

Temperature-dependent effects of cadmium and purine nucleotides on mitochondrial aconitase from a marine ectotherm, *Crassostrea virginica*: a role of temperature in oxidative stress and allosteric enzyme regulation

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Summary

Temperature and heavy metals such as cadmium (Cd) are important environmental stressors that can strongly affect mitochondrial function of marine poikilotherms. In this study, we investigated the combined effects of temperature (20°C and 30°C) and Cd stress on production of reactive oxygen species (ROS) and oxidative stress in a marine poikilotherm *Crassostrea virginica* (the eastern oyster) using mitochondrial aconitase as a sensitive biomarker of oxidative damage. We also assessed potential involvement of mitochondrial uncoupling proteins (UCPs) in antioxidant protection in oyster mitochondria using purine nucleotides (GDP, ATP and ADP) as specific inhibitors, and free fatty acids as stimulators, of UCPs. Our results show that exposure to Cd results in elevated ROS production and oxidative damage as indicated by aconitase inactivation which is particularly pronounced at elevated temperature. Unexpectedly, oyster mitochondrial aconitase was inhibited by physiologically relevant levels of ATP (IC_{50} =1.93 and 3.04 mmol l⁻¹ at 20°C and 30°C, respectively), suggesting that allosteric regulation of aconitase by this nucleotide may be involved in regulation

of the tricarboxylic acid flux in oysters. Aconitase was less sensitive to ATP inhibition at 30°C than at 20°C, consistent with the elevated metabolic flux at higher temperatures. ADP and GDP also inhibited mitochondrial aconitase but at the levels well above the physiological concentrations of these nucleotides (6–11 mmol l⁻¹). Our study shows expression of at least three UCP isoforms in *C. virginica* gill tissues but provides no indication that UCPs protect mitochondrial aconitase from oxidative inactivation in oysters. Overall, the results of this study indicate that temperature stress exaggerates toxicity of Cd leading to elevated oxidative stress in mitochondria, which may have important implications for survival of poikilotherms in polluted environments during seasonal warming and/or global climate change, and suggest a novel temperature-dependent mechanism of allosteric regulation of TCA flux in oyster mitochondria.

Key words: aconitase, cadmium, temperature, uncoupling proteins, oxidative stress, tricarboxylic acid cycle, ectotherms, bivalve.

Introduction

Mitochondrial bioenergetics plays a key role in environmental adaptation and stress tolerance because of the central role of mitochondria in providing ATP to cover energy expenses of an organism, which are required for survival, growth and reproduction. In poikilotherms, mitochondrial function is very sensitive to environmental temperature because of the direct effects of temperature on the rate of all biochemical and physiological reactions as well as its indirect effects on mitochondria through changes in their intracellular milieu (for reviews, see Willmer et al., 2000; Hochachka and Somero, 2002). Estuarine organisms such as oysters can experience strong seasonal and diurnal fluctuations of the environmental temperature in their habitats, with amplitude

reaching up to 20–30°C (Sokolova et al., 2000; Helmuth et al., 2002). Oysters are also exposed to elevated levels of heavy metals including Cd in polluted estuaries (GESAMP, 1987). Owing to oysters' ability to bioaccumulate and bioconcentrate metals, Cd levels in their tissues can exceed environmental concentrations by orders of magnitude (Roesijadi, 1996; Frew et al., 1997; Sokolova et al., 2005). Body levels of Cd in natural oyster populations range from 0.4 to 40 µg g⁻¹ dry mass (Roesijadi, 1996; Frew et al., 1997) corresponding to the intracellular concentrations of ~1–90 µmol l⁻¹ of total Cd. During acute exposure to elevated Cd concentrations in water or sediments, oysters can accumulate even higher loads of this metal, up to 300–600 µg g⁻¹ dry mass, which corresponds to intracellular concentrations of ~670–1350 µmol l⁻¹ total Cd

(Roesijadi, 1996) (A. S. Cherkasov, S. Grewal and I. M. Sokolova, manuscript submitted for publication). Thus, a combination of temperature and Cd stress is an environmentally relevant situation for populations of intertidal poikilotherms (including oysters) from polluted environments.

Our earlier studies have shown that Cd strongly affects mitochondrial bioenergetics of eastern oysters *Crassostrea virginica*, resulting in reduced mitochondrial efficiency, lower rates of ATP synthesis and potential energy deficit (Sokolova, 2004; Cherkasov et al., 2006a,b). Cd-induced discrepancy between energy demand and energy supply is especially strong at elevated temperatures resulting in general physiological stress and high mortality in oysters (Lannig et al., 2006; Cherkasov et al., 2006a,b). Thus, impaired energy production appears to be an important aspect of Cd toxicity in poikilotherms, especially at elevated temperatures. However, other aspects of Cd toxicity in poikilotherm mitochondria are less well understood. In particular, effects of Cd and temperature stress on mitochondrial production of reactive oxygen species (ROS) and oxidative stress in poikilotherms have not been extensively studied. Elevated temperatures are known to increase the rate of ROS production in molluscan mitochondria (Abele et al., 2002; Heise et al., 2003), and Cd is known to induce ROS formation in mammals (Wang et al., 2004); however, it is not known how these two potentially pro-oxidant stressors interact in poikilotherm mitochondria during environmentally relevant exposures to temperature and Cd.

Mitochondrial proteins are often among the first targets of ROS attack in cells because of their immediate proximity to the ROS-generating sources, therefore, they may serve as sensitive markers to detect oxidative stress in mitochondria. Among the mitochondrial proteins, a Krebs cycle enzyme aconitase is commonly used as a sensitive and specific marker of oxidative stress [(Bota et al., 2002; Talbot and Brand, 2005) and references therein]. Mitochondrial aconitase contains a cubane [4Fe-4S] cluster in the active center which is open to attack by ROS, causing a release of a labile iron atom (Fe- α) and inactivation of the enzyme (Beinert and Kennedy, 1993; Talbot and Brand, 2005). This enzyme has been shown to be a selective target for oxidation by reactive oxygen and nitrogen species including superoxide ion, hydrogen peroxide, hydroxyl radical and peroxynitrite (Gardner and Fridovich, 1991; Castro et al., 1994; Andersson et al., 1998; Bota et al., 2002). Therefore, inhibition of mitochondrial aconitase may be used as a measure of mitochondrial oxidative damage, on one hand, and as an indicator of the adverse effects of a stressor on mitochondrial substrate cycles that could have important implications for mitochondrial energy production, on the other.

Owing to the constant exposure to ROS in the process of aerobic respiration, mitochondria are protected by multiple enzymatic and non-enzymatic mechanisms of ROS detoxification (Halliwell and Gutteridge, 1999; Goglia and Skulachev, 2003; Miwa and Brand, 2003) which may, to a certain degree, offset pro-oxidant effects of environmental

stressors. In addition to the traditionally recognized antioxidants such as antioxidant enzymes, some vitamins and glutathione, mitochondrial uncoupling proteins (UCPs) have recently emerged as potentially important players in antioxidant protection. UCPs are the members of anion carrier family, which are located in the inner mitochondrial membrane and can facilitate proton transport into the matrix (Goglia and Skulachev, 2003). Their activity appears to be tightly regulated with free fatty acids and superoxide acting as stimulators, and purine nucleotides, especially GDP – as inhibitors in all organisms studied so far, including plants, mammals and protists [(Rafael et al., 1994; Navet et al., 2005; Talbot and Brand, 2005; Dlaskova et al., 2006; Vercesi et al., 2006) and references therein]. Antioxidant protection has been proposed as a key function for UCP2 and 3; these proteins were found to be upregulated in response to pro-oxidant conditions, and elevated oxidative damage was observed in genetic or functional knockouts for these proteins (Nedergaard et al., 2001; Cadenas et al., 2002; McLeod et al., 2005). Our recent studies have shown that eastern oysters (*C. virginica*) express uncoupling proteins homologous to UCP2 and 3 (Sokolova and Sokolov, 2005) raising questions about possible functions of these proteins in oyster mitochondria. Antioxidant defense appears a feasible candidate for the ancestral function of UCPs, which may also be expected to be found in oysters, given that mitochondrial generation of ROS and thus the need of antioxidant protection is an early and universal feature of aerobic eukaryotes.

The goals of the present study were to study the combined effects of temperature and a toxic metal, Cd, on mitochondrial oxidative stress in eastern oysters using mitochondrial aconitase as a marker of oxidative damage, and to assess the potential role of UCPs in antioxidant protection of oyster mitochondria. We hypothesized that if UCPs are involved in antioxidant protection of oyster mitochondria, their inhibition by purine nucleotides will result in elevated oxidative stress and thus inhibition of mitochondrial aconitase, whereas stimulation by free fatty acids will alleviate these effects. We also hypothesized that exposure to Cd will result in oxidative stress in oyster mitochondria, and so tested whether the role of UCPs in antioxidant protection may be more prominent under these pro-oxidant conditions. To the best of our knowledge, this is the first study to address the functional role of UCPs in invertebrate mitochondria and describe the unusual sensitivity of mitochondrial aconitase to allosteric inhibition by purine nucleotides.

Materials and methods

Animal collection and maintenance

Adult oysters, *Crassostrea virginica* (Gmelin) (70–120 mm shell length) were collected in spring 2006 from Stump Sound, North Carolina, USA. The study site has very low background concentrations of heavy metals and organic pollutants (Mallin et al., 1999) (J. Schwarzenberg and B. Aquafood, personal communication). Animals were transported to the University

of North Carolina at Charlotte within 8 h and acclimatized at 20°C and 30‰ in recirculating aerated tanks with artificial sea water (Instant Ocean®, Kent Marine, Acworth, GA, USA) for at least 4 weeks prior to experimentation. They were fed *ad libitum* with a commercial algal blend (2 ml per oyster every other day) containing *Nannochloropsis*, *Tetraselmis* and *Isochrysis* spp. (PhytoPlex®, Kent Marine, Acworth, GA, USA).

Mitochondrial isolation

Mitochondria were isolated from oyster gills by differential centrifugation as described (Sokolova, 2004). Isolation buffer consisted of 400 mmol l⁻¹ sucrose, 100 mmol l⁻¹ KCl, 50 mmol l⁻¹ NaCl, 16 mmol l⁻¹ EGTA, 30 mmol l⁻¹ Mops (pH 7.5), 2 mg ml⁻¹ protease inhibitor aprotinin, 0.5 mmol l⁻¹ DL-dithiothreitol, 5 mmol l⁻¹ citric acid (trisodium salt) and 0.1% β-mercaptoethanol. Citric acid, dithiothreitol and mercaptoethanol were omitted in isolations used for determination of ROS production and mitochondrial oxygen consumption (see below). The resulting mitochondrial pellet was washed and resuspended in ice-cold EGTA-free isolation buffer to minimize Cd²⁺ binding by the chelator. All assays were completed within 2 h. Respiratory control ratios (RCR) in oyster mitochondria isolated using this technique averaged 2–2.5, which is within the range of values earlier reported in marine mollusks (Abele et al., 2002; Heise et al., 2003; Sokolova, 2004; Cherkasov et al., 2006b). Protein concentrations in mitochondrial suspensions were measured using a modified Biuret method with 1% Triton X-100 (Bergmeyer, 1985).

Mitochondrial respiration and ROS production

Mitochondrial rate of oxygen consumption (\dot{M}_{O_2}) at 20°C was measured using Clarke-type electrodes in the presence of an ATPase inhibitor oligomycin (2.5 µg ml⁻¹) as described by Sokolova (Sokolova, 2004). ROS production was measured with a fluorescence spectrophotometer (Hitachi F-2500, Hitachi Ltd, Japan) in a water-jacketed cuvette at 20°C by an increase in the fluorescence of dihydrorhodamine (DHR123) (4 µmol l⁻¹) at excitation wavelength 505 nm and emission wavelength 534 nm (excitation and emission slits 10 nm) as described elsewhere (Abele et al., 2002). Both ROS generation and mitochondrial \dot{M}_{O_2} were measured in the absence of Cd (control) or after addition of 50 µmol l⁻¹ Cd as CdCl₂ to mitochondrial suspensions. Our earlier studies have shown that Cd is strongly accumulated by oyster mitochondria (Sokolova et al., 2005b); therefore, it can affect external as well as matrix-side mitochondrial sites. ROS generation and \dot{M}_{O_2} were measured using 1.6 mmol l⁻¹ malate and 2.5 mmol l⁻¹ pyruvate for the full electron transport chain (complexes I, III and IV) and 20 µmol l⁻¹ of reduced decyl-ubiquinol (DBH₂) in the presence of a complex I inhibitor, rotenone (2 µmol l⁻¹) for complexes III and IV. Background (non-mitochondrial) \dot{M}_{O_2} and ROS generation was measured after inhibition of complex IV with 1 mmol l⁻¹ KCN. ROS production was calibrated with xanthine/xanthine oxidase ROS-generating system. Preliminary tests with xanthine/xanthine oxidase ROS-

generating system have shown that these substrates and inhibitors do not interfere with the detection of ROS. All values were corrected for electrode drift and background respiration or ROS production. The rates of ROS formation were expressed as nmol H₂O₂ min⁻¹ mg⁻¹ protein, and the percentage of oxygen converted to ROS was determined by dividing the rate of ROS formation by mitochondrial \dot{M}_{O_2} .

Aconitase activity

Aconitase activity was measured spectrophotometrically using an NADPH-coupled assay (Talbot and Brand, 2005) with a UV-Vis spectrophotometer (VARIAN Cary 50 Bio, Cary NC, USA) equipped with a temperature-controlled cuvette holder at 20°C or 30°C (±0.1°C). Assay medium consisted of 200 mmol l⁻¹ sucrose, 250 mmol l⁻¹ mannitol, 150 mmol l⁻¹ KCl, 150 mmol l⁻¹ NaCl, 1 mmol l⁻¹ MgCl₂, 10 mmol l⁻¹ KH₂PO₄ and 30 mmol l⁻¹ Hepes (pH 7.4), 38.5 mmol l⁻¹ succinate, 3.25 mmol l⁻¹ citrate, 0.52 mmol l⁻¹ NADP and 0.3–0.5 i.u. ml⁻¹ isocitrate dehydrogenase (IDH). Aconitase activity was determined as a rate of increase in NADPH absorbance at 340 nm.

We performed two sets of experiments to determine indirect (ROS-mediated) and direct effects of cadmium, purine nucleotides or fatty acids on aconitase activity. For ROS-mediated effects, mitochondrial suspensions in the assay medium were incubated for 20 min at the respective assay temperature in the absence of additives (control) or with addition of the varying amounts of cadmium (10–250 µmol l⁻¹ Cd as CdCl₂), purine nucleotides (0.5–10 mmol l⁻¹ ATP, ADP and GDP), free fatty acids (0.5–5 µmol l⁻¹ of oleic and linoleic acid) or 2% fatty acid free bovine serum albumin (BSA). After the incubation, background absorption was recorded and mitochondria were solubilized with 10% Triton X-100 solution to release the enzyme. Preliminary studies have shown that addition of up to 25% Triton X-100 does not interfere with the aconitase assay (data not shown). To determine whether Cd, nucleotides or fatty acids have direct (non-ROS-mediated) effects on aconitase activity, 50–100 µmol l⁻¹ Cd, 1–3.5 mmol l⁻¹ ATP or GDP and 2–5 µmol l⁻¹ of oleic or linoleic acid were added to mitochondria after solubilization with Triton X-100, and aconitase activity was recorded. In our assays, no aconitase activity was detected in mitochondrial suspensions prior to addition of Triton X-100 confirming that mitochondria were intact. After Triton X-100 addition, aconitase activity was linear and constant for at least 10–15 min, and we used data for the initial 3–5 min of reaction. At the end of the recording, Fe-Cys buffer (154 mmol l⁻¹ Tris, 10 mmol l⁻¹ cysteine, 10 mmol l⁻¹ Fe²⁺ as FeSO₄, pH 7.4) was added to reactivate aconitase through substitution of Fe²⁺ in the enzyme active center lost as a result of oxidative stress (Rose and O'Connell, 1967). Only slight reactivation (<10%) was typically found in control samples at 20°C or 30°C, indicating that isolation conditions were adequate to protect aconitase from oxidative damage (data not shown). The degree of reactivation in Cd-treated samples varied depending on Cd concentrations (see Results).

mRNA expression of mitochondrial uncoupling proteins

Total RNA and mRNA were extracted from the gill tissue of oysters as described elsewhere (Sokolova et al., 2005b). Target fragments were amplified from 30–50 ng of *C. virginica* mRNA using OneStep RT-PCR kit (QIAGEN, Valencia CA, USA) under the following conditions: reverse transcription step of 30 min at 50°C; initial polymerase activation step of 15 min at 95°C; 25–30 cycles of 45 s at 95°C, 45 s at 55°C, 45 s at 72°C; final extension step of 10 min at 72°C. To check for possible DNA contamination of the mRNA samples, we performed RT-PCR with the same reaction mixture omitting the reverse transcription step. No product was obtained, indicating that our samples were not contaminated with DNA (data not shown). Primer sequences were:

For UCP4: UCP4-F15, 5' TGT GAA CAT GGG AGA CTT GTG CAC TTA TGA TA

UCP4-R520, 5' AAT CAC TGA TTG TCT TTA CAG ATA GGC TGA GGC

For UCP5: UCP5-2F117, 5' AGA CTT GTA GAT GGG TGC AGC CTC

UCP5-2R453 5', GCA AGC TCA AAG GGA GAA TGG A

For UCP6: UCP3-6F262, 5' CCA AAA CAA TGA AGG TGG GCG TCC 3'

UCP3-6R574, 5' CAG TGG TCA CTC CCG CGA AGA CA 3'

Amplified fragments were resolved on 1.5% agarose gel, stained with ethidium bromide and photographed using Kodak Documentation System EDAS 290 with Kodak 1D Image analysis software. Amplified fragments were randomly selected, gel-purified, cloned and sequenced as described (Sokolova and Sokolov, 2005) to confirm their identity with expected UCP products.

Calculations and statistics

Concentrations of free nucleotides in solution ($[ATP]_{\text{free}}$ and $[GDP]_{\text{free}}$) were calculated at 20°C and 30°C using a computer program 'Bound And Determined' (Brooks and Storey, 1992). Apparent inhibition constants (IC_{50}) for Cd, free and total nucleotides were calculated assuming noncompetitive inhibition model as concentrations of inhibitors resulting in a 50% decrease of the maximal enzyme velocity (V_{max}) (Segel, 1976). IC_{50} was determined from the intercept and slope of the respective linear regressions, and standard errors for IC_{50} were determined using approximation derived from Taylor expansion (Sokal and Rohlf, 1995) (Z. Y. Zhang, Department of Mathematics, UNC, Charlotte, personal communication). It is worth noting that determination of the apparent IC_{50} in isolated intact mitochondria involves an unknown amount of binding of Cd and nucleotides to mitochondrial proteins, which can reduce concentrations of free inhibitors. However, such binding is also likely to occur *in vivo*, allowing us to assume that the reported inhibition patterns are physiologically relevant.

Repeated-measures ANOVA were used to test the effects of Cd, purine nucleotides and fatty acids at different temperatures on aconitase specific activity after testing the assumptions of

normality of data distribution and homogeneity of variances. Owing to non-homogeneity of variances, a non-parametric one-way analysis of variance (NPAR-ANOVA) was used to analyze the effects of Cd on the rate of ROS production and on percentage conversion of oxygen to ROS. Dunnett tests were used for *post-hoc* comparisons, and LSD (least squares difference) tests for planned comparisons of sample means, as appropriate. Statistical analyses were performed using SAS 9.1.3 software (SAS Institute, Cary, NC, USA). Differences were considered significant if the probability for Type II error was less than 0.05.

Chemicals

All chemicals were purchased from Sigma Aldrich (St Louis, MO, USA) or Fisher Scientific (Suwanee, GA, USA) and were of analytical grade.

Results

Cadmium effects

Cd exposure considerably increased ROS production in oyster mitochondria in the full (complexes I, III and IV) and the partial chain (complexes III and IV) (Fig. 1A). Efficiency

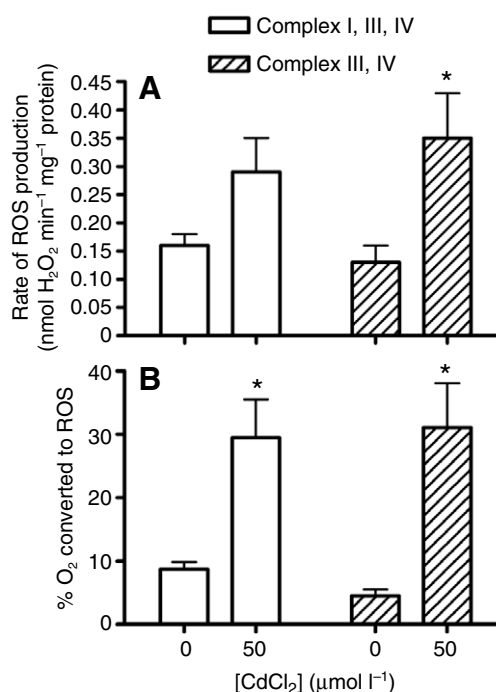


Fig. 1. Cadmium-induced ROS production in oyster mitochondria. (A) Total rate of ROS production in control mitochondria and those exposed to 50 μmol l⁻¹ Cd at 20°C. (B) Percentage oxygen converted to ROS in oyster mitochondria, which was calculated by dividing the rate of ROS production by the rate of mitochondrial oxygen consumption measured with the same substrates at 20°C. ROS production and \dot{M}_{O_2} of complete and partial ETC was measured with complex-specific substrates as described in 'Materials and methods'. *Values significantly different from the respective controls ($P < 0.05$). Values are means \pm s.e.m., $N = 5-7$.

of the full two-electron reduction of oxygen also decreased in Cd-exposed mitochondria, as indicated by a significant rise of the percentage of the consumed O_2 converted to ROS (Fig. 1B). In control mitochondria, only 5–8% of consumed oxygen was converted to ROS which is within the range typical for bivalves (Abele et al., 2002; Heise et al., 2003), whereas in Cd-exposed mitochondria nearly 30% of oxygen was converted to ROS, reflecting an elevated net rate of ROS production, on the one hand, and decreased mitochondrial \dot{M}_{O_2} , on the other.

As expected, aconitase activity increased with increasing temperature in control mitochondria, with activation energy of $77.2 \pm 7.5 \text{ kJ mol}^{-1}$. Cd exposure led to a significant inhibition of aconitase at 30°C but not 20°C (Fig. 2A). At 30°C, a significant decrease in aconitase activity was detected at Cd levels at or above $25 \mu\text{mol l}^{-1}$, and at the highest tested Cd concentration ($200 \mu\text{mol l}^{-1}$) aconitase activity was inhibited by 52%. It is worth noting that a strong inhibition of aconitase achieved at higher Cd concentrations ($\geq 50 \mu\text{mol l}^{-1}$ at 30°C) could not be reversed by addition Fe-cysteine buffer, whereas effects of $10\text{--}25 \mu\text{mol l}^{-1}$ Cd were fully reversible (data not shown). By contrast, at 20°C exposure to $200 \mu\text{mol l}^{-1}$ Cd resulted in only 34% decrease in aconitase activity, and this decrease was statistically non-significant because of large variation. Inhibition constants (IC_{50}) for Cd, which result in 50% decrease in aconitase activity, were $\geq 200 \mu\text{mol l}^{-1}$ (extrapolated $IC_{50}=326 \mu\text{mol l}^{-1}$) and $171 \mu\text{mol l}^{-1}$ at 20°C and 30°C, respectively. Importantly, the observed decrease in aconitase activity was not due to the direct effects of Cd on this enzyme. Cd was found to inhibit aconitase activity only when incubated with intact mitochondria; when added to Triton-

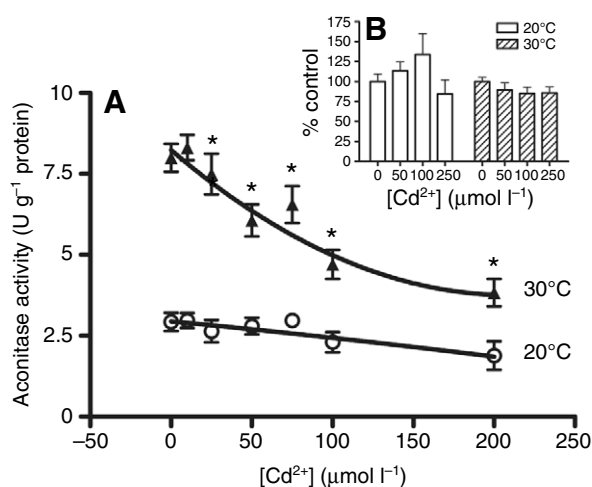


Fig. 2. Effects of Cd exposure on aconitase activity in intact (A) and permeabilized (B) mitochondria of oysters *Crassostrea virginica*. Cd was either incubated with intact mitochondria (A), or directly added to Triton X-100 permeabilized mitochondria (B). *Values significantly different from the respective controls ($P < 0.05$). Aconitase activity in B is presented as a percentage of the respective control values. Cd added to permeabilized mitochondria had no effect on aconitase activity ($P > 0.28$). $N=5\text{--}11$, except for $10 \mu\text{mol l}^{-1}$ and $75 \mu\text{mol l}^{-1}$ Cd ($N=3\text{--}4$).

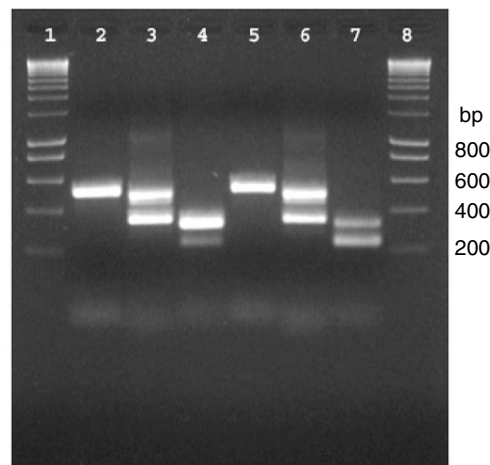


Fig. 3. Expression of mRNA of mitochondrial uncoupling proteins in gill tissues of *Crassostrea virginica*. Lanes 1 and 8: DNA size markers; lanes 2 and 5: UCP4; lanes 3 and 6: UCP5; lanes 4 and 7: UCP6. Samples in lanes 2–4 and 5–7 were mRNA isolated from two individual oysters, respectively.

solubilized mitochondria, this metal had no effect on aconitase (Fig. 2B).

UCP expression and effects of purine nucleotides and fatty acids

Our data show that the mRNA for three homologs of UCPs (UCP4, 5 and 6) are expressed in gill tissues of oysters (Fig. 3). Cloning and sequencing of all the amplified fragments confirmed their high similarity with the expected UCPs. UCP5 and UCP6 were present in two isoforms in oyster transcriptome. Thus, RNA message for at least three UCPs is expressed in gills, indicating that these proteins may be functional in gill mitochondria.

Purine nucleotides significantly inhibited activity of mitochondrial aconitase, with ATP being a considerably stronger inhibitor than GDP or ADP (Table 1). Significant inhibition of aconitase activity was detected at $0.5\text{--}1 \text{ mmol l}^{-1}$ total ATP, whereas it required $3.5\text{--}5 \text{ mmol l}^{-1}$ total GDP or ADP to cause a significant decrease in aconitase activity (Fig. 4). Interestingly, elevated temperatures decreased sensitivity of mitochondrial aconitase to ATP as indicated by the higher inhibition constant at 30°C compared with 20°C (Table 1). Unexpectedly, effects of purine nucleotides on mitochondrial aconitase were similar irrespective of whether they were added to intact or Triton-permeabilized mitochondria ($P > 0.30$) indicating that purine nucleotides act directly as allosteric inhibitors of oyster mitochondrial aconitase.

In order to test for the potential involvement of UCPs in protecting aconitase against ROS-induced inactivation and to avoid the direct inhibitory effects of nucleotides on aconitase, we modified experimental procedure by incubating mitochondria in the presence of a specific UCP inhibitor, GDP or activators (oleic or linoleic acid, $0.5\text{--}5 \mu\text{mol l}^{-1}$) and then removing the additives by wash with the assay buffer. Under

Table 1. Purine nucleotides inhibition constants for mitochondrial aconitase from *C. virginica*

Temperature (°C)	IC ₅₀ (mmol l ⁻¹)		
	[ATP]	[ADP]	[GDP]
20	1.93±0.19 (0.46±0.04)	5.63±1.00* (2.53±0.48)*	6.53±0.56* (2.01±0.17)*
30	3.04±0.37 [†] (0.72±0.06) [†]	6.88±1.25* (3.06±0.53)*	11.03±2.71* (3.41±0.82)*

IC₅₀, 50% inhibition constant.

Values in parentheses are concentrations free nucleotides.

*IC₅₀ values for GDP and ADP significantly different from the respective IC₅₀ for ATP ($P<0.05$); [†]values significantly different from the IC₅₀ for the respective nucleotide at 20°C ($P<0.05$).

these conditions, neither GDP nor free fatty acids had an effect on aconitase activity in control or Cd-exposed mitochondria (Fig. 5, data for linoleate not shown). Also, removal of free

fatty acids by incubation with 2% fatty acid-free BSA did not significantly affect aconitase activity (data not shown). The only exception was addition of 5 $\mu\text{mol l}^{-1}$ oleic acid in Cd-exposed mitochondria, which slightly stimulated aconitase activity, but this stimulation was only statistically significant at

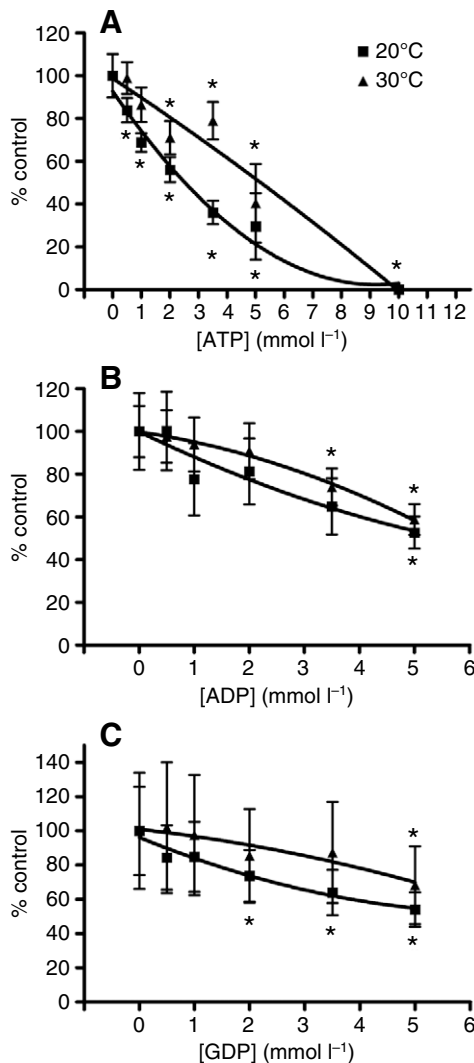


Fig. 4. Effects of purine nucleotides on aconitase activity in oyster mitochondria. (A) ATP, (B) ADP and (C) GDP. Total nucleotide concentrations are given, and aconitase activity is expressed as a percentage of the respective control values. *Values significantly different from the controls ($P<0.05$). $N=3-9$, except for 10 mmol l^{-1} ATP ($N=2$).

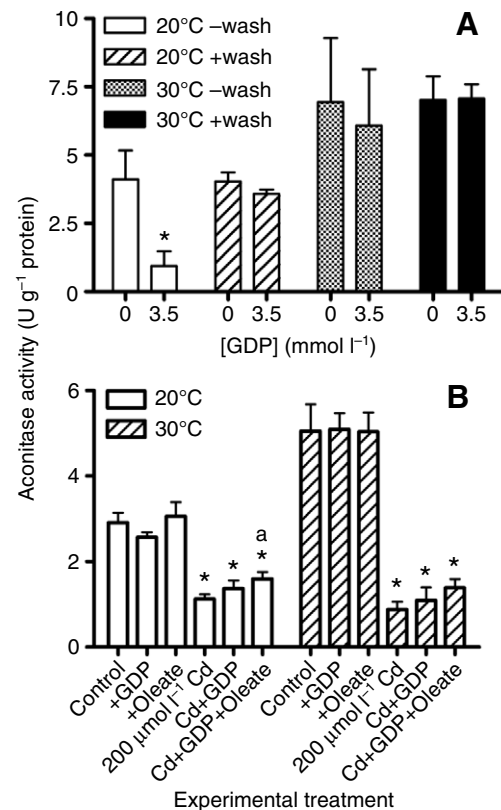


Fig. 5. Uncoupling protein (UCP) involvement in antioxidant protection of aconitase activity in oyster mitochondria. (A) GDP (3.5 mmol l^{-1}) was incubated with intact mitochondria and either removed by washing in nucleotide-free buffer (+wash) or left in the assay medium (–wash) prior to the determination of aconitase activity. *Value significantly different from the respective controls ($P<0.05$). (B) Mitochondria were incubated with GDP (3.5 mmol l^{-1}), oleate (5 $\mu\text{mol l}^{-1}$) and/or Cd (200 $\mu\text{mol l}^{-1}$) and washed to remove the additives prior to the measurements of aconitase activity. *Values significantly different from the respective controls (no Cd; $P<0.05$). ^aValue significantly different from the Cd only value at the respective temperature ($P<0.05$). Values are means \pm s.e.m.; $N=3$ (A), $N=5$ (B).

20°C (Fig. 5). Moreover, it was observed in the presence of GDP, suggesting that this weak protective effect of oleic acid is independent of UCPs.

Discussion

Cd-induced oxidative stress in oyster mitochondria

Earlier studies on oyster aconitase have either failed to detect activity (Jodrey and Wilbur, 1955) or reported extremely low activities of aconitase in oyster tissues (Shoukry, 1982). This may be partially due to the oxidative damage of this enzyme during isolation and purification as indicated by a 35–50% increase in activity of the purified enzyme after addition of Fe^{2+} and cysteine (Shoukry, 1982). In this study, we isolated intact mitochondria instead of obtaining enzyme homogenate or purified enzyme, and released aconitase from mitochondria immediately before testing for its activity, which resulted in the recovery of >90% of aconitase activity and indicated that our method provided adequate protection of aconitase from oxidative damage during isolation.

Exposure of isolated oyster mitochondria to Cd resulted in considerable oxidative stress as indicated by elevated production of reactive oxygen species and decreased activity of mitochondrial aconitase. A decrease in mitochondrial respiration and a simultaneous increase in the net rate of ROS production in response to Cd result in a disproportionately large increase in the percentage of oxygen converted to ROS up to 30%. Notably, Cd effects on mitochondrial aconitase appear to be purely ROS mediated, because direct addition of Cd to aconitase in detergent-solubilized mitochondria had no effect on activity of this enzyme. This finding agrees with an earlier report of mammalian cytosolic aconitase, or iron regulatory protein 1 (IRP-1), in which the holoenzyme of IRP-1 (i.e. cytosolic aconitase) was insensitive to up to 1 mmol l^{-1} Cd, in contrast to the RNA-binding and enzymatically inactive apoform of IRP-1 (Martelli and Moulis, 2004). Similarly, up to 10 mmol l^{-1} Zn^{2+} failed to inhibit purified aconitase from *C. virginica*, whereas transition metals (Ni^{2+} and especially Cu^{2+}) at millimolar concentrations resulted in a decrease of aconitase activity (Shoukry, 1982). Because Shoukry did not measure ROS production in his study (Shoukry, 1982), it is impossible to tell whether Ni- and Cu-induced inactivation of aconitase is due to the direct effects of these metals or, more likely, due to the production of ROS in Fenton-like reactions. Our findings are also in line with the results of earlier studies which have shown that activity of mitochondrial aconitase is a sensitive and specific biomarker of oxidative stress [(Yan et al., 1997; Das et al., 2001) and references therein]. It is worth noting that Cd levels that induce significant ROS production and inhibition of aconitase in oysters are within physiologically relevant intracellular levels in Cd-exposed organisms (Costello et al., 2000; Martelli and Moulis, 2004; Sokolova et al., 2005a), suggesting that elevated mitochondrial oxidative stress may be an important mechanism of Cd toxicity *in vivo*.

Cd-induced oxidative stress in oyster mitochondria was particularly strong and pronounced at elevated temperatures as

indicated by significantly stronger loss of aconitase activity in Cd-exposed mitochondria at 30°C as compared to 20°C. Notably, at 20°C there was no significant damage to mitochondrial aconitase even at the highest tested Cd level ($200 \mu\text{mol l}^{-1}$) despite a strong increase in the rate of ROS generation. This suggests that mitochondrial antioxidants offer adequate protection against the Cd-induced increase in ROS production at this temperature. By contrast, at 30°C, antioxidant systems appear to be incapable of coping with Cd-induced ROS generation, and considerable oxidative damage to aconitase ensues. These findings support the results of our earlier study, which showed that exposure of oysters to Cd *in vivo* results in a significant accumulation of malondialdehyde (MDA, a by-product of lipid peroxidation) at elevated temperatures (24°C and 28°C) but not at 20°C (Lannig et al., 2006). The present study suggests that elevated mitochondrial ROS production in response to Cd may be a mechanism accounting for the observed lipid peroxidation at higher temperatures. The findings of this study also support the view that elevated temperatures can exacerbate Cd-induced oxidative stress in oyster mitochondria and stresses the importance of the thermal context for accessing metal toxicity in poikilothermic organisms.

Oxidative inactivation of aconitase (such as was observed as a result of Cd exposure in this study) may have important implications for the mitochondrial function and can serve as a ‘circuit breaker’, which decreases flux through the TCA cycle and limits the supply of reducing equivalents to the electron transfer chain of mitochondria, slowing down the rate of ROS formation (Gardner and Fridovich, 1991). By contrast, when conditions require elevated flux through TCA (e.g. in actively phosphorylating mitochondria), mitochondrial aconitase can be reactivated by Fe^{2+} and cellular thiols, thus restoring the circuit (Vasquez-Vivar et al., 2000). Our experiments with addition of Fe^{2+} and cysteine to oxidatively inactivated aconitase suggest that similar mechanism may also be functional in oysters. However, at high levels of Cd-induced oxidative inactivation, Fe^{2+} and cysteine fail to completely restore aconitase function, suggesting that excessive oxidative stress may result in the ‘point of no return’ for this enzyme. The mechanisms of this irreversible inactivation of oyster aconitase are not known but it may reflect formation of aggregates of aconitase apoprotein, which are resistant to reactivation, such as shown for aconitase from other organisms (Bota et al., 2002; Grune et al., 1998; Bota and Davies, 2002; Martelli and Moulis, 2004). Irreversible damage of aconitase in Cd-exposed mitochondria may have important implications for Cd toxicity through effects on mitochondrial energy production, as well as on stability of mitochondrial DNA, which can quickly degrade when mitochondrial aconitase activity decreases (Chen et al., 2005; Shadel, 2005).

Allosteric effects of purine nucleotides on aconitase

The present study revealed a unique property of mitochondrial aconitase in oysters, which to the best of our knowledge has not been reported in other organisms – namely,

its sensitivity to allosteric inhibition by purine nucleotides. It is worth noting that in this study the apparent inhibition constants IC_{50} for purine nucleotides were determined at saturating substrate concentrations and further research is needed to determine IC_{50} at physiological concentrations of aconitase substrates and products. However, relative sensitivity of aconitase to inhibition by purine nucleotides strongly suggests that ATP but not GDP or ADP is likely to play a role in allosteric regulation of aconitase *in vivo*. The degree of inhibition of mitochondrial aconitase by purine nucleotides decreased in the order ATP>>ADP>GDP, and effects of purine nucleotides appeared to be direct and did not require intact mitochondrial membrane. Notably, IC_{50} for ATP for mitochondrial aconitase is close to physiological levels of this nucleotide (Traut, 1994; Sokolova et al., 2005b). This suggests that inhibition of mitochondrial aconitase by ATP may be relevant under physiological conditions, helping to maintain mitochondrial redox balance and preventing excessive production of NADH during periods of high substrate supply and low energy demand. Interestingly, IC_{50} for ATP is significantly higher (by ~60%) at 30°C than at 20°C suggesting that ATP-induced inhibition of aconitase may be partially released at elevated temperatures. A decreased sensitivity of aconitase to ATP inhibition at high temperatures may be important for ectotherms such as oysters, allowing them to maintain increased aerobic metabolic rates as the temperature rises. By contrast, IC_{50} for ADP and GDP of oyster mitochondrial aconitase are an order of magnitude higher than physiological levels of these nucleotides (Traut, 1994; Sokolova et al., 2005b) and do not significantly change with the temperature.

Overall, our data provide an insight into a possible novel mechanism of regulation of metabolic flux in mitochondria of a model marine invertebrate. In mammals, mitochondrial aconitase is normally considered to catalyze a near-equilibrium reaction and thus not to play an important role in flux regulation in resting cells (Gardner and Fridovich, 1991). The situation may be different in oysters where allosteric inhibition of mitochondrial aconitase by ATP may provide an additional checkpoint for TCA flux regulation. Further investigations are required to determine whether temperature-dependent allosteric regulation by ATP is a general feature of ectotherm aconitases and whether the degree of this temperature dependence may differ between eurytherms (such as oysters) and stenotherms.

Are oyster UCPs involved in antioxidant defense?

Our data show that at least three UCP homologs are expressed in oyster gill tissues. Although protein expression levels could not be determined because of the absence of appropriate antibodies, high levels of mRNA expression suggest that these proteins are functional in oyster gills. One of the oyster UCP homologs (UCP6) is closely related to the mammalian UCP1-3 branch (Sokolova and Sokolov, 2005), two members of which (UCP2 and 3) have been implicated in antioxidant defense in mammalian mitochondria (Goglia and

Skulachev, 2003; Jezek et al., 2004; Nicholls, 2006). In mice, UCP2 or UCP3 knockout results in elevated oxidative damage in their tissues (Nedergaard et al., 2001; Cadenas et al., 2002; McLeod et al., 2005), and inhibition of UCP2 by GDP in mouse mitochondria results in elevated oxidative stress and damage to mitochondrial aconitase (Talbot and Brand, 2005). Two other oyster UCP homologs found in this study (UCP4 and UCP5), are closely related to mammalian UCP4 and UCP5, respectively. The functions of these UCPs are less well understood; although they have been suggested to play a role in antioxidant protection in brain and nervous tissues (Kim-Han et al., 2001; Hagenauer et al., 2005; Andrews et al., 2005), direct evidence for this is lacking and there is no general consensus as to their physiological function.

We used a functional approach to address the question of the possible involvement of UCPs into the antioxidant defense based on the fact that fatty acids are known UCP activators, and purine nucleotides, especially GDP – specific inhibitors of UCPs in all organisms studied so far including mammals, plants and protists [(Talbot and Brand, 2005; Nicholls, 2006; Vercesi et al., 2006) and references therein]. We found no evidence that mitochondrial uncoupling proteins play a significant role in antioxidant protection in oyster mitochondria either under conditions of background ROS generation in resting mitochondria, or during Cd-induced oxidative stress. Incubation with purine nucleotides (ATP, ADP and GDP) had no effect on aconitase inactivation in oyster mitochondria provided that nucleotides were removed prior to the determination of aconitase activity to prevent direct allosteric inhibition. Free fatty acids had a slight stimulatory effect on aconitase activity in Cd-exposed mitochondria, which was significant at 20°C suggesting that they may provide a low level of antioxidant protection, possibly due to the mild uncoupling. However, this effect is unlikely to be UCP-mediated because it is also observed in the presence of a UCP inhibitor, GDP. Although definitive answers about the role of UCPs in oyster mitochondria must await the development of novel genetic tools such as genetic or functional UCP knockouts of oysters, our data are highly suggestive and allow us to hypothesize that UCPs are either not functionally involved in regulation of ROS generation in oyster gills and are specialized on other functions (such as fatty acid transport or regeneration of free coenzyme A in mitochondrial matrix) (Ledesma et al., 2002; Jezek et al., 2004) or that, despite sequence similarity to other UCPs (Sokolova and Sokolov, 2005), regulation of oyster UCPs is cardinaly different from all studied plant and animal UCP isoforms. In any case, our data provide important information that may serve as a starting point for further investigations to elucidate the functions of oyster UCPs and to shed light on the functional evolution of this important family of mitochondrial carriers in invertebrates.

As a corollary, this study showed that temperature can significantly modulate sensitivity of a key mitochondrial enzyme, aconitase, to allosteric regulation by ATP and to Cd-induced oxidative inactivation. These findings suggest a novel mechanism of regulation of TCA flux in mitochondria in

oysters and requires further investigation to test its universality in poikilotherms. Our data also indicate that temperature increase may exaggerate toxicity of Cd so that mitochondrial antioxidant defenses are overwhelmed, leading to elevated oxidative stress. This provides another mechanism for synergistic effects between environmental temperature and pollutants, which may have important implications for survival of poikilotherms in polluted environments during seasonal warming and/or global climate change.

List of abbreviations

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
DBH ₂	decyl-ubiquinol
DHR123	dihydrorhodamine 123
EGTA	ethylene glycol-bis-(beta-aminoethyl ether)-N,N'-tetraacetic acid
GDP	guanosine 5'-diphosphate
ROS	reactive oxygen species
\dot{M}_{O_2}	oxygen consumption rate
UCP	uncoupling protein

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