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Sulfate Assimilation in *Rhodopseudomonas globiformis*

Johannes F. Imhoff, Johann Then, Fuad Hashwa*, and Hans G. Trüper**

Institut für Mikrobiologie der Rheinischen Friedrich-Wilhelms-Universität, Meckenheimer Allee 168, D-5300 Bonn 1, Federal Republic of Germany

Abstract. *Rhodopseudomonas globiformis* is able to grow on sulfate as sole source of sulfur, but only at concentrations below 1 mM. Good growth was observed with thiosulfate, cysteine or methionine as sulfur sources. Tetrathionate supported slow growth. Sulfide and sulfite were growth inhibitory. Growth inhibition by higher sulfate concentrations was overcome by the addition of O-acetylserine, which is known as derepressor of sulfate-assimilating enzymes, and by reduced glutathione. All enzymes of the sulfate assimilation pathway, ATP-sulfurylase, adenylylphosphate-sulfotransferase, thiosulfonate reductase and O-acetylserine sulfhydrylase are present in *R. globiformis*. Sulfate was taken up by the cells and the sulfur incorporated into the amino acids cysteine, methionine and homocysteine. It is concluded, that the failure of *R. globiformis* to grow on higher concentrations of sulfate is caused by disregulation of the sulfate assimilation pathway. Some preliminary evidence for this view is given in comparing the activities of some of the involved enzymes after growth on different sulfur sources and by examining the effect of O-acetylserine on these activities.

Key words: Rhodospirillaceae – *Rhodopseudomonas globiformis* – Sulfate assimilation

Rhodopseudomonas globiformis was isolated by Pfennig (1974) from enrichments of a warm acidic sulfur spring of Yellowstone Park, USA. Pfennig found a requirement for reduced sulfur compounds for growth in this organism. Best growth occurred with thiosulfate or cysteine as sulfur source while sulfide was inhibitory at concentrations of 0.4 mM. The excellent growth observed with thiosulfate is explained by the fact that thiosulfate is not only assimilated but the bulk of it is oxidized to tetrathionate thus yielding additional electrons for photosynthesis (Then and Trüper 1981). Pfennig (1974) concluded, that the organism lacks the enzymes of assimilatory sulfate reduction. After preliminary experiments in our laboratory had shown, that sulfate is taken up by *R. globiformis* and that growth occurred with sulfate as sole sulfur source in rather low concentrations also in the absence of yeast extract, we started a more thorough investigation of sulfate assimilation in this species. The results are presented in this paper.

* Biology Department, University of Jordan, Amman, Jordan
** To whom offprint requests should be sent

Abbreviations: DTE, DL-dithioerythritol; APS, adenosine 5′-phosphosulfate, adenylyl sulfate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate, 3′-phosphoadenylylsulfate

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Materials and Methods

Rhodopseudomonas globiformis (DSM 161) cultures were maintained and grown as described by Then and Trüper (1981). Growth on different sulfur sources was tested in 50 ml screw cap bottles. Experiments for measuring sulfate uptake and incorporation were performed in the described medium at 30° C and 4000 lux in 2 l carboys closed with rubber plugs and stirred magnetically. The gas phase was flushed with sterile oxygen-free nitrogen. Samples were removed by nitrogen pressure. Experiments with O-acetylserine to derepress the sulfate assimilating enzymes were performed in a 10 l glass fermenter at controlled temperature (30° C), with strong stirring at 8000 – 12000 lux under nitrogen. Precultures were grown with thiosulfate, washed with sulfate-free medium and then incubated in sulfate-free medium with 1 mM O-acetylserine. The initial cell density was 1.2 g wet weight/l.

Optical density of cell suspensions was measured at 650 nm against medium blanks. Protein was determined according to Lowry et al. (1951). For dry weight determinations cells were centrifuged, washed with distilled water and dried over night at 95° C.

Thiosulfate was determined in the supernatant after centrifugation of cell suspensions as described by Urban (1961). The total sulfur content of the cells was determined as sulfate after complete digestion in $HClO_3/H_2O_2$ according to Novozamsky and van Eck (1977). Cells were washed with distilled water, dried and then oxidized. The digested cells were made up to a standard volume and sulfate was determined in aliquots of this solution either chemically or in experiments with ^{35}S-labeled sulfate by measuring the radioactivity. In the latter case total sulfur content was calculated from total radioactivity and specific radioactivity of the added sulfate.

The APS sulfotransferase assay was performed according to Schmidt (1972), the thiosulfonate reductase assay according to Schmidt (1973).

For the analysis of amino acids, acid hydrolysis of *R. globiformis* cells was performed after Spackman et al. (1958). The amino acids were separated by thin layer chromatography according to Haworth and Heathcote (1969). Cysteine was oxidized prior to acid hydrolysis as described by Moore (1963).

Measurement of radioactivity in water samples was done in the liquid scintillation counter "Betascint" (Beckman, USA) in the "Lumagel" scintillation cocktail (Baker Chemicals, Holland) with 0.5 ml sample and 5 ml cocktail. The activity of radioactive spots on thin layer plates was recorded with a thin layer scanner (Berthold LB 2723, Germany).

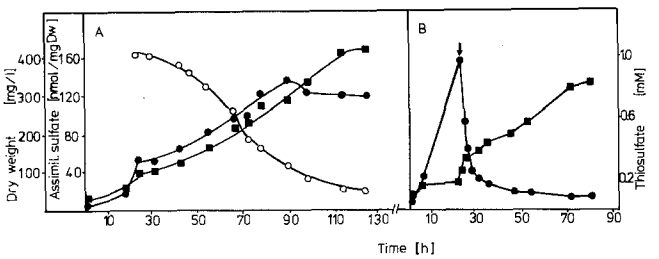

Fig. 1A and B. Assimilation of sulfate by *Rhodopseudomonas globiformis* during growth on ^{35}S-labeled sulfate (0.5 mM, 35 μCi/mmol). **A** With addition of 1 mM thiosulfate after 23 h; **B** with addition of 0.8 mM cysteine after 23 h; thiosulfate (○); dry weight (■); assimilated sulfate-sulfur (●); time of cysteine addition (⇩)

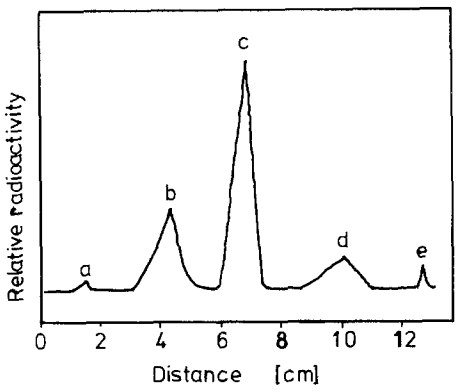

Fig. 2. Thin layer chromatography of radioactive labeled amino acids from *Rhodopseudomonas globiformis* after growth on ^{35}S-sulfate. The amino acids were identified by cochromatography of reference substances. Start (*a*); homocysteine (*b*); cysteic acid (*c*); methionine (*d*); front (*e*)

Results

Growth Experiments

Growth of *Rhodopseudomonas globiformis* on different sulfur sources was followed over three passages by determining optical density at 650 nm and dry weight. Best growth occurred at 0.1 mM sulfate. Higher sulfate concentration were inhibitory. In the presence of 5 mM sulfate, O-acetylserine and reduced glutathione enhanced the growth considerably. Growth under these conditions was equal or better than growth with thiosulfate or cysteine as sulfur source.

Growth with glutathione alone was very poor. Optimal thiosulfate concentration (in the range from 0.1 – 10 mM) was 1 mM and the optimal cysteine concentration (in the range from 3.4 – 13.4 mM) was 13.4 mM. Growth was also possible with methionine (0.3 – 3.4 mM tested, optimum at 0.7 mM) and tetrathionate (0.05 – 5 mM tested, optimum at 0.5 mM). Sulfide and sulfite were growth inhibitory at concentrations above 0.4 and 0.1 mM respectively. Thus sulfate, which is growth inhibitory at normal concentrations supports growth at very low concentrations. Growth inhibition by sulfate is overcome by addition of O-acetylserine or reduced glutathione.

Sulfate Uptake and Incorporation

To demonstrate the uptake of sulfate into cells of *R. globiformis*, cells were precultured in a medium without sulfur source for three days and then incubated in a medium with 0.5 mM ^{35}S-labeled sulfate (35 μCi/mmol). Samples were removed at different intervals of time for the determination of dry weight and cellular sulfur content. Sulfate uptake parallels growth and the uptake rate is 1.15 nmol/mg dry weight and h. The sulfur content of the cells after 4 days was 1.9% of the dry weight. Additions of 1 mM thiosulfate or 0.5 mM tetrathionate had no significant influence on the uptake rate of sulfate, which was 1.2 nmol/mg dry weight and h with thiosulfate (cf. Fig. 1A). Sulfate uptake was, however, inhibited by yeast extract, and the addition of 0.8 mM cysteine to cells growing on ^{35}S-sulfate blocked the uptake of sulfate immediately (Fig. 1B). At concentrations of 0.1 and 0.5 mM cysteine the uptake of sulfate after 6 h was only 26% and 20%, respectively, as compared with a control without cysteine. Carbonyl-cyanide 3-chlorophenylhydrazone (CCCP) at 10 μM blocked sulfate uptake completely. To obtain evidence for the incorporation of sulfate sulfur into the amino acids, acid hydrolysates of ^{35}S-sulfate grown cells were developed in one- and two-dimensional thin layer chromatography. Cysteine and cystine were oxidized prior to hydrolysis with paraformic acid. Three components were identified as cysteic acid (59%), homocysteine (25%) and methionine (16%). Figure 2 shows the scan of a one-dimensional separation of the hydrolysate on thin layer plates.

Table 1. Reduction of sulfonucleotides and sulfate by cell extracts of *Rhodopseudomonas globiformis*

Substrate	Relative reduction rate (%)
$AP^{35}S$	100
$AP^{35}S$ + ATP	85
$AP^{35}S$ + $PAP^{32}S$	58
$PAP^{35}S$	131
$PAP^{35}S$ + $AP^{32}S$	2
^{35}S-sulfate + ATP	2

The test assay contained in 1 ml: 100 mM Tris-HCl pH 7.6, 10 mM DTE, 10 mM $MgCl_2 \cdot 6\,H_2O$, 100 mM Na_2SO_4, 1.8 mg protein, 0.005 ml purified thioredoxin from spinach leaves and if indicated, 10 mM ATP, 0.1 mM $AP^{35}S$ and $PAP^{35}S$ (8440 cpm/nmol and 2560 cpm/nmol), 1 mM $AP^{32}S$ and $PAP^{32}S$ and 20 mM ^{35}S-sulfate (13800 cpm/nmol).

Enzymes and Their Regulation

For measurement of the sulfotransferase activity cells were incubated with 1 mM O-acetylserine to derepress the sulfate-assimilating enzymes. O-acetylserine has been successfully used for this purpose in other Rhodospirillaceae (Imhoff 1980). The reduction of ^{35}S-sulfate, $AP^{35}S$ and $PAP^{35}S$ to acid volatile radioactivity was tested. The rate of APS reduction was 1.34 nmol/mg protein and h, and thus comparable with the rates in other Rhodospirillaceae grown on sulfate (Imhoff 1980). Addition of 10 mM ATP to the enzyme assay did not increase this activity while addition of a tenfold higher concentration of unlabeled PAPS led to a decrease of the activity to 58% (Table 1). With $PAP^{35}S$ a higher reduction rate was found, which is attributed, however, to the

Table 2. Activities of APS-sulfotransferase and thiosulfonate reductase under different growth conditions

Sulfur source	Sulfotransferase	Thiosulfonate reductase
Thiosulfate	1.8	600
Cysteine	1.2	660
Sulfate + yeast extr.	0.4	320
Sulfate	0.1	160
O-Acetylserine (without sulfur source)	10 – 30	150 – 500

Test conditions for APS-sulfotransferase were the same as in Table 1 with $AP^{35}S$ as substrate. The assay for thiosulfonate reductase contained in 1 ml: 100 mM Tris-HCl pH 8.0, 25 mM dithionite, 1 mM methylviologen, 10 mM DTE, 0.01 ml purified thioredoxin from spinach leaves and 1.8 mg soluble protein. Values are given in μU/mg protein.

decomposition of $PAP^{35}S$ to $AP^{35}S$ and subsequent reduction of the latter. The same decomposition reaction caused the lower activity with $AP^{35}S$ in the presence of unlabeled PAPS, because unlabeled APS is formed and dilutes the pool of radioactive $AP^{35}S$. Increasing the concentration of sulfate up to 100 mM the assay may not be sufficient to completely prevent hydrolysis of APS to AMP and sulfate.

If in turn the concentration of unlabeled APS is tenfold higher than that of radioactive $PAP^{35}S$, only 2% of the activity with $AP^{35}S$ alone can be found. These data indicate, that the reduction pathway is starting with APS rather than with PAPS as the substrate for the sulfotransferase. Activities of ATP-sulfurylase and thiosulfonate reductase were measured in connection with experiments performed on enzyme regulation. O-acetylserine sulfhydrylase had been found earlier (Hensel and Trüper 1976).

In order to study regulatory phenomena of the enzymes involved in the assimilation of sulfate, cells were grown in thiosulfate medium, washed with sulfate-free medium and then incubated in sulfate-free medium with 1 mM O-acetylserine. Over 10 h samples were taken and the activities of ATP-sulfurylase, APS-sulfotransferase, APS-kinase and thiosulfonate reductase were measured in cell free extracts. The first response after the addition of O-acetylserine was an increase of ATP-sulfurylase and APS-sulfotransferase activities, but a decrease of thiosulfonate reductase activity by about three times of the initial activity. This is in contrast to similar experiments with other Rhodospirillaceae in which thiosulfonate reductase was regulated similar to sulfotransferase (Imhoff 1980). The activity of ATP-sulfurylase was nearly constant over the observed time at about 1.5 mM/mg protein. Activities of both sulfotransferase and thiosulfonate reductase in *R. globiformis* were higher in cells grown in the presence of yeast extract, cysteine or thiosulfate, as compared with sulfate grown cells (Table 2). Under similar conditions these enzymes are repressed in other Rhodospirillaceae. The highest activities of both enzymes were found in cells grown on thiosulfate. Only under derepressing conditions with O-acetylserine higher activities of sulfotransferase can be observed, whereas thiosulfonate reductase activities under these conditions are lower than in cells grown on cysteine or thiosulfate; its lowest level during "derepression" – observed 4 h after addition of O-acetylserine – is even lower than in sulfate grown cells (Table 2).

Discussion

The results show, that *Rhodopseudomonas globiformis* is able to grow on sulfate as sole sulfur source at concentrations below 1 mM. The organism is able to take up sulfate into the cells and to incorporate sulfate sulfur into the amino acids cysteine, cystine, methionine and homocysteine. All enzymes necessary for the incorporation into cysteine, ATP-sulfurylase, APS-sulfotransferase, thiosulfonate reductase and O-acetylserine sulfhydrylase (Hensel and Trüper 1976) are present. Thus the reasons for the growth inhibitory effect by higher sulfate concentrations should be found in certain regulatory anomalities in the enzymatic steps of the sulfate assimilation pathway. First evidence for this assumption was gained from growth experiments with different sulfur sources. These experiments showed that growth inhibition by sulfate, at the inhibitory concentration of 5 mM, could be reduced by addition of O-acetylserine or reduced glutathione, which – as sulfur source – did not support growth. Under such conditions the highest cell densities were obtained.

Sulfate uptake is inhibited by yeast extract and strictly inhibited by cysteine, as it has been found also in Enterobacteriaceae (Jones-Mortimer et al., 1968). In the latter all enzymes of the sulfate assimilation pathway are repressed by cysteine, which is not the case in *R. globiformis*. Activities of APS-sulfotransferase and thiosulfonate reductase were lowest in cells grown on sulfate in *R. globiformis*. They increased 2 – 4 fold with the addition of yeast extract and even higher, when cells were grown with cysteine as sulfur source. The highest activities of these enzymes were found with thiosulfate as sulfur source (Table 2). The activity of O-acetylserine sulfhydrylase increased about three times, too, if cells were grown on 0.8 mM thiosulfate instead of 1.7 mM sulfate. On cysteine only 40% of the activity with sulfate were found (Hensel and Trüper 1976). In all other species of the Rhodospirillaceae tested this activity is repressed with thiosulfate as sulfur source. *R. globiformis* does not possess S-sulfocysteine synthase or cysteine desulfhydrase (Hensel and Trüper 1976). It is assumed, that during growth on sulfate the sulfate-assimilating pathway in *R. globiformis* is repressed. As repressor bound sulfide may act rather than cysteine. This may accumulate during growth on sulfate, if thiosulfonate reductase is not inhibited or repressed by its own product and if serine transacetylase is not present or only present at very low activities. Thus the O-acetylserine concentration remains so low, that even with high activities of O-acetylserine sulfhydrylase (5.9 U/mg protein after growth on sulfate; Hensel and Trüper 1976) bound sulfide will accumulate. The effect of cysteine (yeast extract may act in the same way because of its content of cysteine and cystine) can be explained by assuming that it inhibits only sulfate uptake and synthesis of O-acetylserine sulfhydrylase, but does not repress the whole sulfate assimilation pathway. Thus the intracellular sulfate concentration and subsequently also the concentration of the repressing intermediates decrease. Then the enzymes of sulfate assimilation though present at rather high activities are not effective because of lack of substrate. A summary of these thoughts is given in Fig. 3.

O-acetylserine is known for its derepressing action on all enzymes involved in sulfate assimilation in *Escherichia coli* and *Salmonella typhimurium* (Jones-Mortimer et al. 1968). In most species of the Rhodospirillaceae it was found to act this way (Imhoff 1980), but in *R. globiformis only* ATP-sulfurylase and APS-sulfotransferase showed increasing activities on

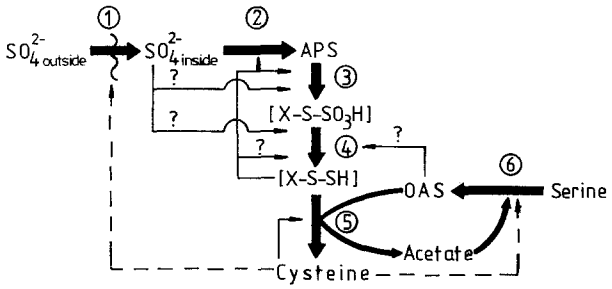

Fig. 3. Scheme for the possible regulation of sulfate assimilation in *Rhodopseudomonas globiformis*. *1*, Sulfate permease; *2*, ATP-sulfurylase; *3*, APS-sulfotransferase; *4*, thiosulfonate reductase; *5*, O-acetylserine sulfhydrylase; X, carrier; OAS, O-acetylserine; ---→ inhibition, ⟶ repression

incubation with O-acetylserine, whereas thiosulfonate reductase activities decreased first with O-acetylserine. It is concluded, that O-acetylserine is taken up by *R. globiformis* and enables proper action of O-acetylserine sulfhydrylase by enlarging the intracellular pool of O-acetylserine. The concentration of bound sulfide will be diminished, so that the enzymes of sulfate assimilation with exception of thiosulfonate reductase become derepressed.

The enzyme with the most peculiar properties is thiosulfonate reductase. Its lowest activities were found after addition of O-acetylserine where other Rhodospirillaceae exhibit highest thiosulfonate reductase activities. But even this low activity is several times higher, than those found in *Rhodospirillum tenue* (75 μU/mg protein) or *Rhodopseudomonas sulfidophila* (15 μU/mg protein) after derepression (Imhoff 1980).

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