Arch Microbiol (1982) 132:197–203

Archives of
Microbiology

Occurrence and Evolutionary Significance of Two Sulfate Assimilation Pathways in the Rhodospirillaceae

Johannes F. Imhoff

Institut für Mikrobiologie, Meckenheimer Allee 168, D-5300 Bonn, Federal Republic of Germany

Abstract. The ability to use adenosine 5′-phosphosulfate (APS) or 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as the substrate for the initial reductive step in sulfate assimilation has been tested in most of the known Rhodospirillaceae species and in some chemotrophic bacteria. Improved and optimized methods for the synthesis and purification of the sulfonucleotides APS and PAPS are described. The production of acid volatile radioactivity from ^{35}S-APS and ^{35}S-PAPS was measured under various conditions in the presence and absence of non-labeled sulfate. Specific differences in the ability to reduce APS or PAPS were observed among the Rhodospirillaceae species and also the chemotrophic bacteria. APS was found to be the substrate of the thiolsulfotransferase in *Rps. acidophila*, *Rps. globiformis*, *Rm. vannielii*, *Rc. purpureus*, *R. tenue*, *Rps. gelatinosa*, in *Alcaligenes eutrophus* and *Pseudomonas aeruginosa*. PAPS was the substrate in *Rps. capsulata*, *Rps. sphaeroides*, *Rps. sulfidophila*, *Rps. palustris*, *Rps. viridis*, *R. rubrum*, *R. fulvum*, in *Paracoccus denitrificans* and in several Enterobacteriaceae. The presence of different enzymatic systems for sulfate reduction in the Rhodospirillaceae family is compared with their taxonomical grouping and their possible phylogenetic relatedness.

Key words: *Rhodopseudomonas* – *Rhodospirillum* – *Rhodomicrobium* – *Rhodocyclus* – Sulfate assimilation – Sulfonucleotide specifity – Biochemical preparation of sulfonucleotides

Sulfate can be used as sole sulfur source by numerous bacteria. To be incorporated into amino acids and some other cellular components, sulfate has to be activated and – except for the sulfocompounds – has to be reduced to the thiol level. Activation by ATP-sulfurylase yields adenosine 5′-phosphosulfate (APS), which may be further phosphorylated to 3′-phosphoadenosine 5′-phosphosulfate (PAPS). Either APS or PAPS are reduced. The reduction via APS has been established in green plants and some algae (Tsang and Schiff 1975; Schmidt 1975), whereas the reduction via PAPS has been found in yeasts (Wilson et al. 1961), *Escherichia coli* (Tsang and Schiff 1975) and several other Enterobacteriaceae (Peck 1961; Dreyfuss and Monty 1963). In view of the earlier results Tsang and Schiff (1975) speculated that sulfate may be reduced via APS in all photosynthetic organisms and via PAPS in chemotrophic organisms. Another generalizing hypothesis could have been that green plants and algae reduce APS, and fungi and bacteria PAPS. In the first case phototrophic bacteria should use APS, in the second case they should use PAPS. To prove both of these hypotheses a survey on the ability to reduce APS or PAPS within the Rhodospirillaceae family was undertaken in the present study. As all of the Chlorobiaceae and many of the Chromatiaceae species are not able to grow on sulfate as the sole sulfur source, but nearly all of the Rhodospirillaceae species are, this was the family of choice for the present investigation.

Non-standard Abbreviations. APS, adenosine 5′-phosphosulfate; PAPS, 3′-phosphate adenosine 5′-phosphosulfate; DTE, dithioerythrol, Rc., *Rhodocyclus;* R., *Rhodospirillum;* Rm., *Rhodomicrobium;* Rps., *Rhodopseudomonas*

Material and Methods

Organisms and Growth Media. Bacterial strains and their sources are listed in Table 1. All growth media contained in 1 l: 1 ml vitamin solution "VA", 1 ml sulfate free trace element solution "SLA" (Imhoff and Trüper 1977), 0.71 g Na_2SO_4 and unless otherwise indicated 0.2 g $MgCl_2 \cdot 6\,H_2O$ and 0.1 g $CaCl_2 \cdot 2\,H_2O$. For Rhodospirillaceae 1 g KH_2PO_4, 0.5 g NH_4Cl, 3 g $NaHCO_3$, 0.5 g Na-ascorbate, 1.8 g Na-malate and for marine strains 30 g NaCl were added; pH was adjusted to 6.9, for *Rps. acidophila* to 5.1. *Rps. globiformis* was cultivated in the medium after Pfennig (1974) with sulfate or thiosulfate as sulfur source. *Paracoccus denitrificans* was grown after Burnell et al. (1975) and *Alcaligenes eutrophus* after Gottschalk (1964) with lactate as carbon source and the above mentioned modifications. Other bacteria were grown on a mineral salts-glucose medium.

Growth and Preparation of Cell Extracts. Phototrophic bacteria were grown at 28° C and 1,000–2,000 lux in screw cap bottles, harvested in late logarithmic growth phase, suspended in 50 mM Tris-HCl pH 7.6 with 1 mM DTE, and ruptured by passing through a French pressure cell at about 140 MPa. Cell fragments were removed by centrifugation (Sorvall RC 2B, SS34 rotor, 10 min at 10,000 rpm), and ultracentrifugation of the crude extract was used to sediment photosynthetic membranes (Beckman L5-50, 60 Ti rotor, 90 min at 45,000 rpm). The supernatant (soluble protein) and the washed pellet (membrane fraction) were separately tested always immediately after centrifugation. Protein was determined after Beisenherz et al. (1953) after extracting the precipitated proteins three times with ice cold acetone.

0302-8933/82/0132/0197/$01.40

Sulfotransferase Assay. The formation of acid volatile ^{35}S-sulfite from ^{35}S-sulfate, ^{35}S-APS and ^{35}S-PAPS was measured. The standard assay modified from the method of Schmidt (1972) contained 100 mM Tris-HCl pH 7.6, 10 mM DTE, 10 mM $MgCl_2$, 100 mM Na_2SO_4, 20 µl purified spinach thioredoxin, 1 – 10 mg protein and one of the radioactive substrates ^{35}S-APS, ^{35}S-PAPS (0.1 mM each) or ^{35}S-sulfate (20 mM) in a total volume of 1 ml. As indicated 10 mM ATP, 1 mM ^{32}S-APS or ^{32}S-PAPS were added. Assays were incubated under nitrogen for 1 h at 37° C and stopped by transfere into ice. After addition of 0.1 mmol Na_2SO_3 the assays were quantitatively transferred into convay dishes and acidified with 1 ml 1 N HCl. Volatile sulfite was trapped into 1 ml 1 M triethanolamine. After complete destillation aliquotes of the triethanolamine were counted in a scintillation counter (Beckman, typ LS-230).

Purification of Spinach Thioredoxin (modified after Buchanan et al. 1971). Spinach leafs were homogenized, soluble proteins separated from the tissue and acidified with 1 N HCl to pH 2.0. The supernatant was neutralized with 1 M Tris-HCl pH 8.0 and proteins precipitated with ammonium sulfate. The fraction between 50 – 90% saturation was resuspended in 20 mM Tris-HCl and ice cold acetone was added up to 75% (v/v). After 1 h at −20° C the precipitate was redisolved and dialysed against 20 mM Tris-HCl pH 8.0. The protein was passed through a Sephadex G-50 column and the thioredoxin containing fractions were identified by activation in the PAPS-sulfotransferase assay. The active fractions were pooled and used as "spinach thioredoxin". This preparation was free of interfering activities, but still contained about 20 different proteins as revealed by preparative isoelectric focussing in the pH-range from 2.0 – 4.0 (Servalyt on LKB multiphor 2117).

Identification of Nucleotides. Nucleotides were identified by their characteristic UV-absorption, by their radioactive label and by their R_f-values in thin layer chromatography and high voltage electrophoresis. Thin layer plates (HPTLC-plates, 10 × 10 cm, coated with silica gel 60 F_{254}, Merck, Darmstadt, FRG) were developed in isopropanol/NH_3/H_2O = 6/3/1. In high voltage paper electrophoresis (CAMAG-HVE system 63051, 15 min at 4,000 V) with 25 mM citrate buffer pH 5.8 the fastet moving nucleotide was PAPS, followed by ATP, PAP, APS, ADP, and AMP in this order. Sulfate moved about twice as fast as PAPS.

Synthesis of Sulfonucleotides. Chemical methods (Baddiley et al. 1957; Cherniak and Davidson 1964) and biochemical methods (Hodson and Schiff 1969; Tsang et al. 1976; Cooper and Trüper 1979) for the synthesis of sulfonucleotides have been described. Most of these methods have too low yields or are too time consuming. In the following optimized synthetic procedures for APS and PAPS and optimized purification procedures are described.

^{32}S-APS synthesis was carried out – as by Adams et al. (1971) and by Cooper and Trüper (1979) – with cell extracts of *Thiobacillus denitrificans* RT grown in the medium described by Schedel (1978). Cells were suspended in 50 mM Tris-HCl pH 7.6, passed through a French pressure cell, centrifugated, and used for the synthesis after dialysis with Sephadex G-25. The synthesis was carried out with 50 mM Tris-HCl pH 7.6, 20 mM AMP, 5 mM Na_2SO_3 (dissolved in 1 mM EDTA), 5 mM $K_3(Fe(CN)_6)$ and approximately 5 U APS-reductase/ml at 30° C. Concentrations of ferricyanide and sulfite were measured during the synthesis. After consumption of one or the other of these limiting substrates another 0.5 mmol were added. In this way a total of 3.0 mmol Na_2SO_3 and 4.0 mmol $K_3(Fe(CN)_6)$ were normally added in a synthesis of 100 ml. The APS yield was usually more than 90% of the AMP in the reaction mixture. Proteins and ferrocyanide were removed on Sephadex G-25 (column 4.8 × 80 cm, equilibrated with 20 mM Tris-HCl pH 7.6). The fractions containing APS were pooled and separated on DEAE-Sephadex A-25 with a linear gradient of Tris-HCl (see Fig. 1). Concentrations of APS in the fractions of the elution maximum were approximately 3 mM. If necessary the APS containing fractions were combined and rechromatographed on Biorex 5 (column 2 × 35 cm) with a linear 1 l-gradient of Tris-HCl pH 7.6 (0.5 – 1.5 M). From this column APS could be eluted with maximal concentrations of more than 10 mM.

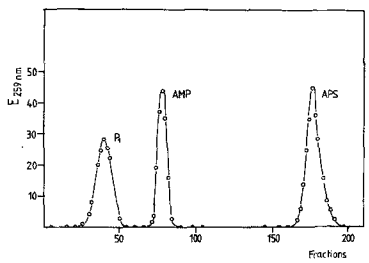

Fig. 1. Separation of the nucleotide fractions containing ^{32}S-APS, AMP and P_i (adenosine 5′-monophosphoramidate) of a Sephadex G-25 column on DEAE-Sephadex A-25; 2 l gradient, 250 – 550 mM Tris-HCl, pH 7.6; fractions a 10 ml; column 50 × 3 cm

Synthesis of ^{35}S-APS was carried out in an enzymatic reaction with yeast ATP-sulfurylase (Sigma, München, FRG) and inorganic pyrophosphatase (Boehringer, Mannheim, FRG). Although the yields were much lower than in the reaction with *Thiobacillus* extracts, this reaction, had much advantage in obtaining high specific activities of the sulfur label. The highly labeled APS was usually diluted with non-labeled APS to the desired specific radioactivity. The synthesis was carried out with 100 mM ATP, 50 mM $MgCl_2$, 300 mM Tris-HCl pH 9.0, 5 mM Na_2SO_4, 0.5 mCi ^{35}S-sulfate/ml, 2.5 U ATP-sulfurylase/ml, 20 U inorganic pyrophosphatase/ml in a total volume of 10 ml. Incubation was 8 – 10 h at 30° C. The yield was 4 – 10% of the applied sulfate. The purification procedure was – despite a few modifications – identical to that used for unlabeled APS. The reaction mixture was diluted twofold with destilled water, applied on a DEAE-Sephadex A-25 column and eluted with a linear gradient of Tris-HCl (see Fig. 2).

For the synthesis of ^{32}S-PAPS and ^{35}S-PAPS ATP-sulfurylase and APS-kinase enriched extracts of baker's yeast, separated from interfering nucleotidases by a modified method of Robbins (1963), were used. Cells were suspended in 20 mM Tris-HCl pH 8.0 (containing 0.5 mM EDTA and 1 mM mercaptoethanol), passed several times through a French pressure cell and kept at 4° C. Cell fragments and mitochondria were removed by centrifugation and the soluble proteins were desalted with Sephadex G-25. The proteins were titrated with 1 N acetic acid to pH 5.7 and precipitated with ammonium sulfate (46% saturation at pH 7.7). The precipitate was redisolved, desalted with Sephadex G-25 and used for the synthesis. The reaction mixture contained: 300 mM Tris-HCl pH 8.1, 10 mM $MgCl_2$, 10 mM Na_2SO_4,

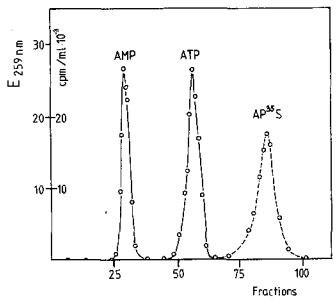

Fig. 2. Separation of ^{35}S-APS, AMP and ATP on DEAE-Sephadex A-25. Amounts of AMP and ATP applied on the column were 0.1 mmol. 1 l gradient, 250 – 550 mM Tris-HCl, pH 7.6; fractions a 10 ml; column 40 × 2 cm

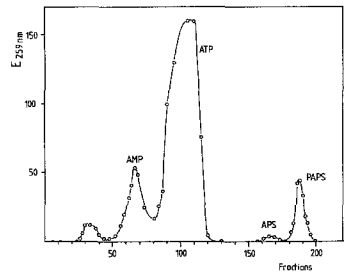

Fig. 3. Separation of a total ^{32}S-PAPS synthesis on DEAE-Sephadex A-25. 2 l gradient, 200 – 800 mM Tris-HCl, pH 7.6; fractions a 10 ml; column 50 × 3 cm

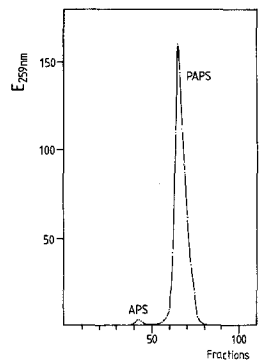

Fig. 4. Separation of the combined PAPS fractions of three DEAE-Sephadex A-25 columns on Biorex 5; 1 l gradient, 0.5 – 2.0 M Tris-HCl, pH 7.6; fractions a 10 ml; column 34 × 2 cm

50 mM ATP, 2 U inorganic pyrophosphatase/ml and approximately 10 mg protein/ml. The total volume was 100 ml and the final pH was 7.5. The mixture was incubated at 30° C for 8 – 10 h and yielded between 20 – 30 % PAPS calculated from the initial amount of sulfate. The reaction was stopped by heating to 90° C for 5 min, proteins were removed, the supernatant was twofold diluted, applied on DEAE-Sephadex A-25, and eluted with a 2 l-gradient of 200 – 800 mM Tris-HCl (Fig. 3). The PAPS containing fractions were either used directly for enzyme assays or concentrated with Biorex 5 (Fig. 4).

All syntheses and purifications can be carried out within 2 – 3 days. The sulfonucleotides were stored at – 20° C and checked for purity before enzyme assays.

Results

Considering the equilibrium conditions and the energetics of the reactions between sulfate, APS and PAPS, it is obvious that transformations of PAPS to APS (with 3′-nucleotidase and possibly APS-kinase) and of APS to sulfate (with ATP-sulfurylase) is always possible and favored without ATP consumption. The opposite way from sulfate to APS and from APS to PAPS, however, is only possible by activation with ATP and can easily be blocked by omitting ATP from the assay. Sulfate reduction via PAPS to acid volatile sulfite includes the reactions of ATP-sulfurylase, APS-kinase and PAPS-thiolsulfotransferase. If APS is the reduced substrate, ATP-sulfurylase amd APS-thiolsulfotransferase are involved and activation is only necessary with sulfate as substrate. Reduction of PAPS would be possible in this system after hydrolysis to APS. Because of the instability of the sulfonucleotides in cell extracts, the mere application of APS and PAPS under otherwise identical test conditions would hardly give reliable informations about the actually reduced sulfonucleotide. Therefore a test system based on isotope dilution of the degradation products of the sulfonucleotides with non-labeled sulfur compounds was applied. The reduction of ^{35}S-APS and ^{35}S-PAPS was tested with and without addition of 100 mM non-labeled sulfate. The activities with the actually reduced sulfonucleotide were generally higher in the presence of 100 mM sulfate, but the activities with the other sulfonucleotide were lower. In strains, which were found to reduce PAPS, activities with APS in the presence of sulfate were 23 – 75 % of the activities without sulfate; activities with PAPS increased to 110 – 380 % with addition of sulfate. The data of assays carried out in the presence of sulfate with addition of ATP and non-labeled APS or PAPS are shown in Table 1. As expected, activities with ^{35}S-sulfate in the absence of ATP were low (they may be taken as blank values and are probably due to the endogeneous ATP level in the undialysed extracts). In all strains addition of ATP increased the reduction rate of sulfate considerably. Two groups of organisms can be distinguished on the basis of their reduction rates of sulfate and APS. In the first group the APS reduction rate under the applied conditions is lower than that of sulfate, in the second group it is several fold higher. For better comparison five different ratios of the data shown in Table 1 were calculated and are shown in Table 2.

1. ^{35}S-sulfate/^{35}S-APS

If a specific strain is able to reduce PAPS only, APS and sulfate have to be activated and the reduction rates with both substrates are expected to be comparable low in the absence of ATP. If the strain is able to reduce APS, however, no activation is necessary with APS and this activity should therefore be significantly higher than that with sulfate. As shown in Table 2, the ratio is 0.4 – 5.7 in group 1 and lower than 0.1 in group 2 (with three exceptions: *Rps. gelatinosa* 0.51 and 2.42, *Rc. purpureus* 0.81).

2. ^{35}S-APS + ATP/^{35}S-APS

Because of the necessary activation in the PAPS-reducing system, the addition of ATP should increase this activity in these strains; no increase of the activity should appear in strains reducing APS. In the first group values higher than 5 (in half of the strains higher than 10, in *Rps. viridis* only 3.1)

Table 1. Rates of reduction of ^{35}S-sulfate, ^{35}S-APS and ^{35}S-PAPS in phototrophic and chemotrophic bacteria (all values are pmol SO_2 formed/mg protein and h)

Bacterial species and strains	Source	^{35}S-sulfate		^{35}S-APS				^{35}S-PAPS		
		O	+ATP	O	+ATP	+PAPS	+PAPS +ATP	O	+APS	+APS +ATP
Rhodopsudomonas capsulata	DSM 155	50	790	20	340	10	10	930	620	550
Rhodopseudomonas sphaeroides	DSM 158	30	180	9	90	9	7	410	220	210
Rhodopseudomonas sulfidophila	DSM 1374	58	200	12	110	6	24	502	521	384
Rhodopseudomonas sulfidophila	BN 196	17	400	6	81	6	16	351	328	204
Rhodopseudomonas viridis	BN 170	25	297	63	196	7	14	1,486	997	651
Rhodopseudomonas palustris	DSM 126	5	81	5	125	1	n.b.	328	287	n.b.
Rhodopseudomonas palustris	1a1	39	627	9	61	7	18	138	194	153
Rhodospirillum rubrum	DSM 467	n.b.	n.b.	19	395	5	5	1,925	1,763	1,762
Rhodospirillum rubrum	DSM 107	17	80	3	17	10	7	93	88	84
Rhodospirillum fulvum	BN 211	210	420	500	2,720	30	150	2,490	2,000	1,410
Serratia marcescens	Stille	51	962	189	3,400	194	167	4,762	4,703	3,567
Escherichia coli	DSM 498	16	1,533	11	680	27	354	13,407	12,883	12,090
Paracoccus denitrificans	DSM 145	30	203	11	50	6	n.b.	186	182	n.b.
Rhodospirillum tenue	BN 230	34	412	4,200	4,027	1,551	1,839	2,788	43	107
Rhodopseudomonas gelatinosa	DSM 149	95	386	186	248	76	64	219	116	108
Rhodopseudomonas gelatinosa	BN 151	138	77	323	237	91	89	404	180	268
Rhodocyclus purpureus	DSM 168	12	24	68	67	45	n.b.	73	28	n.b.
Rhodopseudomonas acidophila	DSM 137	5	151	1,263	982	277	n.b.	1,173	73	n.b.
Rhodopseudomonas globiformis	DSM 161	79	34	1,343	1,152	775	n.b.	1,747	28	n.b.
Rhodomicrobium vannielii	DSM 162	27	654	902	760	435	573	1,523	78	21
Pseudomonas aeruginosa	Stille	28	631	17,228	20,922	9,941	18,925	12,466	928	288
Alcaligenes eutrophus	DSM 428	85	3,359	7,513	8,926	2,181	n.b.	5,054	354	n.b.

O: no addition

Table 2. Nucleotide specifity of sulfate reduction in phototrophic and chemotrophic bacteria

	$\frac{^{35}\text{S-sulfate}}{^{35}\text{S-APS}}$	$\frac{^{35}\text{S-APS+ATP}}{^{35}\text{S-APS}}$	$\frac{^{35}\text{S-APS}}{^{35}\text{S-PAPS}}$	$\frac{^{35}\text{S-APS+PAPS}}{^{35}\text{S-APS}}$	$\frac{^{35}\text{S-PAPS+APS}}{^{35}\text{S-PAPS}}$
Rps. capsulata	2.5	17.0	0.022	–	0.67
Rps. sphaeroides	3.3	10.0	0.022	–	0.54
Rps. sulfidophila 1374	4.8	9.2	0.024	–	0.93
Rps. sulfidophila 196	2.8	13.5	0.017	–	1.04
Rps. viridis	0.4	3.1	0.042	–	0.67
Rps. palustris 126	0.83	20.8	0.018	–	0.88
Rps. palustris 1a1	4.3	6.8	0.065	–	1.41
R. rubrum 467	–	20.8	0.010	–	0.92
R. rubrum 107	5.7	5.7	0.032	–	0.95
R. fulvum	0.42	5.4	0.201	–	0.80
Serratia marcescens	0.27	17.9	0.040	–	0.99
Escherichia coli	1.45	61.8	0.001	–	0.96
Paracoccus denitrificans	2.73	4.5	0.059	–	0.98
R. tenue	0.008	0.96	1.51	0.37	0.015
Rps. gelatinosa 149	0.51	1.33	0.85	0.41	0.53
Rps. gelatinosa 151	2.43	0.73	0.80	0.28	0.45
Rc. purpureus	0.18	0.99	0.93	0.66	0.38
Rps. acidophila	0.004	0.78	1.08	0.22	0.06
Rps. globiformis	0.06	0.85	0.77	0.58	0.02
Rm. vannielii	0.03	0.84	0.59	0.48	0.05
Pseudomonas aeruginosa	0.002	1.21	1.38	0.58	0.07
Alcaligenes eutrophus	0.011	1.19	1.49	0.29	0.07

were found. In the second group values of about 1 (always lower than 1.5) were found.

3. ^{35}S-APS/^{35}S-PAPS

In strains, which need to activate APS rather low activities with this sulfonucleotide were expected. Ratios below 0.2, in most strains below 0.05 were found. In strains, which were assumed to reduce APS, values around 1 (0.73 – 1.33) were found. As 3′-nucleotidase is generally present in the extracts (data not shown), ^{35}S-PAPS can be continuously hydrolyzed to ^{35}S-APS, which in turn may be reduced to acid volatile radioactivity in strains reducing APS. Because of the re-

latively high initial concentrations of ^{35}S-APS in the assays, ATP-sulfurylase may catalyze its hydrolysis and thus rapidly reduce the initial concentration. These two reactions may explain the higher activities with PAPS in some of the strains, assumed to reduce APS.

4. ^{35}S-PAPS + ^{32}S-APS/^{35}S-PAPS

In strains reducing PAPS, the addition of ^{32}S-APS should have little influence on the reduction rate, because it can not dilute the pool of labeled PAPS. Accordingly ratios of about 1 (0.54 – 1.41) were found. In strains, which reduce APS, the ratio should be lower than 0.1 (assumed the APS pool is stable and PAPS can be quantitatively transformed to APS). Ratios between 0.01 – 0.06 were found (three exceptions: *Rps. gelatinosa* 0.45 and 0.53, *Rc. purpureus* 0.38). The higher values in these strains may be explained by the reasons given under 3.

5. ^{35}S-APS + ^{32}S-PAPS/^{35}S-APS

In PAPS reducing strains both activities were rather low, so that the ratio is not very conclusive. In strains assumed to reduce APS these values were between 0.2 – 0.7 and thus also indicate hydrolysis of PAPS.

On the basis of these data two groups can be distinguished among the investigated Rhodospirillaceae. Organisms of group 1, comprising strains of *Rps. capsulata*, *Rps. sphaeroides*, *Rps. sulfidophila*, *Rps. palustris*, *Rps. viridis*, *R. rubrum* and *R. fulvum* were found to reduce PAPS. Organisms of group 2, *Rps. acidophila*, *Rps. globiformis*, *Rm. vannielii*, *Rc. purpureus*, *R. tenue* and *Rps. gelatinosa* reduce APS.

Because two different sulfate reducing pathways occur in the Rhodospirillaceae and some of these bacteria seem to be closely related to certain chemotrophic bacteria (Gibson et al. 1979), it seemed likely that also within the chemotrophic bacteria different pathways are present. Therefore several chemotrophic bacteria were included in the present study. *Paracoccus denitrificans* reduced sulfate via PAPS, as *Serratia marcescens* and *E. coli* did. But two other species, *Pseudomonas aeruginosa* and *Alcaligenes eutrophus* used the pathway via APS.

Discussion

Sulfate assimilation has been intensively studied in plants and yeasts (see Tsang and Schiff 1975; Schmidt et al. 1974; Wilson and Bierer 1976). The plant type cells were found to reduce sulfate via APS, the yeasts via PAPS. Among different bacteria most numerous and intensive studies have been performed with *Escherichia coli* and other Enterobacteriaceae (see Dreyfuss and Monty 1963; Kredich 1971; Tsang and Schiff 1976), which reduce PAPS. Incorporation studies of ^{35}S-sulfate into whole cells of *Nitrobacter agilis* (Varma and Nicholas 1971) and the participation of APS-kinase in cysteine biosynthesis of *Paracoccus denitrificans* (Burnell and Whatley 1980) revealed that the pathway via PAPS operates also in these bacteria. The first indication for sulfate assimilation via APS in bacteria came from studies of Tuovinen et al. (1975), who were unable to show PAPS formation in extracts of *Thiobacillus ferrooxidans*. Among phototrophic procaryotes both pathways were found in different cyanobacteria (Schmidt 1977a) and preliminary tests using crude extracts of several anoxygenic phototrophic bacteria and APS or PAPS under otherwise identical assay conditions gave first indications that different pathways of sulfate assimilation might be operative also in this bacterial group (Schmidt and Trüper 1977).

The development of suitable purification procedures for the sulfonucleotides (see Material and Methods) enabled us to perform thorough isotope studies on the alternative reduction of APS or PAPS in a number of phototrophic and non-phototrophic bacteria. Both sulfate reducing pathways, the APS and the PAPS-pathway were found to function in different species of the Rhodospirillaceae as well as in the investigated chemotrophic bacteria: The APS-pathway in species of all four genera of the Rhodospirillaceae, in *Rm. vannielii*, *Rc. purpureus*, *R. tenue*, *Rps. gelatinosa*, *Rps. acidophila* and *Rps. globiformis* and the PAPS-pathway in several *Rhodopseudomonas* and *Rhodospirillum* species. In contrast within five genera of the Enterobacteriaceae, in *Enterobacter aerogenes*, *Proteus mirabilis*, *Proteus vulgaris*, *Salmonella typhimurium*, *Seratia marcescens* and in *E. coli* only the PAPS-pathway is present (Peck 1961; Dreyfuss and Monty 1963; Pasternak et al. 1965; Kredich 1971; Tsang and Schiff 1976; the present work). With the exception of *R. rubrum* the present findings are well in agreement with earlier results. The enrichment of an APS-sulfotranferase from *R. rubrum* 1761-1a, which, however, showed also some activity with PAPS, was reported (Schmidt 1977b). But according to our results PAPS is reduced by *R. rubrum* (also strain 1761-1a, data not shown). Enrichment attempts with different PAPS-sulfotransferases from other Rhodospirillaceae revealed an extremely high instability of this enzyme and it seems possible that during the enrichment procedures carried out by Schmidt the PAPS-sulfotransferase activity was lost. A satisfactory explanation for the observed activity with APS is difficult, but possibly a loss of the PAPS specifity during purification, a certain unspecifity for the sulfonucleotide, or the additional presence of a more stable APS-sulfotransferase in this preparation were responsible for the higher activity with APS in the enriched preparation.

Is the presence of one or the other pathway a phylogenetically stable property? At the first glance the distribution of the two pathways within the Rhodospirillaceae species (sulfotransferases of different strains of the same species use the same substrate) seems to show no correlation to the properties used for their classification. That not only a change in the substrate specifity of the sulfotransferases is responsible for the different substrates used was ruled out by findings of different stabilities of the two enzyme systems, different behaviour during enzyme purification, and different requirements for low molecular weight protein cofactors (data not shown).

If the Enterobacteriaceae are considered as being a rather homogeneous family and the Rhodospirillaceae as an extremely heterogeneous family, the possession of the APS- or PAPS-pathway may indeed be a rather stable property in evolutionary terms. The phylogenetic heterogeneity of the Rhodospirillaceae is indeed supported by a number of other investigations.

The morphological and physiological similar species *Rps. capsulata*, *Rps. sphaeroides*, and *Rps. sulfidophila* use the PAPS-pathway, they are very similar according to their 16 S rRNA homologies (Gibson et al. 1979) and DNA-DNA hybridization studies (De Bont et al. 1981). Furthermore high similarities were found in the 23 S rRNA sequences between *Rps. sphaeroides* and *Paracoccus denitrificans* (MacKay et al. 1979) and between the three dimensional structures of

cytochrome c_{550} from *Paracoccus denitrificans* and cytochrome c_2 from *R. rubrum* (Almassy and Dickerson 1978). All these bacteria use the PAPS-pathway.

Also the species *R. tenue*, *Rps. gelatinosa* and *Rc. purpureus*, form – despite their belonging to three different genera – a defined group on the basis of high similarities of their 16 S rRNA (Gibson et al. 1979), of their cytochrome *c'* sequences and their use of the APS-pathway. The two chemotrophs, *Alcaligenes eutrophus* and *Pseudomonas aeruginosa* have not only in common with these three phototrophs that they reduce APS. *Alcaligenes faecalis* and *Alcaligenes eutrophus* fall into the same cluster of "*Alcaligenes*" strains (De Ley et al. 1978) and among all chemotrophic bacteria tested, *Alcaligenes faecalis* showed the highest similarity to *R. tenue* (Gibson et al. 1979). In addition *Rps. gelatinosa* was found very similar to H_2-oxidizing *Pseudomonas* species and the above mentioned *Alcaligenes* group (De Ley et al. 1978).

Among the budding *Rhodopseudomonas* species *Rps. viridis* and *Rps. palustris*, which are more similar to *Rhizobium leguminosarum* than to each other in terms of their 16 S rRNA (Gibson et al. 1979), reduce PAPS. *Rps. palustris* is also very similar to *Nitrobacter agilis* (Seewaldt et al. 1982), which reduces PAPS as well.

The good correlation between similarities in the cytochrome sequences (Ambler et al. 1979), the 16S rRNA homologies (Gibson et al. 1979) and the species specifity for the sulfonucleotides APS or PAPS in sulfate reduction among phototrophic as well as non-phototrophic bacteria indicates a genetically determined and stable difference between the two sulfate reducing pathways. With the support of its evolutionary stability and the demonstrated relationship between Rhodospirillaceae and chemotrophic bacteria such predictions as follow may be made: *Rhizobium leguminosarum*, *Spirillum itersonii* and *Photobacterium phosphoreum* with related species use the PAPS-pathway; *Sphaerotilus natans* and the organisms related to *Alcaligenes faecalis* and *Pseudomonas acidovorans* use the APS-pathway.

Acknowledgements. The present work was supported by a fellowship from the Studienstiftung des Deutschen Volkes to the author and a grant from the Deutsche Forschungsgemeinschaft to Prof. H. G. Trüper. Valuble discussion with Prof. Trüper and skillful technical assistance of M. Kramer are gratefully acknowledged.

References

Adams CA, Warnes GM, Nicholas DJD (1971) Preparation of labeled adenosine-5'-phosphosulfate using APS-reductase from *Thiobacillus denitrificans*. Analyt Biochem 42:207–213

Almassy RJ, Dickerson RE (1978) *Pseudomonas* cytochrome c_{551} at 2.0 Å resolution: Enlargement of the cytochrome c family. Proc Natl Acad Sci USA 75:2674–2678

Ambler RP, Daniel M, Hermoso J, Meyer TE, Bartsch RG, Kamen MD (1979) Cytochrome c_2 sequence variation among the recognized species of purple non-sulfur photosynthetic bacteria. Nature 278:659–660

Baddiley J, Buchanan JG, Letters R (1957) Synthesis of adenosine-5'-sulfatophosphate. A degradation product of an intermediate in the enzymatic synthesis of sulfuric esters. J Chem Soc 1067–1071

Beisenherz G, Boltze HJ, Bücher T, Czok R, Garbade KH, Meyer-Arendt E, Pfleiderer G (1953) Diphosphofructose-Aldolase, Phosphoglycerinaldehyd-Dehydrogenase, Milchsäure-Dehydrogenase, Glycerophosphat-Dehydrogenase und Pyruvat-Kinase aus Kaninchenmuskel in einem Arbeitsgang. Z Naturforsch 8b:555–557

Buchanan BB, Schürmann P, Kalberer PP (1971) Ferredoxin-activated fructose diphosphatase of spinach chloroplasts. Resolution of the system, properties of the alkaline fructose diphosphatase component, and physiological significance of the ferredoxin-linked activation. J Biol Chem 246:5952–5959

Burnell JN, Whatley FR (1980) Regulation of sulfur metabolism in *Paracoccus denitrificans*. J Gen Microbiol 118:73–78

Burnell JN, John P, Whatley FR (1975) The reversibility of active transport in membrane vesicles of *Paracoccus denitrificans*. Biochem J 150:527–536

Cherniak R, Davidson EA (1964) Synthesis of adenylyl sulfate and adenylyl sulfate 3'-phosphate. J Biol Chem 239:2986–2990

Cooper BP, Trüper HG (1979) Improved synthesis and rapid isolation of millimole quantities of adenylyl-sulfate. Z Naturforsch 34c:346–349

De Bont JAM, Scholten A, Hansen TA (1981) DNA-DNA hybridization of *Rhodopseudomonas capsulata*, *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas sulfidophila* strains. Arch Microbiol 128:271–274

De Ley J, Segers P, Gillis M (1978) Intra- and intergeneric similarities of *Chromobacterium* and *Janthinobacterium* ribosomal ribonucleic acid cistrons. Int J Syst Bacteriol 28:154–168

Dreyfuss J, Monty KJ (1963) The biochemical characterization of cysteine-requiring mutants of *Salmonella typhimurium*. J Biol Chem 238:1019–1024

Gibson J, Stackebrandt E, Zablen LB, Gupta R, Woese CR (1979) A phylogenetic analysis of the purple photosynthetic bacteria. Current Microbiol 3:59–64

Gottschalk G (1964) Die Biosynthese der Poly-β-hydroxybuttersäure durch Knallgasbakterien. II. Verwertung organischer Säuren. Arch Mikrobiol 47:230–235

Hodson RC, Schiff JA (1969) Preparation of adenosine-3'-phosphate-5'-phosphosulfate (PAPS): an improved enzymatic method using *Chlorella pyrenoidosa*. Arch Biochem Biophys 132:151–156

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

Kredich NM (1971) Regulation of L-cysteine biosynthesis in *Salmonella typhimurium*. I. Effects of growth on varying sulfur sources and O-acetyl-L-serine on gene expression. J Biol Chem 246:3474–3484

MacKay RM, Zablen LB, Woese CR, Doolittle WF (1979) Homologies in processing and sequence between the 23 S ribosomal ribonucleic acids of *Paracoccus denitrificans* and *Rhodopseudomonas sphaeroides*. Arch Microbiol 123:165–172

Pasternak CA, Ellis RJ, Jones-Mortimer MC, Crichton CE (1965) The control of sulphate reduction in bacteria. Biochem J 96:270–275

Peck HD (1961) Enzymatic basis for assimilatory and dissimilatory sulfate reduction. J Bacteriol 82:933–939

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

Robbins PW (1963) Preparation and properties of sulfur adenylates. In: Colowick SP, Kaplan NO (eds) Methods in enzymology. Academic Press, New York, vol VI, pp 766–775

Schedel M (1978) Untersuchungen zur anaeroben Oxidation reduzierter Schwefelverbindungen durch *Thiobacillus denitrificans*, *Chromatium vinosum* und *Chlorobium limicola*. PhD Thesis, University of Bonn

Schmidt A (1972) On the mechanism of photosynthetic sulfate reduction. An APS-sulfotransferase from *Chlorella*. Arch Mikrobiol 84:77–86

Schmidt A (1975) Distribution of the APS-sulfotransferase activity among higher plants. Plant Sci Lett 5:407–415

Schmidt A (1977a) Assimilatory sulfate reduction via 3'-phosphoadenosine-5'-phosphosulfate (PAPS) and adenosine-5'-phosphosulfate (APS) in blue-green algae. FEMS Lett 1:137–140

Schmidt A (1977b) Adenosine-5'-phosphosulfate (APS) as sulfate donor for assimilatory sulfate reduction in *Rhodospirillum rubrum*. Arch Microbiol 112:263–270

Schmidt A, Abrams WR, Schiff JA (1974) Reduction of adenosine-5'-phosphosulfate to cysteine in extracts from *Chlorella* and mutants blocked for sulfate reduction. Eur J Biochem 47:423–434

Schmidt A, Trüper HG (1977) Reduction of adenylsulfate and 3'-phosphoadenylsulfate in phototrophic bacteria. Experientia 33:1008–1009

Seewaldt E, Schleifer KH, Bock E, Stackebrandt E (1982) The close phylogenetic relationship of *Nitrobacter* and *Rhodospeudomonas palustris*. Arch Microbiol 131:287–290

Tsang MLS, Lenieux J, Schiff JA, Bojarski TB (1976) Preparation of adenosine-5′-phosphosulfate (APS) from adenosine-3′-phosphate-5′-phosphosulfate (PAPS) prepared by an improved procedure. Analyt Biochem 74:623–626

Tsang MLS, Schiff JA (1975) Studies of sulfate utilization by algae. 14. Distribution of adenosine-3′-phosphate-5′-phosphosulfate (PAPS) and adenosine-5′-phosphosulfate (APS) sulfotransferases in assimilatory sulfate reducers. Plant Sci Lett 4:301–307

Tsang MLS, Schiff JA (1976) Sulfate-reducing pathway in *Escherichia coli* involving bound intermediates. J Bacteriol 125:923–933

Tuovinen OH, Kelley BC, Nicholas DJD (1975) The uptake and assimilation of sulphate by *Thiobacillus ferrooxidans*. Arch Microbiol 105:123–127

Varma AK, Nicholas DJD (1971) Metabolism of ^{35}S-sulfate and properties of APS-kinase and PAPS-reductase in *Nitrobacter agilis*. Arch Mikrobiol 78:99–117

Wilson LG, Asahi T, Bandurski RS (1961) Yeast sulfate-reducing system. I. Reduction of sulfate to sulfite. J Biol Chem 236:1822–1829

Wilson LG, Bierer D (1976) The formation of exchangeable sulfite from adenosine-3′-phosphate-5′-sulfatophosphate in yeast. Biochem J 158:255–270

Received March 15, 1982/Accepted May 19, 1982