Full Paper

Amino acids as main substrates for sulfate-reducing bacteria in surface sediment of a eutrophic bay

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The inner part of Tokyo Bay, Japan, is highly eutrophicated as shown by the frequent occurrence of red tide. The bottom water is anoxic during warm seasons especially at artificially dredged sites. In the sediment slurries prepared from surface sediment samples collected from the dredged sites, substrate addition stimulated the consumption of sulfate during anaerobic incubation. Of the substrates added, the seston composed mainly of diatom stimulated consumption more than lactate and acetate. Its effect was nearly equal to that of casamino acids. Casamino acids and some amino acids also accelerated the rate of sulfate reduction measured by the tracer method in sediment samples more than lactate or acetate. Anaerobic incubation of the sediment slurry amended with casamino acids showed that the consumption of amino acids was retarded by the addition of molybdate (final concentration; 20 mM). In the slurry amended with only molybdate, glutamate was accumulated distinctively and linearly with time. Its accumulation rate in molar base was comparable to the rate of sulfate reduction. These results suggested that amino acids were the main substrates for sulfate-reducing bacteria (SRB) in the sediment. The MPN values of SRB in these sediment samples were often higher with the enumeration medium containing casamino acids instead of lactate. Furthermore, during a week incubation of sediment slurries amended with substrates, casamino acids and seston more greatly stimulated the growth of SRB enumerated by both media than lactate.

Key Words——amino acids; sediment; seston; substrates; sulfate-reducing bacteria; Tokyo Bay

Introduction

Aquatic sediments are important sites for decomposition of organic matter supplied from the water column. In coastal marine sediments where sufficient sulfate is available, sulfate reduction is often the most important process in microbial mineralization of organic matter (Jørgensen, 1982). Immediate substrates for

sulfate-reducing bacteria (SRB) have been considered to be mainly acetate in marine sediments (Christensen, 1984; Fukui et al., 1997; Parkes et al., 1989; Sørensen et al., 1981; Winfrey and Ward, 1983). Furthermore, *Desulfobacter* spp., SRB utilizing only acetate, often predominate in marine sediments (Jørgensen and Bak, 1991; Purdy et al., 2001). However, our preliminary experiment (Takii et al., 2002) showed that sulfate reduction in sediment samples from the inner part of Tokyo Bay was more greatly stimulated by seston collected from surface seawater than by lactate, a preferred substrate for most SRB. Lactate seems to be rapidly oxidized to acetate and propionate in anoxic sediments whether or not SRB

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contribute to its oxidation (Kondo et al., 1990; Purdy et al., 1997). Therefore, the addition of lactate should stimulate the sulfate reduction by acetate-utilizing SRB in addition to lactate-utilizing SRB. Then, substrates other than acetate may be important in this marine sediment.

In eutrophic seawater such as in Tokyo Bay, seston is mainly composed of phytoplankton which contain much protein (Persons et al., 1961). Fermentative bacteria are regarded to hydrolyze protein to amino acids and to further degrade them in anoxic environments (MacInerney, 1988). On the other hand, many SRB can directly metabolize amino acids as carbon, energy and nitrogen sources (Baena et al., 1998; Hansen, 1993; Rees et al., 1998). I examined the possibility that amino acids are utilized as the main substrates for sulfate reduction in the surface sediments of the artificially dredged sites, where sulfate reduction was more active than that on the natural sea floor in Tokyo Bay (Takii et al., 2002).

Materials and Methods

Sampling sites and sampling method. Sediment samples were collected from two sites, sites A and D, in the inner part of Tokyo Bay, Japan. Site A (139°59'20"E, 35°37'17"N) is located in a sailing route where the bottom (about 5 m deep) has been dredged down to about 7 m (water depth: about 12 m). Site D (140°00'2"E, 35°37'19"N) is near the north-east shore, and its bottom has been dredged to about 10 m below the natural bottom to collect sand (water depth: about 20 m). At these dredged sites bottom water is apt to stagnate and to be deoxygenated in summer compared with the natural sea floor (Takii et al., 2002).

Surface sediment samples were collected by an Ekman-Burge dredge or a gravity core sampler (Type MT-2, Rigosha, Tokyo, Japan) equipped with an acrylic resin tube (12 cm in diameter and 50 cm in length). The sediment sample collected by the dredge was filled in a bottle which was plugged with a stopper to avoid contact with air. Sub-core samples were obtained by inserting small tubes (4.5 cm in diameter and 30 cm in length) into the core obtained. Seston samples from the water column at these sites were collected with a plankton net (NXX25). All samples were transported in a cool box to the laboratory. Sampling was carried out from December, 1991 to August, 1993.

Effect of substrate addition on sulfate reduction in

sediment samples. Sediment slurry was prepared by mixing a surface sediment sample with an equal volume of deoxygenated bottom seawater under oxygen-free nitrogen gas. Aliquots of slurry in 200 ml conical flasks plugged with double butyl rubber stoppers were pre-incubated at in situ temperature for about an hour and then amended with collected seston (final concentration: 0.28 g L^{-1}), casamino acids (Difco, Detroit, MI, USA, 0.6 g L^{-1}), and sodium lactate (5 mM). The slurry with no addition was prepared as a control. The slurries were incubated on an orbital shaker at in situ temperature and sub-samples were taken periodically while gassing the flask with oxygen-free nitrogen to maintain anaerobic conditions. These samples were immediately centrifuged at $8,000 \times g$ for 10 min, and the supernatant was stored at -20°C until analysis for residual sulfate.

The effect of substrate addition on sulfate reduction rate in surface sediment was evaluated also by radio-tracer method: The well mixed sediment samples were put into a 2.5 ml-plastic syringe with the needle end removed and sealed with a Suba seal (Wm. Freeman & Co., Ltd., Bernsley, UK) to exclude air. After equilibrium at in situ temperature, the sub-samples in the syringes were injected through the seals with various substrates (final concentration: ca. 10 mM in pore water) and then carrier-free $\text{Na}_2^{35}\text{SO}_4$ solution (20 μl) by a syringe. The syringes were incubated at in situ temperature and sulfate reduction rates were determined in triplicates as described previously (Takii et al., 2002).

Utilization of amino acids by SRB in slurry sediments. Determination of amino acids used by SRB in sediment slurry was carried out using sodium molybdate, a specific inhibitor for sulfate reduction: Two sediment slurry samples (100 ml) supplemented with casamino acids (final concentration, 20 mg L^{-1}) were incubated as described above with or without sodium molybdate (final concentration, 20 mM). The sub-samples were taken periodically under flushing nitrogen gas and centrifuged as described above. The supernatant was stored for analysis of amino acids.

Similar experiments without added casamino acids were also carried out to estimate the utilization of amino acids by SRB in the in situ sediment. At the same time, the sulfate reduction rate in slurry was measured in triplicate by the radio-tracer method (Fukui and Takii, 1990).

Analysis of sulfate and amino acid concentrations in

slurry sediments. Sulfate ion in pore water and sediment slurry samples was determined by ion chromatography (Type Quick, Dionex, Sunnydale, CA, USA).

Free amino acids in sediment slurry samples were analyzed by the AccQ/Tag method according to the manufacturer's instructions (Waters Chromatography, Japan Millipore, Ltd., Tokyo, Japan). Briefly, after amino acids were derivatized by fluorescent reagent AQC, the derivatives were analyzed by a gradient HPLC (Waters 510 and 501 pumps, and a Waters automated gradient controller) equipped with a fluorescence detector (Waters 470 scanning fluorescence detector).

Enumeration and enrichment of SRB. SRB in sediments or slurries were enumerated by the MPN method using Postgate's B medium (Postgate, 1984) and that supplemented with casamino acids instead of lactate. The medium contains (per liter) sodium lactate (70%), 5.0 g or casamino acids, 3.5 g; yeast extract, 1.0 g; KH_2PO_4 , 0.5 g; NH_4Cl , 1.0 g; $\text{CaSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g; NaCl, 20 g and agar, 3 g. After additions of separately sterilized 1% solutions (each 10 mL L^{-1}) of ascorbic acid and thioglycollic acid, each 4 ml of the medium was distributed to small test tubes ($12 \times 105 \text{ mm}$). One milliliter of sediment samples was added to 9 ml sterile salt water (NaCl , 20 g L^{-1} ; $\text{MgCl}_2 \cdot 7\text{H}_2\text{O}$, 3 g L^{-1}), and was dispersed for 1 min by a blender (Polytron Model K, Kinematica, Switzerland). The obtained slurry was diluted serially in 10-fold steps and the diluents were inoculated to five tubes per dilution. The melted medium was solidified in cold water immediately after inoculation, and was overlaid with melted 2% agar solution to prevent contact with oxygen. After incubation for 2 weeks at 30°C , the presence of SRB was evaluated by black precipitates of ferrous sulfide in the tube. The statistical method of Cochran (1950) was used to determine if the MPN value for the casamino acids medium was higher than that for the lactate medium ($p < 0.05$).

Enrichment culture of SRB was carried out by incubation of sediment slurries supplemented with lactate (final concentration: 1 mM) or casamino acids (0.3 g L^{-1}). After a week at 20°C , SRB were enumerated by the MPN method described above.

Results

Effect of substrate addition on sulfate reduction in sediment samples

To estimate which kinds of substrates were preferably used for sulfate reduction in sediment, several substrates were added to the sediment slurries prepared from sediment samples collected from sites A and D, and the uptake rate of sulfate was compared during anaerobic incubation at in situ temperature. A typical example is shown in Fig. 1. All added substrates stimulated the uptake of sulfate in the slurries. The most effective added substrate for sulfate reduction was the seston mainly composed of diatom (mostly *Skeletonema costatum*). Its degree of effectiveness was almost equivalent to that by addition of casamino acids. The stimulation effect by the addition of seston mainly composed of *Noctilca* sp. was relatively small and equivalent to that of lactate. Similar results were obtained repeatedly for the sediment slurry prepared from sediment samples collected at sites A and D (data not shown). These results suggested that the important substrates in these sediments for sulfate reduction were amino acids.

Several substrate solutions (final concentration about 10 mM in pore water) were injected into sediment samples in hypodermic syringes and the rates of sulfate reduction measured by a radio-tracer method were compared with the rate in non-amended samples

Fig. 1. Effect of substrate addition on the consumption of sulfate in sediment slurry prepared from site D sediment sample.

(Table 1). Again, casamino acids provided the most stimulating substrate for sulfate reduction compared with lactate and acetate. The single addition of glutamate and serine also stimulated the rate more strongly than lactate or acetate.

Amino acids in casamino acids or in surface sediment utilized by SRB

To determine which amino acids in casamino acids were utilized by SRB, the sediment slurry was anaerobically incubated with casamino acids (20 mg L^{-1}) in the presence or absence of molybdate (20 mM). The

Table 1. Relative rates (%) of sulfate reduction in sediment samples supplemented with various substrates.

Sampling site	D	A	D	D
Depth of sediment (cm)	0–5	0–5	0–5	0–6
Sampling date	Dec., 91	May, 92		July, 92
Incubation temperature (°C)	13.2	17.2		16.7
No addition	100***	100**	100***	100
Lactate	217***	284*	155**	98*
Acetate	171***		136***	
H ₂ +CO ₂	261*			
Casamino acids	361	492	292	126
Glutamate				175
Serine				142

100% as the rate in the non-supplemented slurry.

The rate in slurry supplemented with casamino acids is greater than that in other slurry statistically at $p < 0.05$ (*), 0.01 (**) or 0.005 (***).

decreasing rates of each amino acid were compared between slurries with and without molybdate. Figure 2 shows the result for the anaerobic incubation (20°C) of slurry prepared from the sediment sample collected at site D on 5 August, 1993. When the decreasing rate of an amino acid is retarded by the presence of molybdate compared with the rate without molybdate, that amino acid should be utilized by SRB because molybdate is considered to be a specific inhibitor for sulfate reduction (Oremland and Capone, 1987). Six amino acids were analyzed in the sediment slurries and five amino acids other than arginine were removed relatively rapidly in both slurries. But the removal rate of each amino acid was different in the presence and absence of molybdate. This difference in removal rates was estimated to be the utilization rate of each amino acid in casamino acids by SRB. Glycine and glutamate seemed to be most rapidly utilized by SRB, followed by alanine, valine, serine and proline (Table 2).

Similar experiments using the same sediment sample were carried out without the addition of casamino acids. Without molybdate, none of the amino acids were accumulated in the slurry. But with molybdate only glutamate was distinctively and linearly accumulated as shown in Fig. 3. The accumulation rate was estimated to be $4.3 \text{ nmol ml}^{-1} \text{ day}^{-1}$ during the first 9 h. The sulfate reduction rate in the slurry was measured to be $5.6 \text{ nmol ml}^{-1} \text{ day}^{-1}$ by the radio-tracer method (24 h incubation). A similar experiment was carried out using the surface sediment sample collected at the same site on 23 August, 1993. Similar results were also obtained (Table 3).

Fig. 2. The consumption of amino acids in the casamino acids-amended (20 mg L^{-1}) sediment slurry in the absence (A) or presence (B) of molybdate (20 mM).

Table 2. Amino acids utilized by sulfate reduction in casamino acids-amended slurry.

Amino acid	Amount utilized during 9 h (μM)
Glycine	$>16.7^a$
Glutamate	10.9
Alanine	$>6.8^a$
Valine	3.9
Serine	$>3.2^a$
Proline	2.4

The amount utilized by sulfate reduction was estimated by difference in the amounts decreased in the slurries with and without molybdate (20 mM) in Fig. 2.

^aLarger than the amount because of complete depletion of the amino acid in the slurry without molybdate.

Fig. 3. The accumulation of amino acids in the non-amended sediment slurry by the inhibition of molybdate (20 mM).

Table 3. Accumulation rate of glutamate in slurry containing molybdate and sulfate reduction rate in slurry without molybdate.

Sampling date	Accumulation rate ($\text{nmol ml}^{-1} \text{ day}^{-1}$)	Sulfate reduction rate ($\text{nmol ml}^{-1} \text{ day}^{-1}$)
5 Aug., 93 ^a	4.3	5.6 (1.7)
23 Aug., 93 ^b	5.2	4.9 (0.2)

Incubation temperature: 20°C. Times for linear accumulation: ^a0–9 h, ^b0–6 h. Figures in parentheses indicate standard deviations.

Fig. 4. Relationship between MPN determinations with casamino acids medium and with lactate medium for sediment samples.

Results from 21 test pairs are plotted and the line showing the 1:1 relationship. Open symbols indicate no significant difference between MPN values. Closed symbols indicate that MPN values for casamino acids medium are significantly greater ($p < 0.05$) than those for lactate medium.

Casamino acids as substrate in media for enumeration and enrichment of SRB

Enumeration of SRB in sediments from the inner part of Tokyo Bay by the MPN method was carried out using two media containing either casamino acids or lactate as the carbon and energy substrates from May, 1992 to March, 1993. The SRB numbers in 22 samples were compared between the two media (Fig. 4). In 9 samples (41%) the MPN value enumerated by the casamino acids medium was statistically ($p < 0.05$) higher than that by the lactate medium.

Sediment slurry from site D was incubated anaerobically at 20°C with amended substrate, and SRB numbers were determined after a week by the MPN method using the medium containing casamino acids or lactate as the carbon substrate. Enrichment with casamino acids gave the largest SRB number with the casamino acids medium. The value was nearly equal to that enriched by the addition of seston, and was more than one order higher than that enriched by lactate (Table 4). On the other hand, the SRB enumeration with the lactate medium gave the highest number in the enrichment by seston, and the SRB number was equal between the slurries enriched with casamino acids and lactate. In all enriched slurries, the MPN values enumerated by the casamino acids medium were statistically ($p < 0.05$) higher than those by the lactate medium.

Table 4. Enrichment of SRB in sediment slurry with different substrates added.

Added substrate ^a	Substrate in medium ^b	Number of SRB (MPN ml ⁻¹)
No addition	Lactate	1.7×10^3 ($0.4-4.9 \times 10^3$) ^c
	Casamino acids	1.8×10^4 ($0.4-5.0 \times 10^4$)
Lactate	Lactate	7.0×10^3 ($2.3-17 \times 10^3$)
	Casamino acids	4.9×10^4 ($1.7-13 \times 10^4$)
Casamino acids	Lactate	7.0×10^3 ($2.3-17 \times 10^3$)
	Casamino acids	1.6×10^6 ($0.6-5.8 \times 10^6$)
Seston	Lactate	3.9×10^4 ($1.3-11 \times 10^4$)
	Casamino acids	9.2×10^5 ($3.0-32 \times 10^5$)

^a Carbon substrate in enrichment (7 days).^b Carbon substrate in enumeration medium.^c Figures in parentheses are 95% confidence limits.

Discussion

Acetate has been frequently reported to be a main substrate for SRB in marine coastal sediments (Christensen, 1984; Fukui et al., 1997; Parkes et al., 1989; Sørensen et al., 1981; Winfrey and Ward, 1983). Several studies also estimated the relative importance of amino acids as direct substrates for sulfate reduction (Burdige, 1989, 1991; Parkes et al., 1989). The common indication is that amino acids are mainly degraded by fermentation processes rather than directly oxidized by sulfate reduction. For example, Burdige (1989) demonstrated by molybdate inhibition experiments that dissolved free amino acids represented only 1–3% of the carbon substrates used for sulfate reduction in coastal sediments, although glutamate, alanine, β -aminoglutarate, aspartate and asparagine were oxidized by SRB. Burdige (1991) also studied the catabolism of alanine and glutamate in anoxic sediments and suggested that amino acids were generally oxidized by fermentative bacteria to volatile fatty acids or H_2 which were used by SRB. Hansen and Blackburn (1995) reported similar suggestions from the results that molybdate treatment did not increase the accumulation rate of protein amino acids, but increased the non-protein amino acids such as δ -amino-valeric-acid. They (Hansen and Blackburn, 1995) also indicated side effects from molybdate that clouded interpretation of the results.

In the present study, the addition of seston composed of mainly diatom stimulated sulfate reduction in the sediment slurry prepared from the surface sedi-

ment of dredged sites in the inner part of Tokyo Bay (Fig. 1). The degree of stimulation was almost equivalent to that caused by added casamino acids, and was greater than that caused by the addition of lactate, a preferred substrate for SRB. Lactate should stimulate even the sulfate reduction by acetate-oxidizing SRB, because lactate is rapidly oxidized to acetate by incompletely oxidizing SRB or fermentative bacteria (Kondo et al., 1990; Purdy et al., 1997). According to Hoshi et al. (1994), particulate organic matter collected in the inner part of Tokyo Bay had a high content of combined amino acids (average about 42%). The cells of diatom *Skelltonema costatum*, a mostly dominant phytoplankton in Tokyo Bay (Hoshi et al., 1994), contain protein at 58% of dry weight (Persons et al., 1961). On the other hand, the stimulation from added seston composed of mainly *Noctilca* was similar to that from lactate (Fig. 1). This might be caused by the low protein content of the dinoflagellates (36% for *Amphidinium carteri* and 35% for *Exuviaella* sp., Persons et al., 1961). The radio-tracer method also showed stronger stimulation on sulfate reduction with the addition of casamino acids or several single amino acids than with lactate or acetate (Table 1). Therefore, the above results suggested that amino acids were preferred substrates for SRB in these sediments.

To determine which amino acids were directly utilized by SRB, the decreasing rates of each amino acid were compared in the presence and absence of molybdate during the anaerobic incubation of casamino acids-amended slurries prepared from a sediment sample collected at site D. The result showed that glycine and glutamate were most rapidly utilized by SRB, and alanine, valine, serine, and proline were also utilized in this order, because the utilization was inhibited by molybdate (Table 2). These amino acids are known to be utilized by SRB (Baena et al., 1998; Hansen, 1993; Rees et al., 1998).

In the slurry amended only with molybdate glutamate accumulated linearly with time (Fig. 3), the accumulation rate in molar base was nearly comparable to the rate of sulfate reduction in the slurry (Table 3). When 1 mol of glutamate is assumed to be completely oxidized to CO_2 by SRB, 2.25 mols of sulfate ion are reduced to sulfide (Rees et al., 1998). If so, the rate of sulfate reduction would be underestimated as less than half its value. This might be caused by the difference in the incubation time, as the rates were estimated by 24 h incubation for sulfate reduction and by

6–9 h incubation for the molybdate inhibition experiment. Another possibility might be that glutamate was incompletely oxidized by SRB with consumption of a smaller amount of sulfate, and the rate was much higher than that of acetate oxidation. Although the concentrations of acetate and lactate were not measured during the incubation, the accumulation of these acids would be estimated to be low from the comparable rates of glutamate accumulation and sulfate reduction. In any event, these results strongly suggested that amino acids were the main substrates for SRB in these sediments.

The reason for the difference in main substrates of SRB between the present result and the previous papers (Christensen, 1984; Fukui et al., 1997; Parkes et al., 1989; Sørensen et al., 1981; Winfrey and Ward, 1983) remains to be resolved. One reason should be the abundant supply of protein-rich particulates to surface sediments of the sampling sites. Dense algal blooms (mainly diatom) were frequently observed in the inner part of Tokyo Bay, and moreover settled particulates on the surrounding sea floor are known to be resuspended and accumulated in concave bottoms such as sites A and D (Hoshika and Tanimoto, 1996). The present results seem to support the active participation of SRB in amino acid oxidation suggested by Hansen et al. (1993). Their report showed that inhibition of sulfate-reducing activity by added molybdate decreased 4.5-fold the rate of amino acid turnover in the incubation experiments of fish food-amended sediment collected below a marine fish farm. However, Holmer and Kristensen (1994) analyzed the mineralization products of fish food added at different amounts to the sediment samples obtained from the same fish farm and concluded that the production of ammonium was related to fermentation processes rather than directly to sulfate reduction.

The numbers of SRB determined by the MPN method using the medium containing casamino acids instead of lactate were often (41%) higher than those by the lactate medium (Fig. 4). The result suggests that casamino acids-utilizing SRB may be different from and more abundant than lactate-utilizing SRB. After 7-day incubation of sediment slurries supplemented with several substrates, the numbers of SRB were enumerated by the MPN methods using the casamino acids medium or the lactate medium. Added casamino acids gave the largest number of SRB enumerated by the casamino acids medium, which was

statistically ($p < 0.05$) equal to that in the slurry added with seston. On the other hand, added lactate did not support the growth of casamino acids-utilizing SRB compared with the control (no addition) slurry, while it stimulated the growth of lactate-utilizing SRB. These results agree with the stimulation effect of added substrates on sulfate reduction (Fig. 1). We succeeded in isolating a new sulfate reducer by repeated enrichment with casamino acids from the sediment sample collected at site D (in preparation), although selection of SRB with amino acids has never been reported (Widdel, 1988). Therefore, the surface sediment of the dredged sites in the inner part of Tokyo Bay seemed to contain abundant SRB which preferentially utilize amino acids in settled seston and amino acids, especially glutamate, were suggested to be main substrates for SRB.

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