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The Role of Thiosulfate in Sulfur Metabolism of *Rhodopseudomonas globiformis*

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Abstract. *Rhodopseudomonas globiformis* is able to assimilate both sulfur moieties of thiosulfate. During growth on ^{35}S-labelled thiosulfate the amino acids cysteine, homocysteine and methionine were labelled. The bulk of thiosulfate, however, was oxidized to tetrathionate and accumulated in the medium. A thiosulfate: acceptor oxidoreductase was partially purified and characterized. The enzyme oxidized thiosulfate to tetrathionate in the presence of ferricyanide. A *c*-type cytochrome isolated from this organism was reduced by this enzyme.

Key words: *Rhodopseudomonas globiformis* – Thiosulfate assimilation – Thiosulfate – Tetrathionate – Thiosulfate: acceptor oxidoreductase

Most species of the Rhodospirillaceae are capable of assimilatory sulfate reduction. But when *Rhodopseudomonas globiformis* was isolated Pfennig (1974) found that this organism could grow only in the presence of growth factors and a reduced sulfur source. Pfennig further reported that sulfate did not support growth and that sulfide was growth inhibitory. In the presence of thiosulfate or cysteine best growth occurred. In the present paper we report a more thorough investigation on the role of thiosulfate in sulfur metabolism of *R. globiformis*.

Material and Methods

Culture Medium

Rhodopseudomonas globiformis, strain 7950 (DSM 161), was grown heterotrophically in the following medium: 0.4 g KH_2PO_4, 0.3 g $MgCl_2 \times 6\,H_2O$, 0.4 g NaCl, 0.05 g $CaCl_2 \times 2\,H_2O$, 0.25 g $Na_2S_2O_3 \times 5\,H_2O$, 1.5 g mannitol, 0.5 g Na-gluconate, 1 ml „VA" and 1 ml „SLA" as described by Imhoff and Trüper (1977) and water to 1 l, pH 5.0.

Experiments with Growing Cultures

The experiment was performed in the medium described above at 4,000 lx and 30° C in 21 carboys which were closed with a rubber plug and stirred magnetically. To achieve anaerobic conditions the gas phase was flushed with sterile nitrogen. Samples were removed by nitrogen pressure.

Enzyme Assay

Thiosulfate: acceptor oxidoreductase was determined in the following assay system: 120 µmol ammonium acetate buffer pH 4.0, 35 µmol $Na_2S_2O_3 \times 5\,H_2O$, 2.5 µmol $K_3Fe(CN)_6$ and approximately 40 µg of the partially purified enzyme in a total volume of 3.0 ml. The assay was performed in a single beam spectrophotometer (Zeiss PM 4). The enzyme activity was determined by the difference between the reaction rates before and after the addition of the enzyme solution. The reduction of ferricyanide was followed at 420 nm and 30° C. The reduction rate of ferricyanide without enzyme was up to 12 nmol per min. One enzyme unit (U) was defined as that amount of enzyme which utilized 1 µmol $S_2O_3^{2-}$/min.

Enzyme Preparation

The cells (about 40 g wet weight) were suspended in 50 ml 50 mM ammonium acetate buffer pH 4.0. The cells were disrupted by passing them three times through an Aminco French pressure cell at $8 \cdot 10^6$ Pa. After ultracentrifugation of the suspension at 144,000 × *g* for 90 min, the supernatant was fractionated with $(NH_4)_2SO_4$. The precipitate formed between 30% and 50% saturation was collected by centrifugation at 12,000 × *g* for 15 min and dissolved in 40 mM phosphate buffer pH 6.0. After desalting by Sephadex G-25, the enzyme was purified by DEAE-cellulose chromatography. Then the active fractions were concentrated by ultrafiltration and were further purified by Sephacryl S-200 gel filtration.

Analytical Methods

Thiosulfate was determined by cyanolysis, as described by Urban (1961) and tetrathionate as described by Kelly et al. (1969). Protein was determined according to Lowry et al. (1951). For dry weight determination cells were centrifuged, washed with distilled water and dried over night at 95° C.

Chromatography

^{35}S-labelled thiosulfate and tetrathionate were separated by ascending thin layer chromatography on cellulose plates in a solvent system composed of 2-propanol and water (75:25) (Garnier and Duval 1959). The radioactive spots were recorded by scanning.

Acid hydrolysis of cells grown on ^{35}S-labelled thiosulfate was performed according to Spackman et al. (1958). The amino acids were separated by thin layer chromatography

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according to Haworth and Heathcote (1969). Before acid hydrolysis the labile amino acid cysteine was protected by oxidation to cysteic acid as described by Moore (1963).

Radioactivity Measurements

Radioactive samples were mixed with scintillation cocktail „Lumagel" obtained from Baker Chemicals, Holland. Radioactivity was measured in the liquid scintillation counter „Betascint" (Beckman, USA). The intensity of the radioactive spots were recorded by scanning the plates with a thin layer scanner (Berthold LB 2723, FRG).

Chemicals

All standard chemicals were of analytical grade and were obtained from Merck GmbH, Darmstadt; *Candida krusei* cytochrome *c* from Sankyo Company Ltd., Tokyo; ^{35}S-labelled thiosulfates from Amersham/Buchler, Braunschweig; biochemicals from Boehringer, Mannheim.

Results

Assimilation of Thiosulfate

Growth experiments with differently labelled ^{35}S-thiosulfates showed that both sulfur moieties, the outer (sulfane) group with the oxidation state of elemental sulfur and the inner (sulfone) group with the oxidation state of sulfite, were assimilated (Fig. 1 A, B). Good growth occurred only in a narrow concentration range of about 1 mM thiosulfate. The uptake rate of thiosulfate by whole cells was about 1 nmol/h per mg dry weight over the first 50 h. After 121 h 51.4 µmol thiosulfate were assimilated. The uptake characteristics for sulfane and sulfone labelled thiosulfate did not differ significantly. During these experiments only 5% of the total amount of thiosulfate present in the medium were assimilated. As cell hydrolysates revealed, the radioactivity appeared in the sulfur amino acids cysteine (as cysteic acid), methionine and homocysteine after incubation of the cells with either labelled thiosulfate. Figure 2 shows these labelled amino acids after incubation with sulfone labelled thiosulfate.

Oxidation of Thiosulfate

Whereas only 5% of the thiosulfate present was assimilated the bulk was oxidized to tetrathionate (Fig. 1 C). No other oxidation product could be found and no assimilation of tetrathionate could be detected.

It is known that below pH 5 to 4 thiosulfate decomposes in a complex manner and the end-product would depend upon the acidity of the reaction mixture (Kurtenacker et al. 1935). In a control experiment we tested the decomposition of thiosulfate in the described medium without any cells. After 80 h only 14% of thiosulfate decomposed but no tetrathionate formation could be found.

The formation of tetrathionate showed that *Rhodopseudomonas globiformis* is obviously capable of deriving electrons from thiosulfate for photosynthesis, i.e. of dissimilatory thiosulfate oxidation. Consequently we looked for the respective enzyme, a thiosulfate: acceptor oxidoreductase. A summary of the purification procedures is given in Table 1.

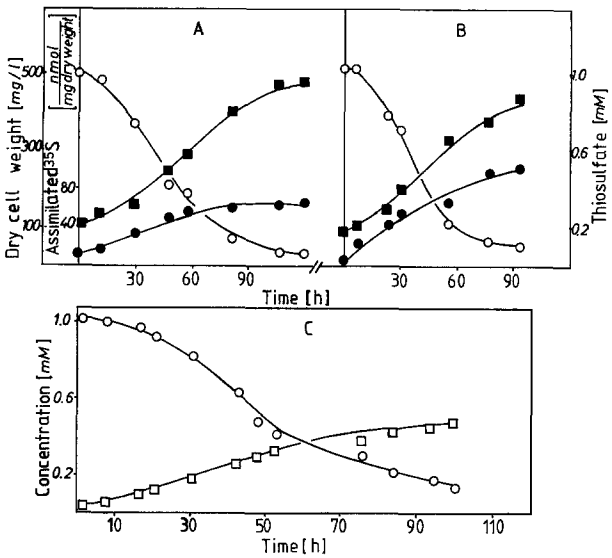

Fig. 1 A—C. Assimilation of cell sulfur by *Rhodopseudomonas globiformis* during growth on ^{35}S-labelled thiosulfate. **A** Assimilation of ^{35}S-sulfane thiosulfate (20 µCi/mmol $S_2O_3^{2-}$). **B** Assimilation of ^{35}S-sulfone thiosulfate (12 µCi/mmol $S_2O_3^{2-}$). **C** Oxidation of non-labelled thiosulfate. From a growing culture samples were removed at different intervals of time and centrifuged. Thiosulfate and tetrathionate were determined in the supernatant. The sediment was washed with distilled water; after determination of its dry weight it was completely oxidized with $HClO_3/H_2O_2$ and made up to a standard volume with distilled water. In an aliquot the radioactivity was measured. Assimilated ^{35}S (●); dry cell weight (■); thiosulfate (○); tetrathionate (□)

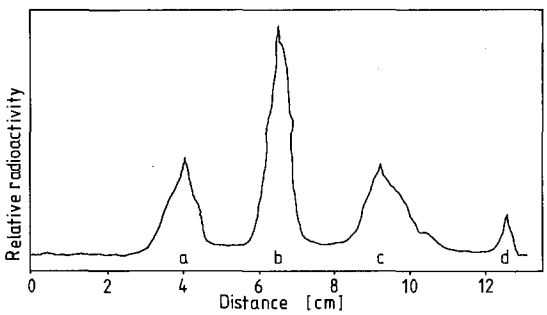

Fig. 2. Thin layer chromatography of radioactive amino acids. The cells were grown on ^{35}S-sulfone thiosulfate (40 µCi/mmol $S_2O_3^{2-}$). Amino acids were identified by cochromatography of reference substances. *a* = homocysteine, *b* = cysteic acid, *c* = methionine, *d* = front

Properties of the Enzyme

The end product of the enzymatic reaction was proven to be tetrathionate (Fig. 3). The radioactive maxima were identified by comparison with reference substances. Table 2 shows the stoichiometry in the reduction of ferricyanide in the presence of limited amounts of thiosulfate. The ratio of ferricyanide reduced to thiosulfate added was unity. The results are in agreement with the following equation:

$$2\,S_2O_3^{2-} + 2\,Fe(CN)_6^{3-} \rightarrow S_4O_6^{2-} + 2\,Fe(CN)_6^{4-}.$$

The molecular weight was determined by gel filtration on Sephacryl S-200 and calculated to be 180,000. The enzyme has a broad pH optimum between pH 4 and 6. Beyond this range only little activity was found.

Table 1. Purification of thiosulfate: acceptor oxidoreductase

Fraction	Total volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (mU/mg protein)	Purification	Yield (%)
Crude extract	64	291.9	1612.8	181.0	1	100
Ultracentrifugation	56	262.0	477.0	549.2	3.0	89
$(NH_4)_2SO_4$ fractionation	5	242.8	100.5	2416.7	13.3	83
DEAE-cellulose	4	202.3	28.2	7174.6	39.6	69
Sephacryl S-200	4	24.4	2.68	9134.0	50.4	8

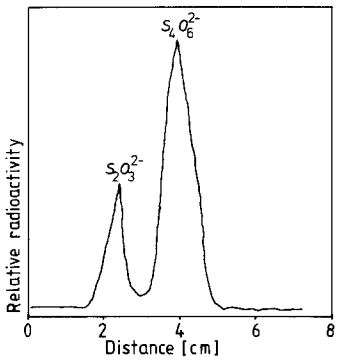

Fig. 3. Reaction products of thiosulfate: acceptor oxidoreductase. The reaction mixture contained 50 mM ammonium acetate buffer pH 4.0, 1.6 mM $K_3Fe(CN)_6$, 11.6 mM ^{35}S-labelled thiosulfate (26 µCi/mmol $S_2O_3^{2-}$), 60 µg partially purified enzyme in a total volume of 3 ml. In order to get a complete transformation at 4 times 15 µmol ferricyanide were added. After 200 min a 20 µl sample was separated by thin layer chromatography

Tabelle 2. Stoichiometry in reduction of ferricyanide catalyzed by thiosulfate: acceptor oxidoreductase in the presence of thiosulfate. The reaction mixture contained 50 mM ammonium acetate buffer pH 4.0, 1.6 mM ferricyanide, 0.4–1.2 µmol thiosulfate and 60 µg partially purified enzyme in a total volume of 3 ml

$Na_2S_2O_3$ added (µmol)	$K_3Fe(CN)_6$ reduced (µmol)
0	0
0.4	0.450
0.6	0.660
0.8	0.855
1.0	1.083
1.2	1.236

The apparent K_m for thiosulfate was determined as 0.62 mM and for ferricyanide as 0.9 mM.

The enzyme did not show sulfite oxidoreductase activity. In search for the natural electron acceptor we found no coupling with horse heart cytochrome *c*, *Candida krusei* cytochrome *c*, NAD, NADP, methylene blue and 2,6-dichlorophenol indophenol.

Preliminary experiments with cytochromes isolated from *R. globiformis* showed that one of the native *c*-type cytochromes was reduced by the purified enzyme.

Discussion

The present work has revealed that *Rhodopseudomonas globiformis* is able to assimilate both of the two sulfur moieties of thiosulfate. Cysteine is synthesized from sulfide by O-acetylserine sulfhydrylase (Hensel and Trüpr 1976). These two facts led us to the conclusion that this organism must possess a thiosulfate-splitting enzyme as well as a sulfite-reducing enzyme for the assimilation of thiosulfate. On the other hand *R. globiformis* is able to oxidize thiosulfate to tetrathionate at the same time. Only a few phototrophic bacteria are able to catalyze this reaction. *Chromatium vinosum* is able to produce tetrathionate at pH 6.25 but at pH 7.3 sulfate is formed (Smith 1966). *Rhodopseudomonas palustris* forms tetrathionate and sulfate from thiosulfate only when the initial concentration of thiosulfate is 10–20 mM; at 2–5 mM thiosulfate only sulfate is formed (Rodova and Pedan 1980). In the family of the Chlorobiaceae so far only *Chlorobium limicola* f. *thiosulfatophilum* has been reported to oxidize thiosulfate to tetrathionate (Larsen 1952). In *R. globiformis* no further oxidation to sulfate occurred, as it has been reported for *C. vinosum* (Smith 1966) and *R. palustris* (Rodova and Pedan 1980). Thus this oxidation pathway seems to be a dead end in *R. globiformis*.

The thiosulfate: acceptor oxidoreductase shows K_m values from 0.1 mM in *Thiobacillus thioparus* (Lyric and Suzuki 1970) up to 3.3 mM in *C. vinosum* (Smith 1966) when ferricyanide is used as electron acceptor. The enzyme from *R. globiformis* shows values within this range. It should be emphasized that decreased K_m values are found when cytochrome *c* is used as electron acceptor (Fukumori and Yamanaka 1979; Lyric and Suzuki 1970). Recently Fukumori and Yamanaka (1979) reported that in *C. vinosum* the partially purified enzyme rapidly reduced HiPIP (high-potential nonheme iron protein) from the same organism in the presence of thiosulfate. Also cytochromes *c* of yeast and tuna were suitable as electron acceptors like ferricyanide, whereas horse heart cytochrome *c* was a poor acceptor. Eley et al. (1971) and Knobloch et al. (1971) report the presence of a thiosulfate: cytochrome *c* oxidoreductase in *R. palustris* grown on thiosulfate.

As tetrathionate is the only detectable product of thiosulfate oxidation and no tetrathionate assimilation could be detected the described thiosulfate: acceptor oxidoreductase may have its physiological function in dissimilatory thiosulfate oxidation. During the formation of tetrathionate only one electron per molecule thiosulfate can be utilized and it is questionable whether this is enough for photolithoautotrophic growth. As tetrathionate is growth inhibitory at concentrations higher than 1 mM good growth under these conditions can be expected only in continuous culture.

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References

Eley JH, Knobloch K, Aleem MIH (1971) Cytochrome *c*-linked reactions in *Rhodopseudomonas palustris* grown photosynthetically on thiosulfate. Arch Biochem Biophys 14:419–429

Fukumori Y, Yamanaka T (1979) A high potential-nonheme iron protein (HiPIP)-linked, thiosulfate-oxidizing enzyme derived from *Chromatium vinosum*. Cur Microbiol 3:117–120

Garnier Y, Duval C (1959) Séparation chromatographique sur papier de quelques anions minéraux renfermant du soufre et leur identification par spectrographie infrarouge. J Chromatog 2:72–75

Haworth C, Heathcote JG (1969) An improved technique for the analysis of amino acids and related compounds on thin layers of cellulose. J Chromatog 41:380–385

Hensel G, Trüper HG (1976) Cysteine and S-sulfocysteine biosynthesis in phototrophic bacteria. Arch Microbiol 109:101–103

Imhoff JF, Trüper HG (1977) *Ectothiorhodospira halochloris* sp. nov., a new extremely halophilic phototrophic bacterium containing bacteriochlorophyll b. Arch Microbiol 114:115–121

Kelly DP, Chambers LA, Trudinger PA (1969) Cyanolysis and spectrophotometric estimation of trithionate in mixture with thiosulfate and tetrathionate. Analyt Chem 41:898–901

Knobloch K, Eley JH, Aleem MIH (1971) Thiosulfate-linked ATP-dependent NAD reduction in *Rhodopseudomonas palustris*. Arch Mikrobiol 80:97–114

Kurtenacker A, Mutschin A, Stastny F (1935) Über die Selbstzersetzung von Polythionatlösungen. Z anorg allg Chem 224:399–419

Larsen H (1952) On the culture and general physiology of the green sulfur bacteria. J Bacteriol 64:187–196

Lowry OH, Rosebrough HJ, Farr AL, Randall RJ (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193:265–275

Lyric RM, Suzuki I (1970) Enzymes involved in the metabolism of thiosulfate by *Thiobacillus thioparus*. Can J Biochem 48:344–363

Moore ST (1963) On determination of cysteine as cysteic acid. J Biol Chem 238:235–237

Pfennig N (1974) *Rhodopseudomonas globiformis*, sp. n., a new species of the Rhodospirillaceae. Arch Microbiol 100:197–206

Rodova NA, Pedan LV (1980) Metabolism of thiosulfate in *Rhodopseudomonas palustris*. Mikrobiologiya 49:221–226

Smith AJ (1966) The role of tetrathionate in the oxidation of thiosulfate by *Chromatium sp. strain D*. J Gen Microbiol 42:357–380

Spackman DH, Stein WH, Moore ST (1958) Automatic recording apparatus for use in the chromatography of amino acids. Analyt Chem 30:1190–1206

Urban PJ (1961) Colorimetry of sulfur anions. I. An improved colorimetric method for the determination of thiosulfate. Z Anal Chem 179:415–426

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