Comparative Enzymology of Sulfite Oxidation in *Thiocapsa roseopersicina* Strains 6311, M1 and BBS under Chemotrophic and Phototrophic Conditions

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The purple sulfur bacterium *T. roseopersicina* is able to grow chemoautotrophically in the dark under micro- to semiaerobic conditions. Under the latter cell yield and growth rate are considerably reduced. During chemo- and phototrophic growth reduced sulfur compounds are metabolized by the same pathway, *i.e.* oxidized to sulfate *via* adenylylsulfate. APS reductase (EC 1.8.99.2), ADP sulfurylase (EC 2.7.7.5) and ATP sulfurylase (EC 2.7.7.4) could be shown in all the strains tested, whereas only strain BBS contained an AMP independent sulfite-oxidizing activity under photo- as well as under chemotrophic conditions. Not only ADP but also ATP sulfurylase perform a dissimilatory function proven by their high specific activities. In contrast to the enzyme of *T. roseopersicina* strain 6311 APS reductases from strains M1 and BBS are strictly membrane-bound. The enzyme from strain M1 was solubilized, enriched and characterized. While the K_M values of purified APS reductase remain unaffected, specific activities of APS reductase, ATP and ADP sulfurylase are increased substantially under chemotrophic growth conditions.

Introduction

Thiocapsa roseopersicina usually is found as one of the main representatives of the anoxygenic phototrophic bacteria at the uppermost region of the sulfide zone of stratified fresh water and laminated microbial sediment ecosystems, where light is still available. Conditions in such environments are characterized by steep and opposing gradients of oxygen – produced by cyanobacteria and algae – and sulfide – produced by sulfate-reducing bacteria in the bottom layer. These gradients are known to shift strongly during the diurnal cycle [1]. In accordance with the requirements of its natural environment *T. roseopersicina* is a well-established facultatively aerobic member of the family of the Chromatiaceae with excellent chemotrophic growth capacities and is capable of growth on sulfide and thiosulfate both aerobically and anaerobically [2–4]. Noteworthy are results obtained by de Wit and van Gemerden [4] which indicate that in the natural environment cells of *T. roseopersicina* do not grow chemolithotrophically regardless of the presence of oxygen, unless the cellular photopigment concentration becomes insufficient to guarantee photosynthesis [5].

The consumption of thiosulfate and sulfide by *T. roseopersicina* under aerobic conditions in the dark is coupled to the reduction of oxygen [6], chemolithotrophic growth of purple sulfur bacteria is therefore comparable to that of the thiobacilli.

Based on experiments carried out with *T. roseopersicina* strain BBS [6–9] we intended to investigate the influence of a switch to chemotrophic growth conditions on specific activities and catalytic properties of central enzymes of dissimilatory sulfur metabolism.

Abbreviations: ADP, adenosin 5′-diphosphate; APS, adenosin 5′-phosphosulfate; ATP, adenosin 5′-triphosphate; DSM, German Collection of microorganisms, Braunschweig; Tris, tris-(hydroxymethyl)aminomethane; U, international units, μmol/min.

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Materials and Methods

Organisms and culture

Thiocapsa roseopersicina strains 6311 (DSM 219), M1 and BBS were used. Strain M1 was obtained from Hans van Gemerden, Rijksuniversiteit Groningen, the Netherlands, strain BBS was kindly provided by Elena Kondratieva, Department of Microbiology, Moscow State University, Moscow, U.S.S.R.

Phototrophic growth of all strains was carried out at 30 °C and at light intensities of 2000 lux in completely filled 1 l screw cap bottles or 10 l carbons. The medium used was that described by Pfennig and Trüper [10] containing 0.075% sulfide. When the cells were free of intracellular sulfur globules the cultures were fed with a neutral sulfide solution [11].

Chemotrophic batch cultures were grown in 2 l or 20 l glass vessels of fermenters ("Biostat L": Braun, Melsungen, F.R.G.; "Kiel": Eschweiler, Kiel, F.R.G.). The medium contained in 1 l: *A:* 0.5 g NH_4Cl, 0.33 g KCl, 0.05 g $CaCl_2 \times 2\ H_2O$, 0.33 g $MgCl_2 \times 6\ H_2O$, 1 ml of the tenfold concentrated trace element solution of Pfennig and Lippert [12]; *B:* 0.5 g KH_2PO_4, 0.62 g K_2HPO_4; *C:* 1.5 g $NaHCO_3$, *D:* 1 g $Na_2S_2O_3$. *A* was dissolved in 750 ml, *B* in 150 ml, *C* and *D* in 50 ml distilled water respectively; all four solutions were autoclaved at 121 °C for 20 min and combined after cooling. Vitamin B_{12} (20 µg/l) was added from a sterile filtered stock solution. Temperature was 30 °C and the pH was kept at 7.2 with the aid of a pH-stat. The oxygen concentration was kept constant at 25% of air saturation, which was guaranteed by a pO_2-stat. No special care was taken to keep the chemolithotrophically growing cultures completely in the dark.

Media for *T. roseopersicina* strains M1 and BBS were supplemented with 2.5% and 2.0% sodium chloride respectively.

Turnover of reduced sulfur compounds

Experiments concerned with the turnover of reduced sulfur compounds were performed in the media described above in a 1 l glas vessel, which was placed in a water bath at 30 °C and magnetically stirred. The vessel was closed by a rubber plug that possessed cotton-stoppered gas in- and outlets and that allowed aliquots of the reaction mixture to be withdrawn *via* a glass-tube reaching to the bottom of the flask. Gas atmosphere was N_2 or air depending on growth conditions. Experiments in the light were carried out at light intensities of 2000 lux.

Preparation of cell extracts

Cells were harvested and crude extracts were prepared as described earlier [13].

Enzyme assays

ATP sulfurylase was measured spectrophotometrically according to Cooper and Trüper [14], ADP sulfurylase was determined as described earlier [13].

APS reductase from *T. roseopersicina* 6311 was measured according to Trüper and Rogers [15]. APS reductase activities from strains M1 and BBS had to be determined as follows: In a total volume of 1 ml the reaction mixture contained: 50 mM Tris-HCl buffer, pH 7.0, 4 mM $K_3Fe(CN)_6$, 7.5 mM EDTA, 10 mM Na_2SO_3, 0.4 mM (strain BBS: 0.6 mM) AMP, pH 7.0 and distilled water. The assay was preincubated at 30 °C for 5 min and the reaction was started by addition of the extract. The reaction was terminated by boiling for 2 min. Denatured protein was removed by centrifugation at $25000 \times g$ for 10 min. An aliquot of supernatant was used for quantitative determination of generated APS. APS was determined in a coupled enzyme assay by reduction of $NADP^+$. The assay contained in 1 ml 100 mM Tris-HCl buffer, pH 8.0, 20 mM β-D-glucose, 10 mM $MgCl_2 \times 6\ H_2O$, 0.5 mM Na-NADP, 1 mM $Na_4P_2O_7$, 5 U glucose-6-phosphatedehydrogenase, 5 U hexokinase, 3 U ATP sulfurylase and up to 120 nmol APS in the sample. The reaction was started with ATP sulfurylase and the $NADP^+$ reduction was measured at 340 nm.

Sulfite oxidoreductase from *T. roseopersicina* BBS was assayed according to Petushkova and Ivanovskii [7].

All extracts were passed through a column of Sephadex G-25 to exclude low molecular weight constituents especially AMP, which would otherwise lead to a simulation of sulfite oxidoreductase activity by APS reductase.

Enzyme purification

APS reductase from *T. roseopersicina* M1 was enriched as follows: All purification steps were performed in 50 mM Tris-HCl, pH 7.5 at 4 °C. To achieve solubilization of the strictly membrane-bound enzyme the supernatant of a first ultracentrifugation was incubated with 1% Triton X-100 for 15 min at room temperature. After a second ultracentrifugation ($140000 \times g$, 2 h) the detergent was removed from the supernatant obtained by chromatography on BioBeads SM2 (column size 2×20 cm, flow rate 18 ml/h). The resuspended pellet of a subsequent 30% ammonium sulfate precipita-

tion was loaded on a Phenyl Sepharose CL-4B column (gel volume 47 ml). A linear Triton X-100 gradient (0–0.8% Triton X-100) was applied and APS reductase was eluted at a calculated detergent concentration of 0.5–0.7%. Active APS reductase fractions were pooled, freed from detergent and concentrated by ultrafiltration using an Amicon PM 30 filter. The concentrated protein solution was further purified by gel filtration on Sephacryl S300 (column size 1.5 × 90 cm).

Analytical methods

Sulfur compounds were determined according to the following methods described in the literature: Sulfide [16], thiosulfate [17], elemental sulfur [18] and sulfate [19]. The protein content of the cultures was determined using a modification of the LOWRY [20] method: Pigments were extracted with 5 ml icecold acetone/methanol (7/2). Acetone/methanol was removed by centrifugation and the pigment-free pellet was dried at 100 °C and dissolved in 0.5 ml distilled water. Bovine serum albumin was used as a standard.

The measurements of protein content in crude and purified extracts were performed as described earlier [13].

Preparative methods

APS was synthesized after Cooper and Trüper [21] modified by Imhoff [22].

Results and Discussion

In accordance with Pfennig [2], Bogorov [3] and de Wit and van Gemerden [4] all three *Thiocapsa* strains tested are capable of chemolithoautotrophic growth.

An average growth yield of 13 g dry cell mass per mol thiosulfate oxidized, which is less than a third of the growth yield obtained under photoautotrophic conditions, was found for *T. roseopersicina* growing under semiaerobic conditions at 25% of air saturation. The thiosulfate required to provide the necessary energy for chemotrophic growth was found to be about 75% of the thiosulfate consumed. The remaining 25% of thiosulfate therefore serve as electron donor for the reduction of CO_2 into cell material. These values are comparable to those determined for chemolithoautotrophically grown thiobacilli [23] and facultatively aerobic Chromatiaceae [24, 25].

Not only growth yields but also growth rates are reduced under semiaerobic conditions. Growth rates of chemotrophically grown cells were in the range of 0.015 h^{-1} for *T. roseopersicina* strain M1 to 0.027 h^{-1} for strain BBS and therefore about 60% lower than those of phototrophically grown cells. This meets results obtained with *T. roseopersicina* strains BBS [6] and M1 [4] and *Thiocystis violacea* [24].

Under chemo- and phototrophic growth conditions thiosulfate oxidation appeared to be biphasic: the sulfane-sulfur is not oxidized to sulfate before it is totally converted to sulfur globules inside of the cells (Fig. 1 and 2).

In accordance with results obtained with *T. roseopersicina* strain M1 by de Wit and van Gemerden [4], who determined a K_s of only 1.5 μM for thiosulfate affinity under semiaerobic conditions, specific rates of thiosulfate oxidation were found to be rather high (290 nmol/min mg protein).

Table I. Partial purification of the APS reductase from photolithoautotrophically with sulfide grown cells of *Thiocapsa roseopersicina* strain M1.

Step	Volume [ml]	Total activity [U]	Total protein [mg]	Specific activity [mU/mg^{-1}]	Purification [fold]	Yield [%]
Crude extract	160.0	64.0	2288.0	28.0	1.0	100.0
Pellet of 1st ultracentrifugation	94.0	57.7	1241.0	46.5	1.7	90.0
Supernatant of 2nd ultracentrifugation	139.2	36.5	730.4	50.0	1.8	56.9
Ammonium sulfate	8.6	15.7	120.4	130.0	4.6	24.4
Phenyl-Sepharose	2.0	5.5	12.0	455.0	16.3	8.6
Sephacryl S300	6.0	3.6	5.6	637.0	22.8	5.5

Experimental details: *cf.* Materials and Methods.

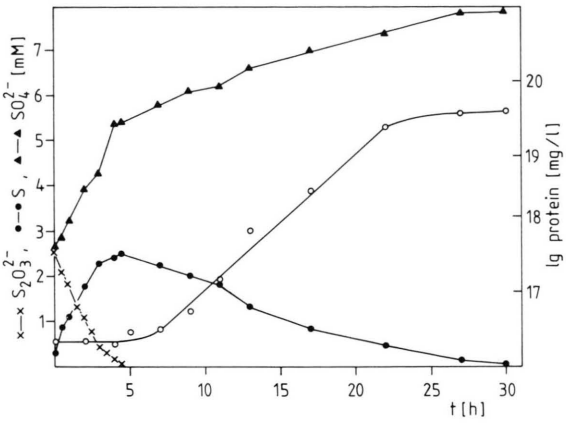

Fig. 1. Thiosulfate oxidation by photolithoautotrophically growing cells of *Thiocapsa roseopersicina* strain M1. Experimental details are described in Materials and Methods.

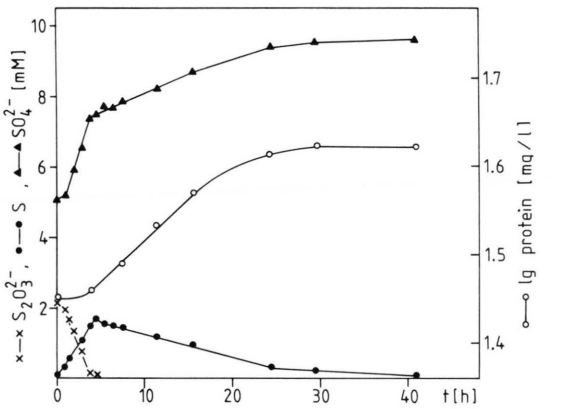

Fig. 2. Thiosulfate oxidation by chemolithoautotrophically growing cells of *Thiocapsa roseopersicina* strain M1. Experimental details are described in Materials and Methods.

During chemo- and phototrophic growth reduced sulfur compounds are metabolized by the same pathway, *i.e.* oxidized to sulfate *via* adenylylsulfate [7].

Both soluble and particulate cell fractions of the strains were monitored for APS reductase, sulfite oxidoreductase (EC 1.8.2.1), ADP sulfurylase and ATP sulfurylase. APS reductase, ADP and ATP sulfurylases could be shown in all three strains, their specific activities were highest during the exponential phase of growth (Fig. 3).

Not only ADP but also ATP sulfurylase perform a dissimilatory function proven by their high specific activities (Table II). ADP sulfurylase is a soluble en-

Table II. Comparison of enzyme activities in crude extracts of *T. roseopersicina* strains 6311, M1, BBS and SL under different conditions of growth. Cells were harvested in the exponential phase of growth.

Enzyme	Strain	Specific activity in photoautotrophic cells [mU/mg protein]	Specific activity in chemoautotrophic cells [mU/mg protein]
Thiosulfate reductase	BBS	36.0[a]	77.0[a]
Rhodanese	BBS	93.0[a]	185.0[a]
APS reductase	BBS	37.0[b]*	128.0[b]*
APS reductase	BBS	26.5	50.4
APS reductase	SL	23.8[b]*	41.5[b]*
APS reductase	M1	38.0	112.0
APS reductase	6311	148.0	348.0
Sulfite OR**	BBS	18.8[b]*	31.5[b]*
Sulfite OR**	BBS	5.3	12.0
Sulfite OR**	SL	10.1[b]*	13.4[b]*
Sulfite OR**	M1	0.0	0.0
Sulfite OR**	6311	0.0	0.0
ADP sulfurylase	BBS	252.7	506.7
ADP sulfurylase	M1	254.0	865.0
ADP sulfurylase	6311	350.0	600.0
ATP sulfurylase	BBS	281.0	536.1
ATP sulfurylase	M1	328.0	657.0
ATP sulfurylase	6311	310.0	720.0

Enzyme activities were determined as described in Materials and Methods.
[a] [7]; [b] [8]; * sulfite-oxidation by membranes of *T. roseopersicina* [nmol Cyt *c*/min mg protein]; ** OR, oxidoreductase.

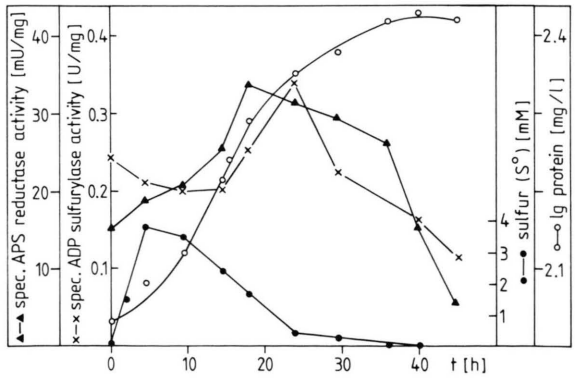

Fig. 3. Relation between specific activities of APS reductase and ADP sulfurylase and the growth phase in *T. roseopersicina* strain 6311. Aliquots from a culture growing phototrophically on sulfide were harvested after different intervalls of time and enzyme activities were determined in crude extracts.

zyme with a molecular weight of 250000, its K_M values are 0.33 mM for APS and 13 mM for phosphate [13].

Neither under photo- nor under chemotrophic conditions could an AMP independent sulfite-oxidizing activity be proven in strains M1 and 6311, whereas in accordance with Petushkova and Ivanovskii [7] strain BBS possesses a sulfite oxidoreductase. The specific activities of sulfite oxidoreductase in strain BBS with a maximum of only 12 mU/mg protein in chemotrophically grown cells are rather low with respect to the fact that the enzyme has a dissimilatory function. In strain BBS sulfite oxidoreductase activity was only measurable with cytochrome *c* – either from horse heart or *Candida crusei* – as electron acceptor. Even after various modifications of the test system no activity could be detected in the ferricyanide-coupled assay after Trüper and Rogers [15] without AMP. Extracts from *T. roseopersicina* strains 6311 and M1 catalyzed sulfite-dependent ferricyanide reduction with specific activities of about 20 mU/mg protein, which appeared to be membrane-associated. This "activity" was proven to be non-enzymatic because it was not destroyable by boiling for 10 min. As it remained in the extract after gel filtration on Sephadex G-25 it was not due to low molecular weight compounds. The sulfite oxidoreductase in strain BBS was, however, destroyed by boiling. Thus the possession of sulfite oxidoreductase besides APS reductase is not a common feature of the facultatively chemotrophic Chromatiaceae as it might have been proposed based on the fact that several representatives of this group contain both sulfite-oxidizing enzymes [26].

In contrast to the enzyme of *T. roseopersicina* strain 6311 APS reductases of strains M1 and BBS are strictly membrane-bound. APS reductases from strains M1 and 6311 function with ferricyanide and cytochrome *c* from *C. crusei* but not with cytochrome *c* from horse heart whereas APS reductase from *T. roseopersicina* BBS is active with both mammalian and fungal cytochrome *c*. Thus the *Thiocapsa* strains investigated do not only differ in the possession of sulfite oxidoreductase but also in possible electron acceptors and the degree of membrane-binding of APS reductases.

APS reductase from strain M1 was solubilized, enriched and characterized. A summary of the purification procedure is given in Table I. PH and temperature optima were determined to be 7.0 and 40 °C. K_M values for AMP and sulfite turned out to be 0.1 mM and 0.34 mM respectively.

K_M values of enriched APS reductase appeared to be independent of growth conditions of the cells from which the enzyme was purified. While under phototrophic growth conditions specific enzyme activities remain unaffected by the reduced sulfur compound offered, namely sulfide or thiosulfate, the activity of the enzymes participating in sulfite oxidation increases substantially when cells of *T. roseopersicina* grow under aerobic conditions on mineral medium with thiosulfate (Table II). These results are in coincidence with data obtained with *T. roseopersicina* BBS [7, 8]. The increase in the activity of these enzymes in chemotrophically grown cells is evidently due to the fact that under such conditions in absence of light as energy donor the sole source of energy is the oxidation of thiosulfate.

Little is known about regulatory mechanisms controlling the effects on activities of the sulfite oxidizing enzymes that accompany the switch from anaerobic photosynthesis to chemotrophic growth with oxygen. Imaginable are mechanisms similar to those that are discussed for the repression of bacteriochlorophyll synthesis by oxygen in Rhodospirillaceae and Chromatiaceae as there are: intracellular ATP level [27, 28] or the level of effectors of central enzymes of bacteriochlorophyll synthesis, as it was reported for trisulfides of cystine or glutathione that activate 5-aminolaevulinate synthetase, the key enzyme for tetrapyrrole biosynthesis, in *Rhodobacter sphaeroides* [29]. Another mechanism that has been taken into consideration is the redox state of a cellular component which interacts with oxygen [30] and works as an effector on the level(s) of transcription, post-transcription, translation or the regulation of enzyme activities.

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