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MITOCHONDRIAL OXYCONFORMITY AND COLD ADAPTATION IN THE POLYCHAETE *NEREIS PELAGICA* AND THE BIVALVE *ARCTICA ISLANDICA* FROM THE BALTIC AND WHITE SEAS

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Summary

The rates of oxygen uptake of the marine polychaete *Nereis pelagica* and the bivalve *Arctica islandica* depend on the availability of ambient oxygen. This is manifest both at the tissue level and in isolated mitochondria studied between oxygen tensions (P_{O_2}) of 6.3 and 47.6 kPa (47–357 mmHg). Oxyconformity was found in both Baltic Sea (Kiel Bight) and cold-adapted White Sea populations of the two species. However, mitochondria isolated from White Sea specimens of *N. pelagica* and *A. islandica* showed a two- to threefold higher aerobic capacity than mitochondria prepared from Baltic Sea specimens. We tested whether mitochondrial oxyconformity can be explained by an additional electron pathway that is directly controlled by P_{O_2}. Mitochondrial respiration of both invertebrate species was inhibited by cyanide (KCN) and by salicylhydroxamic acid (SHAM). The overall rate of mitochondrial oxygen consumption increased at high P_{O_2}. Phosphorylation efficiency (ADP/O ratio) decreased at elevated P_{O_2} (27.5–47.6 kPa, 206–357 mmHg), regardless of whether malate or succinate was used as a substrate. In contrast to the invertebrate mitochondria studied, mitochondria isolated from bovine heart, as an oxyregulating control species, did not show an elevated rate of oxygen uptake at high P_{O_2} in any respiratory state, with the exception of state 2 malate respiration. In addition, rates of ATP formation, respiratory control ratios (RCR) and ADP/O ratios remained virtually unchanged or even tended to decreased. In conclusion, the comparison between mitochondria from oxyregulating and oxyconforming organisms supports the existence of an alternative oxidase in addition to the classical cytochrome *c* oxidase. In accordance with models discussed previously, oxidative phosphorylation does not explain the rate of mitochondrial oxygen consumption during progressive activation of the alternative electron transport system. We discuss the alternative system, thought to be adaptive in confined, usually hypoxic environments, where excess oxygen can be eliminated and oxygen levels can be kept low by an increase in the rate of oxygen consumption, thereby minimizing the risk of oxidative stress.

Key words: *Nereis pelagica*, *Arctica islandica*, oxyconformer, oxyregulation, oxidative phosphorylation, electron transport pathway, oxygen consumption, isolated mitochondria.

Introduction

Marine benthic invertebrates experience considerable temporal and spatial fluctuations in oxygen concentration in their natural environment (Fenchel and Finlay, 1995). Oxygen deficiency in the bottom water or in the underlying sediment is caused by the oxygen consumption of microbial and bottom fauna. However, oxygen can quickly be reintroduced into the system by wind-induced downward mixing of oxygenated surface waters in shallow areas. Invertebrates colonizing the coastal macrophyte belts have to deal with diurnal fluctuations in photosynthetic oxygen production that may range from supersaturation during the day to oxygen deficiency at night. Oxygen availability also depends upon temperature. Low temperature increases the solubility of oxygen in sea water, so that animals in high latitudes and especially polar areas generally have to cope with higher environmental oxygen concentrations at the same P_{O_2}. However, low temperatures can also cause oxygen deficiency because ventilatory and circulatory efficiency are reduced by cold exposure, resulting in an inadequate oxygen supply and cold-induced functional hypoxia below a critical temperature (Pörtner et al., 1998).

In general, two strategies characterize the response of animals to fluctuating environmental P_{O_2} while metabolism is fully aerobic. In contrast to oxyregulators, which are able to adjust their rate of oxygen consumption to a constant level irrespective of ambient P_{O_2}, the rate of oxygen uptake of oxyconformers depends on the environmental oxygen concentration. However, if ambient P_{O_2} falls below a critical level ($P_{C(l)}$; Dejours, 1981), anaerobic metabolism sets in and the rate of oxygen consumption finally decreases in both groups of animals (Pörtner and Grieshaber, 1993).

Nevertheless, only a few examples have been reported that clearly distinguish between aerobic oxyconformity and oxyregulation. In the oxyconforming worms *Arenicola marina* and *Scoloplos armiger*, two critical P_{O_2} values have been identified: a low critical level, $P_{C(l)}$, which marks the onset of anaerobiosis, and a high critical level, $P_{C(h)}$, characterized by the transition from oxyconformity to a constant rate of oxygen consumption that may reflect saturation of the electron transport system (Pörtner and Grieshaber, 1993).

Little is known about the mechanism that forms the basis of oxyconformity between $P_{C(l)}$ and $P_{C(h)}$ in marine invertebrates. In this context, the existence of an additional P_{O_2}-controlled mitochondrial oxidase, which enables marine oxyconformers to increase their rate of oxygen consumption when cytochrome *c* oxidase is oxygen-saturated, has been discussed (Pörtner and Grieshaber, 1993). The ecological idea was that this enzyme would have a lower affinity for oxygen than cytochrome *c* oxidase and would consume excess ambient oxygen, thereby reducing cellular P_{O_2} and the risk of oxygen radicals being produced (respiratory protection; for a review, see Skulachev, 1996).

Several alternative electron pathways have been characterized and described for bacteria (Webster, 1975; D'Mello et al., 1994), plants (Siedow and Moore, 1992; Popov et al., 1997) and, among animals, for oxyconforming free-living and parasitic nematodes (Cheah, 1972; Mendis and Evans, 1984; Paget et al., 1987a,b, 1988a,b). The latter colonize a micro-oxic habitat (the gut) and possess an additional uncoupled electron pathway branching off the classical chain. The alternative oxidase of these predominantly anaerobic nematodes was termed cytochrome *o* and has been characterized as a *b*-type cytochrome (Paget et al., 1988a,b). This cytochrome proved to be insensitive to cyanide, and the authors relate its presence to the frequent occurrence of S^{2-} in the gut, which will impair oxygen reduction *via* cytochrome a/a_3. A possible drawback of this oxidase is the generation of H_2O_2 when two of the electrons of oxygen are reduced (Paget et al., 1987a,b, 1988a,b).

In contrast, some bacterial alternative oxidases have been characterized as *bd*-type cytochromes and are induced at higher P_{O_2} values (D'Mello et al., 1994; Bertsova et al., 1997). *Escherichia coli* contains two terminal oxidases, one of which is termed cytochrome *o*. In this case, oxygen is reduced to water, not to H_2O_2 (Minghetti and Gennis, 1988).

In general, alternative oxidases reduce oxygen at P_{O_2} values above the saturation level of cytochrome *c* oxidase and thereby protect the cell from the formation of reactive oxygen species by autoxidation of reduced respiratory enzyme complexes (mainly complexes I and III; Popov et al., 1997). Under these conditions, respiration is at least partly uncoupled (D'Mello et al., 1994). In consequence, progressive activation of a low-affinity alternative oxidase at high P_{O_2} could support oxyconformity as found in marine invertebrates (Pörtner and Grieshaber, 1993).

The present study aimed to elucidate the concept of an alternative terminal oxidase pathway in two marine benthic invertebrates, the polychaete *Nereis pelagica* and the bivalve *Arctica islandica*, two species abundant in boreal and subpolar coastal areas. Whereas *A. islandica* performs frequent vertical migrations within the upper sediment layers, the polychaete *N. pelagica* is a typical inhabitant of macrophyte belts in water 2–3 m deep. Both species are subject to frequent fluctuations in the P_{O_2} in their natural environment, ranging from anoxic conditions below the sediment redoxcline through normoxic conditions (100 % atmospheric oxygen saturation) in the sediment water interface to hyperoxic conditions of more than 200 % atmospheric oxygen saturation at sites of high photosynthetic activity (Fenchel and Finlay, 1995).

After testing whether the two species act as oxyconformers at the tissue level, inhibitors of classical and alternative electron transport pathways were used to detect the existence of an alternative mitochondrial oxidase. It has been suggested that the activity of the alternative oxidase in endoparasites is concomitant with a partial 'uncoupling' of the electron transport system from phosphorylation (Cheah, 1972), so we compared ADP/O ratios at a variety of P_{O_2} values. To contrast our data on marine invertebrate mitochondria with those for the mitochondria of a typical oxyregulator, we investigated the same variables in mammalian mitochondria, in which an alternative oxidase should not be present. To examine the effects of temperature and mitochondrial modifications in a latitudinal gradient, the present study compares mitochondrial respiration and phosphorylation efficiency of populations of *N. pelagica* and *A. islandica* from the Baltic Sea with those of subpolar specimens from the White Sea.

Materials and methods

Animals

Specimens of the clam *Arctica islandica* (L.) were dredged in muddy sediments in Kiel Bight (Western Baltic) and in Chupa Bay (White Sea) at approximately 20 m and 12 m water depth, respectively. The epibenthic worm *Nereis pelagica* (L.) was dredged in Kiel Bight at approximately 8–12 m water depth, where it lives in sponges or bunches of red algae. White Sea specimens of *Nereis pelagica* were collected in Chupa Bay from *Mytilus* ropes at approximately 1–2 m water depth. All animals were kept in aerated aquaria filled with sea water (salinity 22 ‰), sediment and algae from the respective sampling site at 10 °C (Germany) or 6 °C (Russia) without extra feeding for a maximum of 2 weeks. Bovine hearts were obtained from the slaughterhouse at Bremerhaven (Germany).

Chemicals

All chemicals were obtained from Fluka (Deisenhofen, Germany), Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany), unless stated otherwise. Substrates and stock solutions of KCN (0.1–100 mmol l^{-1}) were dissolved or diluted in deionized water and neutralized to pH 8. Salicylhydroxamic acid (SHAM; 1 mmol l^{-1} to 2 mol l^{-1}) was dissolved in 25 % dimethylsulphoxide (DMSO). Oligomycin A (1 mg) was dissolved in ethanol (99.8 %) and was further

diluted with deionized water to give a final concentration of 1 μg mg^{-1} protein.

Tissue rates of oxygen consumption

The rates of oxygen consumption of isolated body wall tissue of *N. pelagica* and of mantle tissue of *A. islandica* were assayed in a closed system using respiration chambers designed according to O'Brien and Vetter (1990) and equipped with polarographic oxygen electrodes (Eschweiler, Kiel, Germany) connected to a P_{O_2} monitor (Eschweiler, Kiel, Germany) and to a chart recorder (Pharmacia LKB, Uppsala, Sweden). Electrodes were calibrated at constant temperature (15 °C) in a saturated Na_2SO_3 solution and in sea water under normoxic oxygen tensions. Mixtures of N_2, O_2 and CO_2 were supplied by a multi-gas controller (MKS, Munich, Germany). Experiments were carried out in 2 ml of filtered sea water (salinity 22 ‰) containing streptomycin (0.005 mg ml^{-1}) and penicillin (5 units ml^{-1}). Tissue mass ranged from 40 to 100 mg fresh mass for *N. pelagica* and from 30 to 60 mg fresh mass for *A. islandica*. The tissue was fixed in the respiration chamber with small hooks on a plastic frame. The oxygen consumption of an individual sample was recorded for a maximum of 4 h. To calculate tissue rates of oxygen consumption, an O_2 solubility coefficient (α) of 1.799 μmol l^{-1} mmHg^{-1} (at 15 °C and 22 ‰ salinity; 1 mmHg=0.1333 kPa; Boutilier et al., 1984) was used. Initial trials of the maintenance of rates of oxygen consumption and ATP levels demonstrated the viability of the tissues of both species throughout the experimental procedure.

Isolation of mitochondria

Mitochondria were isolated from the body wall tissue of *N. pelagica* and from the mantle tissue of *A. islandica* at 0 °C. For *N. pelagica*, the isolation buffer, modified from that of Schroff and Schöttler (1977), contained 540 mmol l^{-1} glycine, 250 mmol l^{-1} sucrose, 2 mmol l^{-1} EDTA, 20 mmol l^{-1} Tris-HCl (pH 7.4) and 0.2 % bovine serum albumin (BSA, fraction V, essentially fatty-acid-free). Worms were used after removal of the head and the caudal body segments. The body wall was opened, and the internal organs and body fluid were removed. The isolated body wall tissue was placed into 40 ml of ice-cold buffer. A total of 1.5 g fresh mass was cut into small pieces and homogenized in a motor-driven glass/Teflon homogenizer (Heidolph, Kehlheim, Germany) using 3–5 passes. The resulting homogenate was centrifuged at 1250 ***g*** for 8 min (0 °C) to separate cellular debris. The supernatant was collected, and the pellet was resuspended in 10 ml of isolation buffer for a second centrifugation (1250 ***g***, 8 min, 0 °C). The two supernatants were then pooled, and a third centrifugation step (10 000 ***g***, 15 min, 0 °C) yielded the mitochondrial pellet, which was resuspended in 2 ml of isolation buffer. The final suspension contained between 5 and 15 mg protein ml^{-1}.

Coupled mitochondria were prepared from the mantle tissue of *A. islandica* using the isolation buffer of Moyes et al. (1985). Freshly dissected mantle tissue (3 g) was blotted dry, cut into small pieces, placed into 40 ml of ice-cold isolation buffer and homogenized using 3–5 passes in a motor-driven glass/Teflon homogenizer (Heidolph, Kehlheim, Germany). The homogenate was centrifuged at 1500 ***g*** for 8 min (0 °C). The supernatant was collected, the pellet was resuspended in 10 ml of isolation buffer and this centrifugation step was repeated. The two supernatants were pooled and centrifuged at 5000 ***g*** for 15 min (0 °C). This last centrifugation yielded the mitochondrial pellet, which was carefully resuspended in 2 ml of isolation buffer. The mitochondrial protein content of the final suspension varied between 3 and 10 mg ml^{-1}.

A freshly excised bovine heart was transported on crushed ice to the laboratory. Fat and connective tissue were carefully removed. Mitochondria from the bovine heart were prepared according to Brierley et al. (1984) with the following modifications. Muscular tissue (70 g) was passed through a meat grinder maintained at 5 °C. The minced tissue was suspended in 100 ml of ice-cold isolation buffer consisting of 250 mmol l^{-1} sucrose, 10 mmol l^{-1} Tris-HCl (pH 7.2) and 1 mmol l^{-1} EGTA. After adding 10 mg of Nagarse (a bacterial proteinase, E.C.3.4.21.14), the suspension was stirred for 15 min at 5 °C. pH was maintained between 7.0 and 7.5 with 5 mol l^{-1} KOH. The suspension was filtered through cheesecloth and washed with 100 ml of isolation buffer. The filtered tissue was suspended in 100 ml of isolation buffer containing 0.1 % BSA. The solution was homogenized with a motor-driven homogenizer (Heidolph, Kehlheim, Germany) equipped with a loosely fitting Teflon pestle. BSA-containing isolation buffer was added to a final volume of 500 ml, and the homogenate centrifuged at 400 ***g*** for 10 min (0 °C). The supernatant was filtered through cheesecloth and then centrifuged for 15 min at 6000 ***g***. The pellet was resuspended in 100 ml of the isolation buffer (containing BSA) and centrifuged for 10 min at 9500 ***g***. The upper light fluffy layer, which consists of damaged mitochondria, was gently removed. The residual pellet was resuspended in 100 ml of isolation buffer and recentrifuged for 10 min at 9500 ***g***. The final pellet was carefully resuspended in 8 ml of isolation buffer containing BSA to a protein content of 5–12 mg ml^{-1}.

Rates of mitochondrial oxygen consumption

The respiration rates of *N. pelagica* and *A. islandica* mitochondria were assayed at 15 °C for populations from both the Baltic and White Sea to compare temperature-specific rates. This temperature is within the natural temperature range for both areas. The respiration buffer for *N. pelagica* mitochondria contained 550 mmol l^{-1} glycine, 250 mmol l^{-1} sucrose, 2 mmol l^{-1} EDTA, 20 mmol l^{-1} Tris-HCl (pH 7.5), 10 mmol l^{-1} K_2HPO_4, 2 mmol l^{-1} $MgCl_2$ and 0.2 % BSA. Respiration buffer for *A. islandica* mitochondria contained 550 mmol l^{-1} sucrose, 150 mmol l^{-1} KCl, 70 mmol l^{-1} Hepes (pH 7.4), 10 mmol l^{-1} KH_2PO_4 and 0.2 % BSA. The respiration of isolated bovine heart mitochondria was assayed at 30 °C. The buffer contained 250 mmol l^{-1} sucrose, 5 mmol l^{-1} Tris-HCl (pH 7.2), 0.5 mmol l^{-1} EGTA, 12 mmol l^{-1} K_2HPO_4, 5 mmol l^{-1} $MgCl_2$ and 0.1 % BSA.

Respiration chambers were filled with respiration buffer,

previously equilibrated with air or a mixture of N_2, O_2 and CO_2 supplied by the multi-gas controller. The addition of the mitochondrial suspension resulted in a final volume of 1.5 ml. The oxygen tension was re-adjusted to the required oxygen partial pressure (P_{O_2}) by aerating the suspension in the chamber with the appropriate gas mixture. Substrates and inhibitors were added to the closed respiration chamber using a Hamilton syringe. Rates of mitochondrial oxygen consumption were measured with continuous stirring and were calculated using solubility coefficients (α) determined in respiration buffers (without BSA) by the Winkler method (Grasshoff, 1999). Values of α were $1.544\pm0.116\,\mu mol\,l^{-1}\,mmHg^{-1}$ for the medium used with *A. islandica* mitochondria and $1.338\pm0.102\,\mu mol\,l^{-1}\,mmHg^{-1}$ (1 mmHg=0.1333 kPa) for the medium used with *N. pelagica* mitochondria. For bovine mitochondria, a value of $\alpha=1.346\,\mu mol\,l^{-1}\,mmHg^{-1}$ was used, as calculated from the osmolality of the medium according to Boutilier et al. (1984) and verified by Winkler analysis.

In the present study, state 2 respiration is defined as the rate of respiration in the presence of substrate but before the addition of ADP. State 3 and state 4 respiration and ADP/O ratios were defined according to Chance and Williams (1955). The respiratory control ratio (RCR) was determined according to Estabrook (1967) by dividing state 3 respiration rate by state 4 respiration rate.

ATP production was assayed luminometrically using a luciferin/luciferase system (Bio Orbit 1243-102 ATP monitoring kit and Bio Orbit 1250 luminometer, Turku, Finland) at a variety of P_{O_2} values at 15 °C (*N. pelagica* and *A. islandica* mitochondria from Chupa Bay) and at 30 °C (bovine heart mitochondria) following the method of Wibom et al. (1990). The assay consisted of 200 µl of reconstituted adenosine triphosphate monitoring reagent, $25\,\mu mol\,l^{-1}$ ADP, 10 µl of mitochondrial suspension and respiration buffer added to a final volume of 2000 µl. Mitochondrial suspensions were diluted if necessary. The oxygen tensions were adjusted as described above. The reaction vessel was closed with an air-tight rubber stopper, and the reaction was started by adding malate or succinate, unless stated otherwise. ATP production was standardised by the stepwise addition of an internal ATP standard ($10\,\mu mol\,l^{-1}$).

H_2O_2 formation was measured continuously (Perkin Elmer luminescence spectrometer LS 50 B, Buckinghamshire, UK) using the fluorescent scopoletin (7-hydroxy-6-methoxy-2H-benzopyran)/horseradish peroxidase (E.C.1.11.1.7.) assay at 15 °C, as described by Abele-Oeschger and Oeschger (1995) with some modifications. Instead of the phosphate buffer, the respective mitochondrial respiration buffers were used, without BSA and with sucrose replaced by glycine (both substances reduce the fluorescence signal). The modified assay was checked using a system continuously generating H_2O_2 (glucose oxidase, E.C.1.1.3.4., and glucose; Boveris et al., 1977). The amount of oxidase added, which is proportional to the rate of H_2O_2 produced, was calculated by the addition of an internal H_2O_2 standard ($0.5\,\mu mol\,l^{-1}$) and gave a linear relationship between 0.3 and 11 $nmol\,H_2O_2\,min^{-1}$.

Protein concentrations were determined by the biuret method (Kresze, 1983) using BSA as a standard.

Cytochrome *c* oxidase (E.C.1.9.3.1.) activity was determined using the method of Hand and Somero (1983) in mitochondrial suspensions of *N. pelagica*, *A. islandica* and in commercially available cytochrome *c* oxidase from bovine heart. The assay (total volume 1.0 ml) contained $100\,mmol\,l^{-1}$ potassium phosphate buffer (pH 6.0), $0.5\,mmol\,l^{-1}$ EDTA and $50\,\mu mol\,l^{-1}$ reduced cytochrome *c*. The reaction was started by adding mitochondrial suspension or enzyme and was monitored at 550 nm (Biochrom 4060, Pharmacia LKB, Uppsala, Sweden).

Statistical analyses

Data were tested for significance using analysis of variance (ANOVA) for paired samples, unless stated otherwise. When significant differences were indicated by ANOVA ($P<0.05$), data were compared with the control value using the Student–Newman–Keuls *post-hoc* test. Values are presented as means ± S.E.M. Concentrations of KCN and SHAM giving 50 % of maximal inhibition (IC_{50}) were calculated from:

$$f(x) = ax(b + x),$$

where $f(x)$ is relative inhibition (%), a is maximal inhibition (%), x is the concentration of the respective inhibitor ($\mu mol\,mg^{-1}$ or $\mu mol\,l^{-1}$) and b is the inhibitor concentration giving half-maximal inhibition (IC_{50}). Lines were fitted to the data sets using this equation and a curve-fitting programme (SigmaPlot 2.01 for Windows) with the constraints that $a<100$ % and $b>0$ ($\mu mol\,mg^{-1}$ or $0\,\mu mol\,l^{-1}$). The critical P_{O_2} (P_C) was calculated by applying two-phase regressions that compared sequential linear fits. Two intersecting lines were selected that best fitted the data according to the method of least squares following the rationale of Yeager and Ultsch (1989).

Results

Tissue rates of oxygen consumption versus P_{O_2}

The use of isolated tissues for oxygen consumption analyses excluded the influence of variations in ventilation and locomotory activity typical for whole animals. At P_{O_2} values between 39.0 and 6.0 kPa, the rate of oxygen uptake of isolated body wall tissue of *N. pelagica* decreased almost linearly with a mean decrement of $0.24\,\mu mol\,O_2\,h^{-1}\,kPa^{-1}\,g^{-1}$ fresh mass ($r^2=0.844$, $P<0.05$) (Fig. 1A). Below the low critical P_{O_2} ($P_{C(l)}$) of 5.3 kPa, the rate of oxygen uptake decreased at a higher rate of $0.45\,\mu mol\,O_2\,h^{-1}\,kPa^{-1}\,g^{-1}$ fresh mass ($r^2=0.662$, $P<0.05$). $P_{C(l)}$ was determined as the point of intersection of the two regression lines (Fig. 1A). Above $P_{C(l)}$, *N. pelagica* body wall tissue displayed oxyconforming respiration. A high critical P_{O_2} ($P_{C(h)}$) could not be measured within the P_{O_2} range investigated.

Between a P_{O_2} of 34.0 and 6.0 kPa, the rate of oxygen uptake of isolated mantle tissue of *A. islandica* also declined almost linearly at a rate of $0.15\,\mu mol\,O_2\,h^{-1}\,kPa^{-1}\,g^{-1}$ fresh mass ($r^2=0.734$, $P<0.05$) (Fig. 1B). After reaching a low critical

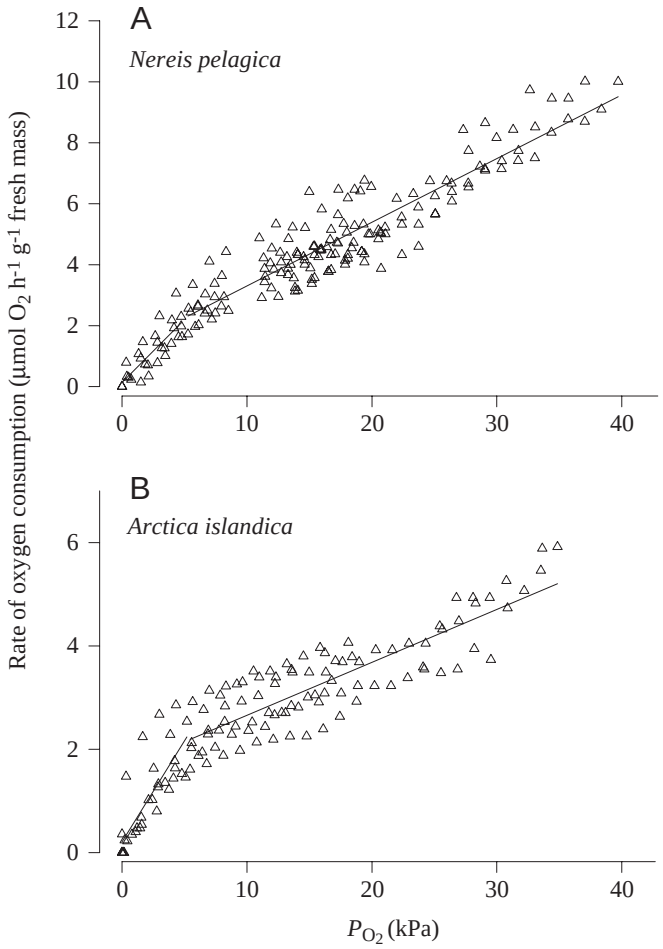

Fig. 1. Rates of oxygen consumption (in $\mu mol\,O_2\,h^{-1}\,g^{-1}$ fresh mass) *versus* P_{O_2} (kPa) at 15 °C and 22‰ salinity by Baltic Sea specimens of (A) *Nereis pelagica* (body wall) and (B) *Arctica islandica* (mantle). Data are from 7–8 independent experiments. The intersection of the regression lines marks the critical P_{O_2}.

value ($P_{C(l)}$) at 4.9 kPa, an enhanced decline in the rate of oxygen uptake of $0.44\,\mu mol\,O_2\,h^{-1}\,kPa^{-1}\,g^{-1}$ fresh mass was found between 4.9 and 0 kPa (r^2=0.633, P<0.05). Thus, the mantle tissue of *A. islandica* is also characterized by aerobic oxyconformity above a $P_{C(l)}$ of 4.9 kPa.

Effects of inhibitors on mitochondrial oxygen uptake and cytochrome c *oxidase activity*

Cytochrome *c* oxidase is characterized by sensitivity to cyanide. In plants, the alternative oxidase pathway is described as being insensitive to cyanide (Bahr and Bonner, 1973), but sensitive to salicylhydroxamic acid (SHAM) (Schonbaum et al., 1971). We tested whether a similar response to these inhibitors also characterizes the alternative electron transport pathway in *N. pelagica* and *A. islandica*.

Inhibition of oxygen uptake and cytochrome *c* oxidase activity in *N. pelagica*, *A. islandica* and bovine heart mitochondria by KCN, measured under normoxia, is depicted in Fig. 2A,B and Table 1. In all cases, KCN caused a maximal inhibition by more than 90 %, except for succinate respiration in *A. islandica*, for which maximal inhibition amounted to only 85.2 %. IC_{50} values, however, differed. Malate and succinate respiration in *N. pelagica* mitochondria were inhibited with an IC_{50} of $17.70\,\mu mol\,l^{-1}$ and $7.01\,\mu mol\,l^{-1}$ for KCN, respectively. In *A. islandica*, sensitivity to KCN was greater, with IC_{50} values of malate and succinate respiration at 1.34 and $1.96\,\mu mol\,l^{-1}$ for KCN respectively. The respiration of bovine heart mitochondria with succinate and malate as substrates was almost completely inhibited when the concentration of KCN was increased to $66\,\mu mol\,l^{-1}$, with an approximate IC_{50} of 10–20 $\mu mol\,l^{-1}$ for KCN. Cytochrome *c* oxidase was highly cyanide-sensitive in all three species, with only minor differences in IC_{50} values: $0.95\,\mu mol\,l^{-1}$ for KCN (*N. pelagica*) and $2.20\,\mu mol\,l^{-1}$ for KCN in bovine heart (Table 1).

Table 1. *IC_{50} values and values of maximal inhibition of respiration and cytochrome* c *oxidase activity in mitochondria from Baltic Sea specimens of* Nereis pelagica *(body wall) and* Arctica islandica *(mantle) and in bovine heart mitochondria in response to the addition of KCN and SHAM*

Inhibitor	Respiration and Cytox activity	IC_{50} ($\mu mol\,l^{-1}$ for KCN) ($\mu mol\,mg^{-1}$ for SHAM)			Maximal inhibition (%)		
		N. pelagica	*A. islandica*	Bovine heart	*N. pelagica*	*A. islandica*	Bovine heart
KCN	Malate	17.70	1.34	ND	94.5	90.3	ND
	Succinate	7.01	1.96	ND	97.6	85.2	ND
	Cytox	0.95	1.24	2.20	98.8	92.6	96.9
SHAM	Malate	1.66	0.19	–	90.1	92.2	23.8
	Succinate	2.94	0.11	20.20	84.2	82.1	7.5
	Cytox	18.77	0.10	2.95	25.6	24.5	21.3

SHAM, salicylhydroxamic acid; Cytox, cytochrome *c* oxidase.
For detailed information, see Figs 2, 3.
ND, not determined; –, calculation not possible.

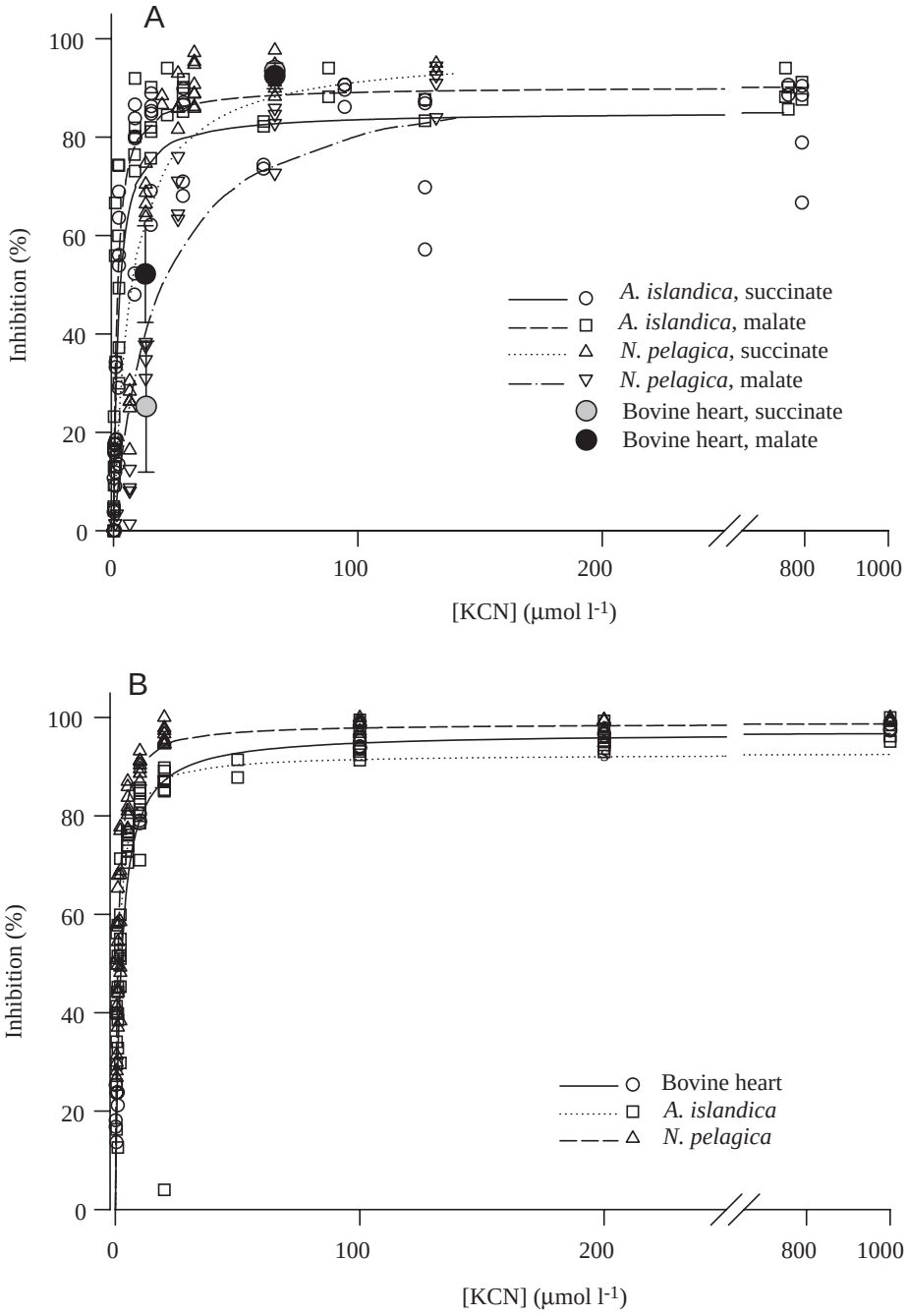

Fig. 2. (A) KCN inhibition of state 3 respiration (%) under normoxia in mitochondria from Baltic Sea specimens of *Nereis pelagica* (body wall) and *Arctica islandica* (mantle) and in bovine heart mitochondria. Inhibition studies were carried out at 15 °C (*N. pelagica* and *A. islandica*) in the presence of succinate (3.3 mmol l^{-1}), malate (3.3 mmol l^{-1}) and ADP (0.67 mmol l^{-1}) (*N*=4–6) or at 30 °C (bovine heart) in the presence of succinate (3.3 mmol l^{-1} plus 0.07 mmol l^{-1} pyruvate), malate (3.3 mmol l^{-1} plus 0.07 mmol l^{-1} pyruvate) and ADP (0.13 mmol l^{-1}) (*N*=4–6). (B) KCN inhibition of cytochrome *c* oxidase activity in *N. pelagica*, *A. islandica* and bovine heart. Measurements were carried out at room temperature (20 °C). Data are from four independent experiments.

Inhibition of mitochondrial oxygen uptake could also be observed after adding SHAM (Fig. 3A; Table 1). Maximal inhibition of *N. pelagica* mitochondria was approximately 90.1 % (IC_{50}=1.66 μmol mg^{-1} protein for SHAM) for malate respiration and 84.2 % (IC_{50}=2.94 μmol mg^{-1} protein for SHAM) for succinate respiration. Again, *A. islandica* mitochondria were more sensitive, with similar maximal levels of inhibition of 92.2 % and 82.1 % but lower IC_{50} values of 0.19 or 0.11 μmol mg^{-1} protein for SHAM, respectively, for malate and succinate respiration. In contrast, SHAM inhibited the respiration of bovine heart mitochondria with succinate as substrate by only 7.5 % (IC_{50}=20.2 μmol mg^{-1} protein for SHAM) and with malate as substrate by 23.8 % (the IC_{50} for malate could not be measured).

Even cytochrome *c* oxidase activity proved sensitive to SHAM (Fig. 3B; Table 1) with a similar inhibition of between 21 and 26 % in mitochondria from all three species. Inhibition was effective at low SHAM concentrations in the bivalve (IC_{50}=0.10 μmol mg^{-1} protein for SHAM in *A. islandica*, see Fig. 3B), with *N. pelagica* cytochrome *c* oxidase being inhibited only at far higher SHAM concentrations (IC_{50}=18.77 μmol mg^{-1} protein). Bovine heart cytochrome *c* oxidase activity was inhibited with an IC_{50} of 2.95 μmol mg^{-1} protein for SHAM. Thus, the inhibition of

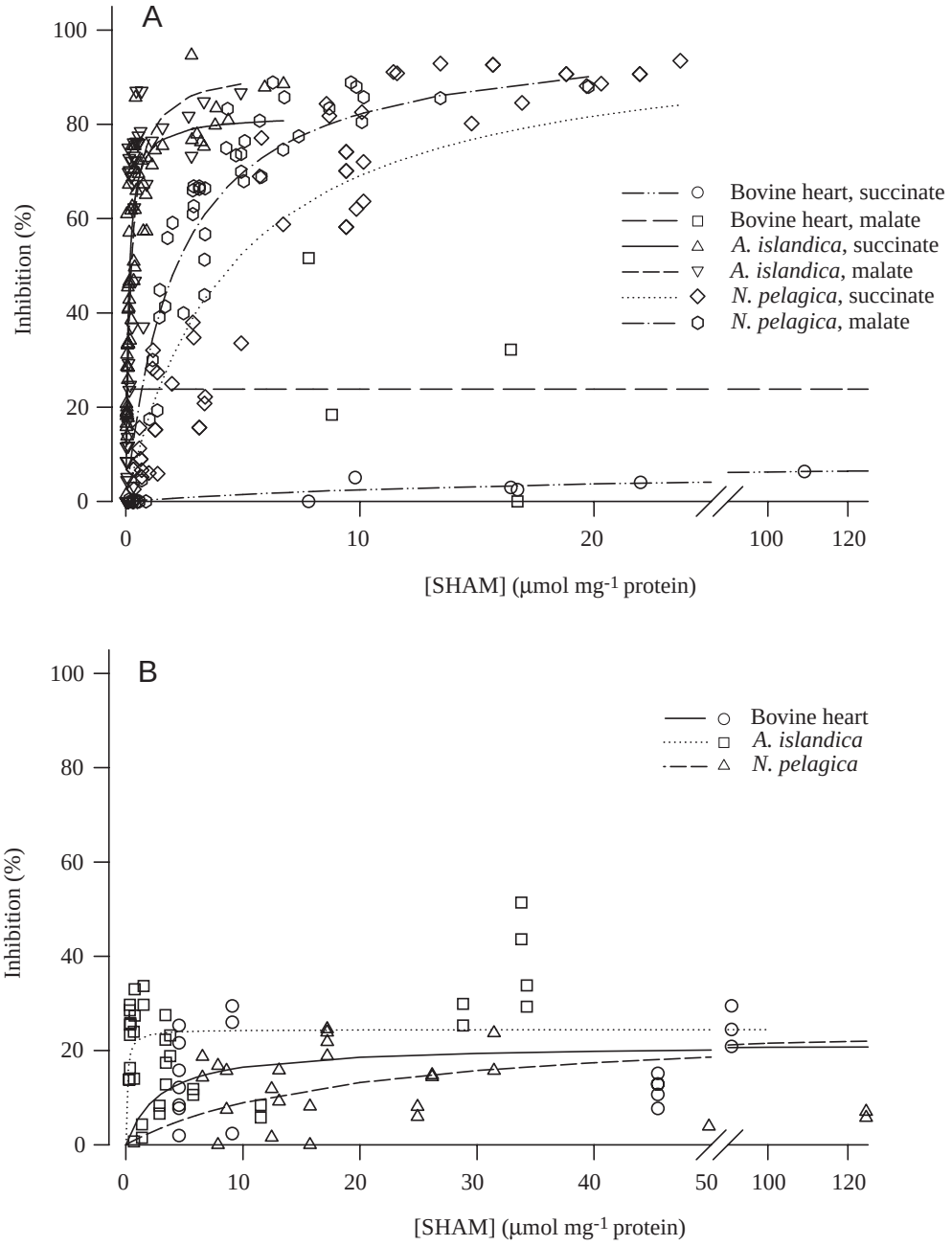

Fig. 3. (A) Inhibition of state 3 respiration (%) by salicylhydroxamic acid (SHAM) during normoxia in mitochondria from Baltic Sea specimens of *Nereis pelagica* (body wall) and *Arctica islandica* (mantle) and in bovine heart mitochondria. Inhibition studies were carried out at 15 °C (*N. pelagica* and *A. islandica*) in the presence of succinate (3.3 mmol l^{-1}), malate (3.3 mmol l^{-1}) and ADP (0.67 mmol l^{-1}) (*N*=4–6) or at 30 °C (bovine heart) in the presence of succinate (3.3 mmol l^{-1} plus 0.07 mmol l^{-1} pyruvate), malate (3.3 mmol l^{-1} plus 0.07 mmol l^{-1} pyruvate) and ADP (0.13 mmol l^{-1}) (*N*=4–5). (B) SHAM inhibition of cytochrome *c* oxidase activity of *N. pelagica*, *A. islandica* and bovine heart mitochondria measured at room temperature (20 °C). Data are from four independent experiments.

bovine heart mitochondria by SHAM is probably due to partial blockage of cytochrome *c* oxidase.

Cyanide-insensitive respiration (sensitive to SHAM) should increase with P_{O_2} in the presence of an alternative oxidase; however, the results of experiments with cross inhibition by KCN were not clear, and the increment observed remained insignificant (data not shown).

Oxygen-dependence of mitochondrial oxygen uptake and H_2O_2 production

Rates of mitochondrial state 2, state 3 and state 4 respiration are depicted as a function of P_{O_2} in Fig. 4. The rate of oxygen consumption of mitochondria from *N. pelagica* increased significantly in states 2 and 3 with increasing P_{O_2}. In addition, state 4 respiration with both substrates also tended to be higher at elevated P_{O_2}, although this increase was not significant. The mitochondria of *A. islandica* showed a similar behaviour with increasing P_{O_2}. State 2 and state 3 respiration with malate and succinate and state 4 respiration with succinate as substrate increased significantly between 7.3 and 47.2 kPa. State 4 respiration with malate showed a non-significant tendency to increase.

H_2O_2 production was not measurable in any respiratory state with either substrate in the invertebrate mitochondria (data not shown) at all three levels of P_{O_2}.

Mitochondria from bovine heart displayed completely

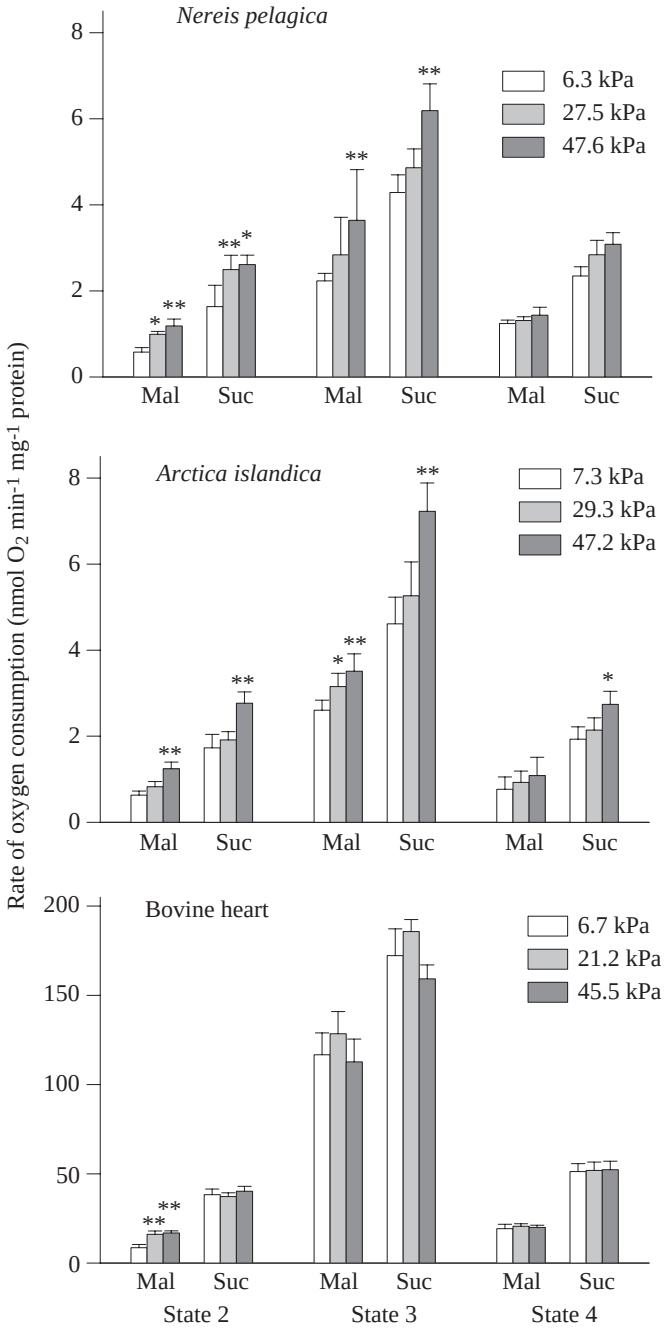

Fig. 4. Mitochondrial rate of oxygen consumption (in nmol O_2 min^{-1} mg^{-1} protein) as a function of P_{O_2} in states 2, 3 and 4 studied in *Nereis pelagica* (body wall) and *Arctica islandica* (mantle) from the White Sea (N=7–9) and in bovine heart mitochondria (N=5). Succinate (Suc) (3.3 mmol l^{-1}) and malate (Mal) (3.3 mmol l^{-1}) oxidation by *N. pelagica* and *A. islandica* mitochondria was measured at 15 °C. State 3 respiration was induced by the addition of 0.07 mmol l^{-1} (*A. islandica*) or 0.13 mmol l^{-1} ADP (*N. pelagica*). Succinate (3.3 mmol l^{-1} plus 0.07 mmol l^{-1} pyruvate) and malate (3.3 mmol l^{-1} plus 0.07 mmol l^{-1} pyruvate) oxidation by bovine heart mitochondria was assayed at 30 °C, and state 3 was induced by the addition of 0.13 mmol l^{-1} ADP. Values are means + S.E.M. Asterisks indicate a significant difference from values measured at the lowest P_{O_2}: *P<0.05, **P<0.01.

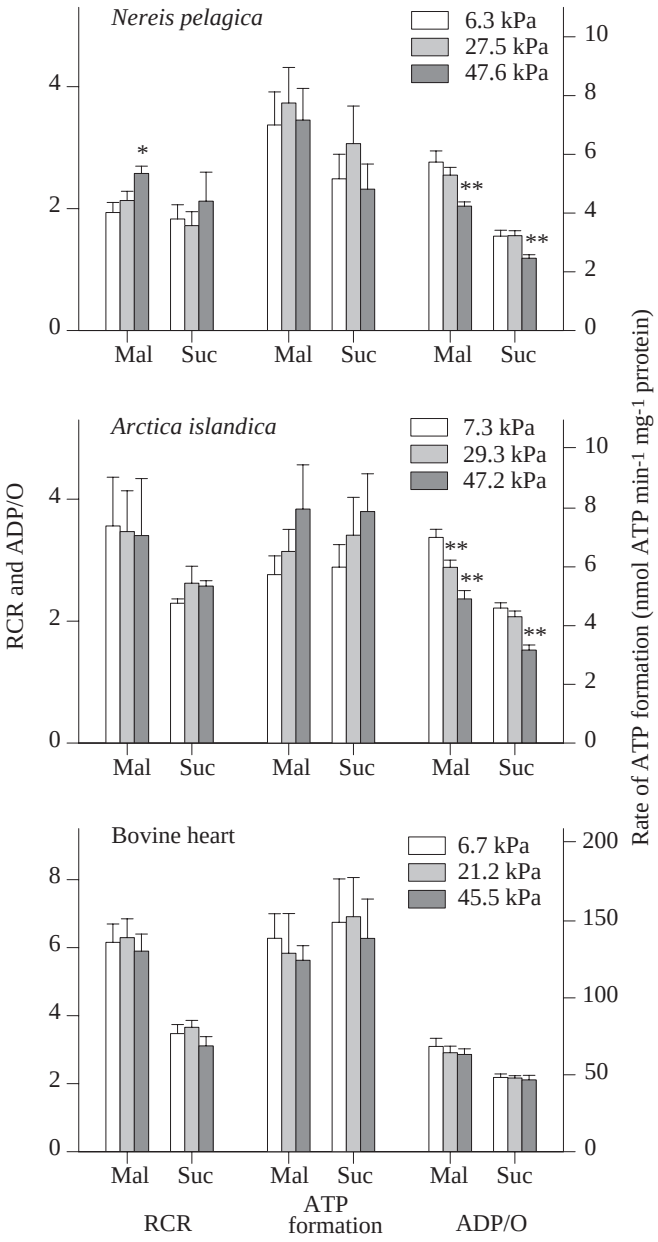

Fig. 5. Respiratory control ratio (RCR), phosphorylation efficiency (ADP/O ratio) and the rate of ATP formation (nmol min^{-1} mg^{-1} protein) as a function of P_{O_2} in mitochondria from White Sea specimens of *Nereis pelagica* (body wall) and *Arctica islandica* (mantle) (N=5–10) (15 °C) and in bovine heart mitochondria (N=5) (30 °C). Substrate concentrations (RCR and ADP/O) are given in the legend to Fig. 4. ATP formation was measured at 15 °C in the presence of succinate (Suc) (2.5 mmol l^{-1}), malate (Mal) (2.5 mmol l^{-1}) and 20 µmol l^{-1} ADP in *N. pelagica* and *A. islandica* mitochondria. In bovine heart mitochondria, ATP formation was assayed in the presence of succinate (2.5 mmol l^{-1} plus 0.05 mmol l^{-1} pyruvate) and malate (2.5 mmol l^{-1} plus 0.05 mmol l^{-1} pyruvate) and 50 µmol l^{-1} ADP at 30 °C. Values are means + S.E.M. Asterisks indicate a significant difference from values measured at the lowest P_{O_2}: *P<0.05, **P<0.01.

different behaviour when exposed to increasing P_{O_2}. Only state 2 respiration with malate in addition to sparking concentrations of pyruvate increased with increasing P_{O_2}. In all other respiratory states, higher P_{O_2} values did not yield higher mitochondrial respiration rates. The H_2O_2 production of bovine heart mitochondria was not investigated.

Oxygen-dependence of mitochondrial control and phosphorylation

The effects of different P_{O_2} values on respiratory control ratio (RCR), phosphorylation efficiency (ADP/O) and rates of ATP formation by *N. pelagica* (body wall), *A. islandica* (mantle) and bovine heart mitochondria are shown in Fig. 5. RCRs of *N. pelagica* mitochondria were not negatively affected by P_{O_2} (Fig. 5). In contrast, a significant increase in RCR was observed at elevated P_{O_2}, but only with malate as respiratory substrate. ADP/O ratios with malate and succinate decreased significantly with increasing P_{O_2}. When calculated according to Chance and Williams (1955), the ADP/O ratio decreased from a mean value of 2.76 to 2.04 (with malate) and from 1.55 to 1.19 (with succinate) when P_{O_2} increased from 6.3 kPa to 47.6 kPa. Accordingly, rates of ATP formation in *N. pelagica* mitochondria, determined in separate assays by luminometric ATP analysis, were not affected by increasing P_{O_2} despite increasing rates of oxygen consumption.

Calculated RCRs of *A. islandica* with malate and succinate as substrate did not change with increasing P_{O_2} (Fig. 5). Between P_{O_2} values of 7.3 and 47.2 kPa, ADP/O ratios (calculated according to Chance and Williams, 1955) with malate and succinate as respiratory substrates decreased significantly, from a mean value of 3.37 to 2.37 for malate and from 2.22 to 1.52 for succinate. Exposure to higher P_{O_2} values caused a non-significant increase in the overall rates of ATP formation determined luminometrically.

In contrast to invertebrate mitochondria, RCR, ADP/O ratio and the rate of ATP formation of isolated mitochondria from bovine heart (Fig. 5) did not change with increasing P_{O_2}.

Latitudinal differences between mitochondria

Rates of oxygen consumption in respiratory states 2, 3 and 4 with both malate and succinate as substrates were significantly greater in mitochondria from White Sea than from Baltic Sea *N. pelagica* and *A. islandica* (Figs 6A, 7A). At a common assay temperature, rates of oxygen consumption in states 3 and 4 were approximately double (per milligram of mitochondrial protein) in subpolar compared with boreal animals. ADP/O ratios and RCR values in mitochondria from *N. pelagica* from the White Sea were slightly lower than those of the Baltic Sea *N. pelagica*. The difference was significant only for RCR determined with succinate as the respiratory substrate (Fig. 6B). However, mitochondrial ADP/O ratios and RCRs were significantly greater in *A. islandica* from the White Sea than in *A. islandica* from the Baltic Sea except for the ADP/O ratio with malate, where the difference was not significant (Fig. 7B).

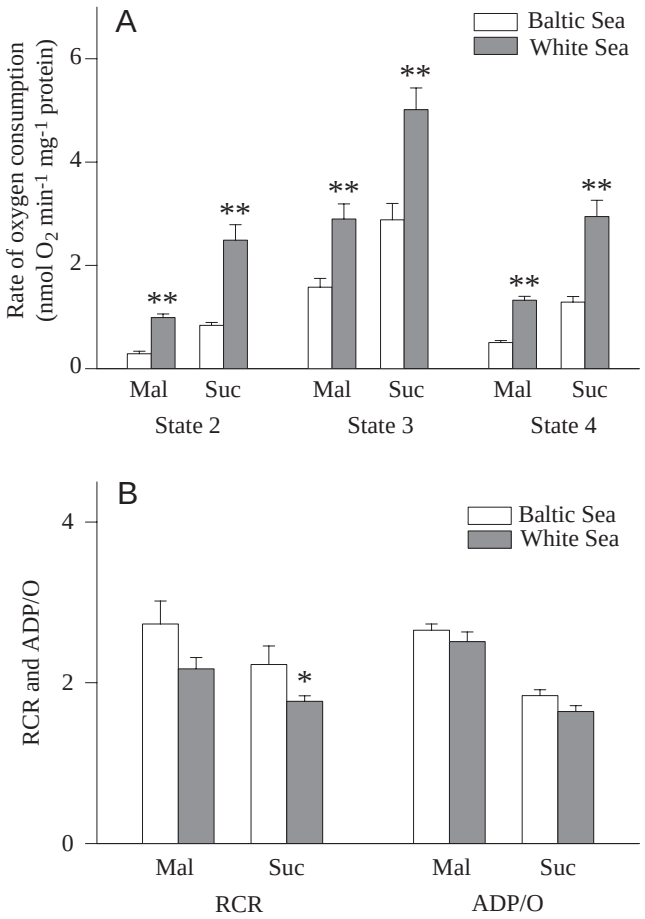

Fig. 6. Rates of oxygen consumption of mitochondria from Baltic and White Sea specimens of *Nereis pelagica* (body wall) in states 2, 3 and 4 (A) and phosphorylation efficiency (ADP/O ratio) and respiratory control ratio (RCR) (B) at 15 °C and under normoxia. For substrate concentrations, see Fig. 4. Values are means + S.E.M. Data are from 6–12 preparations (Baltic Sea) and from 8–11 preparations (White Sea). Asterisks indicate a significant difference from data collected from Baltic Sea specimens: *P<0.05, **P<0.01 (two-tailed t-test for unpaired samples).

Discussion

Nereid polychaetes have previously been characterized as oxyconformers (Shumway, 1979; Kristensen, 1983). Accordingly, the rate of oxygen uptake of isolated body wall tissue from *N. pelagica* showed a linear dependence on P_{O_2} (Fig. 1A). This is reminiscent of the patterns observed in the marine worm *Sipunculus nudus*, in which oxyconformity was found in completely aerobic animals, isolated body wall musculature and isolated cells (Pörtner et al., 1985; Pörtner and Grieshaber, 1993). Why has *A. islandica* been classified as an oxyregulator (Taylor and Brand, 1975a,b) even though isolated mantle tissue displays oxyconforming respiration (Fig. 1B)? The ability to oxyregulate seems to depend on body size. Smaller animals exhibited a reduced regulatory capacity (Taylor and Brand, 1975a,b). Bayne (1971), investigating only small animals, characterized *A. islandica* as a strict oxyconformer. This shelled

animal is more dependent on ventilation for its oxygen supply than the worms, a statement also true for large animals. Oxyregulatory respiration by *A. islandica* tissues in the intact animal may be caused by the maintenance of tissue P_{O_2}, which is controlled by changes in ventilation in intact animals, thereby masking tissue oxyconformity.

In contrast, animals such as *N. pelagica* and *Sipunculus nudus* rely on skin respiration. They are therefore subject to changing P_{O_2} in the body fluids depending on ambient P_{O_2}. In consequence, oxyconformity may be much more evident. As a corollary, the mechanisms of cellular oxyconformity may exist but may not be immediately obvious in species that are able to maintain tissue P_{O_2} and in which whole animals display full oxyregulation.

If oxyconformity and oxyregulation are controlled at the cellular level, these patterns should also become obvious in isolated mitochondria. Processes that could theoretically account for a stimulation of rates of mitochondrial oxygen consumption at high P_{O_2} are an increased electron flux through the classical pathway, implying increased rates of ATP formation at elevated P_{O_2}, non-phosphorylating mitochondrial proton leakiness (Brand et al., 1994) and the formation of reactive oxygen species. The literature on the presence of an alternative oxidase, cytochrome *o*, in endoparasites led Pörtner and Grieshaber (1993) to hypothesize that oxyconformity in marine invertebrates could be related to the presence of this additional mitochondrial electron pathway, which would be P_{O_2}-controlled, lead to partial uncoupling of mitochondria and be activated only at high P_{O_2}, implying an increase in the rate of oxygen consumption without a stoichiometric rise in ATP turnover.

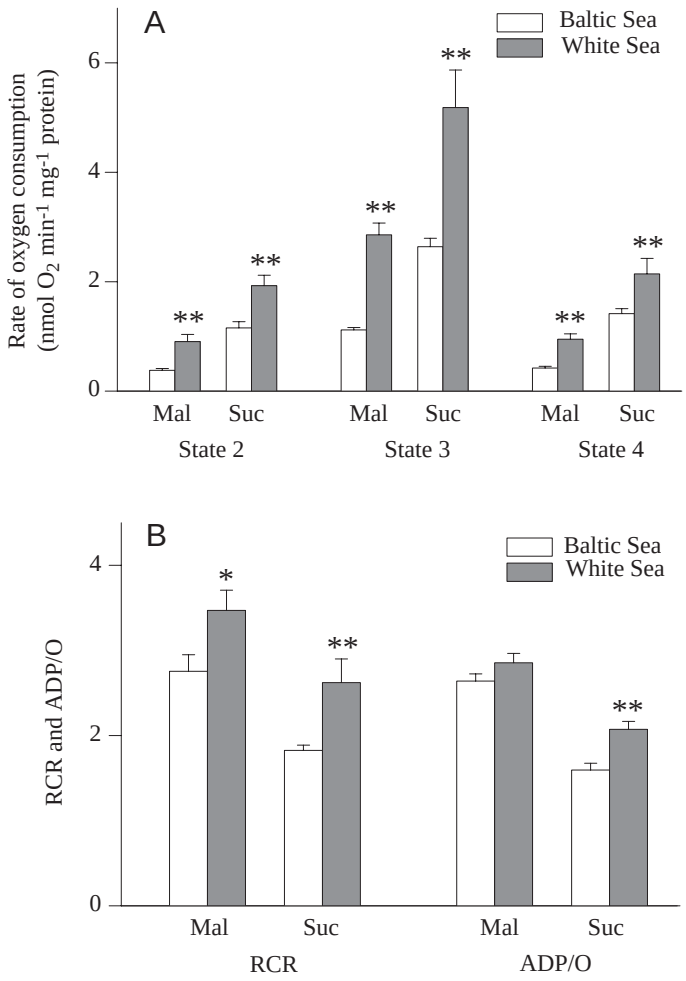

Fig. 7. Rates of oxygen consumption of mitochondria from Baltic and White Sea specimens of *Arctica islandica* (mantle) in states 2, 3 and 4 (A) and phosphorylation efficiency (ADP/O ratio) and respiratory control ratio (RCR) (B) at 15 °C and under normoxia. For substrate concentrations, see Fig. 4. Values are means + S.E.M. Data are from 12–14 preparations (Baltic Sea) and from eight preparations (White Sea). Asterisks indicate a significant difference from data collected from Baltic Sea specimens: **P*<0.05; ***P*<0.01 (two-tailed *t*-test for unpaired samples).

Inhibitory effects of KCN and SHAM on mitochondrial respiration

Studies using KCN and SHAM to characterize alternative respiration pathways have shown that it is often difficult to quantify the relative contributions of the classical and alternative pathways to overall rates of mitochondrial oxygen consumption. This is because, at higher concentrations, cyanide also has some effects on the alternative respiration pathway (Njogu et al., 1980). Similarly, SHAM, when applied at high concentrations (e.g. >5 μmol l^{-1}) inhibits cytochrome *c* oxidase and, thus, the classical respiratory chain (Bingham and Stevenson, 1995; Goyal and Srivastava, 1995).

Inhibition of mitochondrial respiration in *N. pelagica* and *A. islandica* by each of these two inhibitors alone amounted to between 80–90 % (SHAM) and 90–100 % (KCN) (Figs 2, 3; Table 1). With the exception of the sensitivity of cytochrome *c* oxidase for KCN, IC_{50} values for both KCN and SHAM were higher in *N. pelagica* than in *A. islandica* mitochondria (Table 1; Fig. 3). This suggests that the sensitivity of mitochondria to these inhibitors is species-specific and does not provide evidence for the presence of an alternative oxidase. However, the large differences between invertebrate and bovine mitochondria with respect to the maximal inhibition of mitochondrial respiration by SHAM under normoxia are in accord with the hypothesis that an alternative pathway is operative in the mitochondria of both these invertebrates but not in bovine heart. The same degree of cytochrome *c* oxidase inhibition (approximately 20–25 %) with SHAM was found in all three species (Table 1). Maximal inhibition of respiration by SHAM in bovine heart mitochondria amounted to approximately 20 % (Fig. 3) and may exclusively reflect the effect on cytochrome *c* oxidase. As a preliminary estimate, these differences between oxyconforming and oxyregulating mitochondria suggest that a large fraction (up to 70 %, i.e. 80–90 % inhibition of respiration by SHAM minus 20 % inhibition of cytochrome *c* oxidase) of mitochondrial oxygen consumption may involve an alternative respiration pathway (under normoxia) in the two invertebrate species. This result appears to conflict with the relatively high P/O values found in the two invertebrate species, so that further effort is required

to quantify the contribution of an alternative oxidase. The fact that oxyconformity is still observed at the higher values of P_{O_2} used in this investigation suggests that the mechanism involved possesses a very low affinity for oxygen, much lower than is characteristic for mammalian mitochondria (for a review, see Gnaiger et al., 1995) and possibly also lower than observed in bacterial alternative oxidases (D'Mello et al., 1994).

Mechanisms of mitochondrial oxyconformity: alternative oxidase or formation of reactive oxygen species?

In accordance with the existence of an alternative oxidase, respiration of *N. pelagica* and *A. islandica* mitochondria showed oxyconforming behaviour, evident in the stimulation of respiratory states 2, 3 and 4 by increasing P_{O_2} (from 6.3 to approximately 47.6 kPa or 47–357 mmHg) (Fig. 4). Increased rates of oxygen consumption with increasing P_{O_2} were also recorded in experiments with uncoupled mitochondria from the endoparasitic nematodes *Nippostrongylus brasiliensis* and *Ascaridia galli* in the P_{O_2} range between 0.3 and 5.3 kPa (2–40 mmHg, see Paget et al., 1988b). In contrast to the results obtained in the present study, mitochondrial respiration of both nematode species was inhibited above a P_{O_2} of 5.3 kPa (40 mmHg) because of oxidative damage during excessive intramitochondrial production of H_2O_2 (Paget et al., 1987a, 1988b). Both nematode species possess at least one alternative electron pathway in addition to the classical respiratory chain. This pathway is reported as being SHAM-sensitive and to contain cytochrome *o* as a H_2O_2-producing terminal oxidase. In consequence, oxyconforming respiration in these nematodes is thought to involve increasing rates of H_2O_2 production during progressive hyperoxia (up to 255 μmol l^{-1} at 182 mmHg; Paget et al., 1988b) and thereby to contribute to oxidative damage in mitochondria at high P_{O_2}. A comparable observation was made by Wieser et al. (1974) in the free-living marine nematode *Paramonhystera* sp., which exhibited extremely high respiration rates and finally died when exposed to atmospheric P_{O_2} values.

In general, enhanced formation of reactive oxygen species as a result of increased autoxidation of respiratory chain components at high P_{O_2} leads to a net consumption of molecular oxygen (Freeman and Crapo, 1981) and could contribute to mitochondrial oxyconformity. However, the rate of this process is usually two orders of magnitude lower than the maximum rate of reduction of oxygen to water and is unlikely to influence the rate of mitochondrial oxygen consumption (Skulachev, 1996).

The literature cited in the Introduction suggests that several types of alternative oxidase may exist, and some do not cause H_2O_2 production. In general, the function of an alternative oxidase is seen as respiratory protection, a lowering of P_{O_2} to a level that minimizes the risk of formation of oxyradicals (Skulachev, 1996; Popov et al., 1997). In *N. pelagica* and *A. islandica*, mitochondrial oxyconformity goes hand in hand with high RCRs that are not affected by elevated P_{O_2}; H_2O_2 formation could not be measured at any P_{O_2}. Thus, their alternative oxidase is unlikely to produce critical amounts of reactive oxygen species (i.e. H_2O_2). By using this pathway, *N. pelagica* and *A. islandica* could reduce tissue and environmental P_{O_2} and theoretically reduce their susceptibility to increasing oxygen levels (respiratory protection; Khazanov et al., 1992; Paget et al., 1987a, 1988b; Figs 4, 5). The ecological implications are addressed below.

In contrast to the mitochondria from the two invertebrate species, rat liver mitochondria maintain a constant rate of oxygen consumption in the P_{O_2} range between 0.07 and 3.9 kPa (0.5–30 mmHg) (Chance and Williams, 1955; Oshino et al., 1974). The same is true for bovine heart mitochondria (Fig. 4). Thus, increasing P_{O_2} does not lead to higher rates of mitochondrial oxygen consumption in oxyregulators. Moreover, the rate of mitochondrial oxygen uptake and RCR may decrease at high P_{O_2} (approximately 21.3 kPa or 160 mmHg) in oxyregulating mitochondria (e.g. rat brain mitochondria; Khazanov et al., 1992). A similar trend, though non-significant, was found in bovine heart mitochondria in the present study (Figs 4, 5). Khazanov et al. (1992) explained this phenomenon as the onset of oxidative injury affecting mitochondrial coupling at high P_{O_2}. Obviously, oxyregulator mitochondria lack respiratory protection so that an elevated P_{O_2} under experimental conditions may result in oxidative damage. Oxyregulators usually possess a sophisticated system of ventilation and circulation and the respective control mechanisms. Ventilatory and circulatory protection occur at the level of the whole animal, leading to tight control of intracellular P_{O_2} by the fine control of O_2 uptake and delivery. This may eliminate the need for respiratory protection and allow for high phosphorylation efficiencies.

The decreasing ADP/O ratios of mitochondria from the two invertebrate species at high P_{O_2} show that considerably less ATP is synthesized per mole of oxygen consumed under hyperoxia (Fig. 5). Additional oxygen consumption by an alternative oxidase supplementing cytochrome *c* oxidase respiration may explain this finding. The alternative oxidase is described as possessing a lower oxygen affinity than cytochrome *c* oxidase (Henry et al., 1978; D'Mello et al., 1994). Our results concur with this view: in mitochondria from both invertebrate species, ADP/O ratios with malate and succinate were still close to 3 and 2 at the lowest P_{O_2} studied and only fell with increasing P_{O_2} (Fig. 5). According to data in the literature, the branch from the classical respiratory chain *via* an alternative oxidase occurs after the first phosphorylation site (Cheah, 1972, 1973; Mendis and Evans, 1984; Paget et al., 1988a), causing partial uncoupling of mitochondrial phosphorylation (Cheah, 1972; D'Mello et al., 1994). Hence, a decline in ADP/O ratios is to be expected. In our experiments, phosphorylation efficiency (ADP/O) between the highest and the lowest P_{O_2} decreased in invertebrate but not in bovine mitochondria, by approximately –0.4 (succinate) and –0.7 (malate) in *N. pelagica* and by approximately –0.7 (succinate) and –1.0 (malate) in *A. islandica* (Fig. 5). These findings are again in line with the occurrence of an alternative oxidase in the invertebrates. As a result of partial mitochondrial uncoupling, rates of ATP formation will fall behind the increase in the rate of oxygen consumption but may still increase, as seen in *A. islandica* mitochondria at high oxygen tensions (Figs 4, 5).

In contrast to the invertebrate organelles, bovine heart

mitochondria showed constant ADP/O ratios with increasing P_{O_2} (Figs 4, 5). Again, comparison with this oxyregulating reference organism provides evidence for the existence of an alternative oxidase in the two invertebrate species.

Mechanisms of mitochondrial oxyconformity: proton leaks?

A mechanism that might contribute to a higher mitochondrial oxygen turnover at elevated P_{O_2} is a potential augmentation of proton leakage through the inner mitochondrial membrane. The rate of oxygen consumption in state 4 (non-phosphorylating) respiration is mainly due to this process (Hafner et al., 1990; Brand et al., 1994), but it can also include residual ATP turnover and associated rephosphorylation of ADP (Masini et al., 1983). F_1-ATPase activity in state 4 respiration is inhibited by oligomycin (Brand, 1990). Preliminary experiments have shown that state 4 respiration in *N. pelagica* mitochondria in the presence of oligomycin ($1\,\mu g\,mg^{-1}$ protein) increased only non-significantly and by approximately 5 % in the range of P_{O_2} between 6.3 and 47.6 kPa (47–357 mmHg) (data not shown). The rate of proton leak depends on the mitochondrial proton motive force (Δp) and will, therefore, be even lower during state 3 respiration when Δp is reduced.

In conclusion, neither proton leakage nor the formation of reactive oxygen species, the two oxygen-consuming processes unaffected by oligomycin, can account for the very large increase (of more than 35 %) in the overall rate of mitochondrial oxygen consumption in respiratory state 3 at increased P_{O_2} in *N. pelagica* and in *A. islandica* (Fig. 4). These findings support the existence of an alternative oxidase. Since the P_{O_2}-dependent increase in the rate of oxygen consumption depends upon the addition of ADP, it is likely that activity of the alternative oxidase is coupled to ATP synthesis. The reduced oxygen-sensitivity seen in state 4 respiration may relate to some contribution of the alternative oxidase to the residual ATP turnover typically observed in this state (Masini et al., 1983).

Ecological and evolutionary implications

The alternative respiration pathway, which appears to be used by both invertebrate species studied, may represent a characteristic adaptation to their habitat, in which environmental oxygen levels exhibit temporary fluctuations between anoxia and normoxia, in the case of *Arctica islandica*, and even hyperoxia, as in areas of high photosynthetic activity, in the habitat of *N. pelagica*. The lower oxygen affinity of the alternative oxidase provides a simple means of controlling the onset and activity of this pathway. Control of the alternative pathway by ambient P_{O_2} would be useful in confined environments in which the P_{O_2} fluctuates, since this oxidase would reduce excess ambient and tissue oxygen levels during times of high environmental P_{O_2}, thereby minimizing the risk of oxidative stress (Pörtner and Grieshaber, 1993; Skulachev, 1996; Popov et al., 1997). A possible drawback of this strategy would, however, lie in a waste of the nutrients oxidized at low ATP yields whenever P_{O_2} is high. This emphasizes the early phylogenetic origin of this mechanism and makes the use of this alternative oxidase appropriate for permanent life in largely hypoxic environments when phosphorylation efficiency is maintained as high as, and for as long as, possible. The perfection of ventilatory control in higher, oxyregulating animals may explain why this mechanism has been abandoned during evolution in favour of high phosphorylation efficiency.

Cold compensation of mitochondrial capacity

Air-saturated sea water at 0 °C contains 1.6 times more oxygen than at 20 °C. However, a decrease in temperature may affect the mechanisms of ventilation and circulation in such a way that a reduction in oxygen supply to the tissues occurs. Mitochondrial proliferation in the cold may prevent the loss of tissue function (for a review, see Guderley, 1998; Pörtner et al., 1998, 2000). Sommer and Pörtner (1999) found 2.4 times more mitochondria in the body wall of subpolar than in that of North Sea specimens of *Arenicola marina* (cf. Pörtner et al., 2000). The present study demonstrates that not only the number of mitochondria but also the aerobic capacity of individual mitochondria is greater in White Sea *N. pelagica* and *A. islandica*. Specific rates (per milligram protein) in state 2, 3 and 4 respiration are approximately 2–3 times higher in mitochondria from White Sea specimens (Figs 6A, 7A), indicating that respiratory coupling (RCR) is independent of maximum aerobic capacity.

The ratio of state 3 to state 4 respiration (RCR) was somewhat lower in *N. pelagica* but higher in *A. islandica* from the White Sea compared with the Baltic Sea populations, reflecting no consistent pattern of change with latitude. RCR probably reflects the ratio of maximum aerobic capacity and proton leakage. In this context, it is relevant that mitochondrial proton leakage, in general, correlates with the standard metabolic rate (SMR) of an animal (Brookes et al., 1998). SMR is known to be related, by a more-or-less constant factor, to maximum aerobic capacity (Wieser, 1985; Brand, 1990). Our data may suggest that this relationship remains more-or-less unchanged with increasing levels of mitochondrial capacity in a gradient of geographical distribution towards high latitudes. Unchanged and, in the case of *A. islandica*, even slightly higher ADP/O ratios also indicate (Figs 6B, 7B) that the mitochondria of subpolar specimens of *N. pelagica* and *A. islandica* maintain their phosphorylation efficiency at low habitat temperature. Finally, the presence of an alternative oxidase in cold-adapted mitochondria could help both invertebrate species to withstand high ambient oxygen levels at low temperatures.

Concluding remarks

Oxyconformity in *N. pelagica* and *A. islandica* is manifest at the tissue and at the subcellular (mitochondrial) level. The increased respiratory activity of oxyconforming mitochondria at elevated P_{O_2} is characterized by a decrease in phosphorylation efficiency. This indicates that the alternative respiration pathway contributes greatly to the increase in the rate of oxygen consumption and less to phosphorylation and that it is regulated by ambient P_{O_2}, thereby allowing a high phosphorylation efficiency at low oxygen levels. The mitochondria of both

invertebrate species show reduced susceptibility to increasing P_{O_2} (respiratory protection) compared with mammalian mitochondria, indicating either that the presumed alternative oxidase does not produce critical amounts of reactive oxygen species or that an effective antioxidant system is present in these invertebrate mitochondria.

The presence of a P_{O_2}-controlled alternative oxidase may represent a means of providing a flexible response to fluctuating oxygen tension, thereby minimizing oxidative stress. The partial 'uncoupling' of respiration and phosphorylation enables these animals to increase their rate of oxygen uptake without increasing their ATP turnover, at the expense of consuming excess nutrients. We suggest that the presence of the alternative oxidase is adaptive in confined habitats with fluctuating oxygen levels.

With respect to cold adaptation, it can be inferred that the observed increase in mitochondrial aerobic capacity in White Sea compared with Baltic Sea populations of these species is due to alterations in the properties of their mitochondria. The elevated aerobic capacity of mitochondria from the subpolar White Sea populations reflects cold compensation and the maintenance of oxyconformity at low temperature.

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