Possible Roles of Sulfur-Containing Amino Acids in a Chemoautotrophic Bacterium-Mollusc Symbiosis

JOANNA L. JOYNER1*, SUZANNE M. PEYER, AND RAYMOND W. LEE

School of Biological Sciences, Washington State University, P.O. Box 644236, Pullman, Washington 99164-4236

Abstract. Invertebrate hosts of chemoautotrophic symbionts face the unique challenge of supplying their symbionts with hydrogen sulfide while avoiding its toxic effects. The sulfur-containing free amino acids taurine and thiotaurine may function in sulfide detoxification by serving as sulfur storage compounds or as transport compounds between symbiont and host. After sulfide exposure, both taurine and thiotaurine levels increased in the gill tissues of the symbiotic coastal bivalve Solemya velum. Inhibition of prokaryotic metabolism with chloramphenicol, inhibition of eukaryotic metabolism with cycloheximide, and inhibition of ammonia assimilation with methionine sulfoximine reduced levels of sulfur-containing amino acids. Chloramphenicol treatment inhibited the removal of sulfide from the medium. In the absence of metabolic inhibitors, estimated rates of sulfide incorporation into taurine and thiotaurine accounted for nearly half of the sulfide removed from the medium. In contrast, amino acid levels in the nonsymbiotic, sulfide-tolerant molluscs Geukensia demissa and Yoldia limatula did not change after sulfide exposure. These findings suggest that sulfur-containing amino acids function in sulfide detoxification in symbiotic invertebrates, and that this process depends upon ammonia assimilation and symbiont metabolic capabilities.

Introduction

Aquatic habitats such as deep-sea hydrothermal vents, mangrove swamps, eelgrass beds, and sewage outfall sites tend to be characterized by high levels of the metabolic toxin hydrogen sulfide (Fenchel and Riedl, 1970; Felbeck et al., 1981; Cavanaugh, 1983). Hydrogen sulfide diffuses freely across respiratory surfaces and therefore cannot be excluded from tissues (Denis and Reed, 1927; Julian and Arp, 1992). It also reversibly inhibits cytochrome c oxidase (Lovatt Evans, 1967; Nicholls, 1975) and decreases hemoglobin oxygen affinity (Carriço et al., 1978). Consequently, animals living in these environments require physiological mechanisms to cope with hydrogen sulfide toxicity.

Invertebrates that harbor symbiotic chemoautotrophic bacteria also require sulfide, for their symbionts utilize the chemical energy generated by hydrogen sulfide oxidation to fix carbon dioxide into carbohydrates (Felbeck et al., 1981; Ruby et al., 1981; Cavanaugh, 1983). The invertebrate host delivers hydrogen sulfide and oxygen to its symbionts and relies upon the symbiont-produced carbohydrates as a source of nutrition (Rau, 1981; Southward et al., 1981; Felbeck, 1985). This type of symbiotic relationship exists in over 100 species from at least five invertebrate phyla (Cavanaugh, 1994).

Sulfide detoxification and transport strategies have been well studied in the coastal protobranch bivalves of the genus Solemya, which harbor approximately $1 \times 10^9$ intracellular chemoautotrophic symbionts per gram wet weight of gill tissue (Cavanaugh, 1983; Felbeck, 1983). These clams generally form U- or Y-shaped burrows in shallow-water reducing sediments and pump oxygenated water through their burrows (Frey, 1968), which enables them to simultaneously acquire the dissolved oxygen and sulfide needed for chemoautotrophy.

To detoxify sulfide, Solemya velum and S. reidi, two well-studied species, use several strategies apart from utilization by their symbionts. For example, S. velum has two types of cytoplasmic hemoglobins: one binds oxygen, and a second, which combines with sulfide to form ferric hemo-
globin sulfide. may mediate sulfide transport to the symbionts (Doeller et al., 1988). The sulfide-binding form of cytoplasmic hemoglobin is not present in S. reidi (Kraus et al., 1992); rather, sulfide oxidation occurs in the mitochondria, hematin, and sulfide-oxidizing bodies (Powell and Somero, 1985, 1986; Powell and Arp, 1989).

Synthesis of sulfur-containing free amino acids may be an additional strategy for sulfide detoxification and transport in chemosynthetic symbioses (Alberic and Boulegue, 1990; Conway and McDowell Capuzzo, 1992; Pranal et al., 1995; Pruski et al., 1997). In many marine organisms, including solemyid clams and some deep-sea symbiotic species, taurine (2-aminoethanesulfonic acid) is the dominant free amino acid (Alberic and Boulegue, 1990; Conway et al., 1992; Conway and McDowell Capuzzo, 1992; Pranal et al., 1995; Lee et al., 1997; Pruski et al., 1997). Intracellular free amino acid pools function in cell volume regulation (Pierce, 1982; Yancey et al., 1982; Rice and Stephens, 1988), and the primary function of taurine may be as a compatible osmolyte, a function which is conserved throughout the animal kingdom (Allen and Garrett, 1971; Huxtable, 1992).

The exceptionally high levels of taurine and the related amino acid thiotaurine (2-aminoethanesulfonic acid) in several chemosynthetic symbioses have prompted investigators to consider additional roles for these amino acids. For example, taurine appears to be an end product of ammonia assimilation in S. reidi (Lee et al., 1997), and may be involved in sulfur cycling in S. velum (Conway and McDowell Capuzzo, 1992). Similarly, thiotaurine may function in sulfur cycling in deep-sea symbiotic bivalves (Alberic and Boulegue, 1990; Pruski et al., 2000; Pruski, 2001). Taurine and thiotaurine may serve as important sulfide storage compounds, allowing S. velum to maintain low levels of intracellular sulfide. Under conditions where sulfide is low or absent, taurine and thiotaurine may provide sulfide to mitochondrial and symbiont sulfide oxidation pathways. Several species of aerobic gram-negative soil and enteric bacteria use taurine as a sulfur, carbon, and nitrogen source (Stapley and Starkey, 1970; Smiley and Wilkinson, 1983; Seitz et al., 1993; King and Quinn, 1997; Chien et al., 1999; Cook et al., 1999; Reichenbecher and Murrell, 1999), and similar metabolic pathways may be present in the gram-negative chemosynthetic symbionts. Additionally, taurine and thiotaurine production could facilitate sulfide detoxification under anaerobic conditions, thereby preventing deleterious effects such as the reaction of sulfide with metalloproteins. This strategy would be particularly beneficial in the burrow environment of solemyid clams, in which oxygen and sulfide levels vary.

In the present study, we tested the hypothesis that taurine and thiotaurine levels in intact gills of S. velum increase upon exposure to sulfide, which would occur if these amino acids are involved in sulfur storage or cycling. The effects of sulfide exposure on levels of hypotaurine (2-aminoethanesulfonic acid), a possible precursor to taurine and thiotaurine, were also examined. Host- and symbiont-specific metabolic inhibitors were used to distinguish the roles of the S. velum host and its symbionts in ammonia assimilation and the maintenance of taurine, thiotaurine, and hypotaurine pools. Flow-through respirometry studies were conducted with S. velum to quantify rates of ammonia flux and to determine whether sulfide consumption is related to fluctuations in amino acid pools. In parallel studies, we tested for correlations between sulfide and free amino acid levels in two sulfide-tolerant, nonsymbiotic bivalve species: the estuarine mussel Geukensia demissa and the protobranch bivalve Yoldia limatula.

Materials and Methods

Specimen collection and maintenance

Solemya velum Say, 1822, Geukensia demissa (Dillwyn, 1817), and Yoldia limatula (Say, 1831) were collected by the Marine Resources Center of the Marine Biological Laboratory (Woods Hole, MA) and shipped on the day of collection. In our laboratory the clams were maintained for up to 5 days in a flow-through respirometry system (see http://www.wsu.edu/~rlee/respirometer/respirometer.htm and Fig. 1). In each experiment, the temperature of the respirometry system was regulated to match the ambient water temperature at which the clams were collected, which ranged seasonally from 5 to 15 °C over a nearly 2-year period. These temperature differences did not affect the free amino acid composition. Consumption of sulfide, oxygen, and ammonia was measured in a subset of experiments conducted in the late spring and at 15 °C. Solemya velum and Y. limatula were maintained in 0.45-μm filtered, 35‰ artificial seawater (ASW; Instant Ocean, Aquarium Systems, Mentor, OH) supplemented with 50 μM ammonia. Geukensia demissa was maintained in filtered, 30‰ ASW supplemented with 50 μM ammonia. The ASW solution in the chambers was kept mixed with magnetic stir bars (60 to 100 rpm). ASW flow rates ranged from 0.18 to 0.19 ml min⁻¹.

Maintaining the clams in a flow-through respirometry system allowed for constant monitoring of experimental conditions. The oxygen concentrations in the chamber outflows were determined with a polarographic O₂ sensor (POS), whereas the concentrations of sulfide and, in some experiments, ammonia were measured by flow injection analysis (FIA). Throughout this paper, sulfide refers to $\Sigma$H₂S (primarily the sum of HS⁻ and H₂S), and ammonia refers to $\Sigma$NH₃ (the sum of NH₃ and NH₄⁺). Outflows were pumped directly through a flow cell containing a sulfide-insensitive gold microcathode POS (Orbisphere 2120, Geneva) followed by a series of FIA injection sample loops.
Figure 1. Schematic of flow-through respirometry system. (A) \( \text{CO}_2 \), \( \text{N}_2 \), and \( \text{O}_2 \) gas mixture. (B) Seawater-gas equilibration column with pH regulation. (C) Syringe pump for metering sodium sulfide stocks into seawater entering the chambers. The syringe pump is also used to meter inhibitor into chamber 2. (D) Water-jacketed chambers containing clams, with at least one empty chamber to function as a control. Outflows from the chambers are pumped to a pneumatically actuated six-way stream-selector valve (E). The position of this valve determines whether the outflow goes to waste or to the analysis system. The analysis system consists of a flow injection analyzer for sulfide and ammonia determination and an \( \text{O}_2 \) sensor mounted in a flow cell (F). The analyzer and stream-selector valve are linked to a data acquisition and automated control system (G).

(Rheodyne Type 50 valves with Rheodyne 5701 pneumatic actuators, Rohnert Park, CA).

The FIA determination of sulfide involved derivitizing the sample stream with 1.5 mM 2,2'-dithiodipyridine, which forms a stable product with an absorbance maximum at 343 nm (Svenson, 1980). The derivitized sample was loaded into an injection loop and sent to a variable wavelength detector (Hyperquan VUV-20, Colorado Springs, CO). This method produces 343-nm absorbance peaks proportional to sulfide concentration (Svenson, 1980). Ammonia was determined by a modification of the FIA protocol of Willason et al. (1986). The sample was loaded into an injection loop and then treated with NaOH to raise the pH, converting ammonium to ammonia gas. Ammonia passes through a Teflon membrane into a carrier stream containing phenol red, raising the pH of the phenol red solution. The resulting color change (absorbance at 560 nm) is monitored with a detector fabricated from a green LED and a phototransistor. POS and FIA detector outputs were continuously recorded with a data acquisition system (Sable Systems, Datacan V, Henderson, NV), which was also used to control FIA injection valve actuation.

Three respirometer chambers containing clams, one control chamber, and two standards were connected to a six-way stream-selector valve (Fig. 1). Every 0.13 h the valve was automatically switched, allowing sampling from all six channels every 0.8 h. Peak height (for FIA) or average output (for POS) was calculated to determine concentration differences between chambers containing clams and the control chamber. These data were continuously monitored to ensure, in the case of oxygen measurements, that the clams were not exposed to hypoxic conditions. Direct measurements of sulfide in samples taken from the respirometer outflow were used to standardize sulfide data obtained by FIA. Fluxes were calculated as follows: \( \mu \text{mol sulfide} \cdot \text{g}^{-1} \cdot \text{h}^{-1} = (\text{concentration}_{\text{experimental chamber}} - \text{concentration}_{\text{control chamber}}) \times (\text{flow rate in } 1 \cdot \text{h}^{-1}) \times (\text{g}^{-1} \cdot \text{wet weight}) \).

Experimental treatments

In all experiments, the clams were acclimated for 24 h in ASW prior to sulfide or inhibitor exposure. In experiments in which clams were exposed to sulfide, a syringe pump (Harvard Apparatus 944, South Natick, MA) was used to meter a 20 mM \( \text{Na}_2\text{S} \) solution (Fig. 1C) into the ASW solution before its entry into the respirometer chambers. Final sulfide concentration in the chambers was 0.45 ± 0.05 mM. Clams assayed for amino acid levels were exposed to sulfide for 24 h. In some experiments, a metabolic inhibitor was added to the ASW solution after the 24-h acclimation period. The final concentrations of these inhibitors were 0.9 mM chloramphenicol (Sigma, St. Louis, MO), 0.02 mM cycloheximide (Sigma), and 0.25 mM methionine sulfoximine (MSX; Sigma). In experiments in which sulfide consumption was examined, the clams were acclimated for 24 h in ASW, then exposed to sulfide for up to 100 h. During the final 24 h of the sulfide exposure, metabolic inhibitors were added to the ASW solution. The clams were weighed before and after treatment.

Amino acid analyses

After experimental treatment, the bivalves were opened by severing their adductor muscles. The gills and foot of each clam were dissected free of other tissues, blotted briefly on a paper towel, then individually frozen in liquid nitrogen and stored at -80 °C. The gills and feet were individually homogenized in distilled water (1:25, tissue/dH\(_2\)O) on ice. To precipitate proteins, the homogenates were treated with 5% sulfosalicylic acid (Sigma) in a 1:10 ratio of sulfosalicylic acid/homogenate (Lee and Slocum, 1988). The solutions were centrifuged for 5 min at 16,000 × g, and the supernatants were stored at -80 °C.

Total free amino acids were quantified in a Beckman
6300 amino acid analyzer following a protocol modified from Lee and Slocum (1988). The samples were diluted (1:30, by volume) with Li-S buffer (96.8% H2O, 1% LiCl, 1% thioglycolic acid; pH 2.7; Beckman Coulter, Inc., Fullerton, CA). Of the 240–250 µL of sample or standard loaded onto the sample loops, 50 µL of each solution was analyzed. The amino acids were separated in Li-A buffer (98% H2O, 1% Li citrate, 0.5% LiCl, 0.5% HCl; pH 2.8; Beckman Coulter) on a 10-cm ion exchange column and reacted in-line with ninhydrin solution. Absorbances were monitored at 570 nm and 440 nm. After preliminary experiments, only taurine, hypotaurine, and thiotaurine were quantified. The standards were 200 µM taurine (Sigma) and 20 µM hypotaurine (Sigma) in Li-S buffer. The thiotaurine standard was prepared by dissolving 0.0011 g hypotaurine and 0.05 g Na2S · 9H2O (Fisher Scientific, Fair Lawn, NJ) in deionized water, heating to 100 °C, acidifying the solution with 1 M HCl, and evaporating the solution (Cavallini et al., 1963). This standard was verified by mass spectrometry.

The temperature of the column affected the separation of thiotaurine from taurine and the reactivity of hypotaurine with ninhydrin. Thiotaurine could be detected only at 70 °C. The values of hypotaurine reported here are only from analyses run at 45 °C, because hypotaurine was not as reactive with ninhydrin at 70 °C. The temperature did not affect taurine levels. Reported values for taurine are averages between levels detected at 45 °C and 70 °C.

Results are presented as the mean ± the standard error and as average rates of synthesis per gram wet weight over a 24-h period [(amino acid level in g·h−1 wet weight in clams exposed to sulfide) − (amino acid level in clams not exposed to sulfide)/24 h] assuming the synthesis rate was linear over the 24-h period. Differences among means were detected using one-way ANOVA for each amino acid in S. velum samples (Statistica, Statsoft, Inc., Tulsa, OK). The appropriate comparisons were analyzed with Fisher’s LSD procedure (Statistica). The G. demissa and Y. limatitla amino acid data were analyzed with two-sample t tests (Statistica).

**Results**

Sulfide exposure increased taurine and thiotaurine levels in S. velum but not in two nonsymbiotic bivalves

Specimens of Solemya velum exposed to sulfide had significantly more taurine (P = 0.0034) and thiotaurine (P < 0.0001) in their gill tissue than clams not so exposed (Table 1). These values equate to average rates of change in taurine and thiotaurine levels of 0.89 µmol · g−1 · h−1 and 0.22 µmol · g−1 · h−1 over the 24-h incubation period. Hypotaurine levels were not significantly affected (P = 0.064). Cysteine and methionine, two other sulfur-containing amino acids, were below the limits of detection in all samples. Levels of the most abundant non-sulfur-containing free amino acids—alanine, glutamate, and aspartate—were unaffected by sulfide exposure (data not shown). In preliminary experiments with sulfide-exposed clams, free amino acid profiles of S. velum foot (symbiont-free) and gill tissues were similar (data not shown), as observed previously (Conway and McDowell Capuzzo, 1992). In subsequent experiments with metabolic inhibitors, only taurine, hypotaurine, and thiotaurine levels in the symbiont-containing gill tissues of S. velum were quantified.

Gill tissue from the nonsymbiotic bivalve species Geukensia demissa and Yoldia limatitla contained less taurine (P < 0.0001, non-sulfide-exposed) than S. velum (Table 1), but comparable levels of hypotaurine (P = 0.068) and thiotaurine (P = 0.648). The concentrations of these amino acids were the same whether or not the bivalves were exposed to sulfide (all comparisons, P > 0.05).

**Metabolic inhibitors decreased taurine and thiotaurine levels in S. velum gills**

To investigate the role of the chemoautotrophic symbionts, clams were exposed to chloramphenicol, a specific

| Species          | Treatment (n) | Taurine           | Hypotaurine         | Thiotaurine       |
|------------------|--------------|------------------|---------------------|------------------|
| *Solemya velum*  | No inhibitor (16)   | 100.3 ± 5.5a     | 120 ± 5.4b          | 13.7 ± 1.4       | 11.4 ± 1.3       | 0.35 ± 0.2a      | 5.4 ± 0.6b       |
|                  | Chloramphenicol (9) | 96.5 ± 6.5       | 102.2 ± 4.5b        | 12 ± 0.8         | 12.9 ± 1.4       | 0.42 ± 0.2a      | 3.6 ± 0.8b       |
|                  | Cycloheximide (5)  | 100.1 ± 6.6      | 109.5 ± 3.9         | 20.3 ± 2.6       | 15.1 ± 2.2       | 1.4 ± 0.2a       | 5.5 ± 0.7a       |
|                  | MSX (7)         | 91.7 ± 10        | 96.1 ± 5.7b         | 18.5 ± 3         | 12.7 ± 4         | 0.79 ± 0.3b      | 6.5 ± 0.6b       |
| *Geukensia demissa* | No inhibitor (7)  | 37.9 ± 1.9       | 39.9 ± 1.2          | 4.7 ± 0.7        | 4.5 ± 0.6        | 0.65 ± 0.2       | 0.95 ± 0.4       |
| *Yoldia limatitla* | No inhibitor (3)  | 62 ± 5.5         | 56 ± 6.7            | 10.9 ± 1.5       | 9.9 ± 1.3        | 0.16 ± 0.16      | 0.24 ± 0.03      |

Table 1

Effects of sulfide exposure and inhibitors on taurine, hypotaurine, and thiotaurine levels in gills of three bivalve species

Data are mean ± SEM in µmol amino acid/g wet weight of gill tissue. (n), Number of replicates; MSX, methionine sulfoximine.

a Significant differences (P < 0.05) between clams exposed to sulfide (+ Sulfide) and not exposed (− Sulfide).

b Significant differences (P < 0.05) between clams treated with inhibitor and not treated.
inhibitor of bacterial protein synthesis (Burnap and Trench, 1989), at a concentration previously determined to disrupt symbiont metabolism but to be nontoxic to the host (R. W. Lee, unpubl.). To examine the role of the host, clams were exposed to cycloheximide, a specific inhibitor of eukaryotic protein synthesis (Burnap and Trench, 1989), at a concentration nontoxic to the host for the duration of the treatment. In additional experiments, the effects of the ammonia assimilation inhibitor, methionine sulfoximine (MSX; Rees, 1987) were examined: MSX inhibits glutamine synthetase, which has been detected in S. velum tissues (Lee et al., 1999). To ensure complete inhibition of ammonia assimilation, the MSX level was 10-fold higher than that utilized by Rees (1987). Taurine, hypotaurine, and thiotaurine levels in clams not exposed to sulfide were not affected by exposure to any of the inhibitors (Table 1). Additionally, the wet weights of whole clams were not altered by treatment with sulfide or metabolic inhibitors.

When clams were treated with the three metabolic inhibitors, the usual sulfide-induced increase in taurine levels was not observed (P values for comparisons between -sulfide and +sulfide, in the presence of inhibitors: chloramphenicol, P = 0.552; cycloheximide, P = 0.451; MSX, P = 0.707). Hypotaurine levels were not altered by sulfide exposure or treatment with inhibitors (P = 0.064). Thiotaurine levels increased after sulfide exposure, even in the presence of metabolic inhibitors (P values for comparisons between -sulfide and +sulfide in the presence of inhibitors: chloramphenicol, P < 0.001; cycloheximide, P < 0.001; MSX, P < 0.0001). This sulfide-stimulated increase, however, was reduced by treatment with chloramphenicol (P = 0.016, sulfide-exposed control clams versus chloramphenicol-treated sulfide-exposed clams).

**Chloramphenicol and MSX decreased S. velum sulfide and ammonia consumption**

The average rates of sulfide and oxygen consumption in the absence of inhibitors were 2.57 ± 0.06 (5) μmol g⁻¹ wet weight ⋅ h⁻¹ [mean ± SE (n of experiments)] and 3.98 ± 0.01 (5) μmol g⁻¹ ⋅ h⁻¹, respectively. Chloramphenicol exposure completely halted sulfide consumption after 10 h (Fig. 2A, hours 120–130). Cycloheximide treatment did not affect sulfide consumption rates. Treatment with MSX inhibited ammonia uptake (Fig. 2B, hours 10–30).

**Discussion**

This study demonstrates that taurine and thiotaurine levels in Solemya velum gills increase after sulfide exposure. These two amino acids may function as nontoxic sulfide storage compounds. Inhibition of symbiont and host protein synthesis and host ammonia assimilation blocked sulfide-stimulated taurine synthesis; in contrast, only the inhibition of symbiont metabolism decreased the sulfide-stimulated thiotaurine synthesis. The maintenance of free amino acid pools depended upon the presence of functioning symbionts, sulfide consumption, and host ammonia assimilation. Thus, sulfur-containing free amino acids also may be a link between cycling of nitrogen and sulfur in chemosymbiotic symbioses.

The magnitude of changes in taurine and thiotaurine pools observed in the present study is sufficient for these amino acids to be physiologically significant sulfide storage compounds. In experiments in which clams were exposed to sulfide for 24 h, the increases in taurine and thiotaurine levels corresponded to synthesis rates of 0.89 μmol g⁻¹ wet weight ⋅ h⁻¹ and 0.22 μmol g⁻¹ ⋅ h⁻¹, respectively. Since taurine contains one S atom and thiotaurine contains two S atoms, this corresponds to a potential sulfide incorporation rate of 1.33 μmol g⁻¹ ⋅ h⁻¹. The average rate of whole animal sulfide consumption measured under the control (no inhibitor) conditions was 2.57 μmol g⁻¹ ⋅ h⁻¹, which is similar to the sulfide consumption rate of Solemya reidi under similar experimental conditions (Anderson et al., 1987). Therefore, the contribution of taurine and thio-
taurine to sulfide detoxification could account for up to 50% of the total sulfide flux.

Treatment with chloramphenicol, cycloheximide, and MSX prevented the sulfide-induced increases in taurine levels exhibited by the control clams. The lack of detectable taurine synthesis in chloramphenicol-treated clams likely can be attributed to the chloramphenicol-induced cessation of sulfide consumption. Additionally, chloramphenicol may act to prevent synthesis of mitochondrial proteins that are not nuclear encoded. Therefore, effects of chloramphenicol treatment might also be ascribed to impairment of mitochondrial metabolism. However, in preliminary experiments, S. velum tolerated treatment with 0.9 mM chloramphenicol for at least 9 days, suggesting that the effects due to chloramphenicol treatment are most likely the result of disrupted symbiont metabolism rather than toxicity to the host. Treatment with cycloheximide, which inhibits eukaryotic protein synthesis (Burnap and Trench, 1989) and is functionally analogous to chloramphenicol, decreased taurine synthesis in the presence of sulfide, but did not affect sulfide consumption. These results suggest that taurine is synthesized by the host and that the cycloheximide treatment did not affect any sulfide consumption which may occur in host tissues (Powell and Somero, 1986). Exposure to MSX blocked ammonia assimilation, probably contributing to the decreased taurine levels in MSX-treated clams. These results mirror those reported by Lee and coworkers (Lee et al., 1997), who found a direct relationship between external ammonia availability and taurine levels in S. reidi.

Hypotaurine levels were not altered by exposure to sulfide or the metabolic inhibitors. These results suggest that hypotaurine is not directly involved in sulfide detoxification, nor is it an intermediate in the taurine synthesis pathway in S. velum (Cavallini et al., 1976). Alternatively, hypotaurine could have protective functions, such as by serving as a compatible osmolyte (Yin et al., 2000) or by scavenging free radicals (Huxtable, 1992).

Thiotaurine levels were greater in the gills of sulfide-exposed clams than in non-sulfide-exposed clams, regardless of exposure to metabolic inhibitors. Treatment with chloramphenicol reduced, but did not prevent, sulfide-induced thiotaurine synthesis. This reduction likely resulted from the chloramphenicol-induced cessation of sulfide consumption. Inhibition of host metabolic activity with cycloheximide did not affect thiotaurine levels in sulfide-exposed clams. Thiotaurine may be produced abiotically in host tissues, in which case host enzymatic pathways may not be necessary. Despite the MSX-induced inhibition of ammonia assimilation, thiotaurine levels in MSX-treated clams increased following sulfide exposure. Again, these results suggest that thiotaurine may be produced abiotically from precursors already present in the gill tissue and depend less upon ammonia availability.

**Taurine and thiotaurine synthesis in S. velum**

It is not known whether symbiotic bivalves maintain free amino acid pools by absorbing amino acids from the environment or by synthesizing them. We do know that S. reidi can take up free amino acids from sediment interstitial water (Lee et al., 1992). However, sulfur-containing amino acids were not detected in the pore water samples from S. reidi burrows (Lee et al., 1992) and were not present in the incubation medium in the experiments presented here, suggesting that solemyid clams synthesize taurine and thiotaurine. The biosynthesis pathways of taurine and thiotaurine in solemyid clams are unknown, but the results from this study suggest that these pathways require ammonia assimilation, sulfide consumption, and active symbiont metabolism.

Ammonia is present at elevated levels in the burrow environment of solemyid clams (Lee et al., 1992; Krueger, 1996), and the clams assimilate it into amino acids, which may then serve as precursors for sulfur-containing amino acids (Lee and Childress, 1994). The present study indicates that glutamine synthetase is the primary enzyme in the assimilation pathway, since MSX treatment blocked ammonia uptake and caused ammonia excretion, similar to what was demonstrated in an algal-cnidarian symbiosis (Rees, 1987). The product of ammonia assimilation, glutamate, can then be used as a precursor in the production of taurine, which is a major product of ammonia assimilation in S. reidi tissues (Lee et al., 1997; thiotaurine production was not tested for). Therefore, in S. velum, taurine and thiotaurine production in response to sulfide, as demonstrated in this study, may be facilitated by the ability of the bacterium-mollusc association to synthesize glutamate from inorganic nitrogen.

Just as glutamate likely contributes organic nitrogen to the synthesis of taurine and thiotaurine, the probable source of organic sulfur is cysteine. All of the demonstrated taurine synthesis pathways in mammalian and invertebrate tissues incorporate cysteine (Jacobsen and Smith, Jr., 1968; Bender, 1975; Bishop et al., 1983; Huxtable, 1992), which apparently cannot be synthesized by molluscs (Bishop et al., 1983). Although some intertidal molluscs utilize external cysteine sources to maintain taurine pools (Allen and Awapara, 1960; Jacobsen and Smith, Jr., 1968; Allen and Garrett, 1972; Bender, 1975), it is unlikely that solemyid clams take up cysteine from their environment (Lee et al., 1992). Therefore, the most likely source of cysteine or other taurine precursors is the symbiotic bacteria. Gram-negative bacteria cannot synthesize hypotaurine, taurine, or thiotaurine (Jacobsen and Smith, Jr., 1968; Huxtable, 1992), but can make cysteine (Kredich, 1996). Cysteine synthesis in E. coli requires sulfur in the form of sulfide or thiosulfate (Stauffer, 1996) and assimilated ammonia in the form of glutamate (Reitzer, 1996). Translocation of the essential
amino acid cysteine from gram-negative symbionts to host may occur in S. velum gill tissue as modeled in Figure 3. Such translocations have been demonstrated in bacteriaphid and algal-cnidarian associations (Wang and Douglas, 1999; Douglas et al., 2001).

The results of this study suggest that S. velum relies upon its symbionts as a source of taurine precursors, as modeled in Figure 3. The inhibition of symbiont metabolism with chloramphenicol, therefore, may equate to a loss of cysteine metabolism, thus decreasing sulfide consumption and taurine and thiotaurine synthesis by the host. Ammonia limitation, either by MSX treatment or reduced exogenous ammonia resources, may limit glutamate availability in S. velum tissues, thereby limiting cysteine production in the symbionts (Fig. 3). This could result in the lower taurine levels seen in the solemyid clams in this study and in previous work (Lee et al., 1997). Thus, taurine and thiotaurine may be a link between nitrogen and sulfur cycling in the presence of transcriptional and translational inhibitors. Proc. R. Soc. Lond. B 238: 73–87.

Carrico, R. J., W. E. Blumberg, and J. Peisach. 1978. The reversible binding of oxygen to sulfhemoglobin. J. Biol. Chem. 253: 7212–7215.

Cavallini, D., B. Mondovi, and C. de Marco. 1963. Hypotaurine and thiotaurine. Biochem. Prep. 10: 72–75.

Cavallini, D., R. Scandurra, S. Dupré, G. Federici, L. Santoro, G. Ricci, and D. Barra. 1976. Alternative pathways of taurine biosynthesis. Pp. 59–66 in Taurine. R. J. Huxtable and A. Barbeau, eds. Raven Press, New York.

Cavanaugh, C. M. 1983. Symbiotic chemosynthetic bacteria in marine invertebrates from sulphide-rich habitats. Nature 302: 58–61.

Cavanaugh, C. M. 1994. Microbial symbioses: patterns of diversity in the marine environment. Am. Zool. 34: 79–89.

Chien, C.-C., E. R. Leadbetter, and W. Godchaux, III. 1999. Rhodococcus spp. utilize taurine (2-aminoethanesulfonate) as sole source of carbon, energy, nitrogen, and sulfur for aerobic respiratory growth. FEMS Microbiol. Lett. 176: 333–337.

Conway, N. M., and J. F. McDowell Capuzzo. 1992. High taurine levels in the Solemya velum symbiosis. Comp. Biochem. Physiol. 102B: 175–185.

Conway, N. M., B. L. Howes, J. F. McDowell Capuzzo, R. D. Turner, and C. M. Cavanaugh. 1992. Characterization and site description of Solemya borealis (Bivalvia: Solemyidae), another bivalve-bacteria symbiosis. Mar. Biol. 112: 601–613.

Cook, A. M., H. Laue, and F. Junker. 1999. Microbial desulfonation. FEMS Microbiol. Rev. 22: 399–419.

Denis, W., and L. Reed. 1927. The action of blood on sulfides. J. Biol. Chem. 72: 385–394.

Doeller, J. E., D. W. Kraus, J. M. Colacino, and J. B. Wittenberg. 1988. Gill hemoglobin may deliver sulfide to bacterial symbionts of Solemya velum (Bivalvia, Mollusca). Biol. Bull. 175: 388–396.

Douglas, A. E., L. B. Minto, and T. L. Wilkinson. 2001. Quantifying nutrient production by the microbial symbionts in an aphid. J. Exp. Biol. 204: 349–358.

Felbeck, H. 1983. Sulfide oxidation and carbon fixation by the gutless clam Solemya reidi: an animal-bacteria symbiosis. J. Comp. Physiol. 152B: 3–11.

Acknowledgments

We thank Gerhard Munske of Washington State University for his aid in conducting the amino acid analyses. We also thank David Julian, Stephanie Wohlgemuth, Michael J. Greenberg, and two anonymous reviewers for helpful comments on the manuscript. The Marine Resources Center of the Marine Biological Laboratory at Woods Hole collected all of the clams and provided the information on water temperature. Ray Romjue of the WSU Technical Services constructed the respirometry chambers. This work was funded by grants from the National Science Foundation (IBN0076604) and the National Undersea Research Program to R.W.L. and the Western Society of Malacologists to J.L.J.

Literature Cited

Alberic, P., and J. Boulogue. 1990. Unusual amino compounds in the tissues of Calyptogetena phaseoliformis (Japanese Trench): possible link to symbiosis. Prog. Oceanogr. 24: 89–101.

Allen, J. A., and M. R. Garrett. 1971. Taurine in marine invertebrates. Adv. Mar. Biol. 9: 205–253.

Allen, J. A., and M. R. Garrett. 1972. Studies on taurine in the euryhaline bivalve Mya arenaria. Comp. Biochem. Physiol. 41A: 307–317.

Allen, K., and J. Awapara. 1960. Metabolism of sulfur amino acids in Mytilus edulis and Rangia cuneata. Biol. Bull. 118: 173–182.

Anderson, A. E., J. J. Childress, and J. A. Favuzzi. 1987. Net uptake of CO2 driven by sulphide and thiosulphate oxidation in the bacterial symbiont-containing clam Solemya reidi. J. Exp. Biol. 133: 1–31.

Bender, D. A. 1975. Amino Acid Metabolism. John Wiley & Sons, New York.

Bishop, S. H., L. L. Ellis, and J. M. Burcham. 1983. Amino acid metabolism in molluscs. Pp. 243–327 in The Mollusca: Metabolic Biochemistry and Molecular Biomechanics, P. W. Hochachka, ed. Academic Press, New York.

Burnap, R. L., and R. K. Trench. 1989. The biogenesis of the cyanellae of Cyanothpha paradoxa. II. Pulse-labeling of cyanellar polypeptides in the presence of transcriptional and translational inhibitors. Proc. R. Soc. Lond. B 238: 73–87.

Carrico, R. J., W. E. Blumberg, and J. Peisach. 1978. The reversible binding of oxygen to sulfhemoglobin. J. Biol. Chem. 253: 7212–7215.

Cavallini, D., B. Mondovi, and C. de Marco. 1963. Hypotaurine and thiotaurine. Biochem. Prep. 10: 72–75.

Cavallini, D., R. Scandurra, S. Dupré, G. Federici, L. Santoro, G. Ricci, and D. Barra. 1976. Alternative pathways of taurine biosynthesis. Pp. 59–66 in Taurine. R. J. Huxtable and A. Barbeau, eds. Raven Press, New York.

Cavanaugh, C. M. 1983. Symbiotic chemosynthetic bacteria in marine invertebrates from sulphide-rich habitats. Nature 302: 58–61.

Cavanaugh, C. M. 1994. Microbial symbioses: patterns of diversity in the marine environment. Am. Zool. 34: 79–89.

Chien, C.-C., E. R. Leadbetter, and W. Godchaux, III. 1999. Rhodococcus spp. utilize taurine (2-aminoethanesulfonate) as sole source of carbon, energy, nitrogen, and sulfur for aerobic respiratory growth. FEMS Microbiol. Lett. 176: 333–337.

Conway, N. M., and J. F. McDowell Capuzzo. 1992. High taurine levels in the Solemya velum symbiosis. Comp. Biochem. Physiol. 102B: 175–185.

Conway, N. M., B. L. Howes, J. F. McDowell Capuzzo, R. D. Turner, and C. M. Cavanaugh. 1992. Characterization and site description of Solemya borealis (Bivalvia: Solemyidae), another bivalve-bacteria symbiosis. Mar. Biol. 112: 601–613.

Cook, A. M., H. Laue, and F. Junker. 1999. Microbial desulfonation. FEMS Microbiol. Rev. 22: 399–419.

Denis, W., and L. Reed. 1927. The action of blood on sulfides. J. Biol. Chem. 72: 385–394.

Doeller, J. E., D. W. Kraus, J. M. Colacino, and J. B. Wittenberg. 1988. Gill hemoglobin may deliver sulfide to bacterial symbionts of Solemya velum (Bivalvia, Mollusca). Biol. Bull. 175: 388–396.

Douglas, A. E., L. B. Minto, and T. L. Wilkinson. 2001. Quantifying nutrient production by the microbial symbionts in an aphid. J. Exp. Biol. 204: 349–358.

Felbeck, H. 1983. Sulfide oxidation and carbon fixation by the gutless clam Solemya reidi: an animal-bacteria symbiosis. J. Comp. Physiol. 152B: 3–11.
Felbeck, H. 1985. CO2 fixation in the hydrothermal vent tube worm Riftia pachyptila (Jones). Physiol. Zool. 58: 272–281.

Felbeck, H., J. J. Childress, and G. N. Somero. 1981. Calvin-Benson cycle and sulphide oxidation enzymes in animals from sulphide-rich habitats. Nature 298: 291–293.

Fenchel, T. M., and R. J. Riedl. 1970. The sulfide system: a new biotic community underneath the oxidized layer of marine sand bottoms. Mar. Biol. 7: 255–268.

Frey, R. W. 1968. The Lebensspuren of some common marine invertebrates near Beaufort, North Carolina. J. Pelecypod. Bull. J. Paleontol. 42: 570–574.

Huxtable, R. J. 1992. Physiological actions of taurine. Physiol. Rev. 72: 161–163.

Jacobsen, J. G., and L. H. Smith, Jr. 1968. Biochemistry and physiology of taurine and taurine derivatives. Physiol. Rev. 48: 424–511.

Julian, D., and A. J. Arp. 1992. Sulfide permeability in the marine invertebrate Usechis caupo. J. Comp. Physiol. 162B: 59–67.

King, J. E., and J. P. Quinn. 1997. The utilization of organosulfonates by soil and freshwater bacteria. Lett. Appl. Microbiol. 24: 474–478.

Kraus, D. W., J. E. Doeller, and J. B. Wittenberg. 1992. Hydrogen sulfide reduction of symbiotic cytochrome cSS in gills of Solemya reidi (Mollusca). Biol. Bull. 182: 435–443.

Kredich, N. M. 1996. Biosynthesis of cysteine. Pp. 514–527 in Escherichia coli and Salmonella: Cellular and Molecular Biology, F. C. Neidhardt, ed. ASM Press. Washington. D.C.

Krueger, D. M. 1996. Ecology and evolution of Solemya spp., marine bivalves living in symbiosis with chemosynthetic bacteria. Ph.D. dissertation, Harvard University, Cambridge, MA.

Lee, P. L. Y., and R. H. Slocum. 1988. A high-resolution method for amino acid analysis of physiological fluids containing mixed disulfides. Clin. Chem. 34: 719–723.

Lee, R. W., and J. J. Childress. 1994. Assimilation of inorganic nitrogen by marine invertebrates and their chemosynthetic and methanotrophic symbionts. Appl. Environ. Microbiol. 60: 1852–1858.

Lee, R. W., E. V. Thuesen, and J. J. Childress. 1992. Ammonium and free amino acids as nitrogen sources for the chemosynthetic symbiosis Solemya reidi Bernard (Bivalvia: Protobranchia). J. Exp. Mar. Biol. Ecol. 158: 75–91.

Lee, R. W., J. J. Childress, and N. T. Desaulniers. 1997. The effects of exposure to ammonia on ammonia and taurine pools of the symbiotic clam Solemya reidi. J. Exp. Biol. 200: 2797–2805.

Lee, R. W., J. J. Robinson, and C. M. Cavanaugh. 1999. Pathways of inorganic nitrogen assimilation in chemosynthetic bacteria-marine invertebrate symbioses: expression of host and symbiont glutamine synthetase. J. Exp. Biol. 202: 289–300.

Lovatt Evans, C. 1967. The toxicity of hydrogen sulfide and other sulphides. Q. J. Exp. Physiol. 52: 231–248.

Nicholls, P. 1975. The effect of sulfide on cytochrome aα2: isoelectric and allosteric shifts of the reduced alpha-peak. Biochim. Biophys. Acta 396: 24–35.

Pierce, S. K. 1982. Invertebrate cell volume control mechanisms: a coordinated use of intracellular amino acids and inorganic ions as osmotic solute. Biol. Bull. 163: 405–419.

Powell, M. A., and A. J. Arp. 1989. Hydrogen sulfide oxidation by abundant nonhemoglobin heme compounds in marine invertebrates from sulfide-rich habitats. J. Exp. Zool. 249: 121–132.

Powell, M. A., and G. N. Somero. 1985. Sulfide oxidation occurs in the animal tissue of the gutless clam, Solemya reidi. Biol. Bull. 169: 164–181.

Powell, M. A., and G. N. Somero. 1986. Hydrogen sulfide oxidation is coupled to oxidative phosphorylation in mitochondria of Solemya reidi. Science 233: 563–566.

Pranali, V., A. Fiela-Medioni, and J.-C. Colomines. 1995. Amino acid and related compound composition in two symbiotic mytilid species from hydrothermal vents. Mar. Ecol. Prog. Ser. 119: 155–166.

Pruski, A. M. 2001. Carrier of reduced sulfur is a possible role for thiotauroine in symbiotic species from hydrothermal vents with thiotrophic symbionts. Hydrobiologia 460: 9–13.

Pruski, A. M., A. Fiela-Medioni, and J.-C. Colomines. 1997. High amounts of sulphur-amino acids in three symbiotic mytilid bivalves from deep benthic communities. C. R. Acad. Sci. Paris. 320: 791–796.

Pruski, A. M., A. Fiela-Medioni, R. Prodon, and J.-C. Colomines. 2000. Thiotaurine is a biomarker of sulfide-based symbiosis in deep-sea bivalves. Limnol. Oceanogr. 45: 1860–1867.

Rau, G. H. 1981. Hydrothermal vent clam and tube worm 13C/12C: further evidence of nonphotosynthetic food sources. Science 213: 338–339.

Rees, T. A. V. 1987. The green hydra symbiosis and ammonium. 1. The role of the host in ammonium assimilation and its possible regulatory significance. Proc. R. Soc. Lond. B. 229: 299–314.

Reichenbecher, W., and J. C. Murrell. 1999. Linear alkanesulfonates as carbon and energy sources for gram-positive and gram-negative bacteria. Arch. Microbiol. 171: 430–438.

Reitzer, L. J. 1996. Ammonia assimilation and the biosynthesis of glutamine, glutamate, aspartate, asparagine, t-alanine, and t-alanine. Pp. 391–407 in Escherichia coli and Salmonella: Cellular and Molecular Biology, F. C. Neidhardt, ed. ASM Press, Washington, D.C.

Rice, M. A., and G. C. Stephens. 1988. Influx, net flux and transmethylal flow of amino acids in the hardshell clam Mercenaria mercenaria (Linne): influence of salinity. Comp. Biochem. Physiol. 89A: 631–636.

Ruby, E. G., C. O. Wirsen, and H. W. Jannasch. 1981. Chemolithotrophic sulfur-oxidizing bacteria from the Galápagos Rift hydrothermal vents. Appl. Environ. Microbiol. 42: 317–324.

Seitz, A. P., E. R. Leadbetter, and W. Godchaux, III. 1993. Utilization of sulfonates as sole sulfur sources by soil bacteria including Comamonas acidovorans. Arch. Microbiol. 159: 440–444.

Smiley, D. W., and B. J. Wilkinson. 1983. Survey of taurine uptake and metabolism in Staphylococcus aureus. J. Gen. Microbiol. 129: 2421–2428.

Southward, A. J., E. C. Southward, P. R. Dando, G. H. Rau, H. Felbeck, and H. Flügel. 1981. Bacterial symbionts and low 13C/12C ratios in tissues of Pogonophora indicate unusual nutrition and metabolism. Nature 293: 616–620.

Stapley, E. O., and R. L. Starkey. 1970. Decomposition of cysteic acid and taurine by soil micro-organisms. J. Gen. Microbiol. 63: 77–84.

Stauffer, G. V. 1996. Biosynthesis of serine, glycine, and one-carbon units. Pp. 506–513 in Escherichia coli and Salmonella: Cellular and Molecular Biology, F. C. Neidhardt, ed. ASM Press, Washington, D.C.

Svensson, A. 1980. A rapid and sensitive spectrophotometric method for determination of hydrogen sulfide with 2,2′-dipyridyl disulfide. Anal. Biochem. 107: 51–55.

Wang, J. T., and A. E. Douglas. 1999. Essential amino acid synthesis and nitrogen recycling in an alga-invertebrate symbiosis. Mar. Biol. 135: 219–222.

Willason, S. W., and K. S. Johnson. 1986. A rapid, highly sensitive technique for the determination of ammonia in seawater. Mar. Biol. 91: 285–290.

Yancey, P. H., M. E. Clark, S. C. Hand, R. D. Bowlus, and G. N. Somero. 1982. Living with water stress: evolution of osmolyte systems. Science 217: 1214–1222.

Yin, M., H. R. Palmer, A. L. Fyfe-Johnson, J. J. Bedford, R. A. J. Smith, and P. H. Yancey. 2000. Hypotaurine. N-methyltaurine, taurine, and glycine betaine as dominant osmolytes of vestimentiferan tubeworms from hydrothermal vents and cold seeps. Physiol. Biochem. Zool. 73: 629–637.
Joyner, Joanna L, Peyer, Suzanne M, and Lee, Raymond W. 2003. "Possible Roles of Sulfur-Containing Amino Acids in a Chemoautotrophic Bacterium-Mollusc Symbiosis." The Biological bulletin 205, 331–338. https://doi.org/10.2307/1543296.