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Sulfate assimilation in *Rhodopseudomonas sulfidophila*

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Abstract. The mechanism of sulfate assimilation was investigated in *Rhodopseudomonas sulfidophila*, a bacterium able to grow either photoautotrophically, with sulfide as electron donor, or photoheterotrophically with sulfate as sole sulfur source. ATP sulfurylase, adenosine-5′-phosphosulfate kinase, 3′-phosphoadenosine-5′-phosphosulfate sulfotransferase, thiosulfonate reductase and cysteine synthase were present. Reduced sulfur compounds, especially sulfide and sulfite repressed all steps of sulfate activation and reduction including sulfate uptake. Adenosine-5′-phosphosulfate kinase activity in contrast to the other activities was high in the presence of cysteine or reduced glutathione in the growth medium. Sulfur was incorporated into the cellular sulfolipid from sulfate and also from reduced sulfur compounds like cysteine and thiosulfate. The activity of 3′-phosphoadenosine-5′-phosphosulfate sulfotransferase was rapidly lost during gel filtration or dialysis. From comparison with other sulfotransferases and from the specific cofactor requirement for the enzyme of *R. sulfidophila* it is concluded that two different low molecular weight cofactors are required in this system. A reaction sequence is proposed involving thioredoxin as the reductant of another dialysable low molecular weight cofactor, that binds to the protein.

Key words: *Rhodopseudomonas sulfidophila* – Sulfate uptake – PAPS sulfotransferase – Sulfolipid – Regulation of sulfate assimilation

Rhodopseudomonas sulfidophila was first isolated and described from the Dutch Waddensea and found to tolerate sulfide at rather high concentrations (Hansen and Veldkamp 1973). It can grow photolithotrophically with sulfide or thiosulfate as electron donors, oxidizing these compounds to sulfate without intermediate accumulation of elemental sulfur, but does not depend on reduced sulfur compounds like *Rhodopseudomonas sulfoviridis* (Keppen and Gorlenko 1975; Neutzling and Trüper 1982) and *Rhodopseudomonas* spec. strain 51 (Hansen 1974), which do not grow with sulfate as sole sulfur source. *R. sulfidophila* has been chosen for an investigation of the sulfate assimilation pathway, because of its ability to either use sulfide as electron donor and sulfur source or to assimilate and use sulfate as sole sulfur source. In a previous paper it has been shown that among the species of the Rhodospirillaceae some reduce sulfate via adenosine-5′-phosphosulfate (APS) and others via 3′-phosphoadenosine-5′-phosphosulfate (PAPS) and that *R. sulfidophila* belongs to the latter (Imhoff 1982). Regulation of its sulfate uptake, of the enzymatic activities involved in the assimilation of sulfate and evidence for the synthesis of a sulfolipid are reported in this communication.

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Abbreviations. APS, adenosine-5′-phosphosulfate; PAPS, 3′-phosphoadenosine-5′-phosphosulfate

Materials and methods

The organisms used during this study, *Rhodopseudomonas sulfidophila* W4 (DSM 1374), *Rhodospirillum rubrum* S1 (DSM 467), and *Rhodomicrobium vannielii* 17100 (DSM 162), were obtained from the Deutsche Sammlung für Mikroorganismen in Göttingen. Methods for cell growth, preparation of cell free extracts, purification of spinach thioredoxin and preparation of sulfonucleotides were essentially the same as reported earlier (Imhoff 1982). ATP sulfurylase was measured after Wilson and Bandurski (1958) with molybdate as substrate. APS kinase was measured with ^{35}S-APS as substrate. The assay contained 100 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 100 mM Na_2SO_4, 10 mM ATP, 0.1 mM ^{35}S-APS (20,000 – 50,000 cpm/nmol) and appropriate amounts of cell extract in a total volume of 1 ml. Incubation was for 1 – 10 min at 30° C. The reaction was stopped in a boiling water bath, the mixture cooled on ice and quantitatively applied on a small Sephadex A-25 column for the separation of ^{35}S-sulfate, ^{35}S-APS and ^{35}S-PAPS. The nucleotides were eluted with 300 – 2,000 mM Tris-HCl pH 7.6. Fractions of 2 ml were collected and the radioactivity counted in a scintillation counter. The rate of PAPS formation was calculated from the specific radioactivity of ^{35}S-APS and the amount of ^{35}S-PAPS formed. Sulfotransferase was tested according to Schmidt (1972) as given in a previous paper (Imhoff 1982). The thiosulfonate reductase assay contained 100 mM Tris-HCl pH 8.0, 25 mM $Na_2S_2O_4$, 1 mM methylviologen, 10 mM dithioerythritol, 0.1 mM ^{35}S-PAPS, 10 µl spinach thioredoxin and 5 – 10 mg protein in 1 ml. Incubation was under nitrogen for 1 h at 30° C. The assay was stopped on ice, 20 µmol sulfide were added and then 1 ml 1 N HCl. Volatile sulfide was trapped in 1 ml 1 M triethanolamine and the radioactivity counted in a scintillation counter. The physiological substrate of the thiosulfonate reductase is thought to be cofactor-bound sulfite and PAPS is converted to this substrate in the presence of sulfotransferase. Therefore this assay was used to measure thiosulfonate reductase activity. For measurements of sulfate uptake, cells were taken at different time intervals, cooled on ice, centrifuged and the radioactivity determined in the

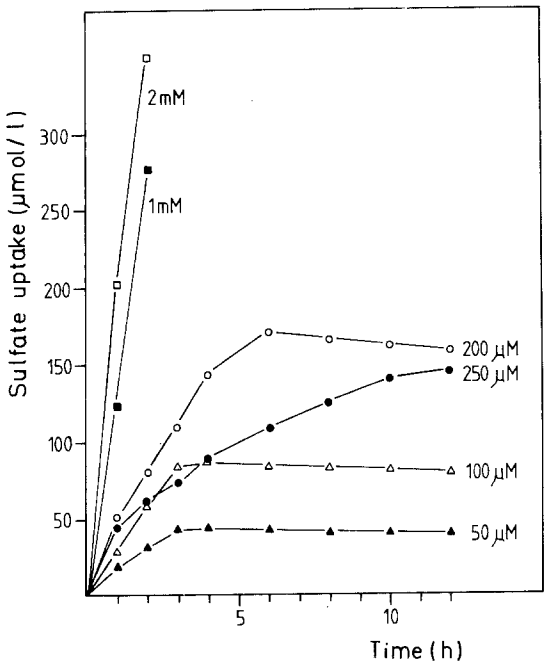

Fig. 1. Time dependence of sulfate uptake at different sulfate concentrations in sulfur starved cells of *Rhodopseudomonas sulfidophila* W4. The inoculum of the cultures contained 300 mg protein/l

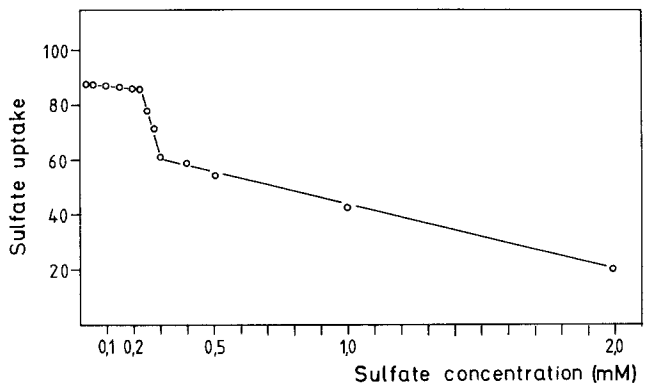

Fig. 2. Relative amounts of sulfate taken up by sulfur starved cells of *R. sulfidophila* after 10 h incubation (values in % of the initial amount present in the cultures prior to inoculation)

supernatant. Extraction and separation of lipids were performed using the methods described earlier (Imhoff et al. 1982). Radioactivity on thin layer plates was detected with a Berthold Scanner. For quantitative work known amounts of lipids were applied to the plates, after developing the radioactive spots were scraped off the plates and counted in a Beckman Liquid Scintillation Counter.

Results and discussion

In the presence of organic substrates good growth of *Rhodopseudomonas sulfidophila* was found with sulfate, sulfite, thiosulfate, sulfide, cysteine and reduced glutathione as sulfur sources.

Sulfate uptake

Sulfate uptake was measured in sulfur starved cells, precultured in a sulfur free medium for 12 h and than inoculated

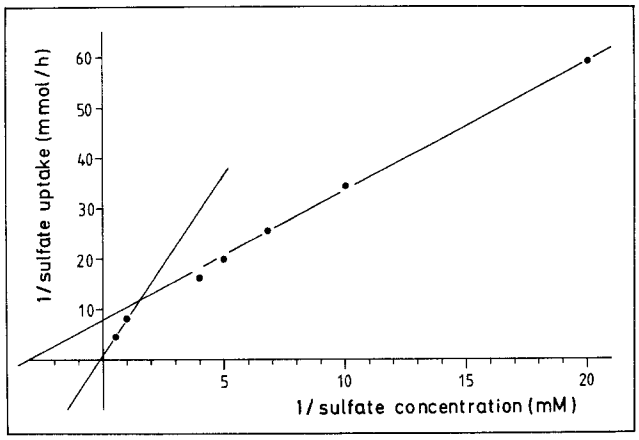

Fig. 3. Lineweaver-Burk-plot of sulfate uptake in *R. sulfidophila*

into media containing 0.01 – 2.0 mM sulfate (^{35}S-labeled, 50 µCi/50 ml culture). All cultures were inoculated from the same preculture and incubated anaerobically in the light. Uptake was measured on samples withdrawn at different time intervals. As shown in Fig. 1, the uptake rate depended on the sulfate concentration; its maximum was at 2 mM sulfate. At concentrations below 250 µM the medium was rapidly depleted of sulfate; more than 85% of the supplied sulfate were taken up within less than 4 h and it is obvious that sulfate became growth limiting at these concentrations. Only at 1.0 and 2.0 mM sulfate did the uptake continue for up to 10 h. At this time, although in the experiments with lower sulfate concentrations uptake had stopped earlier, with concentrations up to 225 µM more than 85% were assimilated; the portion assimilated of the supplied sulfate decreased rapidly up to 300 µM to about 60% and then only slowly to 20% at 2 mM (Fig. 2). If the initial uptake rates were used to determine the K_m-value of the uptake system (Fig. 3), two apparently different K_m-values were obtained: 0.32 and 5.6 mM, respectively. It is not known whether this property reflects the presence of two different sulfate transport enzymes or the change of one enzyme's catalytical properties dependent on the extracellular and/or intracellular sulfate concentration. When cultures were incubated for 10 h in the presence of 0.25 mM sulfate (4 mCi/mmol) and different concentrations of reduced sulfur compounds, the strongest inhibition of sulfate uptake occurred in the presence of even very low concentrations of sulfide (0.1 mM). Cysteine, reduced glutathione and methionine also exerted more than 50% inhibition at 0.5 mM (Fig. 4A). O-acetylserine, known as a derepressor of sulfate-assimilating enzymes in *Escherichia coli* and *Salmonella typhimurium* (Pasternak 1962; Jones-Mortimer et al. 1968; Kredich 1971), caused activation of sulfate uptake after 3 h and only slight inhibition after 10 h as compared to a culture without additions. The slight inhibitory effect of O-acetylserine at very low concentrations decreased with increasing concentrations (Fig. 4B). The time course of the experiments with addition of different cysteine concentrations showed oscillations in uptake and release of sulfate (Fig. 5). This oscillation was also observed with other reduced sulfur compounds. With O-acetylserine sulfate uptake was retarded periodically and excretion was only minimal. The periodically occurring changes may demonstrate a regulatory property of the sulfate transport system(s) to control the intracellular sulfate concentration; they depend on the presence of reduced sulfur compounds

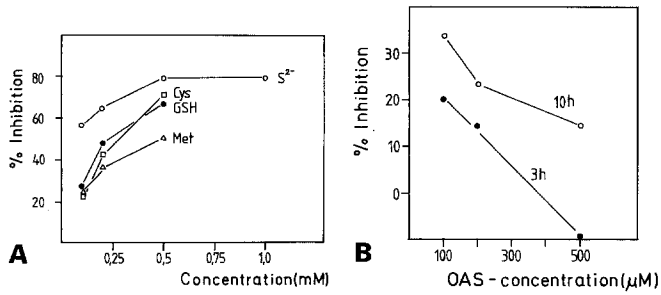

Fig. 4. **A** Inhibition of sulfate uptake in *R. sulfidophila* W4 by sulfide, glutathion *GSH*, cysteine *Cys*, and methionine *Met*. Values are given as % inhibition after 10 h incubation time compared to a culture without reduced sulfur compounds. Sulfate concentration was 0.25 mM; **B** The effect of O-acetylserine on the sulfate uptake after 3 and 10 h. Experimental conditions were the same as in the experiments shown in Fig. 4A

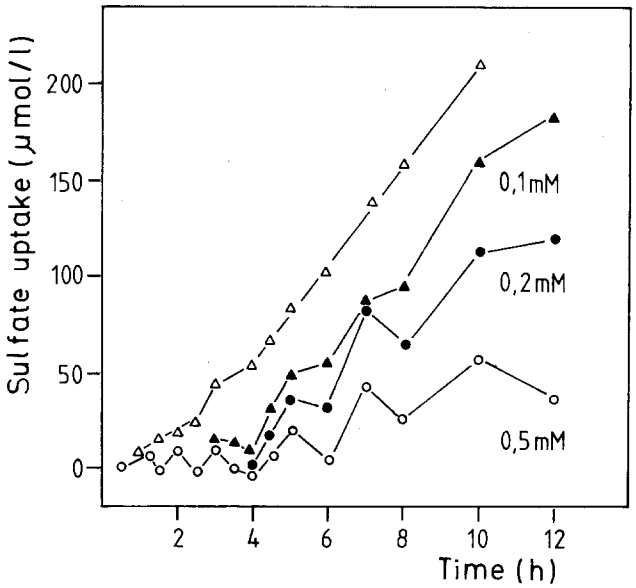

Fig. 5. Influence of different concentrations of cysteine on the sulfate uptake in sulfur starved cells of *R. sulfidophila* W4. Sulfate concentration was 0.25 mM. △: Control experiment without cysteine

and also on the sulfate concentration and were not observed in similar experiments with lower sulfate concentration (0.1 mM). In this context it is important to remember that *R. sulfidophila*, while growing on sulfide or thiosulfate as electron donors, has to excrete sulfate, the oxidation product of these compounds. It seems unlikely that the observed inhibition of sulfate uptake by reduced sulfur compounds is due to a direct action on the sulfate permease, but rather reflects the action of other cellular metabolites, the concentrations of which increased under the applied conditions. At 0.1 mM glutathione and sulfite the excretion of sulfate began 0.5–1.5 h after their addition, and with cysteine and O-acetylserine sulfate uptake slowed down after 1–2 h. With O-acetylserine the initial uptake rate was resumed thereafter. An immediate response of the uptake was observed with sulfide only. It may, therefore, be concluded, that with the exception of sulfide the action of the other sulfur compounds on the uptake of sulfate is an indirect one. The inhibition by O-acetylserine at low concentrations can be explained by its rapid conversion into cysteine, leading to increased uptake rates only if its cellular concentration remains significantly high over reduced sulfur compounds, especially its enzymatic reaction product cysteine.

Sulfate activation

All enzymatic activities for the conversion of sulfate into cysteine were found in the soluble particle free protein fraction; washed chromatophores did not show any activity. This was true also for the activities in other Rhodospirillaceae species (Hensel and Trüper 1976; Cooper 1980; Imhoff 1982) and is in contrast to the early report by Ibanez and Lindstrom (1962), who found synthesis of PAPS by a so-called "chromatophore fraction" from *Rhodospirillum rubrum*. The formation of APS and PAPS from sulfate in cell extracts of *R. sulfidophila* has been demonstrated earlier (Imhoff 1982). The ATP sulfurylase of *R. sulfidophila* has been purified, its molecular weight determined as 290,000 and its K_m-values as 0.33 mM for sulfate and 0.26 mM for MgATP (Cooper 1980). Competitive inhibitors of this enzyme were AMP (K_i = 2.1 mM), ADP (K_i = 1.15 mM), APS (K_i = 0.8 μM), sulfite (K_i = 0.4 mM) and sulfide (K_i = 0.66 mM) (Cooper 1980). The presence of APS kinase is indicated by the formation of PAPS from sulfate or APS.

Sulfolipid

Small amounts of sulfolipids have been found in *Rhodospirillum rubrum* (Benson et al. 1959) and *Rhodomicrobium vannielii* (only 0.01 % of the cell dry weight, Park and Berger 1967). In *Rhodopseudomonas sphaeroides* the content of the "plant sulfolipid" (sulfoquinovosyl diglyceride) was 2.6 % (Radunz 1969). In a recent survey on the lipid compositions of phototrophic purple bacteria the presence of a sulfolipid in *R. sulfidophila* has been suggested from similarities of R_f-values and staining behavior to the sulfolipid from spinach leaves (Imhoff et al. 1982). It was demonstrated by labeling with ^{35}S-sulfur that this lipid, which is present in *R. sulfidophila* in rather high concentration indeed contains sulfur. In cells grown with ^{35}S-sulfate (200 μM) it was highly labeled, but in the simultaneous presence of 1 mM non-labeled sulfide, thiosulfate or cysteine the label was reduced to less than 10 % compared to the control. The proportion of the lipid, however, was not significantly reduced under these conditions. The same lipid was also labeled in cells grown on a sulfate free medium containing 250 μM ^{35}S-cysteine (100 μCi/μmol). From the cysteine taken up by the cells about 22 % were found in the methanol/water-phase and about 5 % in the lipid containing chloroform phase. The remaining percentage is supposed to represent that part incorporated into the protein of the cells. There was no apparent difference in the label of the sulfolipid from thiosulfate labeled in the inner or in the outer sulfur. It is evident from these data that *R. sulfidophila* does contain a sulfolipid, which is most probably sulfoquinovosyl diglyceride and that the sulfur moiety can be derived from sulfate as well as from reduced sulfur compounds. Under conditions where sulfate uptake is inhibited by the presence of reduced sulfur compounds, these compounds supply the sulfur for sulfolipid biosynthesis. The incorporation of the sulfur moiety from reduced sulfur compounds may involve their oxidation to sulfate and PAPS as the donor of the sulfo group. An alternative reaction sequence with the involvement of cysteic acid, 3-sulfopyruvate and 3-sulfolacaldehyde as intermediates had been

proposed for the synthesis of 6-sulfoquinovose in plants (Harwood 1980). Although cleavage of cysteine into sulfide and pyruvate was demonstrated in *R. sulfidophila*, a conversion into cysteic acid and the incorporation via this proposed reaction sequence into the sulfolipid cannot be excluded. Similar mechanisms – oxidation of reduced sulfur compounds within the cell via PAPS or cysteic acid – have to be considered for the sulfolipid synthesis in *Rhodopseudomonas sulfoviridis* (Imhoff, unpublished), which is unable to take up sulfate from the medium, but able to activate sulfate to PAPS and also cleave cysteine (Neutzling and Trüper 1982).

Reduction of PAPS

The highest rates obtained for production of acid volatile sulfite were 200 pmol/h × mg protein from sulfate (20 mM) and 520 pmol/h × mg protein from PAPS (0.1 mM) (Imhoff 1982). Attempts to purify PAPS sulfotransferase from *R. sulfidophila* failed because of the small activities, the extreme instability, and the complexity of this enzyme system. In contrast to the APS sulfotransferase activities of other Rhodospirillaceae, the PAPS sulfotransferase of *R. sulfidophila* was lost after gel filtration, dialysis or isoelectric focussing and even after overnight storage on ice. Some of the properties of the activity reducing PAPS to acid volatile sulfite were therefore determined in freshly prepared soluble protein immediately after ultracentrifugation. Like all so far tested sulfotransferases the enzyme from *R. sulfidophila* needs thiol compounds for activity. The highest activity was found with dithioerythritol. Less than 10% of this activity were obtained with the same concentration (10 mM) of cysteine, mercaptoethanol, reduced glutathione and 2,3-dimercaptopropanol (BAL). The nucleotides 5′-AMP, 3′-AMP and 3′,5′-ADP (10 mM) were tested as possible competitive inhibitors of PAPS hydrolysis in the sulfotransferase assay, because of the presence of nucleotidases in the soluble protein. There was a tenfold increase in the sulfotransferase activity with 3′,5′-ADP compared to the control activity and about twofold increase with the two other nucleotides. Similar effects were observed within other PAPS reducing Rhodospirillaceae, but strains reducing APS showed slight activation only with 3′-AMP. This finding may suggest the presence of a 3′,5′-nucleotidase in *R. sulfidophila* able to convert PAPS to APS, which may be similar to the activity found in *Chlorella pyrenoidosa* (Tsang and Schiff 1976). Its function, however, cannot be explained by a model similar to that proposed for *Chlorella*, because *R. sulfidophila* does not reduce APS (Imhoff 1982).

First evidence for the presence of a low molecular weight cofactor for the sulfotransferase reaction in *R. sulfidophila* came from measurements on the correlation of activity and protein concentration. While this relationship was linear in the APS reducing *Rhodomicrobium vannielii*, it was sigmoid in *R. rubrum* and *R. sulfidophila* (Fig. 6) and the activities decreased rapidly at low protein concentrations in the latter two species. The addition of boiled and dialysed "own" cell extract increased the activity about 1.5 times in *R. sulfidophila*, whereas similar "cofactor preparations" from *E. coli* and yeast did not increase this activity, purified spinach thioredoxin did only slightly (about 8%). A cofactor requirement was found in the well investigated sulfotransferases of *E. coli* (Tsang and Schiff 1976), *Chlorella* (Schmidt and Schwenn 1971; Abrams and Schiff 1973) and also in other

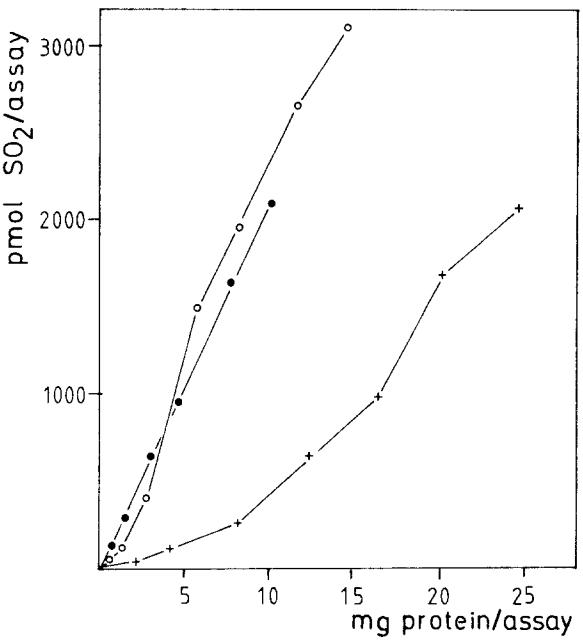

Fig. 6. Dependence of the sulfotransferase activity on the protein concentration. Cells of *Rhodomicrobium vannielii* and *Rhodospirillum rubrum* were preincubated with 1 mM O-acetylserine. Activities of *R. vannielii* are 10 times as high as indicated in the scale. Assay conditions were as given under Material and methods. ○: *R. rubrum*, +: *R. sulfidophila*, ●: *R. vannielii*

investigated organisms like the cyanobacterium *Synechococcus* (Schmidt and Christen 1978). In yeast the presence of two different low molecular weight protein cofactors has been demonstrated (Wilson et al. 1961; Wilson and Bierer 1976), one of them more tightly bound to the sulfotransferase. That the situation may be similar in *R. sulfidophila* is concluded from different observations and comparison with some APS sulfotransferases. Results obtained during molecular weight determinations and isoelectric focussing with APS sulfotransferases from different species of the Rhodospirillaceae also revealed, that these enzymes contained a cofactor, which was partly lost during isoelectric focussing. It was highly acidic and had a molecular weight from 15,000 to 30,000 depending on the species. The greater stability of the APS sulfotransferases and the failure to find activation with spinach thioredoxin may be due to stronger binding of the cofactors to these proteins as compared to the PAPS sulfotransferases or the possible replacement of thioredoxin by dithioerythritol. Some evidence for the presence of thioredoxin in *R. sulfidophila* has been obtained by activation with boiled extract in the sulfotransferase assay of *Synechococcus* 6301 (Schmidt and Christen 1978). Its involvement in the sulfotransferase reaction of *R. sulfidophila* is likely from the small activation obtained with spinach thioredoxin and the "own" dialyzed cofactor preparation. The rapid loss of activity during gel filtration and dialysis in *R. sulfidophila* may indicate that a second cofactor is necessary for the reaction, which cannot be replaced by spinach thioredoxin and which is rapidly lost during dialysis. A reaction scheme for cysteine biosynthesis in *R. sulfidophila* is proposed, which involves the direct binding of a low molecular weight component to the sulfotransferase (Fig. 8). This component may be reduced by thioredoxin. As reductant for thioredoxin in the assay system dithio-

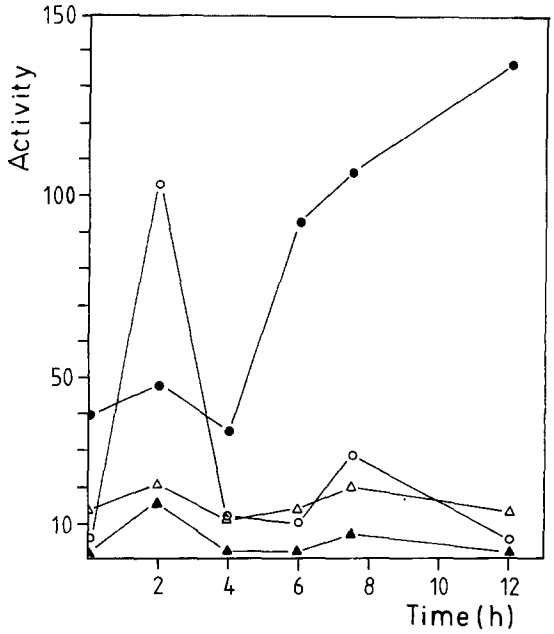

Fig. 7. Derepression of sulfate assimilation enzymes by O-acetylserine in *R. sulfidophila* W4. ● ATP sulfurylase (mU/mg protein), △ APS kinase (mU/mg protein), ○ PAPS-sulfotransferase (μU/mg protein), ▲ thiosulfonate reductase (μU/mg protein)

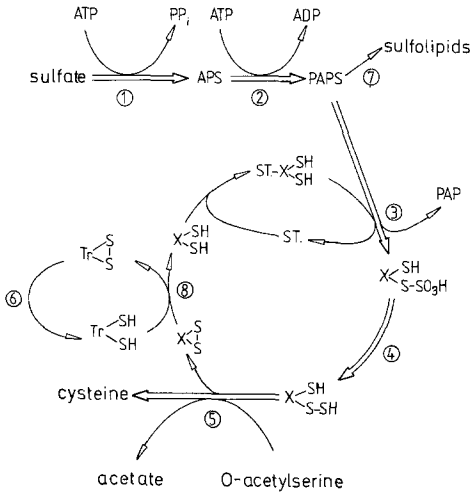

Fig. 8. Sulfate assimilation pathway in *R. sulfidophila* W4. *1* ATP sulfurylase, *2* APS kinase, *3* PAPS-sulfotransferase, *4* thiosulfonate reductase, *5* O-acetylserine sulfhydrylase, *6* thioredoxin reductase, *7* sulfotransferases, *8* cofactor reducing enzyme, *Tr* thioredoxin, *X* low molecular weight cofactor, *ST* inactive form of the PAPS-sulfotransferase, which reacts with the reduced low molecular weight cofactor to form the active enzyme

erythritol is used, which also may be able to directly reduce the second cofactor and thereby replacing thioredoxin. This proposed reaction sequence implies the participation of bound intermediates in the reactions of sulfate reduction.

Like sulfate uptake and activation, the enzymatic reduction activities also depend on growth conditions and are repressed by reduced sulfur compounds. Highest activity of the sulfotransferase was found with sulfate as sulfur source under photoheterotrophic growth conditions. Cells grown under the same conditions, but with addition of 0.05 % yeast extract had about 70 % and photoautotropically grown cells (sulfide as electron donor) only 13 % of this activity. Cells grown in the presence of 1 mM O-acetylserine had about twice the activity of cells grown with sulfate. Strongest repression of both the sulfotransferase and the thiosulfonate reductase occurred with sulfide and sulfite. In contrast to these enzymes the APS kinase had high activity, if cells were grown with cysteine or glutathione as sulfur source.

The effect of O-acetylserine, known to induce the enzymatic activities for sulfate activation and reduction in *E. coli* and *Salmonella typhimurium* (Pasternak 1962; Jones-Mortimer et al. 1968; Kredich 1971), on these enzymes in *R. sulfidophila* during a growth experiment is shown in Fig. 7. Cells were precultured under sulfate limitation, incubated for 2 h with 2 mM cysteine, washed with sulfate free medium and then incubated in a sulfate free medium containing 1 mM O-acetylserine at 5,000 – 10,000 lux and 28° C in a glass fermenter. The initial cell density was 1.5 – 2.0 g wet weight/l. There was an immediate strong increase in activities of PAPS sulfotransferase and thiosulfonate reductase with a maximum after 2 h and a second, smaller maximum after 7.5 h. APS kinase showed much smaller increases and only ATP sulfurylase activity after a small first maximum increased continuously during the time of the experiment. The activities found after 2 h were 49 mU ATP sulfurylase, 20 mU APS kinase, 0.1 mU sulfotransferase and 0.016 mU thiosulfonate reductase. The time dependent course of the activities forming acid volatile sulfite from sulfate was similar to that of the sulfotransferase activity, which makes it likely that this is the rate limiting step during conversion of sulfate to enzyme bound sulfite.

A similar effect than with O-acetylserine, but much smaller, was found by the addition of djenkolic acid to the culture in an otherwise identical experiment.

Cysteine synthesis

O-acetylserine sulfhydrylase has been found in *R. sulfidophila* in the soluble protein fraction in photoheterotrophically grown cells (22,100 mU/mg protein). In cells grown with 4 mM thiosulfate or 4.2 mM sulfide this activity was only 17 % and 9 % of the activity in cells grown with sulfate (Hensel and Trüper 1976).

The striking differences of several orders of magnitude in the activities involved in the synthesis of cysteine from sulfate are difficult to explain. One would expect specific activities of about 0.5 mU considering the overall incorporation into cysteine, a protein content of 60 %, a sulfur content of 0.6 % of the cellular dry weight and a growth rate of 0.11 h^{-1}. Considering the conditions used for the enzymatic assays, the ATP sulfurylase assay using molybdate is expected to give somewhat higher activities than the enzyme would have in the physiological reaction. APS kinase activities should, however, be more reliable. Higher activities of these two enzymes would also physiologically be necessary to pull the energetically and kinetically unfavorable activation of sulfate. Sulfotransferase activity, to a small extent, and thiosulfonate reductase may be seriously inhibited by their own reaction products. This would explain the low activities measured in the assay. High activities of O-acetylserine sulfhydrylase would be necessary to immediately remove the reaction product of thiosulfonate reductase and thereby enhance this activity to a reasonable level under physiological conditions.

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