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Marine invertebrate mitochondria and oxidative stress

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TABLE OF CONTENTS

1. Abstract
2. O_2 and respiration in the marine environment
2.1. Oxyregulating vs oxyconforming respiration
3. Possible role of mitochondrial oxyconformity to control cellular PO_2 in water breathers
4. Unbalanced tissue PO_2 and mitochondrial reactive O_2 species formation under thermal stress
5. What mechanisms control reactive O_2 species formation in marine invertebrate mitochondria?
6. Alternative oxidase activity in marine invertebrate mitochondria
7. Conclusions and perspectives
8. Acknowledgement
9. References

1. ABSTRACT

The marine environment confronts its inhabitants with a wide variety of O_2 concentrations, as well as with fluctuations of small scale local O_2 availability over time. This review analyzes the respiratory response of marine animal ectotherms to fluctuating environmental O_2 availability in their specific habitats and reactive O_2 species generation under environmental stress, with a special emphasis on temperature. Specifically we compare mitochondrial functioning and reactive O_2 species formation in these animals to what is known from endothermal, mammalian species. Among the strategies employed by marine invertebrates to maintain tissue PO_2 low and within tolerable margins, the possible role of mitochondrial oxyconformity to control cellular PO_2 in water breathers is discussed. Mitochondrial generation of reactive O_2 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 O_2 species formation. The effect of nitric oxide, which in mammals controls the reduction state of the electron transport by lowering cytochrome oxidase O_2 affinity**,** remains to be investigated in marine invertebrates. A new concept is proposed, showing how the cross talk of reactive O_2 species in metabolically low marine invertebrates could support their outstanding hypoxia tolerance under non-stressed conditions.

2. O_2 AND RESPIRATION IN THE MARINE ENVIRONMENT

Water contains 30-times less O_2 per litre of respiratory medium than air, and O_2 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 ectothermal bottom dwelling (benthic) species, like marine worms, bivalves and small sedimentary crustaceans, from the point of avoiding O_2 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 O_2 levels from almost zero O_2 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, O_2 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 O_2 demand. At all events, compared to a rather constant PO_2 in air (at one and the same sea level), the marine environment confronts its inhabitants with a wide variety of O_2 concentrations and with considerable fluctuations of local small scale O_2 availability over time.

2.1. Oxyregulating vs oxyconforming respiration

Exposure to hyperoxic conditions is a problem for an organism, as cellular reactive O_2 species formation increases linearly with O_2 concentration (6,7). Especially in soft bodied animals, which can take up a major proportion of O_2 via the body surface (1,8) elevated O_2 concentrations are linked to elevated oxidative stress levels. However, several marine invertebrates respire in an oxyconforming manner (respiration positively correlated with $P\mathbf{O}_2$) above the low critical PO_2 (= $P_c(O_2)$: PO_2 below which mitochondrial respiration is limited by cellular O_2

availability) which might help to maintain low both O_2 concentration and O_2^- production in the tissue (9-11). In some animals, respiration seems to be oxyconforming above $P_c(O_2)$ 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 PO_2, above which O_2 uptake becomes independent of medium PO_2 ((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 O_2 sedimentary environments (14). The animals die when exposed to higher PO_2 levels, presumably because of excessive intra-mitochondrial H_2O_2 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 PO_2 dependent increase of the mitochondrial H^+ leak, the futile cycling of H^+ through the inner mitochondrial membrane which constitutes the major O_2 consuming process in state 4 (chapter 3).

In contrast, most if not all vertebrates are oxyregulators above $P_c(O_2)$, which means that they maintain a constant rate of O_2 consumption against variable environmental PO_2 (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 O_2 affinity of cytochrome a/a_3 (cytochrome oxidase, EC 1.11.1.7), with an O_2 partial pressure of half-maximal saturation (P_{50}) estimated to range below 0.2 Torr (0.03 kPa in resting cardiomyocytes 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 PO_2 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 O_2 affinity between rat liver mitochondria, and mitochondria of hypoxia-tolerant brine shrimp, *Artemia franciscana*, reports a higher P_{50} in the brine shrimp (P_{50}= 0.057 kPa PO_2) than the rat heart (P_{50}= 0.024 kPa PO_2) (24). This would indicate brine shrimp mitochondria to require higher O_2 partial pressure for half-maximal saturation under low O_2 conditions and in non-phosphorylating state 4. Although the difference seems small, we are talking here of gradients of O_2 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 PO_2 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 PO_2 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 O_2 is low but also largely fluctuating in space and over time compared to the rather constant PO_2 in air, control tissue oxygenation to avoid the risk of oxidative stress?

3. POSSIBLE ROLE OF MITOCHONDRIAL OXYCONFORMITY TO CONTROL CELLULAR PO_2 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 O_2 solubility in the water is high (8,25). Various strategies are employed by marine invertebrates to maintain tissue PO_2 low and within tolerable margins (see the "low tissue oxygen strategy" proposed by Massabuau (6)). A prime behavioural strategy of many infaunal sediment dwellers is the retreat to low O_2 sediment horizons, in search for a constant low O_2 environmental atmosphere, preferably between 3 and 7 kPa (26). Thus, podocopid crustaceans move vertically through the sediment, following the O_2 profile in search for the adequate low PO_2 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 PO_2 is effected by ventilation in larger crustaceans (28, 29), as well as in bivalve molluscs (30) and fish (31). Similarly low O_2 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 PO_2 falling to between 10 and 5 kPa during ventilation pauses that lasted over 5 min. PO_2 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 PO_2 sensitive glass fibre optode into the shell, we measured O_2

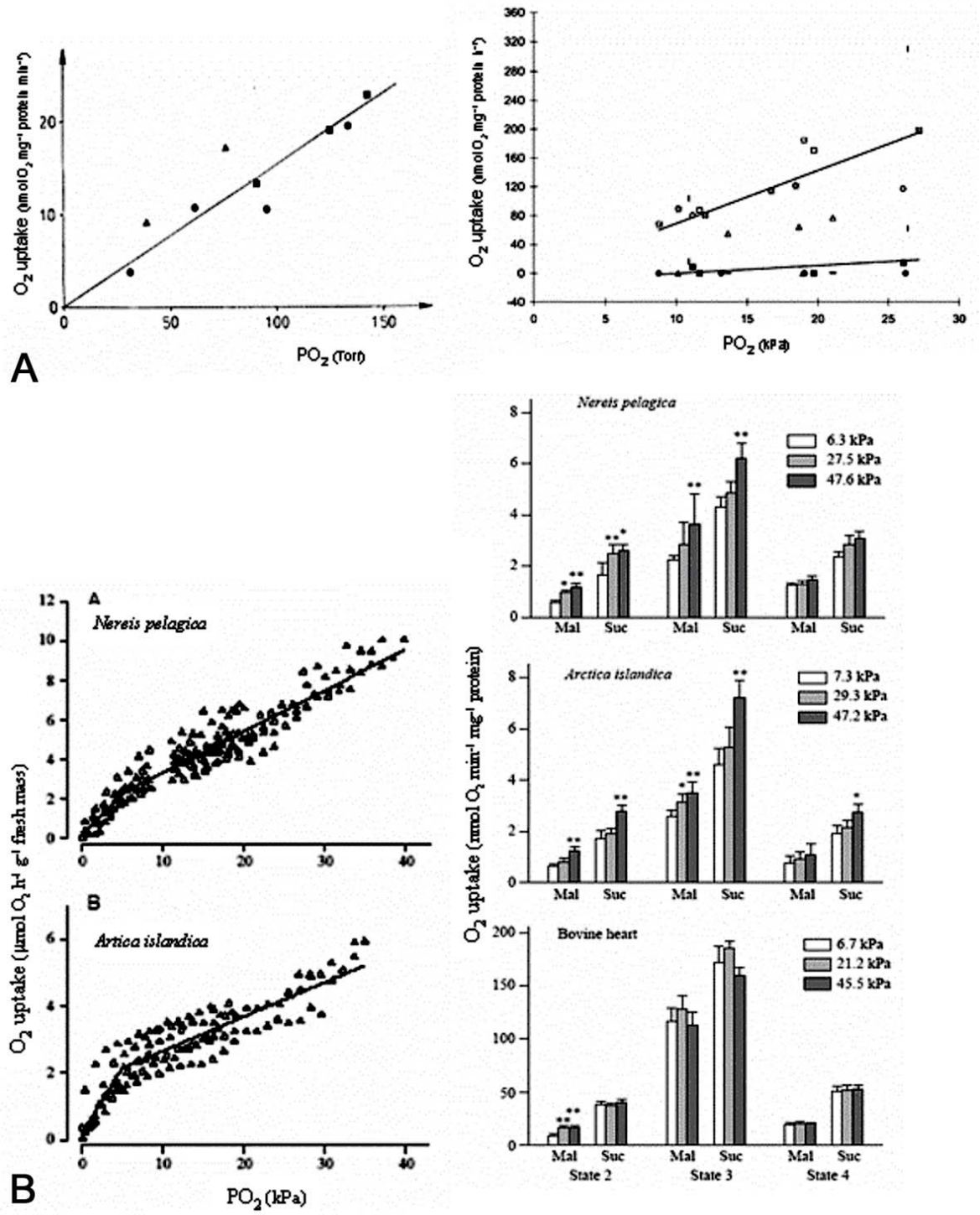

Figure 1. Oxyconforming respiration in marine invertebrates on different organismal levels. A. *Left*: Respiration isolated muscle cells from sipunculide body wall *vs* PO_2, from (10); *Right*: Sipunculide isolated mitochondria, respiring in state 3 *vs* PO_2. Upper graph (open symbols) shows respiration without and lower graph (closed symbols) with oligomycin. Slopes significantly different with $p<0.05$, from (16). B. *Left*: Whole animal 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 stubstrates. Both graphs taken after (12).

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 PO_2 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 PO_2 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 O_2 species on re-oxygenation/resurfacing, is currently debated (37,38).

4. UNBALANCED TISSUE PO_2 AND MITOCHONDRIAL REACTIVE O_2 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 O_2 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 O_2 supply is compromised at critically high temperature, because thermally stressed pericardial mitochondria consume more O_2 than can be delivered by accelerated ventilation and by the circulatory system (25,44-45).

We have studied H_2O_2 formation in marine mollusc mitochondrial preparations and found that it increases critically at elevated temperatures. Above the critical temperature, mitochondrial H_2O_2 formation increased to about 6 and 8% of O_2 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 H_2O_2 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 O_2^- activating the mitochondrial uncoupling protein UCP (48). Just recently UCPs have been identified in marine invertebrates (49) and may add to control reactive O_2 species generation by altering the mitochondrial membrane potential (membrane potential and reactive O_2 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 O_2 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 O_2 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 O_2 SPECIES FORMATION IN MARINE INVERTEBRATE MITOCHONDRIA?

Mitochondria stem from anaerobically functioning proterozoic ancestors, alpha-proteobacteria, that became associated with archaeabacterian host cells during early evolution (53) in an atmosphere, low in O_2 (around 3 kPa) and mildly sulphidic owing to slow rates of sulphate reduction (54). In the low O_2 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

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 PO_2 in diffusion limited organisms, by reducing O_2 to water, and keeping the production of reactive O_2 species low (57-58).

Mitochondria consume about 95% of the cellular O_2 in unstressed cells ((59) for resting rat hepatocytes) and are considered the major cellular sites of reactive O_2 species production (60-65) especially under stress (62, 66). In vertebrate mitochondria the conversion of O_2 to univalently reduced O_2^- anions *in vitro* is estimated to about 2%. The extent to which reactive 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 O_2^- and H_2O_2 production has been shown to depend on the magnitude of the mitochondrial membrane potential (delta-Psi_m) in vertebrates (57,70) and invertebrates like the polychaete *Arenicola marina* (43), rather than to be linear to electron transport rates. The delta-Psi_m-threshold value for significant O_2^- and H_2O_2 production is just above state 3 (i.e. ADP stimulated oxidative phosphorylation) delta-Psi_m level (70). Indeed, most investigations do not detect substantial O_2^- and H_2O_2 production under phosphorylating state 3 conditions. Mild uncoupling of the H^+ gradient through futile cycling of H^+ through the inner mitochondrial membrane dissipates delta-Psi_m and reduces H^+ motive force (57,70-72), thereby preventing overflow of electrons from mitochondrial complexes I and III. High H^+ motive force slows respiratory electron transport and leads to an increased reduction of complex III ubiquinone (QH), which then leaks O_2^- into the matrix and presumably also to the intermembrane space (43,67). As stated by Brookes and Darley-Usmar (73), it is growing consensus that O_2^- formation in the mitochondria is a function of the reduction of the respiratory chain, as it was early pointed out by Boveris and Chance (60).

We have recently summarized literature data on H_2O_2 production reported for mammalian and marine invertebrate mitochondrial preparations, which indicated absolute rates in nmol H_2O_2 per mg of mitochondrial protein to be about an order of magnitude lower in marine invertebrates (38). Turrens and Boveris (61) summarized literature data on overall reactive O_2 species production in mammalian mitochondria of approximately 0.6-1 nmol H_2O_2 min^{-1} mg^{-1} protein or 2-3 nmol O_2^- min^{-1} mg^{-1} protein of mitochondrial membranes, which is roughly equivalent considering that two O_2^- radicals dismutate to one mol of H_2O_2 ($2O_2^- + 2H^+ \Rightarrow H_2O_2 + O_2$). However, in the vast majority of the reported mammalian studies, O_2^- and H_2O_2 formation in mitochondrial isolates was stimulated by the addition of inhibitors like antimycin A, myxothiazol or rotenone (60,64,74-75). These inhibitors block electron transport at complex I (NADH-dehydrogenase; rotenone) and complex III (ubiquinone-cytochrome b; myxothiazol, antimycin A) and thereby foster O_2^- formation from autoxidizing ubisemiquinone molecules and from reversed electron flow via complex I (61). However, rates determined with mitochondrial inhibitors represent maximal capacities of state 3 and 4 reactive O_2 species formation. In studies omitting inhibitors, reactive O_2 species formation ranged at the lower end of the above range. Thus, rat heart mitochondria produced around 0.6 nmol H_2O_2 min^{-1} mg^{-1} protein with succinate as respiratory substrate and no added inhibitor (75). This production was largely abolished by addition of the inhibitor rotenone to the assay and thus related to complex I formation. Other studies detected even less H_2O_2 formation, unless the mitochondria were stimulated with antimycin A (62), and more recent work by St-Pierre and colleagues (67) reported H_2O_2 formation by rat mitochondria ranging as low as 0.01 nmol H_2O_2 min^{-1} mg^{-1} protein, where no inhibitor was added. These data are much closer to the marine invertebrate mitochondria, which produce reactive O_2 species at rates of 0.1 nmol H_2O_2 min^{-1} mg^{-1} mitochondrial protein and less (42-43,76).

So what controls mitochondrial O_2^- and H_2O_2 formation in marine invertebrates *in vitro*? In a recent study comparing mitochondrial functions of seasonally warm (summer) and cold (winter) adapted marine lugworms (*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 H_2O_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° *vs* 1°C) with an immediate increase of H^+ leakage, whereby reducing membrane potential and H^+ motive force, and ameliorating thermally accelerated reactive O_2 species formation. Thus, the mild uncoupling effect of H^+ leak on mitochondrial membrane potential (57) seems to be an important controlling factor of reactive 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 H_2O_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 O_2 conversion to reactive 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 O_2 min^{-1} mg^{-1} mitochondrial protein) in the mud clam *Mya arenaria*, and H_2O_2 formation ranged low but well detectable at 0.12-0.07 nmol H_2O_2 min^{-1} mg^{-1}, with a percent conversion of O_2 to reactive O_2 species amounting to up to 20% of state 2/4 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, H_2O_2 can be formed only at complex III and, possibly to a minor extent, complex II.

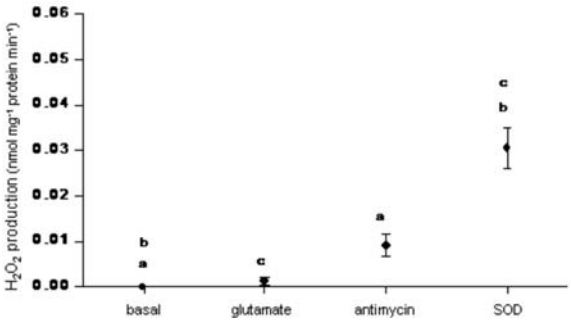

Figure 2. H_2O_2 generation of isolated adductor muscle mitochondria of the scallop *Aequipecten opercularis* measured at 10°C. Given are mean values and standard errors of 11 individual isolations (with 1-2 repetitions per isolate). SOD was added to convert mitochondrial O_2^- to H_2O_2 for peroxidase/homovanillic acid detection (E. Philipp and D. Abele, unpublished data).

In another series of experiments we used scallop adductor muscle, as we assumed that H_2O_2 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 O_2^- to H_2O_2, in order to achieve a significant H_2O_2 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 H_2O_2 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, H_2O_2 can be formed at complex I and III and on both, matrix and cytoplasma side of the mitochondrial membrane (7,67).

This leads us to the conclusion that O_2^- and H_2O_2 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 ectotherms and scallops, capable of burst swimming activity. A controlling function of the H^+ leak towards forward reactive O_2 species formation could be an advantage not only under thermal stress in small ectotherms, but also during periods of limited O_2 availability or inhibition of cytochrome oxidase by microbial H_2S, 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 O_2^- and H_2O_2 formation from reduced electron carriers, like complex III ubiquinone (57,62). Also, these reduced carriers presumably autoxidize, to generate more O_2^- and H_2O_2, during onset of re-oxygenation (see ischemia-reperfusion injury). Theoretically, mild uncoupling might limit excessive O_2^- and H_2O_2 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-oxidoreductase 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 O_2 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 O_2 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 O_2 binding site of cytochrome oxidase, raising the P_{50} for the affinity of mitochondrial respiration for O_2 ($K_{0.5}$ (82-84)) in peripheral cells. This facilitates O_2 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 O_2-competitive inhibition of cytochrome oxidase (86). For mammals it is increasingly accepted that mitochondrial, as well as cellular O_2 uptake are regulated by adenosine diphosphate (ADP), O_2^- and NO, and, moreover, that the rate of cellular ATP formation depends on the O_2/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 O_2 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), H_2O_2 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 H_2O_2 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 O_2^- and H_2O_2 forming capacities, but that also O_2^- and H_2O_2 quenching capacities by mitochondrial superoxide dismutase and

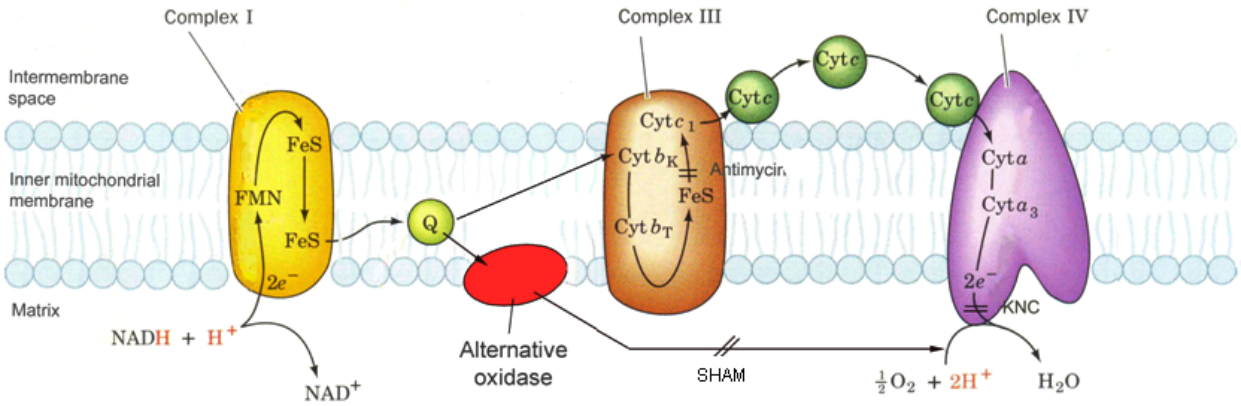

Figure 3. Schematic diagram of the mitochondrial membrane showing the alternative oxidase pathway in marine invertebrate mitochondria. KCN can be replaced by either H_2S or NO.

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 O_2 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 O_2^- and H_2O_2 formation in scallop mitochondria may also be rooted in a more favorable, i.e. higher ratio of complex IV (cytochrome a/a_3) 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 O_2^- and H_2O_2 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, O_2^- and H_2O_2 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, O_2 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), O_2^- and H_2O_2 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 O_2^- and H_2O_2 formation should be expected when extrapolating to lower temperatures, as is already seen from our comparison of O_2^- and H_2O_2 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 O_2 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, O_2 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 PO_2 under ADP limiting, metabolically down regulated conditions, or when O_2 levels within the cell start to rise because the classical respiratory chain is O_2 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, Nematoda 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 protists 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 PO_2 is confined and controlled within a low and narrow range in these animals (12,26). Regarding the O_2^- and H_2O_2 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 H_2O_2 somewhere along the alternative electron transport pathway (15). In contrast, some bacterial AOXs have been characterized as bd-type cytochromes and are induced at PO_2 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, O_2 is reduced to water, not to H_2O_2 (107). Again, in anoxia tolerant animals, like the nematodes studied by Paget and co-authors, it seems conceivable that H_2O_2 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 O_2 to H_2O but not H_2O_2 or O_2^- (108). Induction or over-expression (109) of the AOX were reported to be protective under different forms of stress (110), and to reduce H_2O_2 generation in isolated plant cells (111) and mitochondria (112).

Mammalian mitochondria are sensitive targets of cytotoxic peroxynitrite, forming from the interaction of O_2^- with NO (73). Like H_2O_2, 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 O_2 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 PO_2. 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 (IC_{50}) 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 O_2^- and H_2O_2 formation (71). No studies of NO effects on mitochondrial O_2^- and H_2O_2 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 PO_2 between anoxia, normoxia, and even hyperoxia (12,53), we suggest that the NO/O_2 ratio may be an important modulator, shunting mitochondrial electron flux between classical and alternative transport chain. The lower O_2 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 PO_2 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 PO_2 is high appears adequate for micro-oxophilic animals. These are highly sensitive to elevated environmental PO_2 and, thus, striving at all means to maintain cellular O_2 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 O_2 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 PO_2, 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 organismal and cellular hardware, but also

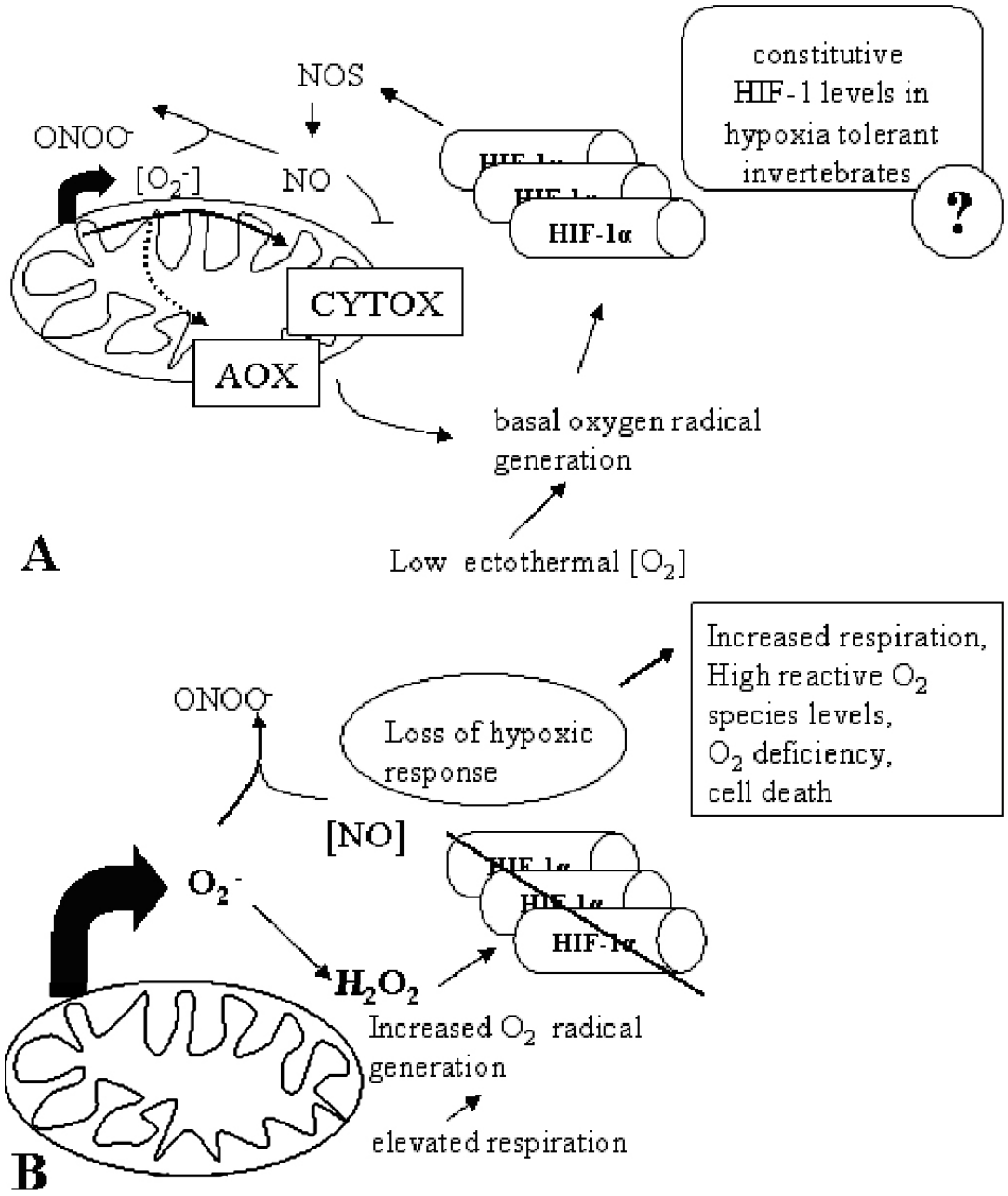

Figure 4. A model describing biochemical cross talk between reactive O_2 species formation, NO, the AOX and hypoxic response factor (HIF-1), designed to explain the extreme tolerance of marine invertebrates to live under environmental low O_2 conditions. A. Non stress situation are characterized by low 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_2O_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 O_2 levels. To early anaerobic and micro-oxophilic organisms, O_2 was a toxic gas, and it seems not too far fetched to imagine that O_2 and its noxious derivatives attained signal function in early organisms. Thus, O_2 and not CO_2 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 O_2 will become more important whenever cytochrome oxidase activity is saturated or (partly or fully) inhibited by H_2S, NO or yet unknown natural inhibitors. Inhibition of cytochrome oxidase causes reduction of classical respiratory chain electron transporters and increases the formation of O_2^- and H_2O_2. 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 O_2^- and H_2O_2 in marine animal mitochondria under exposure to elevated temperatures (within the natural range (43)) whereby again leading to non-phosphorylating O_2 consumption and preventing intracellular hyperoxia and oxidative stress.

Prolonged environmental hypoxia and exposure to minor concentrations of H_2S 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 O_2, O_2^- 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 PO_2 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 O_2 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 H_2S elimination (120) and the formation of peroxynitrite, to counteract the NO signal as schematically described in figure 4.

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