokinase activity (data not shown). Instead, during each malonate addition, we added 0.15 µmol of ADP per 1 ml of the assay medium to prevent ADP depletion. Our pilot studies showed that these additions were sufficient to maintain the maximum respiration rates of oyster mitochondria and that up to 2 mM ADP or ATP (the maximum possible concentrations after all additions) did not affect mitochondrial oxygen consumption (data not shown). The oxygen consumption due to the proton leak was subtracted from state 3 respiration at the respective ΔΨ values to obtain the kinetics of the phosphorylation subsystem only (see Data analysis and statistics).

Data analysis and statistics. The data from the kinetic analyses (i.e., plots of the oxygen consumption rates vs. ΔΨ for each subsystem) were described using second- or third-order polynomials; the best fit was determined by the significance of the second- or third-order coefficients of the regressions using a standard procedure as implemented in SAS. Briefly, we started with linear regressions (zero-order polynomials) and increased the polynomial order until a further increase of the order did not result in a significantly improved fit (indicated by the lack of significance of the corresponding higher-order coefficient of the regression). As a result, second-order polynomials were used for kinetics of the substrate oxidation subsystem, and third-order polynomials were used for kinetics of the proton leak and phosphorylation subsystems. All regressions were significant (P < 0.05) and provided an excellent fit to the experimental data (R² =...
0.969–0.998, except for the kinetics of the substrate oxidation subsystem for Cd-exposed mitochondria in Fig. 3A, where $R^2 = 0.687$.

We also fitted the experimental data with exponential regressions. Exponential regressions were significant ($P < 0.05$) for 11 out of 14 analyzed kinetic curves, but compared with the polynomials, generally provided a poorer fit to the experimental data ($R^2 = 0.008–0.953$). Nevertheless, we tested for significant differences of the kinetic responses of the respective subsystems in control and Cd-exposed mitochondria using both exponential and polynomials fits. For exponential fits, regressions were linearized prior to analysis using log-transformed values of the oxygen consumption rates of the respective mitochondrial subsystems. For polynomial regressions, no transformation was used.

To test for significant differences between the kinetic responses between the control vs. Cd treatment groups, we used contrasts generated for the respective exponential or polynomial curves for each of the three subsystems (proton leak, substrate oxidation, and phosphorylation) using the GLM procedure of SAS (60). These contrasts tested the collective differences between the curves (including both the intercepts and slopes, with 3 and 4 degrees of freedom for quadratic and cubic polynomials, respectively). Separate statistical comparisons of intercepts and slopes of the respective curves were not conducted, because we were interested in the overall differences in the kinetic responses of different subsystems between the treatments, rather than in the exact parameters of the empirical curves. Because we only used orthogonal contrasts appropriate for the pairwise comparisons of the regression lines of oxygen consumption rates on $\Delta \Psi$ between the control and Cd-exposed groups, no adjustments of the alpha levels were required.

Polynomial regressions were used to calculate respiration rates in control and Cd-exposed mitochondria at a common membrane potential and to correct the kinetic response of the phosphorylation subsystem by subtracting the contribution of the proton leak to oxygen consumption at a given membrane potential. The polynomial regressions were also used to calculate elasticities (from the first derivatives of the equations) and the flux control coefficients as described by Brand et al. (9) and Hafner et al. (33). Elasticities and the flux control coefficients, as well as the respiration rates at the common membrane potential, were calculated at the highest respective $\Delta \Psi$ for state 3 and 4 of control or Cd-exposed mitochondria (see Figs. 3–5).

The effects of Cd on state 3 and 4 respiration, $\Delta \Psi$ and RCR were tested using a repeated-measures ANOVA followed by post hoc procedures (Fisher’s least significant difference test for unequal $N$), after testing for the normality of data distribution and for homogeneity of variances among the treatment groups. Repeated-measures ANOVA was used because these parameters were measured in two separate aliquots of the same mitochondrial isolate in the presence or absence of Cd. All assays were completed within 2 h of mitochondrial isolation, and our pilot studies showed no change in the measured mitochondrial characteristics within this time frame (data not shown).

All differences were considered significant if the probability of Type I error ($P$) was less than 0.05. In adherence with the guidelines for publications of the American Physiological Society (22), we present precise $P$ values for statistical tests whenever available. Data are expressed as means ± SE unless stated otherwise.

RESULTS

Cd effects on mitochondrial bioenergetics. Cd exposure resulted in a slight, but significant, reduction of mitochondrial membrane potential in resting (state 4) and ADP-stimulated (state 3) mitochondria by 12% and 6%, respectively (Fig. 2). Cd exposure had opposing effects on state 3 and state 4 respiration, inhibiting the former and stimulating the latter; these changes resulted in a significant reduction of the RCR of mitochondria from 2.2 to 1.5 (Fig. 2).

Exposure to 12.5 μM Cd significantly affected both the flux and kinetic response of the substrate oxidation subsystem (Fig. 3A; $P < 0.0001$ and $P = 0.0001$ for polynomial and exponential fits, respectively, for the difference in the kinetic response between control and Cd-exposed mitochondria). Uncoupled respiration of oyster mitochondria was strongly inhibited by Cd, decreasing from 28.9 ± 1.4 to 13.6 ± 2.1 nmol

![Fig. 2. Effects of Cd exposure (12.5 μM) on bioenergetic characteristics of oyster mitochondria. A: respiratory control ratio (RCR). B: mitochondrial membrane potential. C: oxygen consumption of ADP-stimulated (state 3), resting (state 4), and resting in the presence of oligomycin (state 4ol) mitochondria. *Significantly different from the respective controls ($P < 0.05$). Vertical bars show means ± SE.](http://ajpregu.physiology.org/)

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O₂·min⁻¹·mg⁻¹ protein. At any membrane potential, the flux through the substrate oxidation subsystem was considerably lower in Cd-exposed mitochondria but the difference was especially prominent in the low ΔΨ range (Fig. 3A).

The kinetic response of the proton leak subsystem indicated a nonlinear increase in proton conductance with increasing membrane potential (Fig. 3B). Cd strongly affected kinetic response of the proton leak subsystem \( P < 0.0001 \) and \( P = 0.899 \) for polynomial and exponential fits, respectively, for the difference between control and Cd-exposed mitochondria; the high \( P \) values for comparisons of exponential fits for the proton leak kinetics is likely due to a poor fit of the exponential curve to the data for Cd-exposed mitochondria \( (R^2 = 0.65) \) compared with the polynomial curve \( (R^2 = 0.97) \). At the physiologically relevant membrane potentials (above 160 mV), proton leak was higher in Cd-exposed mitochondria compared with the controls, indicating that Cd stimulated proton conductance of the mitochondrial membrane. At low membrane potentials (around 155–160 mV), proton conductance of the control and Cd-exposed mitochondria converged on similar low values (Fig. 3B). At 170 mV (the average ΔΨ achieved by the resting mitochondria in the presence of 12.5 μM Cd), proton conductance was almost double in Cd-exposed compared with their control counterparts \( 0.38 \) vs. \( 0.21 \) nmol \( \text{H}^+\cdot\text{min}^{-1}\cdot\text{mg}^{-1}\cdot\text{protein} \cdot\text{mV}^{-1} \). At the ΔΨ typical for the resting control mitochondria \( (190 \text{ mV}) \), the predicted differences in the proton conductance between the Cd-exposed and control mitochondria were even greater \( (0.95 \text{ vs. } 0.37 \text{ nmol} \text{H}^+\cdot\text{min}^{-1}\cdot\text{mg}^{-1}\cdot\text{protein} \cdot\text{mV}^{-1} \).

Although the regressions for the kinetic response of the phosphorylation subsystem were significantly different between control and Cd-exposed mitochondria \( (P = 0.011 \text{ and } P = 0.048 \text{ for polynomial and exponential regressions, respectively}) \), this was mostly due to the differences in the nonoverlapping ΔΨ range between the control and Cd-exposed mitochondria (Fig. 3C). In the range of the common membrane potentials \( (135–155 \text{ mV}) \), the curves for control and Cd-exposed mitochondria were essentially superimposed (Fig. 3C). This indicates that the relationship between the flux through the phosphorylation subsystem and ΔΨ is not considerably affected by Cd in the physiological ΔΨ range.

Consistent with the above-described kinetic responses, a comparison of Cd effects on the three studied mitochondrial subsystems at a common membrane potential \( (170 \text{ mV}) \) indicates that Cd exposure results in a considerable decrease \( (31\%) \) in the substrate oxidation flux and an increase \( (80\%) \) in proton conductance, but no change in the flux through phosphorylation subsystem (Fig. 3D).

**Flux control coefficients.** Control coefficients over the state 3 mitochondrial respiration were similar in control and Cd-exposed mitochondria, with the maximum control (over 75%) exerted by oxidation subsystem followed by the phosphorylation subsystem (Fig. 4A). Proton leak exerted a low degree of control over mitochondrial respiration in state 3 (3–6%) (Fig. 4B).
Phosphorylation rate in state 3 mitochondria was also largely controlled by the substrate oxidation subsystem with a relatively modest degree of control exerted by phosphorylation and proton leak subsystems (Fig. 4B). Proton leak in state 3 mitochondria was predominantly controlled by the proton leak subsystem in the absence of Cd, whereas in the presence of 12.5 µM Cd all three subsystems significantly contributed to the control of the proton leak flux (Fig. 4C).

In the absence of Cd, control over the state 4 respiration and proton leak was shared between the substrate oxidation and proton leak subsystems, with a higher contribution of the proton leak subsystem (Fig. 4D and E). In contrast, in Cd-exposed state 4 mitochondria both oxygen consumption and proton leak fluxes were almost exclusively controlled by the substrate oxidation subsystem (Fig. 4D and E).

**MitovitE effects on kinetic responses of mitochondrial subsystems.** Because Cd exposure had the strongest effects on the substrate oxidation and proton leak subsystems (cf. Fig. 3, A, B, and C), we tested whether Cd effects on these two subsystems could be alleviated by a mitochondrially targeted antioxidant, MitovitE (10 µM). In the absence of Cd, MitovitE did not significantly affect mitochondrial respiration (11.93 ± 0.68 vs. 15.29 ± 3.47 nmol O₂·min⁻¹·mg⁻¹ protein in the presence or absence of MitovitE, respectively; P = 0.55).

Cd exposure significantly affected the kinetic response of the substrate oxidation subsystem irrespective of the presence or absence of MitovitE (P < 0.0001 for both polynomial and exponential fits; cf. Figs. 3A and 5A), causing inhibition of the flux especially noticeable in the range of low ΔΨ. When calculated at the common ΔΨ (170 mV), Cd exposure suppressed the substrate oxidation flux by 60% in oyster mitochondria, and this suppression remained practically unchanged in the presence of MitovitE (Fig. 5C). In the presence of MitovitE, Cd exposure also significantly affected the kinetic responses of the proton leak subsystem (P = 0.007 and P < 0.0001 for polynomial and exponential fits, respectively), and elevated the proton leak by ~35% at 170 mV (Fig. 5B and C).

**DISCUSSION**

Top-down control analysis clearly shows that negative effects of Cd on bioenergetics of oyster mitochondria are predominantly caused by 1) inhibition of the substrate oxidation...
subsystem and 2) stimulation of proton conductance and subsequent proton leak across the inner mitochondrial membrane. In contrast, the phosphorylation subsystem was not affected by Cd exposure. Earlier research conducted in potato tuber mitochondria also yielded similar results, showing suppressed substrate oxidation and stimulated proton leak but no effects on phosphorylation subsystem (37, 41, 42). This similarity of mitochondrial responses to Cd between oysters and plants suggests that the sites of Cd action in mitochondria are evolutionarily conserved and that the high sensitivity of substrate oxidation and proton leak subsystems to Cd effects can be expected in other animals.

Cd-induced inhibition of the substrate oxidation subsystem may be due to at least two potential mechanisms: 1) direct inhibition of ETC or TCA enzymes and/or substrate transporters by Cd binding; and 2) oxidative damage to these enzymes or substrate transport system by Cd-induced ROS. Our earlier studies showed that Cd exposure strongly stimulated ROS production in oyster mitochondria, leading to inhibition of ROS-sensitive enzymes, such as aconitase (18). However, this study showed that even high concentrations (10 μM) of a potent mitochondrial ROS scavenger MitoVitE do not restore or augment the substrate oxidation flux in oyster mitochondria respiring on succinate. This suggests that ROS are not likely to be involved in Cd-induced inhibition of substrate oxidation and that the inhibitory effects of Cd may be due to the direct binding of Cd to the thiol group of ETC enzymes (and/or other proteins involved in the substrate oxidation pathways) and/or the displacement of metals (such as Cu or Fe) from their active centers.

We previously showed that ETC complexes are sensitive to Cd in oysters and can be directly inhibited by this metal in vitro (35). Inhibition of ETC by Cd was also shown in other animal and plant models (13–14, 50–51, 75), suggesting that this may be a common toxic mechanism responsible for the Cd-induced reduction of the substrate oxidation flux. Complex II was the most sensitive of the four studied ETC complexes to Cd in oyster gills (35) similar to the mammalian Complex II (75), suggesting that this enzyme may be a conserved site for Cd action in ETC. Potentially, Cd-induced inhibition of succinate transport (i.e., dicarboxylate carrier) can also contribute to the observed decline in the substrate oxidation flux. Currently, there are no studies demonstrating Cd effects on substrate transport, including dicarboxylate transporters in mitochondria. However, given that succinate transport is not considered to be rate-limiting in energized animal mitochondria and the fact that we used saturating concentrations of succinate, it is less likely that the observed Cd-induced inhibition of substrate oxidation reflects insufficient substrate transport. Nevertheless, this possibility cannot be presently excluded and warrants further investigation. Moreover, the toxic effects of Cd on substrate oxidation may be compounded in vivo with the suppression of the Krebs cycle flux due to the inhibition of enzymes such as NAD-dependent isocitrate dehydrogenase and citrate synthase, which are also highly sensitive to Cd in oysters (18, 35).

Proton leak was strongly stimulated by the presence of Cd in oyster mitochondria, suggesting that Cd either increases the passive permeability of the inner mitochondrial membrane to protons (i.e., proton conductance) and/or leads to a decrease in
H⁺/O stoichiometry of the ETC pumps (i.e., proton slip). Previous studies have shown that proton slip does not significantly contribute to mitochondria proton leak under the normal conditions (in plant and animal mitochondria) or in the presence of Cd (studied in plants only) (8, 10, 43), suggesting that most of the observed proton leak is due to proton conductance. The proton conductance of the inner mitochondrial membrane was \( \Delta \Psi \)-dependent, and ranged from 0.21 to 0.24 nmol H⁺-\( \text{mg}^{-1} \)-protein mV⁻¹ in oyster mitochondria at 160 mV and 20°C. This is the first estimate of mitochondrial proton conductance in marine mollusks, but it is in a close agreement with the values obtained for insect (0.30 nmol H⁺-\( \text{mg}^{-1} \)-protein mV⁻¹) and frog (0.26 nmol H⁺-\( \text{mg}^{-1} \)-protein mV⁻¹) mitochondria measured at similar \( \Delta \Psi \) and temperature (159–160 mV and 25°C) (16, 69). This comparison of three unrelated taxa indicates that proton conductance of the inner mitochondrial membrane might be a conserved feature among ectotherms. However, further extensive sampling of major invertebrate and vertebrate taxa would be required to test this hypothesis.

Elevated proton conductance of the inner mitochondrial membrane appears to be the most feasible explanation of the Cd-induced increase in the flux of the proton leak subsystem. Given that the observed responses of proton leak to Cd occur within minutes of exposure, it is unlikely that the changes in intrinsic membrane properties (such as alterations of the fatty acid composition of the membrane phospholipids by phospholipases and/or changes in the surface area of the inner mitochondrial membrane) significantly contribute to this response. More likely, this change in proton conductance reflects upregulation of the preexisting mechanisms of proton leak.

Interestingly, earlier studies in mammalian mitochondria showed that superoxide produced in the mitochondrial matrix induces an increase in proton conductance of the inner mitochondrial membrane due to the activation of uncoupling proteins (UCPs) that allow influx of protons into the mitochondrial matrix (26, 29, 46, 70–72). This UCP- and superoxide-dependent increase in proton conductance of mammalian mitochondria is alleviated by the mitochondrial targeted antioxidants (such as MitoVitE and MitoQ) but not by nontargeted antioxidants (such as vitamin E or TROLOX) (26). Notably, UCPs are also expressed in oyster tissues (36, 66), and Cd is known to stimulate ROS production in oyster mitochondria (18), suggesting that a similar mechanism can potentially be involved in the regulation of the proton leak in Cd-exposed oyster mitochondria. However, in this study, a mitochondrially targeted antioxidant MitoVitE did not abolish Cd-induced increase of proton leak in oyster mitochondria (cf. Fig. 3 and 5), indicating that Cd-stimulated proton leak in oyster mitochondria is to a large extent due to the direct effects of Cd on proton conductance. Our data do not allow us to determine the degree of contribution of the ROS-dependent mechanisms to Cd-induced proton leak in oyster mitochondria, and determination of such mechanisms and their role in mitochondrial proton conductance of ectotherms under physiological and pathological conditions would represent an exciting new avenue for future research.

Other potential mechanisms of elevated proton leak may include activation of the adenine nucleotide transporter (ANT), UCP-independent cycling of protonated/unprotonated fatty acids, Ca²⁺ cycling and/or opening of the mitochondrial permeability transition (MPT) pore by Cd (12, 53). The first two mechanisms are unlikely to play a significant role in our in vitro system that was devoid of fatty acids (FFAs) due to the presence of fatty acid-free BSA used to scavenge FFAs during mitochondrial isolation and assays, and did not contain AMP, the most potent allosteric stimulator of ANT (53). Our earlier studies also indicate that Cd does not induce opening of the MPT pore in oyster mitochondria (67). We did not measure the rates of Ca²⁺ cycling in Cd-exposed oyster mitochondria; however, earlier studies in mammalian and plant systems show that Cd²⁺ and other bivalent metals such as Hg²⁺ may interfere with Ca²⁺ homeostasis stimulating Ca²⁺ efflux from intracellular storage sites (27, 45, 63, 74). This suggests that Cd-induced increase of cation (e.g., Ca²⁺) cycling may be another possible mechanism for elevated proton conductance. Irrespective of the precise molecular mechanisms involved, the Cd-induced increase in proton leak results in a decrease in phosphorylation efficiency of oyster mitochondria. Thus, in the physiological range of membrane potentials (170–180 mV), only about 13–16% of respiration of phosphorylating control mitochondria is due to the futile proton leak, whereas during exposure to 12.5 \( \mu \text{M} \) Cd²⁺ this proportion rises to 35–55%. A similar decrease in phosphorylation efficiency in response to Cd was observed in potato tuber mitochondria (41), suggesting that this may be another common mechanism of Cd effects on mitochondrial bioenergetics.

Surprisingly, the phosphorylation subsystem was not affected by Cd in oyster mitochondria under the assay conditions used in this study. This finding agrees with earlier research in potato tuber mitochondria, which showed no effect of Cd on the phosphorylation subsystem in a free Cd²⁺ concentration range from 3.5 to 21 \( \mu \text{M} \) (41). This indicates that of the three major subsystems of mitochondrial reactions, the phosphorylation subsystem is the least sensitive to Cd. It also suggests that any subtle effects of Cd on this subsystem (if present) are likely to be eclipsed by the strong effects on the substrate oxidation and proton leak subsystems.

Cadmium exposure significantly affected the distribution of control over the respiration flux in state 4 but not in state 3 mitochondria. The substrate oxidation subsystem conferred over 75% of control over state 3 respiration of oyster mitochondria. This high level of control over respiration is similar to that observed in insect (17) and plant (44) mitochondria but is significantly higher than in mammalian mitochondria (32, 59). In resting (state 4) oyster mitochondria, control of respiration was shared between the substrate oxidation and proton leak subsystems with the proton leak subsystem conferring 63% of control. This situation resembles that observed in midgut mitochondria of the tobacco hornworm larvae during early development (16–17). Interestingly, in later, premetamorphic larvae of the tobacco hornworm, state 4 respiration was predominantly controlled by the substrate oxidation subsystem; this change went hand-in-hand with a decreased flux through the substrate oxidation subsystem (16–17). Changes in mitochondrial state 4 respiration in response to Cd in oysters closely resemble the above-described situation, with a decrease in the flux through the substrate oxidation subsystem paralleled by a considerable increase in the degree of control conferred by this subsystem upon state 4 oxygen consumption.

The phosphorylation rate in oyster mitochondria was mostly controlled by the substrate oxidation subsystem with moderate...
contributions of the proton leak and phosphorylation sub-systems, similar to insect mitochondria (16). Cd exposure did not significantly change the distribution of control over phosphorylation flux in oyster mitochondria. In contrast, control over the proton leak flux was dramatically changed by Cd, with a significant increase of the degree of control conferred by the substrate oxidation subsystem, especially in state 4 (16). Cd exposure also increased the degree of the negative control exerted by the phosphorylation subsystem on the proton leak flux. These data indicate that Cd exposure induces the strongest changes in the flux control patterns of the proton leak (both in states 3 and 4), as well as of the overall oxygen consumption in state 4, which is mostly driven by the proton leak. In contrast, contribution of the different mitochondrial sub-systems to the control over the substrate oxidation in state 3 is not affected by Cd, despite the fact that the overall oxidation flux significantly decreases.

**Perspectives and Significance**

Given the high affinity of Cd to nitrogen- and sulfur-containing ligands and thus its potential for interaction with a broad spectrum of mitochondrial proteins, it is perhaps not surprising that Cd has multiple sites of action in oyster mitochondria and elicits both direct and indirect, ROS-mediated effects. Notably, Cd’s effects on mitochondrial bioenergetics are conserved between plant and animal (oyster) mitochondria and include inhibition of the substrate oxidation subsystem and an increase in the proton conductance of the inner mitochondrial membrane. This can provide a mechanistic explanation for the Cd-induced suppression of mitochondrial respiration and decreased phosphorylation efficiency commonly observed in a variety of systems, from plants to invertebrates and mammals. An increase in proton conductance in oyster mitochondria during Cd exposure raises an intriguing possibility that this may be an ancient and highly conserved mechanism preventing excessive ROS buildup in animal mitochondria under the stressed conditions. Future studies are needed to determine the molecular mechanisms responsible for the modulation of the physiological and pathologic proton leak in mitochondria of oysters and other ectotherms. Irrespective of whether the elevated proton leak has an adaptive value in preventing oxidative stress or is simply a side effect of Cd-induced mitochondrial dysfunction, it is likely to represent a significant energy liability for an organism due to the lower efficiency of ATP synthesis, higher basal metabolic costs, and potential energy trade-offs between the basal maintenance and other energy-demanding functions, such as growth or reproduction. This may have important consequences for sustainability of oyster populations in metal-polluted estuaries.

**ACKNOWLEDGMENTS**

The authors thank Dr. Mary Chamberlin for the help with the elasticity analysis and for the useful discussion, and three anonymous reviewers for their comments on earlier drafts of this manuscript.

**GRANTS**

This work was supported by the National Science Foundation awards IOS-0921367 and IBN-0347238 to I. M. Sokolova.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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