Marine invertebrate mitochondria and oxidative stress

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1. ABSTRACT

The marine environment confronts its inhabitants with a wide variety of O2 concentrations, as well as with fluctuations of small scale local O2 availability over time. This review analyzes the respiratory response of marine animal ectotherms to fluctuating environmental O2 availability in their specific habitats and reactive O2 species generation under environmental stress, with a special emphasis on temperature. Specifically we compare mitochondrial functioning and reactive O2 species formation in these animals to what is known from endothermal, mammalian species. Among the strategies employed by marine invertebrates to maintain tissue PO2 low and within tolerable margins, the possible role of mitochondrial oxyconformity to control cellular PO2 in water breathers is discussed. Mitochondrial generation of reactive O2 species in ectotherms has been shown to depend on the magnitude of the mitochondrial membrane potential and the mitochondrial H+ leak. Alternative mitochondrial oxidases are described in marine ectotherms and might add to ameliorate reactive O2 species formation. The effect of nitric oxide, which in mammals controls the reduction state of the electron transport by lowering cytchrome oxidase O2 affinity, remains to be investigated in marine invertebrates. A new concept is proposed, showing how the cross talk of reactive O2 species in metabolically low marine invertebrates could support their outstanding hypoxia tolerance under non-stressed conditions.

2. O2 AND RESPIRATION IN THE MARINE ENVIRONMENT

Water contains 30-times less O2 per litre of respiratory medium than air, and O2 diffusion is 8000-times slower in water than in air (1). While this is certainly a constraint that has set limits to water breather metabolic capacities and aerobic scope in many ectotherm bottom dwelling (benthic) species, like marine worms, bivalves and small sedimentary crustaceans, from the point of avoiding O2 toxicity it seems that water breathers may be on the safer side. However, in some environments, like marine intertidal pools, animals can be exposed to extremely fluctuating ambient O2 levels from almost zero O2 at night to hyperoxic conditions during daytime, as micro- and macroalgae start photosynthesizing (2-5). Moreover, animals that are subject to diurnal tidal submersion often experience periodic hypoxia as they close their shells to avoid desiccation. The incoming tide then causes shell opening and re-oxygenation. But also in the open ocean, O2 is not homogeneously concentrated, and animals may pass oxygen minimum layers during vertical migration, or encounter very low oxygenation in the sediment water interface, due to intense chemical and microbial O2 demand. At all events, compared to a rather constant PO2 in air (at one and the same sea level), the marine environment confronts its inhabitants with a wide variety of O2 concentrations and with considerable fluctuations of local small scale O2 availability over time.

2.1. Oxyregulating vs oxyconforming respiration

Exposure to hyperoxic conditions is a problem for an organism, as cellular reactive O2 species formation increases linearly with O2 concentration (6,7). Especially in soft bodied animals, which can take up a major proportion of O2 via the body surface (1,8) elevated O2 concentrations are linked to elevated oxidative stress levels. However, several marine invertebrates respire in an oxyconforming manner (respiration positively correlated with PO2) above the low critical PO2 (= Pc(O2); PO2 below which mitochondrial respiration is limited by cellular O2
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availability) which might help to maintain low both O₂ concentration and O₂ production in the tissue (9-11). In some animals, respiration seems to be oxyconforming above P₅₀(O₂) straight to hyperoxic conditions (>20 kPa (10,12)). In others, especially micro-oxic, hypoxia tolerant species, oxyconformity is only observed within a low range of PO₂ above which O₂ uptake becomes independent of medium PO₂ ((13) for parasitic nematods, (11) for the sedimentary polychaete Heteromastus filiformis). Oxyconformity below 7 kPa was found for example in endoparasitic (13) and free living nematodes from low O₂ sedimentary environments (14). The animals die when exposed to higher PO₂ levels, presumably because of excessive intra-mitochondrial H₂O₂ formation (15), bound to cause cellular damage. In some marine invertebrates, oxyconformity was documented also for isolated cells (see Figure 1 A. Sipunculus nudus haemocytes and muscle cells (10) and mitochondria (16), nematode mitochondria (13), and worm and bivalve mitochondria (12). Oxyconformity in states 2/4 and state 3 was recorded in experiments with mitochondrial isolates from two marine invertebrates: the mud clam (Arctica islandica, mantle) and the polychaete worm (Nereis pelagica, body wall) (12)). Applying the F1-ATPase inhibitor oligomycin, as well as inhibitors for cytochrome oxidase (cyanide) and for the alternative oxidase (salicylhydroxamic acid, SHAM), we could confirm that mitochondrial oxyconformity of Arctica and Nereis mitochondria in resting state 2/4 and in phosphorylating state 3 most likely relates to existence of an alternative oxidase (see chapter 6), and not to PO₂ dependent increase of the mitochondrial H⁺ leak, the futile cycling of H⁺ through the inner mitochondrial membrane which constitutes the major O₂ consuming process in state 4 (chapter 3).

In contrast, most if not all vertebrates are oxyregulators above P₅₀(O₂), which means that they maintain a constant rate of O₂ consumption against variable environmental PO₂ (17,10). Again this applies to mitochondrial (12) for bovine heart mitochondria, see Figure 1) and cellular systems (18-20). Constant respiration in mitochondrial preparations has been attributed to high O₂ affinity of cytochrome a/a₃ (cytochrome oxidase, EC 1.11.1.7), with an O₂ partial pressure of half-maximal saturation (P₅₀) estimated to range below 0.2 Torr (0.03 kPa in resting cardiomycocytes isolated from rat heart (21)). Indeed, the enzyme was found to be fully saturated already at values around 1 Torr (0.13 kPa) (17,19,22). These values are to be compared with the intracellular PO₂ range of 0.2-3 Torr (0.03-0.4 kPa) in mammalian cellular environment (endothelial cells, myocytes, hepatocytes, (21,23-24) and papers cited herein), with lowest values anticipated to reside in the vicinity of the mitochondria.

The only paper so far providing a comparison of cytochrome oxidase O₂ affinity between rat liver mitochondria, and mitochondria of hypoxia-tolerant brine shrimp, Artemia franciscana, reports a higher P₅₀ in the brine shrimp (P₅₀ = 0.057 kPa PO₂) than the rat heart (P₅₀ = 0.024 kPa PO₂) (24). This would indicate brine shrimp mitochondria to require higher O₂ partial pressure for half-maximal saturation under low O₂ conditions and in non-phosphorylating state 4. Although the difference seems small, we are talking here of gradients of O₂ from the outside of the cell/the animal to the vicinity of cytochrome oxidase, so that a small difference at intracellular level may mean a large PO₂ difference in the extracellular medium.

Thus, oxyconformity of respiration on all levels of organismal organization (whole animals, cells and mitochondria) could be one important characteristic at least in several hypoxia tolerant marine invertebrates, and in all oxyregulators below Pc. And it is crucial for survival, because reduced aerobic activity of an animal at low environmental PO₂ that transfers to the mitochondrial level will prevent, or at least delay, early onset of fatal tissue hypoxia and cause a reduction of mitochondrial free radical formation in these animals.

The question is: how do hypoxia tolerant marine animal ectotherms, colonizing sedimentary environments in which O₂ is low but also largely fluctuating in space and over time compared to the rather constant PO₂ in air, control tissue oxygenation to avoid the risk of oxidative stress?

3. POSSIBLE ROLE OF MITOCHONDRIAL OXYCONFORMITY TO CONTROL CELLULAR PO₂ IN WATER BREATHERS

An important characteristic of water breathing animals is the presence of external respiratory organs and the comparative importance of surface respiration (1), especially at low temperatures, when O₂ solubility in the water is high (8,25). Various strategies are employed by marine invertebrates to maintain tissue PO₂ low and within tolerable margins (see the "low tissue oxygen strategy" proposed by Massabau (6)). A prime behavioural strategy of many infaunal sediment dwellers is the retreat to low O₂ sediment horizons, in search for a constant low O₂ environmental atmosphere, preferably between 3 and 7 kPa (26). Thus, podocopid crustaceans move vertically through the sediment, following the O₂ profile in search for the adequate low PO₂ range and, in so doing, avoid higher, and for them hyperoxic, as well as anoxic sediment layers (27). While this strategy is reserved to small, diffusion limited meiofauna species of less than a 1 mm in diameter, apt to migrate within the sediment in search for the adequately oxygenated horizon, the same low tissue PO₂ is effected by ventilation in larger crustaceans (28, 29), as well as in bivalve molluscs (30) and fish (31). Similarly low O₂ levels can be reached in shell water of the marine mud clams like Arctica islandica during prolonged shell closure. In this hypoxia tolerant bivalve, Brand and Taylor (32) reported shell water PO₂ falling to between 10 and 5 kPa during ventilation pauses that lasted over 5 min. PO₂ of 5 kPa was the lowest value reported before the animals opened the shell again and seems to be the lower limit for the active animal. However, Arctica islandica is known for its ability to perform metabolic reduction during which the animals do not ventilate for several hours and days (33), while on the other hand, switching to a hypometabolic state with heart rates as low as 10% of active rates. Inserting a PO₂ sensitive glass fibre optode into the shell, we measured O₂...
Figure 1. Oxyconforming respiration in marine invertebrates on different organismal levels. A. Left: Respiration isolated muscle cells from sipunculide body wall vs PO\(_2\), from (10); Right: Sipunculide isolated mitochondria, respiring in state 3 vs PO\(_2\). Upper graph (open symbols) shows respiration without and lower graph (closed symbols) with oligomycin. Slopes significantly different with p<0.05, from (16). B. Left: Whole animal \(\text{O}_2\) consumption of the marine polychaete Nereis pelagica and the mud clam Arctica islandica vs PO\(_2\). Right: Mitochondrial respiration in both animal ectotherms and in bovine heart mitochondria in different respiratory state measured at different PO\(_2\) levels with malate (Mal) and succinate (Suc) as respiratory substrates. Both graphs taken after (12).
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partial pressure of 5 kPa and even lower during prolonged shell closure lasting several hours in Arctica islandica (D. Abele and C. Guerra, unpublished results).

Control of inner shell water in molluscs and haemolymph PO₂ in crustaceans and molluscs with open circulatory systems (i.e. where inner organs are less vasculated and surrounded by the respiratory fluid) appears of specific importance in oxyconforming species, to control cellular rates of respiration. By keeping PO₂ in respiratory fluids within tolerated margins, the animals avoid an increase in cellular respiration of oxyconforming mitochondria (26). As a drawback, hypoxia-tolerant aquatic ectotherms are prone to disaster when their environment becomes prooxidant. Straightly normoxic conditions (21 kPa) may already be perceived as hyperoxic by these animals ((34) chironomide larvae and Tubifex, (14) free-living micro-oxic nematodes, (15) and (13) parasitic nematodes, (11) the marine polychaete Heteromastus filiformis; (27) podocopid ostracods). Whether their reduced metabolic activity under hypoxic exposure moreover grants these animals to avoid the hypoxia-reoxygenation phenomenon, known from many hypoxia tolerant air breathers (35, 36) and involving burst formation of toxic reactive O₂ species on re-oxygenation/resurfacing, is currently debated (37,38).

4. UNBALANCED TISSUE PO₂ AND MITOCHONDRIAL REACTIVE O₂ SPECIES FORMATION UNDER THERMAL STRESS

Oxygen solubility decreases markedly upon warming of ambient water, whereas respiration rates of ectothermal animals (39-40) and especially of isolated mitochondria (40-43) increase at higher temperature. It results that ectotherms, subject to pronounced environmental warming, become O₂ limited. The phenomenon of temperature induced functional hypoxia has been broadly studied in marine invertebrates and fish in various groups, and can be summarized in the finding that especially in fish, the hearts own O₂ supply is compromised at critically high temperature, because thermally stressed pericardial mitochondria consume more O₂ than can be delivered by accelerated ventilation and by the circulatory system (25,44-45).

We have studied H₂O₂ formation in marine mollusc mitochondrial preparations and found that it increases critically at elevated temperatures. Above the critical temperature, mitochondrial H₂O₂ formation increased to about 6 and 8% of O₂ turnover in state 4, presumably as mitochondria became thermally damaged (41-42). At the same time, state 4 respiration increased over-proportionally (41), indicating increased mitochondrial H⁺ leak, and less efficient energy conservation in critically heated mitochondria. The higher H⁺ leak may reflect induction of the mitochondrial transmission pore by the forming H₂O₂ and may represent a first indication of a presumably irreversible mitochondrial loss of function and apoptosis induction at high temperature (46-47). On the other hand, higher H⁺ leak can also be induced by O₂; activating the mitochondrial uncoupling protein UCP (48). Just recently UCPs have been identified in marine invertebrates (49) and may add to control reactive O₂ species generation by altering the mitochondrial membrane potential (membrane potential and reactive O₂ species see chapter 5).

We have previously shown that elevated oxidative stress accompanies critical warming of marine animal ectotherms, specifically molluscs (39,41,50). Also, oxidative stress was identified to cause coral bleaching on exposure to critically high temperatures (51). In the North Sea eelpout Zoarces viviparus, transfer to critically high temperatures (18, 22 and 26°C) caused an increase of lipid and protein oxidative damage parameters. The effect was even more pronounced when the fishes were returned to control temperature (12°C) (52). Onset of functional hypoxia was confirmed by the induction of a hypoxic response (hypoxia inducible factor binding to DNA probe in gel shift assay) at 18°C, but as temperatures approached to upper critical limit (22°C), was abolished possibly by heat induced elevated free radical formation (52). Thus, oxyconforming marine ectotherms are exposed to elevated cellular formation of reactive O₂ species not only under conditions of increasing tissue oxygenation, but moreover during exposure to stressfully high temperatures and subsequent cooling, therein mimicking hypoxia-reoxygenation injury in mammals. Temperature induced functional hypoxia is further complicated as antioxidant defense systems fail, or are not induced to counterbalance elevated reactive O₂ species production. In Zoarces viviparus neither SOD nor glutathione peroxidase were induced during warming, nor during return to control temperature (52). In the stenothermal mud clam Yoldia eightsi we found a decline of SOD activity above the critical temperature, which led to elevated lipid peroxidation levels under critical warming (39). The sum of experimental data on induction of antioxidant enzymes during heat stress indicates that while these enzymes can be induced within the thermal tolerance range of a species, above the critical temperature enzyme activities are thermally compromised, as is protein stability and synthesis in general, giving rise to elevated oxidative stress (summarized in (38)).

5. WHAT MECHANISMS CONTROL REACTIVE O₂ SPECIES FORMATION IN MARINE INVERTEBRATE MITOCHONDRIA?

Mitochondria stem from anaerobically functioning proterozoic ancestors, alpha-proteobacteria, that became associated with archaeabacterial host cells during early evolution (53) in an atmosphere, low in O₂ (around 3 kPa) and mildly sulphidic owing to slow rates of sulphate reduction (54). In the low O₂ environment, the anaerobic capacities of the endosymbiont were partly conserved and enabled the evolution of anaerobically working mitochondria as a second prototype, besides the entirely aerobic vertebrate mitochondrion. To date, anaerobically functioning mitochondria are found in parasitic helminthes and hypoxia tolerant marine
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invertebrates (55). Marine invertebrate mitochondria are endowed with especially adapted electron transport systems for anaerobic fermentation of malate, resulting in succinate and short chained organic acids, acetate and propionate, as end-products (reviewed by Grieshaber and colleagues (56)). It is by now a well accepted view that a prime function of early mitochondrial activity in an atmosphere of changeable oxygenation was, to control intracellular \( \text{PO}_2 \) in diffusion limited organisms, by reducing \( \text{O}_2 \) to water, and keeping the production of reactive \( \text{O}_2 \) species low (57-58).

Mitochondria consume about 95% of the cellular \( \text{O}_2 \) in unstressed cells ([59] for resting rat hepatocytes) and are considered the major cellular sites of reactive \( \text{O}_2 \) species production (60-65) especially under stress (62, 66). In vertebrate mitochondria the conversion of \( \text{O}_2 \) to univalently reduced \( \text{O}_2^- \) anions in vitro is estimated to about 2%. The extent to which reactive \( \text{O}_2 \) species formation occurs in vivo, and the rate of escape of radicals to the cytoplasm are still under debate (24,67-69). Mitochondrial matrix and presumably also to the intermembrane space complex III ubiquinone (QH), which then leaks \( \text{O}_2 \) (i.e. ADP stimulated oxidative phosphorylation) delta-\( \text{P}_{\text{im}} \) for significant \( \text{O}_2 \) in unstressed cells ((59) for resting rat hepatocytes) and the reported mammalian studies, \( \text{O}_2 \) (2O2) formation in marine invertebrates (55). Marine invertebrate mitochondria are considered the major cellular sites of reactive \( \text{O}_2 \) species formation ([43]) from the North Sea, we found a lower membrane potential (mp: 147 mV, Figure 1) in mitochondria isolated during winter (43). These mitochondria produced only one quarter of the \( \text{H}_2\text{O}_2 \) released by summer animal mitochondria (mp: 161 mV) under the same in vitro conditions. Possibly in response to rapid temperature fluctuations on intertidal mudflats during summer, lugworm mitochondria from summer animals responded to warmer experimental temperature (10°C vs 1°C) with an immediate increase of \( \text{H}^+ \) leakage, whereby reducing membrane potential and \( \text{H}^+ \) motive force, and ameliorating thermally accelerated reactive \( \text{O}_2 \) species formation. Thus, the mild uncoupling effect of \( \text{H}^+ \) leak on mitochondrial membrane potential (57) seems to be an important controlling factor of reactive \( \text{O}_2 \) species formation with an ecological function in marine, but also in air-breathing invertebrates. Miwa and Brand (65) observed that a reduction of membrane potential by only 10% reduced \( \text{H}_2\text{O}_2 \) production at complex I (from reverse electron flow) by as much as 70% in insect flight muscle mitochondria. Contradictory, however, we measured highest rates of \( \text{O}_2 \) conversion to reactive \( \text{O}_2 \) species in marine mud clams with the lowest membrane potential so far reported for a marine ectotherm (130 mV in state 4). In fact, to our knowledge, mud clams had the lowest membrane potential so far reported for endo- or ectotherms (43,77). Along with this low membrane potential, state 4 respiration was extremely low (1.5 nmol \( \text{O}_2 \) min\(^{-1} \) mg\(^{-1} \) mitochondrial protein) in the mud clam \( \text{Mya arenaria} \), and \( \text{H}_2\text{O}_2 \) formation ranged low but well detectable at 0.12-0.07 nmol \( \text{H}_2\text{O}_2 \) min\(^{-1} \) mg\(^{-1} \) protein, with a percent conversion of \( \text{O}_2 \) to reactive \( \text{O}_2 \) species amounting to up to 20% of state 2/4 \( \text{O}_2 \) consumption. In lugworm (body wall) and bivalve (mantle tissue) mitochondria we used 3 mM succinate as respiratory substrate, rotenone to prevent (reverse) electron flow via complex I, and no antimycin A, but oligomycin to suppress residual ATPase activity in state 4. Under these conditions, \( \text{H}_2\text{O}_2 \) can be formed only at complex III and, possibly to a minor extent, complex II.

So what controls mitochondrial \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) formation in marine invertebrates in vitro? In a recent study comparing mitochondrial functions of seasonally warm (summer) and cold (winter) adapted marine lugworms (\( \text{ Arenicola marina} \)) from the North Sea, we found a lower membrane potential (mp: 147 mV, Figure 1) in mitochondria isolated during winter (43). These mitochondria produced only one quarter of the \( \text{H}_2\text{O}_2 \) released by summer animal mitochondria (mp: 161 mV) under the same in vitro conditions. Possibly in response to rapid temperature fluctuations on intertidal mudflats during summer, lugworm mitochondria from summer animals responded to warmer experimental temperature (10°C vs 1°C) with an immediate increase of \( \text{H}^+ \) leakage, whereby reducing membrane potential and \( \text{H}^+ \) motive force, and ameliorating thermally accelerated reactive \( \text{O}_2 \) species formation. Thus, the mild uncoupling effect of \( \text{H}^+ \) leak on mitochondrial membrane potential (57) seems to be an important controlling factor of reactive \( \text{O}_2 \) species formation with an ecological function in marine, but also in air-breathing invertebrates. Miwa and Brand (65) observed that a reduction of membrane potential by only 10% reduced \( \text{H}_2\text{O}_2 \) production at complex I (from reverse electron flow) by as much as 70% in insect flight muscle mitochondria. Contradictory, however, we measured highest rates of \( \text{O}_2 \) conversion to reactive \( \text{O}_2 \) species in marine mud clams with the lowest membrane potential so far reported for a marine ectotherm (130 mV in state 4). In fact, to our knowledge, mud clams had the lowest membrane potential so far reported for endo- or ectotherms (43,77). Along with this low membrane potential, state 4 respiration was extremely low (1.5 nmol \( \text{O}_2 \) min\(^{-1} \) mg\(^{-1} \) mitochondrial protein) in the mud clam \( \text{Mya arenaria} \), and \( \text{H}_2\text{O}_2 \) formation ranged low but well detectable at 0.12-0.07 nmol \( \text{H}_2\text{O}_2 \) min\(^{-1} \) mg\(^{-1} \) protein, with a percent conversion of \( \text{O}_2 \) to reactive \( \text{O}_2 \) species amounting to up to 20% of state 2/4 \( \text{O}_2 \) consumption. In lugworm (body wall) and bivalve (mantle tissue) mitochondria we used 3 mM succinate as respiratory substrate, rotenone to prevent (reverse) electron flow via complex I, and no antimycin A, but oligomycin to suppress residual ATPase activity in state 4. Under these conditions, \( \text{H}_2\text{O}_2 \) can be formed only at complex III and, possibly to a minor extent, complex II.
In another series of experiments we used scallop adductor muscle, as we assumed that H₂O₂ formation might be higher in muscle fibers, supporting burst swimming activity. In these experiments we used either 24 mM succinate or 30 mM glutamate as substrate, and applied complex III inhibitor antimycin A and SOD, which converts extra-mitochondrially generated O₂⁻ to H₂O₂, in order to achieve a significant H₂O₂ signal (E. Philipp and D. Abele unpublished data, Figure 2). Hydrogen peroxide formation under succinate, succinate/rotenone and glutamate respiration remained below the detection limit (<0.01 nmol H₂O₂ mg⁻¹ protein min⁻¹), but was markedly increased with both substrates, when antimycin A was added and significant after SOD addition (Figure 2). In this case, H₂O₂ can be formed at complex I and III and on both, matrix and cytoplasmic side of the mitochondrial membrane (7,67).

This leads us to the conclusion that O₂⁻ and H₂O₂ production in marine invertebrates seems to occur mainly during forward electron transport and is sensitive to mild uncoupling of H⁺ motive force. This mechanism matches the need for high metabolic flexibility in oxy- and thermoconforming eucaryotes and scallops, capable of burst swimming activity. A controlling function of the H⁺ leak towards forward reactive O₂ species formation could be an advantage not only under thermal stress in small eucaryotes, but also during periods of limited O₂ availability or inhibition of cytochrome oxidase by microbial H₂S, typical for the North Sea intertidal environment of Arenicola marina and Mya arenaria (78-80). Environmental hypoxia below cytochrome oxidase saturation levels may slow electron transport in the lower part of the respiratory chain and propagate O₂⁻ and H₂O₂ formation from reduced electron carriers, like complex III ubiquinone (57,62). Also, these reduced carriers presumably autoxidize, to generate more O₂⁻ and H₂O₂, during onset of re-oxygenation (see ischemia-reperfusion injury). Theoretically, mild uncoupling might limit excessive O₂⁻ and H₂O₂ formation during re-oxygenation by re-oxidizing mitochondrial respiratory complexes, including b-type cytochromes (65). Presently unknown, while quite possible, reverse electron flow to NADH-oxidoredutase complex I could be fuelled by anaerobic succinate, accumulating during the first 2 h of hypoxia exposure of Arenicola marina (81). This could cause complex I reactive O₂⁻ species formation in hypoxic worms, before they induce metabolic reduction, as hypoxic electron transport is slow, but not blocked, and membrane potential presumably kept high, in order to sustain maximal energetic coupling.

Another mechanism to maintain electron flow and prevent reduction of the electron transport systems in a diffusion limited tissue under environmental O₂ limitation would be a nitric oxide (NO) effect which lowers cytochrome oxidase oxygen affinity. For mammals it has recently been recognized that NO interacts with the O₂ binding site of cytochrome oxidase, raising the P₅₀ for the affinity of mitochondrial respiration for O₂ (Kₐ₅ (82-84)) in peripheral cells. This facilitates O₂ transport to cells, located deeper within the tissue and remote from surface diffusion. When supplemented with the nitric oxide synthase (NOS) substrate L-arginine, isolated mammalian mitochondria show a significant decrease in their respiratory rates, whereas mitochondria, supplemented with NOS inhibitors, exhibit a significant respiratory increase (85). The two effects are explained by the continuous production of NO by mitochondrial NOS (mt-NOS) and by the reversible and O₂-competitive inhibition of cytochrome oxidase (86). For mammals it is increasingly accepted that mitochondrial, as well as cellular O₂ uptake are regulated by adenosine diphosphate (ADP), O₂⁻ and NO, and, moreover, that the rate of cellular ATP formation depends on the O₂/NO partitioning through the mitochondrial membrane (82). NOS activity has recently been confirmed in several marine (87,88) and freshwater molluscs (89) and characterized, using specific inhibitor sets known from mammalian NOS isoforms. However, up to present, NO effects on marine invertebrate mitochondria wait to be studied and no mt-NOS activity in these animals has so far been reported. It seems intriguing to speculate that NO could be involved in metabolic down regulation (see (82)) which would minimize reactive O₂⁻ species formation under environmental hypoxia in marine ectotherms.

It is interesting and yet unexplained, why in more actively swimming scallops (mantle tissue (90) and unpublished results for adductor muscle mitochondria, Figure 2), as well as in fish mitochondria isolated from the liver of North Sea eelpout (Zoarces viviparus, K. Heise & D. Abele unpublished results), H₂O₂ formation was below detection limit in both respiratory states. With a respiratory control ratio (RCR) of 3.0 (succinate respiration), scallop mitochondria were slightly better coupled than mud clam mitochondria, the latter exhibiting significant H₂O₂ formation under the same in-vitro conditions (76). The high RCR was due to higher state 3 respiration of scallop mitochondria, recorded at the same low membrane potential as in mud clams: 100 to 130 mV. We conjecture that mitochondria from actively swimming marine ectotherms differ not only with respect to O₂⁻ and H₂O₂ forming capacities, but that also O₂⁻ and H₂O₂ quenching capacities by mitochondrial superoxide dismutase and...
glutathione peroxidase (91) might be higher in more active species. This makes sense, since higher respiratory intensity in both states 3 and 4 would otherwise cause elevated reactive O₂ species formation. In keeping with this, mitochondrial Mn-SOD activity was absent in gill tissues of hypoxia tolerant mud clams (Astarte borealis, Cardium edule and Arctica islandica (92)). The mechanistic basis for the extremely low O₂ and H₂O₂ formation in scallop mitochondria may also be rooted in a more favorable, i.e. higher ratio of complex IV (cytochrome a/a₃) to complex I-II components within the respiratory chain than in the anaerobically functioning mitochondria of hypoxia tolerant animals. However, quantification of respiratory chain complexes and ratios of respiratory complex molecular amounts still wait to be investigated in marine species.

So, mitochondrial O₂⁻ and H₂O₂ formation seems to be less controlled in sluggish than in more active marine invertebrates. This makes sense if the system is analyzed in the cellular and the whole animal context. To begin with, O₂⁻ and H₂O₂ formation rates in marine ectotherms are thermally slowed because of their low body temperature, when compared to endotherms with a constant body temperature above 35°C. Moreover, O₂ turnover and mitochondrial densities are much lower in sluggish marine invertebrates than in most cell types of vertebrates (41,43), but can reach considerably higher densities in fish muscle (93,94) and presumably scallop adductor muscle. However, as stated by Brand (57), O₂⁻ and H₂O₂ production is not a linear function of the electron transport rate, but can be modulated by other features of the electron transport system. Thus, no linearity of O₂⁻ and H₂O₂ formation should be expected when extrapolating to lower temperatures, as is already seen from our comparison of O₂⁻ and H₂O₂ formation rates between endo- and ectotherms, investigated at habitat temperature conditions.

6. ALTERNATIVE OXIDASE ACTIVITY IN MARINE INVERTEBRATE MITOCHONDRIA

Cyanide-insensitive respiratory pathways are electron transport chains that terminate in so called alternative end oxidases (AOX). They branch off the classical respiratory chain after complex I and transfer electrons from reduced ubiquinone (Coenzyme Q) onto an alternative cytochrome oxidase, which reduces O₂ to water (95,96). The system is located in the inner mitochondrial membrane (97) and differs from the classical electron transport pathway as it does not pump H⁺ from the matrix to the inter-membrane space. Also, O₂ affinity of the AOX (Km > 0.08 kPa) is considerably lower than that of cytochrome oxidase (Km < 0.00078 kPa) (98). Moreover, AOX pathways are insensitive not only to cyanide, but also to azide, CO, antimycin A, and myxothiazol (99) and, instead, specifically inhibited by salicylhydroxamic acid (SHAM) (100). The prospective function of AOX is dual: (i) to maintain respiratory electron flux, substrate oxidation and cellular redox potential when the classical cytochrome oxidase is inhibited by cyanide, sulfide or other respiratory inhibitors, (ii) to maintain redox balance and intracellular PO₂ under ADP limiting, metabolically down regulated conditions, or when O₂ levels within the cell start to rise because the classical respiratory chain is O₂ saturated (see (12)). This implies that the AOX system minimizes the risk of oxyradical formation and therefore it can be assigned with an antioxidant function (70). AOX pathways fuel phosphorylation only via complex I NAD⁺-linked substrates at the first coupling site (NADH-dehydrogenase complex I) (Figure 3). We postulated that the mitochondrial AOX system in hypoxia tolerant marine invertebrates enables the cell to maintain some level of respiratory activity under conditions of unfavorably high ATP/ADP ratio (during metabolic reduction), when the electron transport activity via the main respiratory chain declines and electrons from respiratory substrate oxidation can be deviated via the non- or less phosphorylating AOX pathway, to maintain cellular redox balance and energetic homeostasis (12,16).

Alternative oxidase expression is influenced by stress-stimuli cold, oxidative stress, pathogen attack, and by factors restricting electron flow through the cytochrome pathway of respiration, like hydrogen sulphide or cyanide inhibition ((80) marine polychaete mitochondria, (101) Euglena gracilis mitochondria). Control over the AOX...
activity is exerted at the levels of gene expression, as well as in response to availability of carbon and reducing potential. Post-translational control involves reversible covalent modification of the AOX and activation by specific carbon metabolites (101).

The presence of an AOX has previously been indirectly confirmed by its CN-resistance and SHAM sensitivity (12,16,80). Recently the gene has been detected in marine ectotherms from the phyla Mollusca, Nematoidea and Urochordata, and expression confirmed in different tissues of the tunicate Ciona intestinalis and the oyster Crassostrea gigas (102). This corroborates earlier reports of cyanide-resistant respiration in similar species. AOX have further been revealed in plants, fungi and prokaryotes and in the alphaproteobacterium Novosphigobium aromaticivorans (103). Phylogenetic analysis is consistent with the animal proteins having originated from vertical inheritance (101). It is still an open question, whether the gene has been lost in mammalian oxyregulators, or whether it is simply not expressed any more, because intracellular PO2 is confined and controlled within a low and narrow range in these animals (12,26). Regarding the O2 and H2O2 generation by AOX reports are contradictory. Several alternative electron pathways have been characterized and described for free living and parasitic nematodes (13,15,104-105), colonizing a micro-oxic/sulphidic habitat: the gut. The AOX of these predominantly anaerobic nematodes was termed cytochrome o and has been characterized as b-type cytochrome (13). A possible drawback of this oxidase is the generation of H2O2 somewhere along the alternative electron transport pathway (15). In contrast, some bacterial AOXs have been characterized as bd-type cytochromes and are induced at PO2 values above saturation levels of cytochrome oxidase (97,106). Escherichia coli contains two terminal oxidases, one of which is termed cytochrome o. In this case, O2 is reduced to water, not to H2O2 (107). Again, in anoxia tolerant animals, like the nematodes studied by Paget and co-authors, it seems conceivable that H2O2 production was so well measurable, because the mitochondria could be very low in antioxidants, but evidence for this idea needs to be obtained. Contrary, plant mitochondria are endowed with an AOX that reduces O2 to H2O but not H2O2 or O2 (108). Induction or over-expression (109) of the AOX were observed in plants, fungi and prokaryotes and in the alphaproteobacterium Novosphigobium aromaticivorans (103). Phylogenetic analysis is consistent with the animal proteins having originated from vertical inheritance (101). It is still an open question, whether the gene has been lost in mammalian oxyregulators, or whether it is simply not expressed any more, because intracellular PO2 is confined and controlled within a low and narrow range in these animals (12,26). Regarding the O2 and H2O2 generation by AOX reports are contradictory. Several alternative electron pathways have been characterized and described for free living and parasitic nematodes (13,15,104-105), colonizing a micro-oxic/sulphidic habitat: the gut. The AOX of these predominantly anaerobic nematodes was termed cytochrome o and has been characterized as b-type cytochrome (13). A possible drawback of this oxidase is the generation of H2O2 somewhere along the alternative electron transport pathway (15). In contrast, some bacterial AOXs have been characterized as bd-type cytochromes and are induced at PO2 values above saturation levels of cytochrome oxidase (97,106). Escherichia coli contains two terminal oxidases, one of which is termed cytochrome o. In this case, O2 is reduced to water, not to H2O2 (107). Again, in anoxia tolerant animals, like the nematodes studied by Paget and co-authors, it seems conceivable that H2O2 production was so well measurable, because the mitochondria could be very low in antioxidants, but evidence for this idea needs to be obtained. Contrary, plant mitochondria are endowed with an AOX that reduces O2 to H2O but not H2O2 or O2 (108). Induction or over-expression (109) of the AOX were reported to be protective under different forms of stress (110), and to reduce H2O2 generation in isolated plant cells (111) and mitochondria (112).

Mammalian mitochondria are sensitive targets of cytoxic peroxynitrite, forming from the interaction of O2- with NO (73). Like H2O2, peroxynitrite causes damage to mitochondrial electron transport systems and mt-DNA, however, cytochrome oxidase has been shown to be resistant to peroxynitrite-inactivation in vitro up to a concentration of 2 mM (113). NO lifetime is significantly longer at physiologically low intracellular O2 tensions (10-60 microM or 1-5 kPa) than under an atmospheric condition (235 microM or 20 kPa) (114). Thus, the inhibitory effect of NO on mitochondrial energy metabolism can be anticipated to be important at physiological PO2. As stated above, state 3 respiration appears more deeply affected by NO than state 4 respiration, so that the inhibitory effect will be more pronounced in actively phosphorylating mitochondria (82, 83) for rat liver mitochondria). The cyanide-insensitive alternative pathway is less sensitive to NO than the cytochrome oxidase pathway (115). Half maximal inhibition (IC50) was found at 0.3 microM NO for cytochrome oxidase and 3.6 microM NO for the AOX of soybean embryonic axes (116). Thus, the branching alternative electron transport pathway offers an additional route for dissipation of electrons, by-passing the single electron reduced components downstream of ubiquinone, thereby avoiding a major site of mitochondrial O2- and H2O2 formation (71). No studies of NO effects on mitochondrial O2- and H2O2 generation in marine invertebrates have so far been performed. As the alternative respiratory pathway seems to be an inherent characteristic of their adaptation to the extreme fluctuations of environmental PO2 between anoxia, normoxia, and even hyperoxia (12,53), we suggest that the NO/O2 ratio may be an important modulator, shunting mitochondrial electron flux between classical and alternative transport chain. The lower O2 affinity of the AOX provides a simple means of controlling onset and activity of this pathway. The effect may moreover be more pronounced under state 3 conditions, cutting cellular phosphorylation and reducing energy turnover.

Elevated activity of the alternative pathway at high ambient PO2 seems useful for diffusion limited invertebrate tissues, since it could be minimizing the risk of oxidative stress. The theoretical drawback of wasteful oxidation of respiratory energetic substrates at low ATP yields whenever PO2 is high appears adequate for micro-oxicophilic animals. These are highly sensitive to elevated environmental PO2 and, thus, striving at all means to maintain cellular O2 concentration at low levels, also at the expense of burning energy reserves via the alternative pathway. This emphasizes the early phylogenetic origin of this mechanism and confirms the appropriateness of the AOX in early O2 avoiding organisms with low aerobic scope.

We further hypothesize that the onset of the hypoxic response under only mildly hypoxic conditions in marine ectotherms (52) might be a remnant of an ancient pathway that induced NO formation by mitochondrial NOS, shunting electrons into an AOX pathway (where present) and causing a relax of the tight energetic coupling in the classical respiratory chain. This could be an adaptive strategy to induce metabolic down regulation in non-active phases in sluggish benthic marine ectotherms. We even think that this strategy might be much more effective than leak opening in mammals, which was shown to be a process, insensitive to changes in PO2, but is presumably controlled by temperature, and by uncoupling proteins in vertebrates as well as invertebrates (Figure 4).

7. CONCLUSIONS AND PERSPECTIVES

Studying early organisms can teach us not only on evolution of organisal and cellular hardware, but also
Figure 4. A model describing biochemical cross talk between reactive O$_2$ species formation, NO, the AOX and hypoxic response factor (HIF-1), designed to explain the extreme tolerance of marine invertebrates to live under environmental low O$_2$ conditions.

A. Non stress situation are characterized by low metabolic rates and reactive O$_2$ species formation, supported also by a mitochondrial AOX, which enables constitutive expression of HIF-1 (based on stability of O$_2$ sensitive subunit HIF-1, as confirmed for fish). The function of HIF-1 could be a PO$_2$ dependent induction of NOS to alter cytochrome oxidase (cytox) affinity for O$_2$ and shunt electrons via the AOX pathway to further minimize O$_2$ formation. Low O$_2$ levels also minimize production of hazardous peroxynitrite (ONOO$^-$).

B. Homeostatic changes expected for a stress situation: Stress (heat, hyperoxia, salinity stress, heavy metal intoxication) will increase mitochondrial O$_2$ formation leading to increased release of H$_2$O$_2$ into the cytosol, oxidizing cellular redox potential, whereby reducing HIF-1 stability to abrogate the hypoxia response. This would entail loss of control of NO over cytox activity such that mitochondrial respiration in central tissues could out power O$_2$ delivery, leading to onset of stress induced hypoxia. NO levels could further be reduced by formation of ONOO$^-$ owing to elevated mitochondrial reactive O$_2$ species release.
about the mechanisms that adjusted the cellular energetic machinery to function optimally under increasing (and also periodically decreasing) environmental O₂ levels. To early anaerobic and micro-oxophilic organisms, O₂ was a toxic gas, and it seems not too far fetched to imagine that O₂ and its noxious derivatives attained signal function in early organisms. Thus, O₂ and not CO₂ is in control of metabolic rates in water-breathing animals. The mitochondrial respiratory chain of hypoxia tolerant marine ectotherms contains ancient, anaerobic and modern aerobic components. In animals, endowed with an additional alternative electron transport pathway, presumably both respiratory chains can be concurrently utilized, but the alternative pathway with less affinity for O₂ will become more important whenever cytochrome oxidase activity is saturated or (partly or fully) inhibited by H₂S, NO or yet unknown natural inhibitors. Inhibition of cytochrome oxidase causes reduction of classical respiratory chain electron transporters and increases the formation of O₂⁻ and H₂O₂. Release of these intermediates from mitochondria can, theoretically, be ameliorated by deviating electrons through the alternative pathway, and it has been shown in plants, but until to date not for animal systems, that oxidative stress is alleviated by induction of an alternative endoxidase. Instead, opening of the mitochondrial H⁺ leak reduces membrane potential and mitigates the formation of O₂⁻ and H₂O₂ in marine animal mitochondria under exposure to elevated temperatures (within the natural range (43)) whereby again leading to non-phosphorylating O₂ consumption and preventing intracellular hyperoxia and oxidative stress.

Prolonged environmental hypoxia and exposure to minor concentrations of H₂S are well tolerated by many aquatic invertebrates and even by hypoxia tolerant fish. It would therefore make sense to switch on a controlled hypoxic response that deviates electrons to an alternative pathway, not inhibitable by hydrogen sulfide. Investigation of the cross talk of O₂, O₂⁻ and NO from hypoxic activation of NOS (117) by hypoxia inducible transcription factor HIF-1 will be an important field in future studies of marine invertebrate hypoxia and sulfide tolerance. This transcription factor, which is progressively degraded under oxidized tissue redox conditions (118), has recently been shown to support hypoxic survival in fish. However in contrast to the mammalian protein, fish HIF-1 becomes stable already at much higher PO₂ and even close to normoxic conditions (52,119). HIF-1 is currently investigated in several invertebrate phyla, but a detailed discussion of this protein would be beyond the scope of this review. Important in this context, however, reactive O₂ species could be involved in this cross talk in numerous ways, the most perspicuous of which would be the modification of the cellular redox potential to abrogate the hypoxic response (118), modification of SH-groups of redox sensitive enzymes, interaction with hemoglobin resulting in met-hemoglobin formation to support H₂S elimination (120) and the formation of peroxyxinitrite, to counteract the NO signal as schematically described in figure 4.

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