Sulphite as Intermediate Sulphur Compound in Anaerobic Sulphide Oxidation to Thiosulphate by Marine Cyanobacteria

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Anaerobic sulphide oxidation in the light was studied with seven cyanobacterial strains isolated from the southern Baltic Sea. All strains oxidized sulphide (1.2–3.5 mM) stoichiometrically to thiosulphate. No elemental sulphur could be detected as end product of anoxygenic photosynthesis. Sulphite as intermediate sulphur compound was released into the growth medium during anaerobic sulphide oxidation by these cyanobacteria. The formation of sulphite from sulphide was inhibited by the photosynthesis inhibitor DCMU.
The cyanobacterium *Oscillatoria* sp. strain BO 32 produced another so far not identified intermediate sulphur compound which was probably stored within the cells.

Introduction

Several species of cyanobacteria are capable to cope with high sulphide concentrations. Some of these cyanobacteria utilize sulphide as electron donor for their anoxygenic photosynthesis with PS I.

Sulphide is oxidized by cyanobacteria in the course of an anoxygenic photosynthesis to at least two known end products. For example, *Oscillatoria limnetica* oxidizes sulphide anaerobically to elemental sulphur which is deposited outside the cells (Castenholz and Utkilen, 1984; Cohen *et al.*, 1975). *Microcoleus chthonoplastes* strain 11 performs anoxygenic photosynthesis resulting in the stoichiometrical formation of thiosulphate from sulphide (De Wit and van Gemerden, 1987). Other sulphur intermediates such as sulphite have not been found so far during anaerobic sulphide oxidation by cyanobacteria. The oxidation of sulphide to sulphate, common to many anoxygenic phototrophic bacteria, could not be demonstrated for the cyanobacterium *Anacystis nidulans*. On the other hand, thiosulphate and sulphite were oxidized to sulphate by this organism (Peschek, 1978). Among anoxygenic phototrophic bacteria *Rhodobacter sulfidophilus* (now *Rhodovulum sulfidophilum*) is the only organism known to excrete sulphite into the medium during anaerobic sulphide or thiosulphate oxidation to sulphate (Neutzling *et al.*, 1985). The utilization of sulphite as electron donor for a photosynthesis including both PS I and II has been reported for *Anacystis nidulans* (Peschek, 1978).

It was the aim of the present study to examine the anaerobic sulphide oxidation and the formation of sulphur compounds by several benthic cyanobacteria isolated from the southern Baltic Sea and to compare the results with those known from other cyanobacteria.

Materials and Methods

Organisms and culture conditions

The marine cyanobacteria *Microcoleus chthonoplastes* strain HI 2, *Arthrospira platensis* strain HI 45, *Lyngbya aestuarii* strains BO 9 and TI 1, *Calothrix* sp. strain HI 41, *Oscillatoria* sp. strain BO 32 and *Anabaena cylindrica* strain HI 24 were all isolated from sampling sites at Hiddensee and Boiensdorf/Wismar at the southern Baltic Sea. The organisms were cultivated in a modified artificial seawater medium ASN III (Rippka *et al.*, 1979) containing half of the normal amounts of NaCl, $MgCl_2$ x 6 H_2O, KCl, $MgSO_4$ x 7 H_2O and $CaCl_2$ x 2 H_2O. The final salinity of the medium was 15‰.

Abbreviations: PS I, photosystem I; PS II, photosystem II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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All cultures were axenic. They are deposited in the culture collection of the „Abteilung für Marine Mikrobiologie, Universität Bremen, Germany" and can be obtained from there on request.

Cells were pregrown aerobically in the growth medium for 2 weeks. The cultures were incubated with sulphide in 25 ml and 110 ml rubber stoppered glass bottles at a light intensity of 35 μmol x s^{-1} x m^{-2} and at room temperature. The inoculated culture media were gased with nitrogen for at least 15 min before sulphide was added to the concentrations required (1.0–3.5 mmol x l^{-1}). At least two parallels of each culture were run. All samples were measured against controls without cells. Chlorophyll *a* and protein contents were estimated at the end of sulphide incubation.

When DCMU (stock solution in pure ethanol) was added, the final concentration in the growth medium was 5 μmol x l^{-1}.

Determination of inorganic sulphur compounds

For the identification of sulphur compounds, samples of 100–300 μl were removed by a sterile syringe.

Sulphide, sulphite, thiosulphate and polysulphides were determined by using a monobromobimane fluorescent labeling assay (Newton *et al.*, 1981; Fahey and Newton, 1987). The bimane derivates were separated by reverse phase high performance liquid chromatography performed on a LiChrospher™-60 RP-select B column (125–4; 5 μm) (Merck, Darmstadt, Germany) using a Merck Hitachi HPLC system with a L-6210 pump, a F-1050 fluorescence detector (excitation 380 nm; emission 480 nm) and a D-2500 chromato-integrator. Solvent A was 0.25 % acetic acid titrated to pH 4.0 with 5 N NaOH; solvent B was methanol (HPLC grade). The elution protocol (room temperature, 1 ml x min^{-1}) employed linear gradients as follows: 0 min 12 % B, 7 min 12 % B, 15 min 30 % B, 19 min 30 % B, 23 min 50 % B, 30 min 100 % B, 33 min 100 % B, 33.1 min 12 % B, 36 min 12 % B, reinjection.

Elemental sulphur was extracted from the growth medium with chloroform. Analysis of elemental sulphur was carried out on the Merck Hitachi HPLC system using a LiChrospher 100 RP-18 column (125–4; 5 μm) (Merck, Darmstadt, Germany) and a L-4250 UV-Vis detector. Elemental sulphur was separated using an isocratic system, methanol/water (20:1; 1 ml x min^{-1}) and detected at 263 nm (Beffa *et al.*, 1988). The retention time of elemental sulphur under these conditions was 5.6 min.

Estimation of chlorophyll a *and protein*

Chlorophyll *a* and protein contents were analysed from harvested cell material obtained by centrifugation at 4,000 x *g* for 10 min. Chlorophyll *a* was extracted in the dark for at least 1 h from the precipitated cells with 1–3 ml methanol. The suspension was centrifuged again (4,000 x *g*, 10 min) and the absorbance of the supernatant was measured at 665 nm against methanol. Chlorophyll *a* content was calculated using the specific extinction coefficient of 74.5 l x g^{-1} x cm^{-1} (McKinney, 1941). The remaining pellets were dried on a low air flow, dissolved in 1–2 ml 1 N NaOH and heated to 90 °C in a water bath. Protein was determined according to Bradford (1976).

Results and Discussion

Macroscopic and microscopic observation

The brownish-green colour of an aerobically grown culture of *Anabaena cylindrica* strain HI 24 changed to brownish-yellow when the cells were incubated in the presence of sulphide. Only a few filament tufts, typical for aerobically grown cultures of this organism, were present and motile filaments were found only in the inner part of the filament tufts. Large amounts of tiny black grains were clustered inside of these filament tufts as observed by light microscopy. The tiny black grains might consist of metal sulphides. The formation of iron- or other metal sulphides at the cell surface may be a common property of cyanobacteria often exposed to anoxic and sulphide-rich conditions. The cyanobacterial polysaccharide sheaths probably play an important role as a first barrier against toxic sulphide concentrations. Highly refractile intracellular bodies, possibly consisting of elemental sulphur or polysulphides, have been observed by phase contrast microscopy in cells of *Anacystis nidulans*, incubated with sulphide or thiosulphate (Peschek, 1978). Similar observations were made in the present study only with cells of *Anabaena cylindrica* strain HI 24 which were incubated in

the presence of sulphide but not with the other six strains examined (data not shown).

Stal (1991) reported that the colour of an *Oscillatoria limosa* culture turned into black after having been exposed to sulphide. The author assumed that this cyanobacterium forms FeS or pyrite which is bound to the cell surface. The colour of the filaments of *Oscillatoria* sp. strain BO 32, *Microcoleus chthonoplastes* strain HI 2, *Lyngbya aestuarii* strains BO 9 and TI 1 studied in the present work became also slightly black during anaerobic growth with sulphide. This behaviour led to the assumption that similar processes must proceed in these organisms as described above for *Oscillatoria limosa* (Stal, 1991).

Cultures of *Lyngbya aestuarii* strains TI 1 and BO 9 formed gas bubbles during the first 24 hours of incubation with sulphide consisting of hydrogen as confirmed by gas chromatography (data not shown). No gas formation was observed in cultures of *Oscillatoria* sp. strain BO 32, *Arthrospira platensis* strain HI 45 and *Calothrix* sp. strain HI 41. Heterocysts of *Anabaena cylindrica* form hydrogen under anaerobic and aerobic conditions (Daday *et al.*, 1977). A sulphide-dependent H_2 evolution was shown for *Oscillatoria limnetica* (Belkin and Padan, 1978a). This cyanobacterium is able to carry out a PS I-driven electron transport obtaining electrons out of sulphide oxidation. The electrons are used for CO_2 photoassimilation or H_2 formation. The cyanobacterium *Aphanothece halophytica* also performs a sulphide-dependent H_2 evolution (Belkin and Padan, 1978b). Sulphide-dependent hydrogen evolution by *Lyngbya aestuarii* strains TI 1 and BO 9 may be a mechanism of detoxification as these strains showed only a limited ability to oxidize sulphide anaerobically.

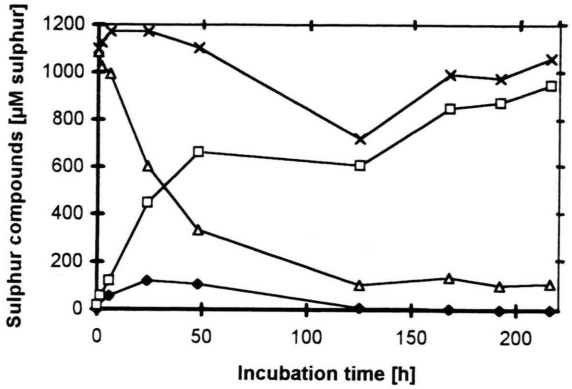

Fig. 1. Anaerobic oxidation of sulphide and formation of thiosulphate and sulphite by *Oscillatoria* sp. strain BO 32 (59 µg protein x ml^{-1}; 0.13 µg chlorophyll *a* x ml^{-1} at the end of sulphide incubation). Sulphide was added at t=0. Symbols: (△) sulphide; (□) thiosulphate sulphur; (◆) sulphite; (X) total sulphur.

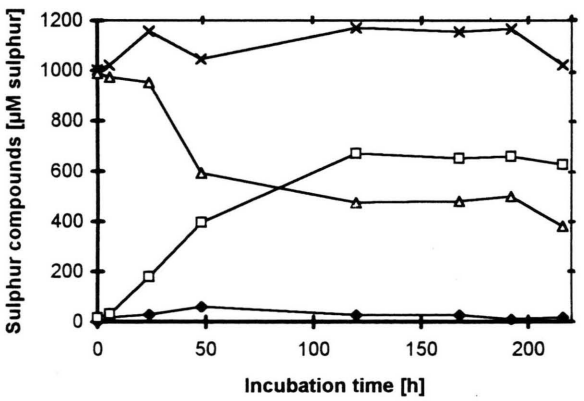

Fig. 2. Anaerobic oxidation of sulphide and formation of thiosulphate and sulphite by *Anabaena cylindrica* strain HI 24 (34 µg protein x ml^{-1} ; 0.07 µg chlorophyll *a* x ml^{-1} at the end of sulphide incubation). Sulphide was added at t=0. Symbols: (△) sulphide; (□) thiosulphate sulphur; (◆) sulphite; (X) total sulphur.

Anaerobic sulphide oxidation

Seven strains of different cyanobacteria were cultivated autotrophically with sulphide under anaerobic conditions. None of the cyanobacteria studied in this work grew under these conditions (data not shown). All strains were able to oxidize sulphide to thiosulphate stoichiometrically (Figs. 1–3). The figures represent typical experiments. Whereas some strains had only a limited ability to oxidize sulphide (Fig. 2), others could cope even with sulphide concentrations above 3000 µmol x l^{-1} (Fig. 3). None of the strains produced elemental sulphur. Small amounts of polysulphides were found as additional intermediate sulphur compounds besides sulphite and thiosulphate in the growth medium of *Arthrospira platensis* strain HI 45 only (data not shown).

For the first time sulphite was found to be an intermediate sulphur compound in photosynthetic anaerobic sulphide oxidation by cyanobacteria. All strains studied in the present work released sulphite into the growth medium during anaerobic

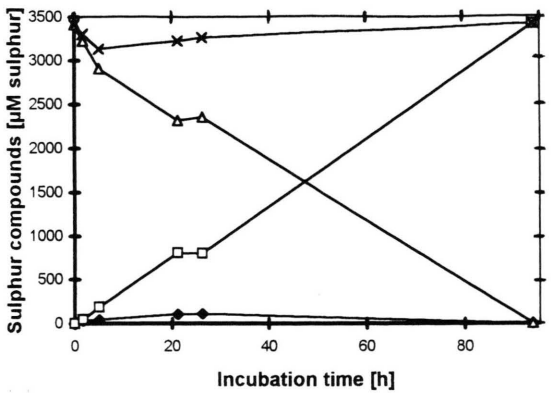

Fig. 3. Anaerobic oxidation of sulphide and formation of thiosulphate and sulphite by *Calothrix* sp. strain HI 41 (159 µg protein x ml^{-1} ; 14.5 µg chlorophyll *a* x ml^{-1} at the end of sulphide incubation). Sulphide was added at *t*=0. Symbols: (△) sulphide; (□) thiosulphate sulphur; (◆) sulphite; (X) total sulphur.

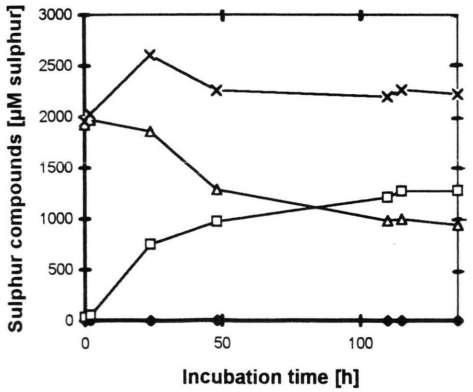

Fig. 4. Anaerobic oxidation of sulphide and formation of thiosulphate by *Oscillatoria* sp. strain BO 32 in the presence of 5 µM DCMU (84.5 µg protein x ml^{-1}; 0.47 µg chlorophyll *a* x ml^{-1} at the end of sulphide incubation). Sulphide was added at *t*=0. Symbols: (△) sulphide; (□) thiosulphate sulphur; (◆) sulphite; (X) total sulphur.

sulphide oxidation. No sulphite formation occurred in growth media without cells. Cells cultivated in the dark neither oxidized sulphide nor released sulphite or thiosulphate into the medium. When DCMU, an inhibitor of PS II, was added, none of the strains formed sulphite as shown for *Oscillatoria* sp. strain BO 32 in Fig. 4. This led to the assumption that sulphite was formed out of sulphide under participation of PS II.

A chemically catalysed formation of sulphite from sulphide with oxygen released from the photosynthetic process of water splitting at PS II could be excluded. As recently demonstrated by Rethmeier (1995), photosynthetic oxygen evolution was totally inhibited at sulphide concentrations above 500 µmol x l^{-1} in cultures of *Oscillatoria* sp. strain BO 32, *Arthrospira platensis* strain HI 45, *Calothrix* sp. strain HI 41, and *Lyngbya aestuarii* strains BO 9 and TI 1. As can be seen from Figs 1–3, *Oscillatoria* sp. strain BO 32, *Anabaena cylindrica* strain HI 24 and *Calothrix* sp. strain HI 41 released sulphite into the medium even at sulphide concentrations higher than 600 µmol x l^{-1}. De Wit and van Gemerden (1987) reported a PS II activity in *Microcoleus chthonoplastes* strain 11 in the presence of up to 1300 µmol x l^{-1} total sulphide. As sulphite formation from sulphide is clearly connected to PS II activity in the cyanobacteria studied in the present work, a very high sulphide tolerance of PS II can be assumed for the organisms examined.

The photosystem of purple bacteria is very similar in structure and function to PS II of cyanobacteria. Both photosystems belong to the phaeophytin-quinone type (Blankenship, 1994). Cells of *Rhodobacter sulfidophilus* grown autotrophically with sulphide or thiosulphate released sulphite into the growth medium as intermediate sulphur compound (Neutzling *et al.*, 1985). Since all cyanobacteria studied in the present work and *Rhodobacter sulfidophilus* released sulphite during anaerobic sulphide oxidation, it could be assumed that for this process the activity of PS II is required.

The unicellular cyanobacterium *Anacystis nidulans* is able to oxidize sulphite by using PS I and II (Peschek, 1978). In the present work, sulphite concentration decreased in the growth medium of *Oscillatoria* sp. strain BO 32 after sulphide concentration had reached a level below 500 µmol x l^{-1} (Fig. 1). Under these conditions, cyanobacteria are able to perform an oxygenic photosynthesis (Rethmeier, 1995). In this case, sulphite could be oxidized chemically with oxygen produced from oxygenic photosynthesis. An abiotic formation of thiosulphate from sulphite produced by the cells and surplus sulphide can be excluded. As can be seen from Fig. 2, sulphite did not disappear totally in the growth medium of *Anabaena cylindrica* strain HI 24 although sufficient sulphide remains available for thiosulphate formation. Oxygenic

photosynthesis was inhibited by the remaining sulphide concentration so that the sulphite produced could not be oxidized chemically as mentioned above for *Oscillatoria* sp. strain BO 32.

After sulphide concentration had decreased to 300 μmol x l^{-1} in cultures of *Oscillatoria* sp. strain BO 32, the release of thiosulphate ceased whereas sulphide oxidation still continued (Fig. 1). This resulted in a decrease of total-sulphur concentration in the growth medium. When sulphide was mostly consumed by the cells, the "missing" sulphur reappeared in the growth medium as thiosulphate. This oxidation behaviour points to the existence of a still not identified intermediate sulphur compound which is stored intracellularly. This assumption was supported by electron microscopic studies which showed the formation of electron dense bodies in cells of *Oscillatoria* sp. strain BO 32 grown with sulphide (data not shown). The formation of the unknown intermediate sulphur compound seemed to be dependent on the activity of PS II. When DCMU was added to cultures of *Oscillatoria* sp. strain BO 32, the decrease of the total-sulphur concentration described above was not observed (Fig. 4).

Cells of *Rhodopseudomonas sulfoviridis* grown autotrophically with sulphide showed a rather similar mechanism (Neutzling *et al.*, 1985). This organism oxidized sulphide to a presently unknown intracellularly stored sulphur compound which was oxidized to sulphate after sulphide in the medium was depleted.

Concerning anaerobic sulphur metabolism in the cyanobacteria examined, it is important to mention that in the presence of DCMU only cultures of *Oscillatoria* sp. strain BO 32 (Fig. 4), *Arthrospira platensis* strain HI 45 and *Calothrix* sp. strain HI 41 were able to oxidize sulphide stoichiometrically to thiosulphate, but not cells of *Anabaena cylindrica* strain HI 24, *Lyngbya aestuarii* strains TI 1 and BO 9, and *Microcoleus chthonoplastes* strain HI 2. Therefore, only the former mentioned three organisms must be able to perform a PS I driven anoxygenic photosynthesis as already described earlier for *Microcoleus chthonoplastes* strain 11 (De Wit and van Gemerden, 1987). This is remarkable, because nearly all of the cyanobacteria examined were isolated from sites of the Baltic Sea which are known for high sulphide concentrations. Further physiological and molecular studies will help to elucidate the different mechanisms of these organisms to cope with high sulphide concentrations.

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