J. exp. Biol. **149**, 133–148 (1990)
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PRODUCTION OF THIOSULPHATE DURING SULPHIDE OXIDATION BY MITOCHONDRIA OF THE SYMBIONT-CONTAINING BIVALVE *SOLEMYA REIDI*

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Accepted 5 October 1989

Summary

Isolated mitochondria of the bivalve *Solemya reidi* Bernard oxidize sulphide and couple this oxidation to ADP phosphorylation. The products of mitochondrial sulphide oxidation were analyzed by HPLC using monobromobimane derivatization. Concurrent measurements of respiration were made using sulphide-insensitive oxygen electrodes. *S. reidi* mitochondria oxidized sulphide exclusively to thiosulphate. The reaction occurred in two steps. One sulphide molecule was first oxidized to sulphite. A second molecule of sulphide was then added oxidatively to form the free product thiosulphate. This oxidation was obligately linked to mitochondrial electron transport and could be inhibited by the cytochrome *c* oxidase inhibitor hydrogen cyanide, or by low oxygen concentration. The site II inhibitor antimycin A did not inhibit thiosulphate production, indicating that sulphide oxidation is linked through only one ATP coupling site (site III). A calculation of the respiratory potential for ATP synthesis by fully intact mitochondria indicated that 2.0–3.25 ATP per sulphide may be synthesized using the proton potential generated by sulphide oxidation. This estimate far exceeds the published phosphorylation ratios for *S. reidi* (0.5–1.2 ATP per sulphide). This difference may be accounted for by partial uncoupling of phosphorylation from sulphide-based respiration. This hypothesis is supported by the observation that the respiratory control ratio of mitochondria respiring on sulphide is 41 % lower than that of mitochondria respiring on succinate. The respiratory control ratio is an index of the tightness of coupling of respiration to ADP phosphorylation. When the adenylate pool of a eukaryotic cell is mostly phosphorylated, respiration is very slow, owing to the maintenance of a high mitochondrial membrane potential. Uncoupling of oxidative phosphorylation from respiration would be an adaptive advantage to the animal in that it allows for continuous, rapid removal of the toxic molecule hydrogen sulphide.

Introduction

The protobranch mollusc *Solemya reidi* inhabits hydrogen-sulphide-rich marine sediments along the west coast of North America. This clam, which lacks a gut,

Key words: *Solemya reidi*, sulphide oxidation, sulphide-oxidizing mitochondria, symbiosis.

contains intracellular symbiotic bacteria in its gills (Felbeck, 1983). The bacteria are believed to be chemoautotrophic sulphur oxidizers and the presence of enzymes involved in sulphur metabolism has been demonstrated (Felbeck *et al.* 1981). Stable carbon isotope measurements (Felbeck, 1983; Conway *et al.* 1989) and CO_2 fixation studies (Fisher and Childress, 1986) confirm that carbon fixed by the bacteria contributes to the nutrition of the clam. Furthermore, Anderson *et al.* (1987) have shown that *S. reidi* are net autotrophs (that is, the intact symbiosis shows net CO_2 consumption) when exposed to $100\,\mu mol\,l^{-1}$ sulphide without oxygen limitation.

The requirements for having a sulphide-based metabolism pose a serious problem for the animal because hydrogen sulphide is highly toxic to aerobic respiration. Cytochrome *c* oxidase, catalase and other haem-containing enzymes are inhibited by sulphide at low micromolar concentrations (National Research Council, 1979). Some animals have been shown to have pseudocatalases which are sulphide-insensitive (Morrill *et al.* 1988) but, in general, the occurrence of sulphide-insensitive forms of enzymes in animals is rare. *S. reidi* has a typical cytochrome *c* oxidase which is poisoned by sulphide (Hand and Somero, 1983). The clam is thus faced with the need to detoxify hydrogen sulphide.

Though the bacterial symbionts are capable of sulphur metabolism, Powell and Somero (1985) discovered that the initial steps of sulphide oxidation occur in the animal tissue. They initially described an organelle, termed a 'sulphide oxidizing body,' in gill cells, that performs sulphide oxidation. They have also shown subsequently (Powell and Somero, 1986) that mitochondria are capable of sulphide oxidation. In fact, isolated *S. reidi* mitochondria couple sulphide oxidation to aerobic respiration and are capable of ATP production with sulphide as the sole respiratory substrate. The extent to which this sulphide-dependent ATP production contributes to the animal's metabolism remains unclear, however.

The pathway of sulphide oxidation by mitochondria and the sulphur products formed have not been studied. Sulphur can exist in at least five different oxidation states so the level of oxidation of the final product of sulphide oxidation has important implications for the potential energetic yield of this process. If sulphide is fully oxidized to the level of sulphate, eight electrons are available for respiration. However, if sulphide is oxidized only to the level of elemental sulphur (S^0), only two electrons are made available for respiratory energy conservation. The existence of many forms of sulphur in different oxidation states makes available to the clam two general metabolic strategies for detoxification of sulphide. If mitochondria oxidize sulphide completely to sulphate, they will detoxify sulphide and make the greatest use of its energetic potential. However, they will also render it unavailable as a substrate for bacterial nutrition. This would represent a competition between the mitochondria and the symbiotic bacteria. If, in contrast, mitochondria partially oxidize sulphide to a non-toxic intermediate, they will prevent poisoning while still providing a useful substrate for bacterial metabolism. The resolution of the strategy used by *S. reidi* will shed light on the nature of the symbiosis. Previous whole-animal studies (Anderson *et al.* 1987

Vetter *et al.* 1989) have shown that thiosulphate accumulates in the blood of *S. reidi* exposed to sulphide. It is not clear whether this thiosulphate is derived from mitochondrial oxidation of sulphide or from oxidation of sulphide by some other system. Our preliminary results (Vetter *et al.* 1989) indicate that mitochondria do produce thiosulphate as the product of sulphide oxidation. In this study, we have investigated how *S. reidi* mitochondria oxidize sulphide and how this oxidation may be coupled to ATP production.

Materials and methods

Experimental animals

Solemya reidi were collected at the Hyperion sewage outfall in Santa Monica Bay, California, at a depth of 100 m using a modified Van Veen grab. The animals were maintained in mud from the site in a flowing seawater aquarium. The aquarium was kept dark at 8°C to mimic natural conditions. The health of the clams and their ability to oxidize sulphide slowly deteriorated during maintenance. All data were taken from experiments conducted within 4 weeks of capture of the animals.

Sprague–Dawley rats were maintained at the animal care facility at the University of California, San Diego. Animals were fed *ad libitum* on rat chow until killed.

Isolation of mitochondria

S. reidi gill mitochondria were isolated by differential centrifugation in a medium consisting of 0.4 mol l^{-1} sucrose, 0.4 mol l^{-1} mannitol, 0.5 mmol l^{-1} EGTA, 10 mmol l^{-1} Hepes, pH 7.4 at 20°C, and 0.4% (w/v) bovine serum albumin (essentially fatty-acid free). One to three grams of gill were homogenized in five volumes of ice-cold isolation medium using an all-glass Dounce homogenizer. The centrifugation procedures were as described in Powell and Somero (1986). Briefly, the homogenate was centrifuged for 10 min at 1000 *g*. Mitochondria were pelleted from the supernatant by centrifugation for 10 min at 9000 *g*. The pellet was resuspended in buffer and washed in an identical high-speed spin. The final pellet was resuspended in a minimum volume of buffer and used immediately. This protocol yields a mitochondrial preparation largely devoid of bacterial symbionts, although a small degree of contamination is unavoidable. Unlike free-living marine bacteria which approach mitochondria in size (0.1–0.5 μm), the symbionts are considerably larger (1–10 μm, Felbeck, 1983; Cavanaugh, 1983). Furthermore, in the healthy animals used, the bacteria frequently contain intracellular elemental sulphur deposits, making them very dense. As a result of both these factors, the bacteria are largely removed in the low-speed spin.

Rat liver mitochondria were isolated in 0.25 mol l^{-1} sucrose by similar procedures. The centrifugation protocol differed in that the low-speed spin was at 700 *g* for 10 min and the high-speed spins were at 6000 *g* for 10 min.

Isolated rat liver mitochondria were used intact for control experiments

concerning sulphide oxidation. They were also used to prepare the inverted inner membrane vesicles (submitochondrial particles or SMPs) which were used for the oxygen assays. Mitochondrial inner membranes were prepared by digitonin fractionation according to Schnaitman and Greenawalt (1968). The mitochondrial outer membranes were stripped away from the inner membranes with a low concentration of detergent and separated by centrifugation. The resulting 'mitoplasts' were used to prepare SMPs by the method of Lemasters (1980). Briefly, purified inner membrane vesicles were washed and swollen in diluted isolation medium. They were then sonicated to break the membranes into small inverted vesicles. Undisrupted inner membranes were removed by centrifugation for 15 min at 9000 *g*. The SMPs were pelleted by centrifugation for 1 h at 210 000 *g*. The vesicles were resuspended in 0.25 mol l^{-1} sucrose to 20 mg ml^{-1} and stored at −80 °C in single-use samples.

Respiration measurements

Oxygen consumption measurements were made with a Clark-type O_2 electrode (Strathkelvin Instruments 1302 microcathode electrode) modified to reduce interference by H_2S. The modification procedure followed that of Revsbech and Ward (1983) for the production of oxygen microelectrodes. The platinum cathode was etched back for 3 min in saturated KCN and washed with 0.1 mol l^{-1} HCl to remove residual cyanide. Gold was electroplated onto the cathode from a solution of 5 % $KAu(CN)_2$ in 0.2 mol l^{-1} ammonium citrate, pH 6.3. A potential of 1.5 V d.c. (negative at the cathode) was applied for 5 min with a 680 kΩ resistance in the circuit. A graphite rod completed the circuit so that the Ag–AgCl anode was not exposed to the plating solution. Following this treatment, the electrode was used according to the manufacturer's instructions. Electrodes modified in this way were largely insensitive to sulphide concentrations up to 1 mmol l^{-1} and had similar oxygen sensitivity to platinum cathode electrodes.

The oxygen electrodes were mounted in acetal holders which fit tightly into the glass water-jacketed chamber (Strathkelvin RC300 respiration chamber). The volume of the chamber could be varied from 0.3 to 1.9 ml by moving the electrode holder up and down. The assay mixture was stirred continuously with a micro stirring bar. A narrow groove machined into the electrode holder stem allowed samples to be taken and additions to be made using Hamilton syringes. It was necessary for us to modify the acetal electrode holders supplied by the manufacturer to accommodate the syringe barrel. This was done by reaming a groove approximately 9 mm wide in the mounting ring of the acetal holder. With this system, it was not necessary to remove the electrode from the solution or introduce air bubbles when making additions or taking samples. Periodically removing samples did not influence the oxygen consumption rate in the experimental mixture.

S. reidi mitochondrial respiration was assayed in a medium consisting of 0.5 mol l^{-1} glycine, 0.15 mol l^{-1} KCl, 5 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} potassium

phosphate, pH 7.2 (at 20°C). Assays were performed at 10°C. Rat liver mitochondrial respiration was assayed in 0.15 mol l^{-1} sucrose, 3 mmol l^{-1} $MgCl_2$, 3 mmol l^{-1} potassium phosphate, pH 7.0 at 20°C. Assays were at 20°C. All assays, unless otherwise indicated, were begun with buffer fully saturated with air at the assay temperature.

Oxygen electrode calibration

Polarographic oxygen electrodes measure the activity of oxygen in a solution (Hale, 1983). This is not strictly comparable to oxygen concentration. A number of factors influence oxygen activity in solution including temperature, partial pressure of O_2 and solute composition of the medium. Extensive published tables are available relating temperature and atmospheric pressure to oxygen concentration in pure water and sea water of varying salinities. However, the oxygen solubility in solutions of nonionic biological osmolytes is different from that in salt solutions. Thus, it is necessary to measure the oxygen concentration of the buffer in order to calibrate the arbitrary full-saturation reading of the electrode. We measured the oxygen concentration of the assay medium each day by a modification of the procedure of Chappell (1964). Rat liver SMPs were added (1 mg ml^{-1} final) to fully air-saturated assay medium to give a final volume of 1.0 ml. 20 μl samples of 5 mmol l^{-1} reduced nicotinamide adenine dinucleotide (NADH) were added sequentially. The stock NADH solution was degassed continuously by bubbling with nitrogen so that no oxygen was added with the solution. The amount of oxygen consumed upon addition of a sample of NADH (measured in % saturation) was linearly dependent on the O_2 concentration of the assay mixture. Each sequential addition of an identical quantity of degassed NADH resulted in less oxygen consumption (see Fig. 1A). It is unclear whether this pattern was a result of nonlinearity in the response of the O_2 electrode to oxygen concentration or to a biochemical property of the system. In either case, direct measurement of O_2 concentration by equating oxygen consumption to NADH oxidation would lead to an overestimate. To minimize this error, the oxygen consumed (in % saturation) during a single addition of NADH was plotted *vs* the mean oxygen concentration (in % saturation) during that addition (Fig. 1B). A regression line was fitted to the data for at least two series of 4–5 additions of NADH. The theoretical oxygen consumption when the mean oxygen concentration was 100% saturation was calculated from this line (see Fig. 1B legend). The result is a measure in % saturation of the oxygen consumed when one sample of degassed NADH is added to the assay mixture. The total oxygen content of the assay mixture was then calculated from this number assuming that one molecule of NADH reduces one atom of O to H_2O and that NADH was fully oxidized. The exact concentration of NADH in the stock solution was measured spectrophotometrically at 340 nm using an extinction coefficient of 6.22 cm^{-1} $mmol^{-1}$. No endogenous oxygen consumption by the SMPs was observed in the absence of added NADH.

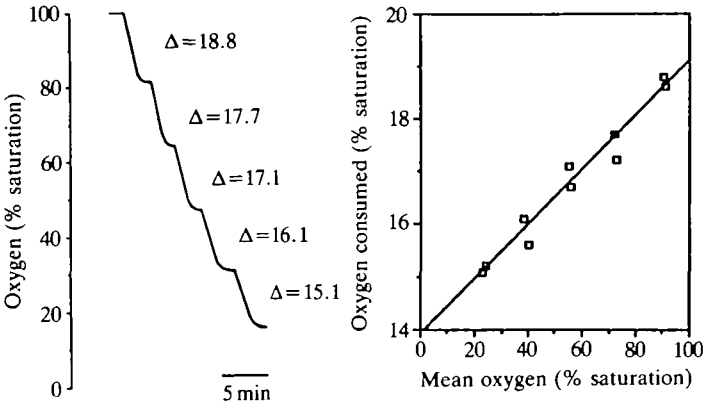

Fig. 1. Oxygen electrode calibration. (A) Oxygen electrode trace during a typical oxygen assay. Each deflection represents the oxygen consumed by submitochondrial particles in 1 ml of buffer after the addition of 100 nmol of NADH. (B) Linear plot of oxygen consumed (in % saturation) on addition of one sample of NADH *vs* mean oxygen concentration (in % saturation) during the respiratory activity following that addition. The intercept at $x=100\%$ saturation gives an estimate of the oxygen which would be consumed on addition of 100 nmol of NADH if the mean oxygen concentration were 100%.

Analysis of the products of $Na_2{}^{35}S$ oxidation

$Na_2{}^{35}S$ was obtained from Amersham. Sulphide oxidation was followed in reaction mixtures starting with a nominal sulphide concentration of 50 μmol l^{-1} in mitochondrial assay medium. The reactions were carried out in the respiration chamber and were started by addition of mitochondria (1.0 mg ml^{-1} final). Our control experiments with different incubation vessels indicated that significant amounts of sulphide were bound to the walls of acrylic vessels. The glass respiration chamber and acetal electrode holders did not bind large amounts of sulphide and could be thoroughly cleaned of protein residues and metal ions which cause sulphide oxidation.

Concentrations of sulphide and its oxidation products were measured by high-performance liquid chromatography using the monobromobimane (mBBr) derivatization technique of Newton *et al.* (1981) as modified by Vetter *et al.* (1989) for the analysis of radioactive sulphur compounds. 100 μl samples of the reaction mixtures were removed and immediately derivatized with mBBr. The reaction of mBBr with the sample rapidly derivatizes most reduced thiols and the reduced sulphur compounds thiosulphate and sulphite. This derivatization of reduced sulphur compounds also prevents their further oxidation. The fully prepared samples were chromatographed on a Gilson HPLC. Fluorescence of the mBBr derivatives was measured with a Gilson fluorometer using a broad-band excitation filter of 305–395 nm and a narrow-band emission filter centred around 480 nm. ^{35}S radioactivity was measured with an in-line flow-through liquid scintillation counter (Raytest Ramona LS) using the ^{14}C window. A non-gelling fluor (Monofluor

National Diagnostics) was used at a ratio of 1:1 with the column effluent. Concentrations of sulphur products were determined by comparison of fluorescence peaks with authentic derivatized standards. Sulphate, the most highly oxidized form of sulphur, is not derivatized by mBBr but was measured as radioactivity in the void volume of the chromatograph. The identity of the sulphate peak can be verified by reacting the sample with barium chloride and rechromatographing. Sulphate is removed as an insoluble barium sulphate precipitate so the radioactive sulphate peak will disappear.

Results

Products of $Na_2{}^{35}S$ oxidation by mitochondria

S. reidi gill mitochondria oxidized $Na_2{}^{35}S$ quantitatively to thiosulphate (Fig. 2A). No other radioactively labelled products accumulated during the course of the reaction. Boiled *S. reidi* mitochondria did not produce thiosulphate over the same period (Fig. 2B). Roughly 40% of the [^{35}S]sulphide disappeared rapidly upon addition of boiled mitochondria but did not reappear as any radiolabelled product. The addition of intact rat liver mitochondria also caused about 50% of the sulphide to disappear initially (Fig. 2C) but the radioactivity did not reappear as soluble products. A small amount of [^{35}S]sulphate (less than 3 μmol l^{-1}) was produced in the reaction mixture but did not account for the sulphide that disappeared. Oxygen consumption continued at a low rate in the presence of sulphide.

Both *S. reidi* and rat mitochondria respired actively on addition of succinate.

Stoichiometry of thiosulphate production

By measuring both radioactivity and fluorescence of the mBBr-derivatized thiols we have been able to determine the origin of both sulphur atoms in the primary oxidation product thiosulphate. Fig. 3 shows that the ^{35}S radioactivity in the form of sulphide was quantitatively replaced by radioactivity in the form of thiosulphate. This means that all the 'sulphide equivalents' were converted to thiosulphate. The measurement of thiosulphate concentration by fluorescence of the mBBr derivative, however, indicated that the concentration of thiosulphate increased at half the rate that sulphide equivalents were incorporated into thiosulphate. This implies that both atoms of thiosulphate were radioactive and hence were derived from free [^{35}S]sulphide.

Linkage of $Na_2{}^{35}S$ oxidation to respiration

The addition of Na_2S to resting (state 2) *S. reidi* mitochondria caused an immediate stimulation of oxygen consumption. When mitochondria were added to buffer containing sulphide, there was also a rapid consumption of oxygen which stopped when the sulphide had been consumed. Fig. 2A shows the stoichiometry of O_2 and Na_2S consumption by *S. reidi* mitochondria. The oxidation of 50 nmol of Na_2S resulted in the consumption of 100 natoms of O.

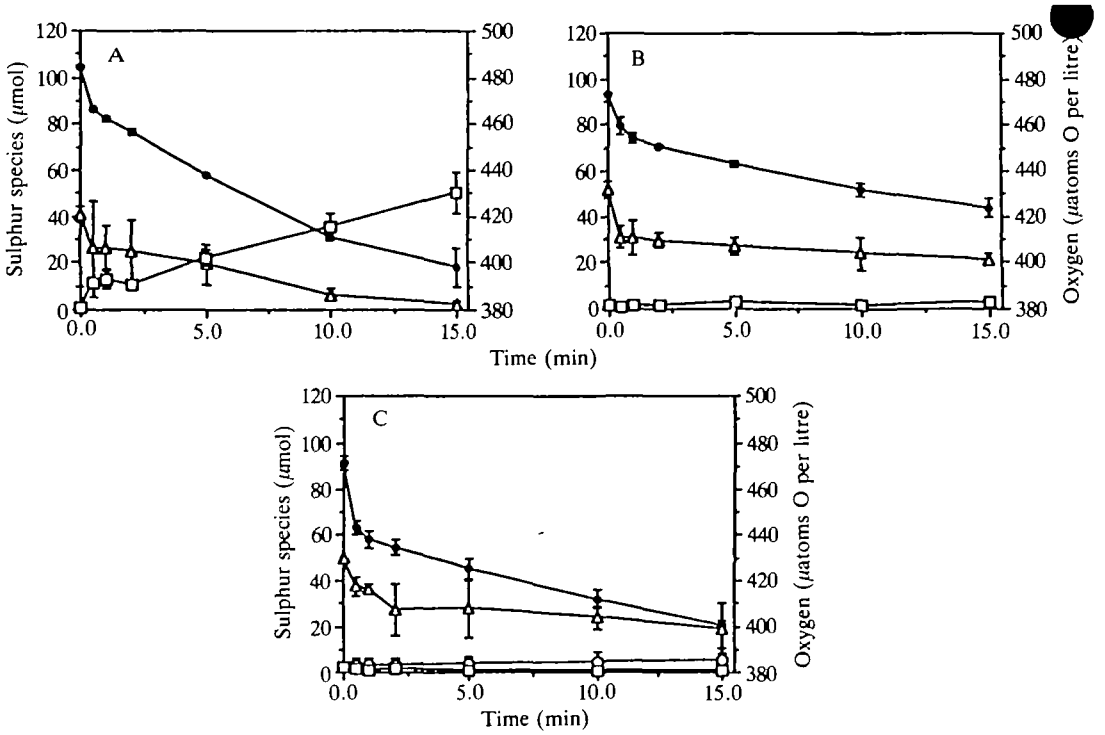

Fig. 2. Oxidation of [^{35}S]sulphide by *Solemya reidi* gill and rat liver mitochondria. Oxygen is represented by solid circles; [^{35}S]sulphide, open triangles; [^{35}S]thiosulphate, open squares; [^{35}S]sulphate, open circles. Isolated mitochondria were given 50 nmol of $Na_2{}^{35}S\,ml^{-1}$. Intact *S. reidi* mitochondria at 10°C (A) oxidized sulphide quantitatively to thiosulphate and consumed two atoms of O per molecule of sulphide oxidized. Boiled mitochondria (B) did not oxidize sulphide to detectable soluble products, though some sulphide disappeared and some oxygen was consumed. Rat liver mitochondria at 20°C (C) removed some sulphide from the medium but did not produce appreciable amounts of soluble products. Oxygen consumption in the presence of sulphide was 80 % of that in *S. reidi* mitochondria. The [^{35}S]thiosulphate line represents the '[^{35}S]sulphide equivalents' incorporated into thiosulphate ($S_2O_3{}^{2-}$; see Fig. 3). Each figure is a mean of three experiments ±1 S.D.

We were unable routinely to eliminate dissolved oxygen from the reaction mixture for the duration of an experiment, but experiments at low O_2 concentrations showed that very little sulphide was oxidized under these conditions. The sulphide that was oxidized was detected in the form of thiosulphate.

The cytochrome *c* oxidase inhibitor hydrogen cyanide (5 mmol l^{-1}) blocked both oxygen consumption and thiosulphate production by *S. reidi* mitochondria (data not shown). Another cytochrome *c* oxidase inhibitor, sodium azide (1 mmol l^{-1}), was ineffective at inhibiting either sulphide oxidation or oxygen consumption, but also failed to inhibit respiration with succinate as the substrate. 67 μmol l^{-1} antimycin A, an inhibitor of the transfer of electrons from cytochrome *b* t

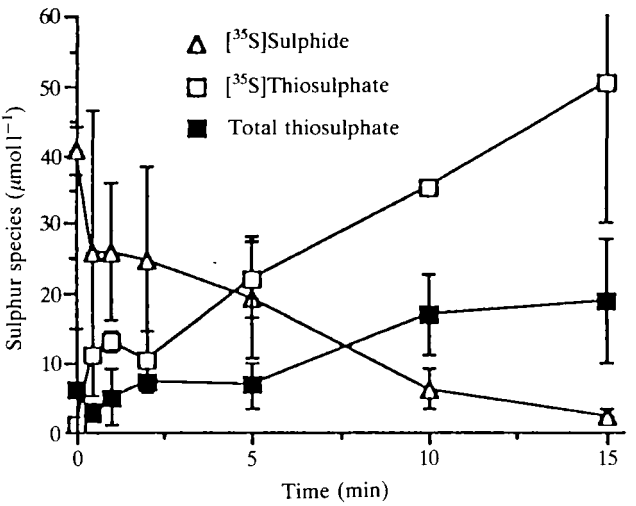

Fig. 3. Stoichiometry of thiosulphate production during [^{35}S]sulphide oxidation by *Solemya reidi* gill mitochondria. [^{35}S]sulphide is represented by the open triangles. ^{35}S radioactivity in the form of thiosulphate (open squares) quantitatively replaces [^{35}S]sulphide during sulphide oxidation. The actual concentration of thiosulphate, measured by fluorescence of the bimane-derivatized product (solid squares), increases at half the rate of incorporation of radioactivity into the compound. Mean of three experiments ±1 S.D.

cytochrome c_1, inhibited respiration with succinate as substrate but did not affect respiration with sulphide as substrate or thiosulphate production.

Sulphide oxidation proceeds through the intermediate sulphite

The two sulphur atoms of thiosulphate are in different oxidation states. The outer or sulphane sulphur is in the S^0 state while the inner sulphur is in the S^{4+} state. There are two ways in which two sulphide molecules may be oxidized to form a thiosulphate molecule. First, a single sulphide molecule may undergo a six-electron oxidation to the level of sulphite, and then a second sulphide molecule may be added with a further two-electron oxidation. Oxidations of sulphide or S^0 to sulphite are well-known in thiobacilli (Kelly, 1982) and involve respiratory-chain-linked oxidation systems. The addition of a sulphane sulphur is similar to the reactions of sulphurtransferases (Westley *et al.* 1983). This two-step process could occur in a membrane-bound state, without the release of sulphite as an intermediate, or sulphite could be released as a free intermediate. Alternatively, two sulphide molecules may interact in a concerted fashion with a net eight-electron oxidation to form thiosulphate directly. No free intermediate is predicted in this reaction, and there is little evidence for this type of reaction in biological systems. The properties of the enzyme(s) that perform sulphide oxidation in the two ways described are expected to be different, so an understanding of this process will shed light on the enzyme(s) involved. Fig. 4 demonstrates that

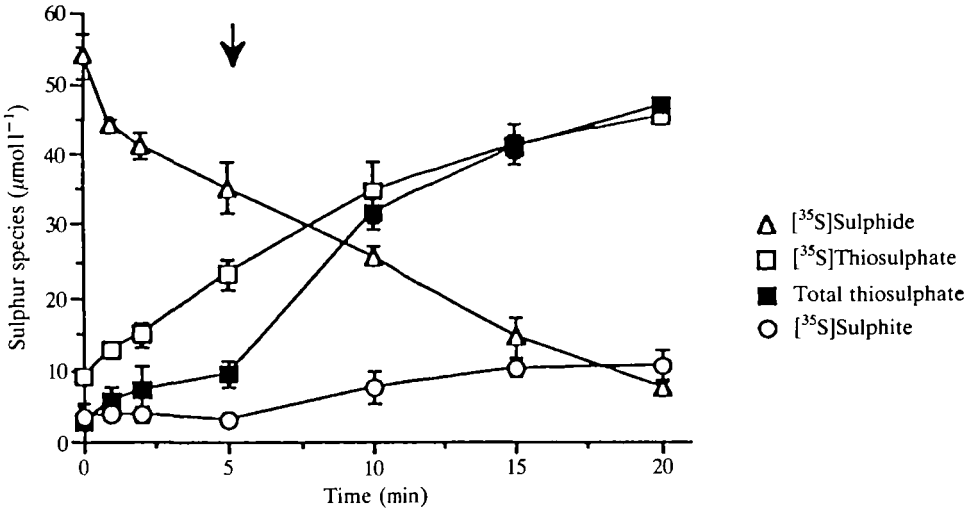

Fig. 4. Competition of complete oxidation of [^{35}S]sulphide (△) with sulphite. *Solemya reidi* gill mitochondria respiring on [^{35}S]sulphide were given a 10-fold excess of unlabelled sulphite at 5.5 min (arrow). Thiosulphate produced after the addition is labelled in only one of the sulphur positions, as indicated by the increase in the rate of production of thiosulphate (■) (measured by fluorescence of the bimane derivative) relative to the rate of incorporation of ^{35}S into thiosulphate (□) (see explanation in Fig. 3). Mean of three experiments ±1 S.D.

sulphide oxidation by *S. reidi* mitochondria proceeds through the intermediate form, sulphite. Mitochondria respiring on 50 μmol l^{-1} $Na_2{}^{35}S$ were presented with a 10-fold excess of unlabelled sodium sulphite. In the absence of sulphite, the product thiosulphate that accumulated was radioactively labelled in both sulphur atoms. This was shown by the accumulation of twice as many '[^{35}S]sulphide equivalents' in thiosulphate as there were thiosulphate molecules (see explanation of Fig. 3). After the addition of excess sulphite, the kinetics of thiosulphate production shifted such that half the thiosulphate sulphur atoms were labelled. Furthermore, the addition of excess unlabelled sulphite caused a slow buildup of labelled sulphite derived from sulphide oxidation. The overall rate of sulphide oxidation did not change when excess sulphite was added and the rate of incorporation of ^{35}S into thiosulphate was also unaffected. The oxygen consumption rate decreased by 29–47 % after addition of sulphite. This is consistent with the replacement of sulphite derived from the six-electron oxidation of sulphide with preformed sulphite. The remaining respiration resulted from the two-electron oxidation of sulphide to the level of S^0 in the final step of thiosulphate formation. The production of thiosulphate from sulphide was abolished by boiling the mitochondria or by omitting mitochondria from the reaction mixture (data not shown). In some control experiments, [^{35}S] sulphide disappeared at a low rate in the presence of excess sulphite, but no soluble products were detected.

Discussion

Solemya reidi mitochondria oxidized sulphide exclusively to thiosulphate. This is similar to the pattern of sulphide oxidation by rat liver extracts (Baxter *et al.* 1958; Baxter and Van Reen, 1958*a*; Sörbo, 1958). The sulphide-oxidizing activity in rat liver has both heat-labile and heat-stable components (Baxter *et al.* 1958). The heat-stable activity is catalyzed by a variety of compounds. Baxter and Van Reen (1958*b*) showed that ferritin, metalloprotein complexes and metal chelates contribute to this component. Free haem compounds have also been implicated in the oxidation of sulphide (Sörbo, 1958; Powell and Arp, 1989). The thiosulphate-producing activity of *S. reidi* mitochondria was greater than 95 % heat-labile. The disappearance of sulphide was only reduced by 55 % by boiling mitochondria. Since healthy mitochondria converted all the sulphide consumed to thiosulphate, we feel that the disappearance of sulphide in boiled preparations is a different phenomenon. It may involve the binding of sulphide to denatured proteins or the chemical oxidation of sulphide to bound polysulphides, which are not detected in our assays. The same processes may account for the disappearance of sulphide in rat mitochondrial preparations which did not yield stoichiometric amounts of soluble products. It should be noted that the rat mitochondria assays were run at 20°C and should show a higher rate of chemical oxidation than do the *S. reidi* mitochondria assays, which were run at 10°C.

The sulphite competition experiment (Fig. 4) indicated that the oxidation of sulphide to thiosulphate involved at least two steps. The presence of excess unlabelled sulphite caused a shift in the ^{35}S labelling pattern of the thiosulphate produced by sulphide oxidation. Thiosulphate produced after addition of unlabelled sulphite was labelled in only one of the two sulphur atoms. We took this to mean that sulphite was formed in the first step(s) of sulphide oxidation and could be replaced by exogenous sulphite in subsequent steps. We cannot conclude from our data whether the sulphite was present as a bound intermediate or was free in solution. Both steps were sensitive to boiling and appear to be enzymically catalyzed.

The oxidation of sulphide to thiosulphate was tightly coupled to respiration in *S. reidi* mitochondria. The cytochrome *c* oxidase (site III) inhibitor hydrogen cyanide inhibits both respiration and ATP production with sulphide as the respiratory substrate (Powell and Somero, 1986). Our study showed that it also inhibited thiosulphate production. Furthermore, low O_2 concentration reduced the rate of thiosulphate production but did not result in the formation of alternative products. This relationship suggests an obligatory link between sulphide oxidation and respiratory oxygen consumption in *S. reidi* mitochondria.

Powell and Somero (1986) found that mitochondrial sulphide oxidation is linked to the respiratory chain at the level of cytochrome *c*. The site II inhibitor antimycin A does not inhibit sulphide-stimulated oxygen consumption whereas cyanide does. Our experiments with thiosulphate production support this conclusion. This means that sulphide oxidation is linked through one 'ATP coupling site,' as was suggested by Powell and Somero (1986). Without knowledge of the product of

sulphide oxidation, prior investigators have not known how many electrons traverse that coupling site per sulphide oxidized, and hence have been unable to predict the potential ATP yield of the process. The results of this study allowed us to make these calculations.

The chemical equation for the oxidation of sulphide to thiosulphate can be written:

$$2H_2S + 3H_2O \rightarrow SSO_3^{2-} + 8e^- + 10H^+. \quad (1)$$

There are four electrons available for respiration per H_2S oxidized. If we assume an $H^+/2e^-$ ratio of 4 for site III (Reynafarje *et al.* 1976, 1986; Beavis, 1987), there should be eight protons ejected from the mitochondrial matrix per H_2S oxidized. In addition to the protons ejected from the mitochondrial matrix, we must take into account the scalar protons produced by sulphide oxidation. These are the protons released by the chemical reaction and are unrelated to those transported across the mitochondrial membrane during respiration. Equation 1 predicts that there are five protons produced per H_2S oxidized. The location of these scalar protons is important to the interpretation of the process. The generation of scalar protons can add to or detract from the electrochemical proton gradient across the mitochondrial inner membrane. The location of the reaction which generates these protons determines what effect they will have. Since sulphide oxidation is linked through cytochrome *c* (Powell and Somero, 1986), it is likely that the oxidation of sulphide occurs outside the mitochondrial inner membrane. Therefore (1) all the respiration-linked protons are available for ATP coupling; (2) the scalar protons may also contribute to the mitochondrial proton gradient and hence be available for ATP coupling.

The number of protons traversing the inner membrane to drive the synthesis of one ATP is termed the proton coupling ratio. The proton coupling ratio of the ATP synthetase is the subject of some controversy. Coupling ratios of 2 and 3 have been proposed (Mitchell, 1966; Alexandre *et al.* 1978; Hinkle and Yu, 1979). A minimum estimate of the ATP production potential of sulphide oxidation will be made by assuming the larger coupling ratio of 3. In addition to the requirement for the translocation of three protons for ATP synthesis, the net inward movement of one additional proton accompanies the exchange of ATP for ADP+Pi (Klingenberg and Rottenberg, 1977). Thus four protons are translocated to effect the synthesis of one ATP from ADP and inorganic phosphate.

If we assume that the oxidation of sulphide occurs at a site distant from the mitochondrial inner membrane, the scalar protons will not contribute appreciably to the electrochemical proton gradient. Then the scalar protons produced by sulphide oxidation would not be used for ATP production. In this case, two ATP can be formed [(eight respiratory protons)/(four protons/ATP)=2ATP] for each sulphide oxidized. This represents the minimum estimate of energy conservation potential if the mitochondria are perfectly coupled.

For isolated mitochondria, a more realistic estimate is made if we assume that sulphide oxidation occurs close to the mitochondrial inner membrane. In this case

the scalar protons can contribute to the electrochemical gradient and 13 protons are available for ATP production. Then (13 protons)/(4 protons/ATP)=3.25 ATP may be produced per sulphide oxidized.

These arguments predict the potential for ATP production from mitochondrial sulphide oxidation to be 2.0–3.25 ATP per sulphide. If a proton coupling ratio of 2 for the ATP synthetase reaction is assumed, then this estimated range increases to 2.7–4.3 ATP per sulphide. In either case, these predictions do not fit well with the published values of 0.5–1.2 ATP produced per sulphide added (Powell and Somero, 1986). A number of factors may contribute to this discrepancy. The mitochondria in the above-mentioned study exhibited poor respiratory control (1.84±0.14 for *S. reidi* gill mitochondria) with sulphide as the respiratory substrate. This indicates poor coupling of respiration with ATP synthesis. Thus, the full potential for ATP production from sulphide may not have been realized. Our preparations show approximately the same respiratory control ratios (1.84±0.24, N=20) with sulphide as the respiratory substrate, while the respiratory controls ratios with succinate as the substrate are substantially higher (3.10±0.54, N=6). Hydrogen sulphide may itself act as an uncoupler of respiration since undissociated H_2S is neutral and readily passes through membranes (National Research Council, 1979). The pK_1 for H_2S is 7.01 at 25 °C (Millero *et al.* 1988). At pH 6, the approximate local pH of the outside of the mitochondrial inner membrane, about 90 % of sulphide is in the form of H_2S. This pool may act as an uncoupler of respiration by carrying H^+ back into the matrix in the form of H_2S. The high pH within the matrix will cause reionization to HS^- and S^{2-}, resulting in the net movement of H^+ into the mitochondrial matrix. This partial collapse of the proton gradient would result in lowered efficiency of oxidative phosphorylation.

Though the factors just discussed may account for the low efficiency of ATP coupling observed, it is also possible that *S. reidi* mitochondria are specifically adapted to reduce ATP coupling efficiency. This argument seems counterintuitive unless one considers the toxicity of H_2S. Powell and Somero (1986) showed that *S. reidi* mitochondria are inhibited by concentrations of sulphide higher than 20 μmol l^{-1}. If sulphide is present in the gill cells of *S. reidi*, then it would be to the animal's advantage to remove it as quickly as possible. Tightly coupled mitochondria *in vivo* are regulated by the availability of cellular ADP. When cellular ATP demand is low, the rate of oxidative phosphorylation is also low. Consequently, if mitochondria are presented with sulphide during a period of low ATP demand, tightly coupled respiration would be slow and hence sulphide oxidation would be slow. The danger of sulphide poisoning would be substantially greater in this case than in the situation in which the mitochondria are loosely coupled. Loosely coupled mitochondria would allow rapid sulphide oxidation without dependency on cellular ATP utilization. A high rate of removal of sulphide may be more important to the animal than is efficient energy conservation. Thus, the poor respiratory control and low ratio of ATP produced to sulphide oxidized may reflect an adaptation for rapid sulphide oxidation.

Other enzymes of sulphur metabolism, such as thiosulphate thiotransferase

(rhodanese) and sulphite oxidase, are normally found in mitochondria (Rajagopalan, 1980; Westley, 1981). It is important to consider why sulphide oxidation might stop at the level of thiosulphate in *S. reidi* mitochondria. Thiosulphate may be the ideal product from the standpoint of the animal–bacterial symbiosis. It is both soluble, a characteristic not shared by elemental sulphur, and non-toxic. Furthermore, a relatively small amount of oxygen is consumed ($1\,O_2$ per H_2S) in the production of thiosulphate from sulphide. This may be important to an animal living in reducing sediments where oxygen may be limiting. Finally, the thiosulphate produced by mitochondria may be further oxidized by the symbiotic bacteria present in *S. reidi* gills. When intact animals are incubated with sulphide, the product released into the water is sulphate (Felbeck, 1983; D. Wilmot, personal communication). *S. reidi* is capable of taking up thiosulphate from the water (Anderson *et al.* 1987), but this is probably not an important route for thiosulphate acquisition. The environment in which the clams live contains virtually no thiosulphate (Vetter *et al.* 1989). Instead, the animal oxidizes sulphide to thiosulphate. Vetter *et al.* (1989) have shown that *S. reidi* accumulate thiosulphate, but not sulphide, in their blood when exposed to sulphide and Anderson *et al.* (1987) have suggested that they may transport this peripherally produced thiosulphate to the symbiotic bacteria. The latter authors have shown that thiosulphate stimulates CO_2 fixation in intact animals and suggest that the bacteria oxidize thiosulphate and use the energy of oxidation to drive CO_2 fixation.

Several other sulphide oxidizing systems have been reported in *S. reidi*. These include 'sulphide oxidizing bodies,' a putative sulphide oxidase enzyme (Powell and Somero, 1985) and free haematin (Powell and Arp, 1989). Any model of sulphide metabolism must take into account all these reported components. Anderson *et al.* (1987) have shown that whole-animal respiration is strongly inhibited above $100\,\mu mol\,l^{-1}$ sulphide and free cytochrome *c* oxidase has a K_i of $0.24\,\mu mol\,l^{-1}$ (Nicholls, 1975). Thus the *in vivo* sulphide detoxification mechanism does not function adequately above $100\,\mu mol\,l^{-1}$ while it must metabolize sulphide rapidly at concentrations below $100\,\mu mol\,l^{-1}$ to prevent inhibition of cytochrome *c* oxidase. The mitochondrial system described here is the only sytem so far described that meets these criteria. The other sulphide-oxidizing systems reported have K_m values above $1\,mmol\,l^{-1}$ and are not inhibited by high sulphide concentrations. How these other sulphide oxidation systems function and how they may transfer electrons to the mitochondria are being studied now. The finding that mitochondria produce only thiosulphate provides a link between observations of high thiosulphate levels in *S. reidi* blood (Powell *et al.* 1987; Vetter *et al.* 1989) and models that suggest that thiosulphate is the form of reduced sulphur transferred between animal and bacteria (Powell *et al.* 1987; Anderson *et al.* 1987).

Finally, the finding that the ATP yield of mitochondrial sulphide oxidation is far below the potential yield for this process indicates that detoxification is the primary function of the system. Though the animal can obtain some ATP from sulphide oxidation, the cost of this ATP is quite high. The ATP to sulphide ratio o

0.5–1.2 (Powell and Somero, 1986) means that 0.5–1.2 ATP are produced per O_2 consumed compared with 4–6 ATP per O_2 for NAD-linked substrates. The primary advantage to the animal must come from its ability to survive in an environment which supports the growth of its chemoautotrophic symbionts but which excludes most other aerobic organisms.

We would like to thank Drs George Somero and Mark Powell for helpful discussions during the course of this work and David Wilmot for assistance with HPLC. We are especially grateful to captains Tom Beattie and Louis Zimm and the crew of the R/V *Robert Gordon Sproul* for their assistance in collecting clams. This research was supported by National Science Foundation grant OCE–86–10513 to George Somero and RDV and ONR grant N00014–88–0012 to RDV.

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