described here are intended to lay the groundwork for the amplification of selectivity between neoplastic and normal cells.

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Hydrogen Sulfide Oxidation Is Coupled to Oxidative Phosphorylation in Mitochondria of *Solemya reidi*

MARK A. POWELL AND GEORGE N. SOMERO

***Solemya reidi*, a gutless clam found in sulfide-rich habitats, contains within its gills bacterial symbionts thought to oxidize sulfur compounds and provide a reduced carbon food source to the clam. However, the initial step or steps in sulfide oxidation occur in the animal tissue, and mitochondria isolated from both gill and symbiont-free foot tissue of the clam coupled the oxidation of sulfide to oxidative phosphorylation [adenosine triphosphate (ATP) synthesis]. The ability of *Solemya reidi* to exploit directly the energy in sulfide for ATP synthesis is unprecedented, and suggests that sulfide-habitat animals that lack bacterial symbionts may also use sulfide as an inorganic energy source.**

MANY MARINE INVERTEBRATE ANIMALS live in sulfide-rich habitats, including the deep-sea hydrothermal vents where geothermally produced sulfide is present (*1*), and reduced sediments where microbial degradation of organic matter leads to the reduction of sulfate to sulfide (*2*). The high sulfide concentrations of these habitats pose a significant threat to these animals because sulfide is a potent inhibitor of aerobic respiration through its effects on the cytochrome *c* oxidase system (*3, 4*). Despite its toxicity, however, sulfide is important for the energy supplies of these animals. Sulfide is a highly reduced compound that can be oxidized by bacteria to yield energy for driving net carbon dioxide fixation. Some, but not all, of the invertebrates of sulfide-rich habitats harbor symbiotic sulfur bacteria in their tissues, and these bacteria are thought to provide their hosts with a substantial source of nutrients (*2*).

The gutless clam *Solemya reidi*, which is found in reduced sediments near sewage outfall zones and pulpmill effluent sites (*5*), contains large numbers of chemolithoautotrophic sulfur bacteria in its gills (*6*) and has a high capacity for aerobic respiration (*7*); nevertheless, the initial steps in sulfide oxidation occur in the animal compartment of the symbiosis, not the bacterial (*6*). This finding led to the present study in which we report that mitochondria of gill and (symbiont-free) foot tissues of *S. reidi* can use sulfide as an inorganic energy source for oxidative phosphorylation. Sulfide oxidation in mitochondria thus protects the aerobic respiration (*6*) of the clam and allows it to exploit, without the direct assistance of bacteria, the energy of the sulfide molecule. This is the first report known to us of an animal that can use an inorganic energy source directly.

To characterize the mitochondria of *S. reidi*, we first examined their ability to use substrates previously reported for marine invertebrate mitochondria (*8*). With succinate and malate as substrates, mitochondria from both tissues respired at rates similar to those reported for mitochondria from other marine invertebrates (*8*), and showed typical mitochondrial acceptor control [stimulation of oxygen consumption by adenosine diphosphate (ADP)] (Fig. 1 and Table 1). Acceptor control, a widely used indicator of the intactness of mitochondria (*9*), is defined as the rate of oxygen consumption when ADP is present divided by the rate when ADP has been consumed. High acceptor control ratios are indicative of intact mitochondria. Our value of 2.5 with succinate as the substrate is in the range reported for marine invertebrates (*8*), which is lower than values common for vertebrates (*9*).

The addition of 20 μM sulfide (*10*) as the sole substrate to mitochondria isolated from gill and foot resulted in oxygen consumption and in acceptor control (Fig. 1 and Table 1). Control experiments without substrates added showed no acceptor control, and control experiments without mitochondria present showed no consumption of oxygen when concentrations of 20 or 50 μM sulfide were added to the system. No oxygen consumption was observed with thiosulfate or sulfite (sulfur compounds more oxidized than sulfide) as substrates. The acceptor control value of 1.8 for sulfide was slightly higher than the value of 1.7 reported for ascorbate plus tetramethylphenylenediamine (*11*), which, like sulfide, are coupled through site III (see below).

Marine Biology Research Division, A-002, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093.

Table 1. Oxygen consumption expressed as mean ± SD (number of samples). State 3 is the rate when ADP is present, state 4 is the rate when all ADP has been phosphorylated. For inhibitors and uncoupler, + means >80% inhibition or uncoupling, and − means <20%, blank means not determined. Abbreviations: R, rotenone; A, antimycin A; C, cyanide; and D, DNP.

Tissue	Substrate	Oxygen consumption (nmol O min^{-1} mg^{-1}) State 3	State 4	Acceptor control ratio	Inhibitors and uncoupler R	A	C	D
Gill	Succinate	13.9 ± 2.9 (8)	5.5 ± 1.2 (8)	2.64 ± 0.88 (8)	−	+	+	+
	Sulfide	8.4 ± 2.4 (7)	4.7 ± 1.3 (7)	1.84 ± 0.14 (7)		−	+	+
	Malate	5.9 ± 0.2 (3)	2.7 ± 0.3 (3)	2.14 ± 0.08 (3)			+	
Foot	Succinate	24.3 ± 3.6 (3)	10.2 ± 1.0 (3)	2.40 ± 0.56 (3)			+	
	Sulfide	19.4 ± 4.6 (4)	11.1 ± 2.1 (4)	1.75 ± 0.20 (4)			+	

The rates of oxygen consumption observed with sulfide as the substrate were higher than with all other substrates tested except succinate. Substrates tested but not shown were glutamate, citrate, pyruvate, proline, and glutamate plus pyruvate. These substrates yielded low rates of oxygen consumption (less than 20% of the rate found with succinate).

The use of sulfide as a substrate required special precautions: above 20 μ*M*, sulfide reversibly inhibited oxygen consumption. To initiate oxygen consumption, we added 20 μ*M* sulfide, which was consumed very rapidly (Fig. 1, A and C). Subsequent 20 μ*M* additions were made only after the initial sulfide was consumed. Because multiple additions of sulfide were required, acceptor control was measured by comparing oxygen consumption rates when sulfide was added just after ADP was added with rates measured when sulfide was added after the ADP had been consumed (Fig. 1, A and C). When sulfide was consumed, no oxygen consumption was observed unless more sulfide or another substrate was added (Fig. 1, A and C).

Ratios of ADP phosphorylated to oxygen consumed (P/O ratio) observed with succinate as the substrate were 1.72 ± 0.24 (SD, $n = 7$). P/O ratios for mitochondria with sulfide as the substrate were 0.76 ± 0.21 ($n = 6$). This finding suggests that coupling of sulfide-based electron transport to oxidative phosphorylation was only through site III of the electron transport chain (the cytochrome *c* oxidase site) (*11*).

Inhibitor studies also indicated a site III coupling of sulfide oxidation (Table 1) (*12*), although the redox potential of sulfide suggests that coupling through sites II and III would be possible in theory (*10*). The site III inhibitor cyanide blocked both sulfide- and succinate-dependent oxygen consumption; the site II (cytochrome *b*) inhibitor antimycin A did not block sulfide-dependent oxygen consumption, but did block succinate oxidation; and the site I (NADH oxidase) inhibitor rotenone had no effect on sulfide or succinate oxidation, but did block malate oxidation. Succinate oxidation by mitochondria from marine invertebrates is coupled through sites II and III, while malate oxidation is coupled through sites I, II, and III (*8*). The mitochondrial uncoupler 2,4-dinitrophenol (DNP) stimulated oxygen consumption with both succinate and sulfide as substrates to state 3 levels (those found with ADP present) in the absence of ADP. This finding is characteristic of mitochondrial oxidative phosphorylation (*12*).

To determine if the oxidation of sulfide was coupled to adenosine triphosphate (ATP) synthesis, we measured the production of ATP by mitochondria with a continuous assay system involving a hexokinase trap of the ATP produced (*13*). Sulfide, succinate, and malate all stimulated ATP synthesis (Fig. 2 and Table 2). Control experiments with no added substrate or with no coupling enzymes showed no ATP production. Thiosulfate or sulfite did not serve as substrates for ATP production. Twenty micromolar sulfide produced the maximal rates of ATP production, as well as maximal oxygen consumption; ATP formation was reversibly inhibited by sulfide levels above 20 μ*M*. The ratio of sulfide added to ATP produced ranged from 0.5 to 1.2. Thus, electron transport from sulfide oxidation probably generated a single ATP per sulfide oxidized.

The effects of the inhibitors cyanide, rotenone, antimycin A, and DNP on ATP production (Table 2) paralleled the results found for oxygen consumption (Table 1): cyanide completely inhibited ATP production from sulfide, antimycin A inhibited ATP production from succinate, and rotenone had no effect on ATP production from succinate, but blocked ATP production

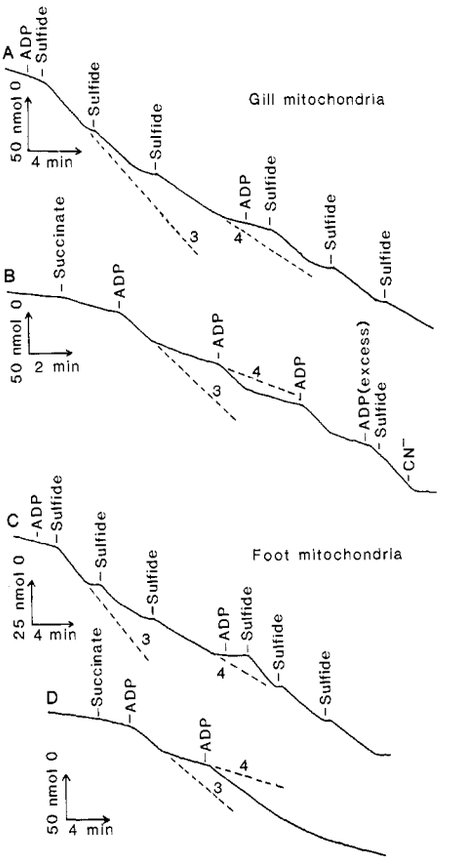

Fig. 1. Oxygen consumption by mitochondria isolated from gill and foot of *S. reidi*. Tracings show the decrease in the oxygen concentration of the medium as a function of time. The slopes used for calculation of the acceptor control ratio are shown (dashed lines). The slope labeled 3 was the state 3 oxygen consumption rate (ADP present), and the slope labeled 4 was the state 4 oxygen consumption rate (after all ADP was phosphorylated to ATP). Mitochondria were prepared by a modification of the procedures described in (*8*). The optimum isolation buffer was 0.5*M* sucrose, 0.15*M* KCl, 20 m*M* Hepes (*p*H 7.4), 0.5 m*M* EGTA, and 0.5% defatted bovine serum albumin (BSA). Centrifugation procedures were: (run 1) 10 minutes at 1000*g*; (runs 2 and 3) 10 minutes at 9000*g*. Run 3 was omitted from some foot mitochondria preparations if the pellet was small after run 2. The following factors decreased the acceptor control ratio: decreased or increased sucrose or KCl concentrations, omission of EGTA, or omission of BSA. Oxygen consumption was assayed with a Clark-type oxygen electrode as described in (*9*), with modifications described in the text for use with sulfide. The optimal assay medium was 0.5*M* glycine, 0.15*M* KCl, 20 m*M* K_2HPO_4, 1 m*M* $MgCl_2$, *p*H 7.4. The reaction volume was 0.5 ml. Gill mitochondria. (A) Additions were 20 μ*M* sulfide plus 20 nmol of ADP. Sulfide was added so that the total sulfide concentration (*10*) never exceeded 20 μ*M*; 1.43 mg of mitochondrial protein was used. (B) Additions were 5 m*M* succinate, 50 nmol of ADP, 100 nmol of ADP (excess), 20 μ*M* sulfide, 1 m*M* sodium cyanide, 1.12 mg of mitochondrial protein. Foot mitochondria. (C) Additions were 20 μ*M* sulfide, 20 nmol of ADP. Mitochondrial protein, 0.24 mg. (D) Additions were 5 m*M* succinate, 50 nmol of ADP (first addition) and 100 nmol of ADP (second addition). Mitochondrial protein, 0.24 mg.

from malate. DNP prevented ATP production from both succinate and sulfide. These findings support the hypothesis that sulfide oxidation is coupled to oxidative phosphorylation only through site III of electron transport.

To test the possibility that sulfide could be used as a substrate by mitochondria from animals that do not encounter high environmental sulfide concentrations, we isolated and examined mitochondria from rat liver (*14*). In the presence of 20 μM sulfide, rat liver mitochondria did not exhibit acceptor control or ATP production. Moreover, with succinate as substrate, ATP production was completely inhibited by 20 μM sulfide. In the absence of sulfide, with succinate as substrate, these mitochondria exhibited acceptor control and synthesized ATP. Thus, the sulfide oxidation system in *Solemya* mitochondria produced ATP and protected cytochrome *c* oxidase against sulfide levels that inhibited rat liver mitochondria.

The findings with mitochondria from rat liver show that the oxidation of sulfide observed with mitochondria from *Solemya* was not merely a consequence of nonenzymatic, sulfide-linked reduction of cytochrome *c*, but rather was due to a specific sulfide oxidase enzyme that is linked to the electron transport chain (probably through cytochrome *c*). Studies of partially purified (*15*) sulfide oxidase from *Solemya* gill indicate the presence of a mitochondrial enzyme functioning as a sulfide:cytochrome *c* oxidoreductase. Ten micromolar sulfide slowly reduced purified horse cytochrome *c*, but the addition of sulfide oxidase–containing mitochondrial fractions (10 μg of protein) stimulated cytochrome *c* reduction approximately fourfold. These findings are similar to those reported for a protein isolated from a phototrophic sulfide-oxidizing bacterium (*16*).

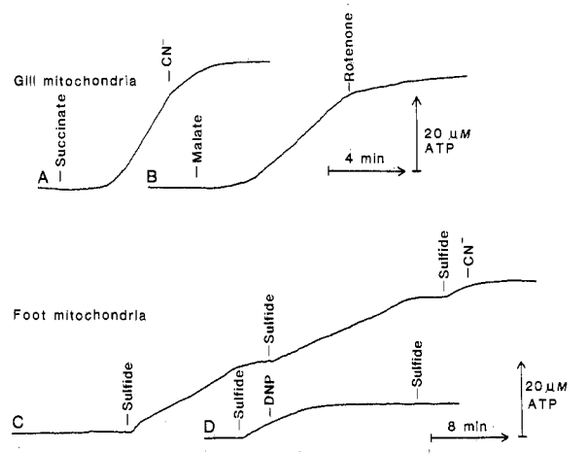

Fig. 2. ATP formation from mitochondria isolated from gill and foot of *S. reidi*. ATP formation was assayed as described in (*14*). The ATP produced was used by hexokinase to produce glucose-6-phosphate (G-6-P) from glucose. Glucose-6-phosphate dehydrogenase (G-6-PDH) then used G-6-P as substrate and reduced nicotinamide adenine dinucleotide phosphate (NADP) to NADPH. Increase in NADPH was monitored photometrically at 340 nm. The assay contained 0.5M glycine, 0.15M KCl, 20 mM K_2HPO_4, 1 mM $MgCl_2$, 2 units of G-6-PDH, 7 units of hexokinase, 10 mM glucose, 1 mM NADP, and 100 μM ADP. Reaction volume was 1 ml. Gill mitochondria. (A) Additions were 5 mM succinate and 1 mM cyanide. Mitochondrial protein, 0.79 mg. (B) Additions were 5 mM malate, 100 μM rotenone, and 0.84 mg of mitochondrial protein. Foot mitochondria. (C and D) Additions were 20 μM sulfide, 1 mM cyanide, and 200 μM DNP. Mitochondrial protein, 0.58 mg.

Table 2. ATP formation expressed as mean ± SD (number of samples). For inhibitors and uncoupler, + means >80% inhibition or uncoupling, − means <20%, blank means not determined. Abbreviations: R, rotenone; A, antimycin; C, cyanide; and D, DNP.

ATP formation (nmol ATP min^{-1} mg $protein^{-1}$)			Inhibitors and uncoupler			
Tissue	Substrate	ATP formation	R	A	C	D
Gill	Succinate	16.9 ± 2.0 (8)	−	+		+
	Sulfide	4.46 ± 0.44 (3)			+	+
	Malate	8.56 (1)	+			
Foot	Succinate	17.9 (1)		+		
	Sulfide	8.94 ± 0.95 (3)		+	+	

Several lines of evidence indicate that the production of ATP with sulfide as the substrate was due to the activity of mitochondria, not to bacterial contaminants. (i) No bacterial endosymbionts occur in foot tissue (*6*), yet sulfide-driven ATP synthesis was as evident as in mitochondria from the symbiont-containing gills. (ii) The finding of acceptor control with sulfide as the added substrate is strong evidence for mitochondrial ATP production. Bacterial oxygen consumption has been reported not to exhibit acceptor control (*17*), while this control is characteristic of intact mitochondria. (iii) Fractions enriched in bacteria from *Solemya* gills (*18*) did not exhibit acceptor control or ATP production with sulfide as the substrate. (iv) Our assay for monitoring ATP production detected only the ATP released into the assay medium; mitochondria are known to export ATP (*19*), but bacteria do not (*17*).

Our findings suggest that mitochondrial oxidation of sulfide in gill and foot tissue of *Solemya* may play two physiologically important roles, the detoxification of sulfide and the exploitation of the molecule's energy for driving ATP synthesis. Thus, sulfide-oxidizing systems in mitochondria not only can rid the cell of a highly toxic molecule, sulfide, but also can couple the energy released during sulfide oxidation to ATP production. Although the contribution of sulfide oxidation to the ATP requirements of *Solemya* cannot yet be quantified, it might be estimated through simultaneous measurements of sulfide and oxygen consumption by the whole animal.

The broader significance of mitochondrial sulfide oxidation in animals from sulfide-rich environments merits investigation. Suggestions of the occurrence of this type of metabolism are found in several studies (*20*). For example, sulfide oxidation by meiofaunal invertebrates that lack bacterial endosymbionts has been reported; however, it has been viewed solely in the context of detoxification, and the roles of mitochondrial ATP synthesis have not been considered. Our findings suggest that, in addition to its exploitation by free-living and symbiotic sulfur bacteria, sulfide may be exploited and detoxified directly by marine invertebrates inhabiting sulfide-rich environments.

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A Macrophage-Derived Factor Required by Plasmacytomas for Survival and Proliferation in Vitro

RICHARD P. NORDAN AND MICHAEL POTTER

Plasmacytoma (PCT) cell lines dependent for proliferation and survival on a factor elaborated by the murine macrophage cell line, P388D1, were established in vitro. Adherent peritoneal cells induced by pristane produced 50-fold greater amounts of this activity in vitro than did resident cells. The molecules responsible for plasmacytoma growth were distinct from a number of characterized factors including interleukin-1, -2, and -3, macrophage colony-stimulating factor, B-cell stimulatory factor–1, B-cell growth factor II, epidermal growth factor, transforming growth factor–β, and γ- and β-interferon, none of which were able to support the growth of the factor-dependent PCT cell lines. These results suggest that PCT growth factor may be a novel factor that has not been previously characterized and, further, that its production is associated with the pristane-induced, chronic peritoneal inflammatory response that precedes plasmacytoma formation.

PLASMACYTOMAS (PCT's) ARE INduced in a high percentage of BALB/cAnPt mice after intraperitoneal injection of mineral oil or pristane (*1*). The intraperitoneal oil evokes the formation of a chronic granulomatous tissue on peritoneal surfaces that consists primarily of macrophages and neutrophils. Plasmacytomas arise exclusively in the granulomatous tissue, which suggests that their growth depends on microenvironmental influences provided by the inflammatory cells (*2*). Factors involved in this process in vivo have not been identified; however, Namba and Hanaoka (*3*) reported a 50-kD protein, produced by an adherent phagocytic cell line, which was required by the MOPC 104E PCT cell line for growth in vitro. This factor relieved a block in the G_1 phase of the cell cycle. Also, Metcalf (*4*) demonstrated that cells from several in vivo PCT's proliferated in soft agar if the cultures were supplemented with mouse serum or with normal or mineral oil–stimulated peritoneal cells. Recently, Corbel and Melchers (*5*) reported that supernatant from the P388D1 macrophage cell line supported the growth of several PCT's in vitro. Aarden *et al.* (*6*) described a human monocyte-derived "hybridoma growth factor" that is required for the growth of a murine B-cell hybridoma. To further characterize factors that may be necessary for PCT growth, we have established several new factor-dependent PCT cell lines in vitro. This adaptation requires the addition of supernatant derived from unstimulated rat spleen cells (RSS) or from the macrophage cell line P388D1. We report here the initial characterization of plasmacytoma growth factors (PCT-GF) produced by P388D1 cells that are clearly distinguishable from other previously described growth factors.

The TEPC 2027 and TEPC 1165 PCT's were induced and serially transplanted as ascites tumors in pristane-conditioned BALB/cAnPt mice. Establishment of the T2027tc cell line was accomplished with medium supplemented with 50% RSS produced by culturing 5×10^6 rat spleen cells per milliliter for 24 hours in RPMI 1640 supplemented with 1% fetal calf serum (FCS). A search for more defined sources of PCT-GF activity led us to the P388D1 cell line. When newly confluent monolayers of P388D1 cells (1×10^6 cells per milliliter, 50 ml per 150-cm^2 flask) were cultured for 5 days in fresh RPMI 1640 and 0% or 1% FCS, PCT-GF activity was found in the low-serum supernatant (P388D1 LSSN) (Fig. 1). The T1165tc cell line was established in vitro by using 5 to 10% P388D1 LSSN as the source of PCT-GF activity. The T1165tc and T2027tc primary cultures were started at densities ranging from 1×10^5 to 5×10^5 PCT cells per milliliter in medium

Table 1. PCT-GF activity found in characterized factors and supernatants.

Factor or supernatant	Amount tested per milliliter	PCT-GF activity (U/ml)	
		T1165tc	T2027tc
Pure factors			
IL-1, human	0.5 to 5,000 U	0	0
rIL-2, human	0.3 to 1,000 U	0	0
IL-3, mouse	0.3 to 350 U	0	0
BSF-1, mouse	0.1 to 560 U	0	0
EGF, mouse	6 pg to 20 ng	0	0
TGF-β, human, with 2.5 ng of EGF	6 pg to 20 ng	0	0
γ-IFN, mouse	0.1 to 500 U	0	
β-IFN, mouse	0.1 to 1,000 U	0	
C3b, human	5 pg to 5 μg	0	0
Partially purified factors			
BCGF-II, mouse	0.1 to 50 U	0	0
Macrophage-CSF, mouse	1 to 10,000 U	0	0
Thymosin F5, bovine	5 pg to 500 μg	0	0
Supernatants			
P388D1 LSSN		100	100
Resident APC-SN, BALB/cAnPt		<0.1	<0.1
Pristane-induced APC-SN, BALB/cAnPt		22	9
Other reagents			
Con A	5 pg to 50 μg	0	0
LPS	5 pg to 50 μg	0	0

Laboratory of Genetics, National Cancer Institute, Bethesda, MD 20892.