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Purification and Properties of Adenylyl Sulfate Reductase from the Phototrophic Sulfur Bacterium, *Thiocapsa roseopersicina*

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Adenylyl sulfate reductase was purified from *Thiocapsa roseopersicina* 60- to 80-fold, and the properties were studied. The molecular weight is 180,000. The enzyme contains, per molecule; one flavine group, two heme groups of cytochrome *c* character, four atoms of nonheme iron, and six labile sulfide groups. Cytochrome *c* and ferricyanide serve as electron acceptors. With ferricyanide as the electron acceptor, the *p*H optimum of the enzyme is at 8.0; with cytochrome *c*, the *p*H optimum is at 9.0. Of the nucleotides studied, adenosine 5'-monophosphate is most effective. The influence of substrate concentrations on the activity of the enzyme was studied, and the K_m values for sulfite, adenosine 5'-monophosphate, ferricyanide, and cytochrome *c* were determined. The properties of the enzyme are compared with those of adenylyl sulfate reductases purified from sulfate-reducing bacteria and thiobacilli.

Like the sulfate-reducing bacteria and some thiobacilli (17), all phototrophic sulfur bacteria tested so far contain adenylyl sulfate (APS) reductase as the sulfite-oxidizing enzyme (21). As shown by Trüper and Peck (21), in phototrophic sulfur bacteria, APS reductase is located in the particulate fraction but is easily leached into the soluble protein fraction upon disruption of the cells.

So far, APS reductase has been purified and characterized only from *Desulfovibrio vulgaris* strain Hildenborough (18), *Thiobacillus denitrificans* (2), and *T. thioparus* (10).

The aim of this study was to purify the enzyme from a phototrophic sulfur bacterium, to characterize it, and to compare it with the APS reductases purified from sulfate-reducing bacteria and thiobacilli. Previous work (21) showed that of the phototrophic bacteria tested, *Thiocapsa roseopersicina* strain SMG219, contained the highest amount of APS reductase activity in the soluble protein fraction: therefore we used this strain for the present study.

MATERIALS AND METHODS

Organism. *T. roseopersicina* strain SMG219 was used throughout this investigation.

Cultivation. Cultivation of the bacteria, preparation of cell-free extracts, and protein determination in crude fractions were carried out as previously reported (21). The purification procedure is described in detail below. The data for column chromatography are given in Fig. 1. All steps were performed at 4 C.

Protein determinations with the purified enzyme were performed by the method of Warburg and Christian (23).

Spectrophotometric tests for APS reductase. The reaction mixture for ferricyanide-coupled assay consisted of (in micromoles): tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride (*p*H 8.0), 125; $K_3Fe(CN)_6$, 1.25; ethylenediaminetetraacetic acid (EDTA), 20; adenosine 5'-monophosphate (AMP), 1.0; Na_2SO_3, 10. (The sulfite solution was always freshly prepared in Tris buffer, *p*H 8.0, containing 0.005 M EDTA.) The assay volume was 2.65 ml. Absorbancy was measured in 1-cm glass absorption cells at 420 nm, 25 C, against a blank containing the reaction mixture minus enzyme.

The reaction mixture for cytochrome *c*-coupled assay consisted of (in micromoles): Tris-hydrochloride (*p*H 9.0), 6.6; AMP, 0.1; Na_2SO_3, 1.0 (solution as given above); cytochrome *c*, 0.1. The assay volume was 1.0 ml. Absorbancy was measured in 1-cm glass cells at 550 nm, 25 C, against a blank containing the reaction mixture minus enzyme.

The assays were carried out in Zeiss PMQ2 or PM4 spectrophotometers. Absorption spectra were measured with a Zeiss DMR21 recording spectrophotometer.

Flavine determination. Flavine was determined by the method of Rao et al. (19), non-heme iron by the bathophenanthroline method (Boehringer test combination TC-FE; Boehringer & Soehne, Mannheim), and labile sulfide by the methylene blue method (15, 22).

Sedimentation experiments. Sedimentation experiments were carried out in an analytical ultracentrifuge

(model E, Beckman-Spinco) equipped with a monochromator, a photoelectric scanner, and a multiplexer. Double-sector cells with sapphire windows and a 12-cm optical path were used. Sedimentation coefficients were calculated by the moving boundary method. The centrifuge was run at 24,000 rev/min and 5 C. Scanner tracings at 280 nm were taken every 12 min. The molecular weight of APS reductase was determined by the low-speed sedimentation equilibrium method at 6,800 rev/min and 5 C.

Chemicals. Chemicals used were of analytical quality.

Biochemicals. Cytochrome *c* from horse heart and all nucleotides used were purchased from Boehringer & Soehne, Mannheim, Germany; iodoacetamide from E. Merck AG, Darmstadt, Germany; *N*-ethylmaleimide and *p*-chloromercuribenzoate from Fluka AG, Buchs, Switzerland; Sephadex from Pharmacia, Uppsala, Sweden; Whatman diethylaminoethyl cellulose from Balston Ltd., Maidstone, Kent, England; cytochrome *c* from *Candida krusei* was a product of Sankyo Co. Ltd., Tokyo, Japan (properties: twice crystallized; molecular weight, 12,523; molar extinction coefficient, oxidized, 8.4×10^3 liters $\times$ $mol^{-1} \times cm^{-1}$).

RESULTS

Purification of APS reductase from T. roseopersicina. As a result of several preliminary experiments, the purification scheme shown in Fig. 1 was found to be most useful and reproducible.

The cells were disrupted by sonic treatment and freed from particulate matter by centrifugation at 105,000 $\times$ *g*. The soluble protein was fractionated by ammonium sulfate precipitation, and the fraction precipitating between 50 and 70% saturation was found to contain the enzyme. This fraction was separated from low-molecular-weight constituents by column chromatography on Sephadex G25. The enzyme activity was located in an orange-red layer. The orange-red eluate was applied to a column of diethylaminoethyl cellulose (Whatman DE52) which had been equilibrated against 0.11 M NaCl in 0.01 Tris-hydrochloride, *p*H 7.0. A linear gradient between 0.11 and 0.33 M NaCl was passed through the column, resulting in the elution of three peaks, each with absorption maxima at 280 nm (protein) and 407 nm (hemoprotein). APS reductase activity was found only in the material comprising peak III (Fig. 2). The combined fractions of this peak were concentrated by ammonium sulfate precipitation (75% saturation) and fractionated on a column of Sephadex G150 or G200 equilibrated against 0.01 M Tris-hydrochloride, *p*H 7.0. Between fractions 29 and 35 (Fig. 3), the maxima for protein and heme absorption and enzyme activity corresponded exactly. The active fractions were combined, and the "purified enzyme" was further characterized. Table 1 shows the efficiency of a typical purification of APS reductase from *T. roseopersicina*.

Stability and storage. By daily enzyme tests over a period of 10 days, it was shown that the purified APS reductase, when stored at 4 C under argon, does not lose more than 8% of its original activity. When frozen (−20 C), the enzyme can be stored for more than 3 months without loss of activity. Thus it is apparently much more stable than the APS reductase recently isolated from *T. thioparus* by Lyric and Suzuki (10).

The APS reductase of *T. denitrificans* isolated by Bowen et al. (2) showed remarkable heat stability. Heat tests with the purified enzyme from *T. roseopersicina* revealed that it could be heated at 65 C for 15 min without loss of activity but was inactivated at 70 C. The enzyme is therefore less heat stable than that of *T. denitrificans* but more stable than that of *T. thioparus* (10).

Molecular weight and purity. The sedimentation coefficient and the molecular weight were determined in an analytical ultracentrifuge (model E, Beckman-Spinco). Figure 4A gives the concentration dependence of the sedimentation coefficient. The line was calculated by the method of the least squares, and the sedimentation coefficient of the enzyme was found to be $S_{20,w}^{c=0} = 9.7 \times 10^{-13}$ (extrapolated to a protein concentration of zero and calculated for the standard conditions, 20 C and water). The molecular weight was determined by the low-speed sedimentation equilibrium method at low protein concentrations in order to minimize possible aggregation. Figure 4B shows the extrapolation of the apparent molecular weight to an APS reductase concentration of zero. The extrapolated molecular weight was found to be 180,000 daltons. The linearity of the curve in the plot log *c* against r^2 (Fig. 5) indicates that the enzyme is homogenous by the criterion of ultracentrifugation.

For the enzyme purified from *D. vulgaris*, Peck et al. (18) found a sedimentation coefficient of 10.88 ($\times 10^{-13}$) and a molecular weight of 220,000 (12). Lyric and Suzuki (10) determined the molecular weight of the *T. thioparus* enzyme by column chromatography and found a value of 170,000 $\pm$ 9,000. Thus the values for the *T. roseopersicina* enzyme are of the same magnitude as those of the APS reductases previously studied.

The purified enzyme (dissolved in 0.01 M Tris-hydrochloride, *p*H 7.0) was further subjected to disc electrophoresis in polyacrylamide gel. After staining with Coomassie blue, a heavy, well-defined protein band and three rather weak bands were observed. This too suggests a high degree of purity of the enzyme.

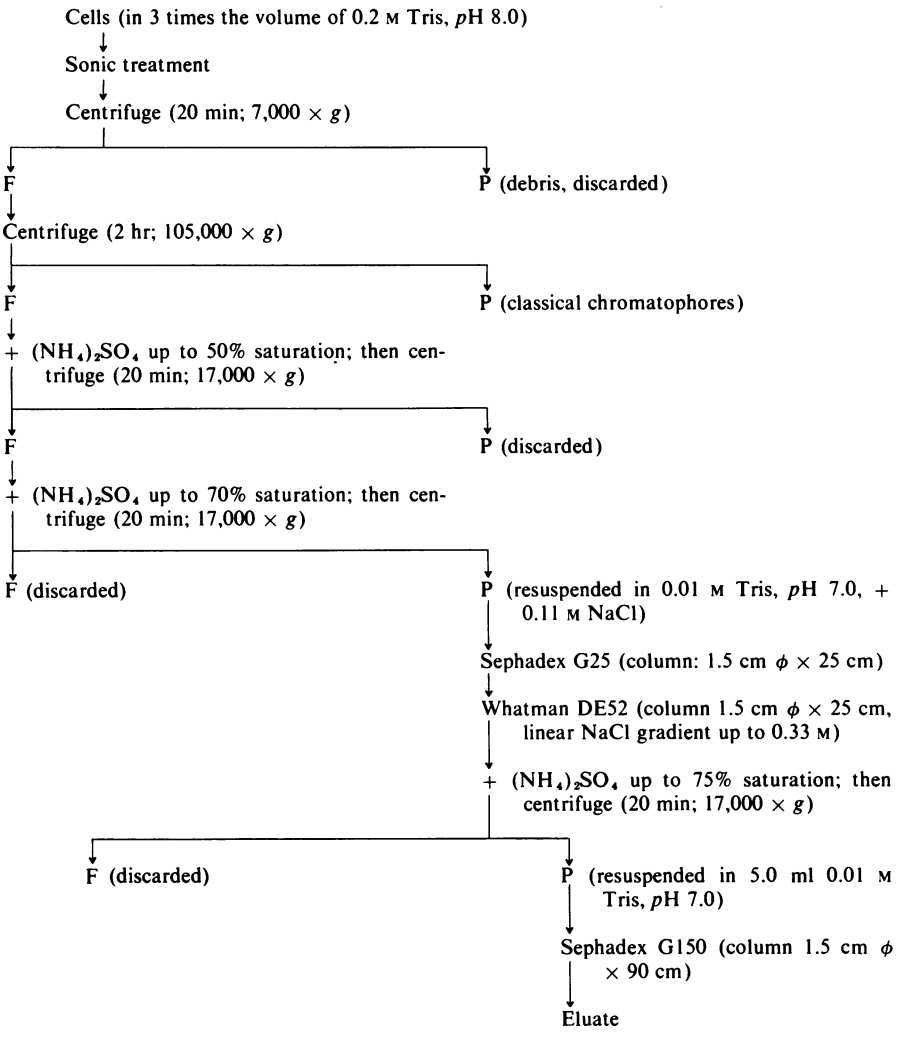

FIG. 1. *Purification procedure for APS reductase from Thiocapsa roseopersicina. F, supernatant fluid; P, pellet.*

Absorption spectrum of the enzyme: cytochrome and flavine constituents. During the purification procedure, it became obvious that the APS reductase of *T. roseopersicina* contained a heme-like group. This finding was confirmed by the absorption spectrum of the purified enzyme in the range of 350 to 700 nm (Fig. 6). The oxidized enzyme shows absorption maxima at 407 and 522 nm and shoulders at 350, 450, and 561 nm. After reduction with dithionite, the enzyme shows maxima at 417, 520, and 550 nm. These spectral characteristics clearly indicate that the heme portion of the enzyme is a *c*-type cytochrome. Based on the specific extinction coefficient for oxidized cytochrome *c* (from *Candida krusei*) at 549 nm (8.4×10^3 liters $\times$ mol$^{-1} \times$ cm^{-1}) the cytochrome content of the purified enzyme was calculated as 13.26 nmoles/mg of protein.

The strong absorption differences in the range from 440 to 500 nm suggested the presence of flavine groups (Fig. 6). Peck et al. (18), Bowen et al. (2), and Lyric and Suzuki (10) have demonstrated flavine adenine dinucleotide (FAD) as a constituent of their purified APS reductases. The *T. roseopersicina* enzyme was treated with saturated urea for 48 hr at 4 C, and the resulting yellow reaction mixture was applied to a Sephadex G25 column and eluted with 0.01 M Tris-hydrochloride, *p*H 7.0. Two colored bands ap-

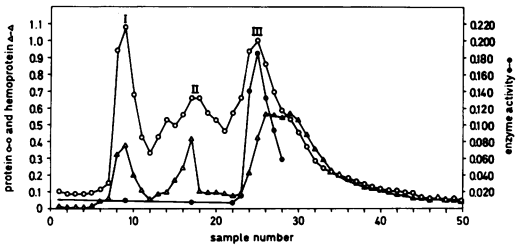

FIG. 2. *Fractionation pattern on diethylaminoethyl cellulose (Whatman DE52). Column proportions: diameter 1.5 cm, length 25 cm. Volume of sample: 1.6 ml. Protein was measured spectrophotometrically at 280 nm in 1-cm cells; hemoprotein at 407 nm. Enzyme activity is given as absorption change per minute for 0.1-ml samples, measured at 420 nm in the ferricyanide assay system.*

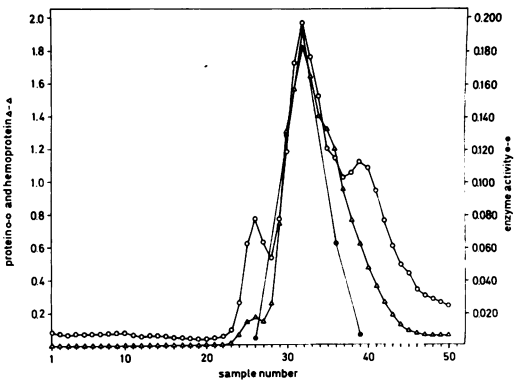

FIG. 3. *Fractionation pattern on Sephadex G150. Column proportions: diameter 1.5 cm, length 90 cm. Volume of sample: 1.1 ml. Measurements as in Fig. 2.*

TABLE 1. *Efficiency of a typical purification of adenylyl sulfate reductase from Thiocapsa roseopersicina*

Fraction[a]	Total protein (mg)	Total activity[b]	Specific activity[b]	Purification factor	Yield (%)
Supernatant fluid of 105,000 × *g*	122.3	19.2	0.157	1.0	100.0
50–70% $(NH_4)_2SO_4$-precipitate	13.6	11.5	0.842	5.4	60.0
DEAE 52 eluate	1.6	7.8	4.860	31.0	40.6
Sephadex 200 eluate	0.9	7.8	8.720	55.4	40.6

[a] DEAE, Diethylaminoethyl.

[b] Activity is expressed as micromoles of $Fe(CN)_6^{3-}$ oxidized per minute.

peared. The first band, orange-yellow in color, was identified by its absorption spectrum as the heme portion (cytochrome *c*) of the enzyme. The second band, clearly separated from the first by

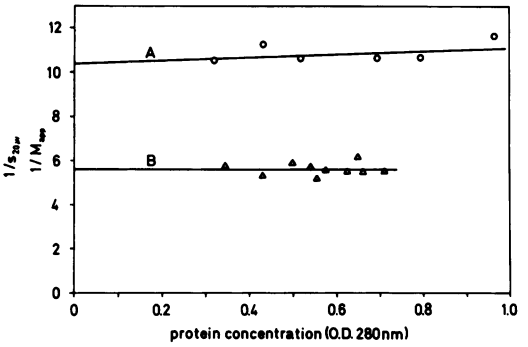

FIG. 4. *Determination of sedimentation coefficient and molecular weight of purified adenylyl sulfate reductase. (A) Reciprocal sedimentation coefficient ($1/S_{20,w}$, multiplied by 10^{-11}) plotted against protein concentration. The sedimentation coefficient is determined by extrapolation of the protein concentration to zero. Enzyme dissolved in 0.01 M Tris-hydrochloride, pH 7.0. (B) Reciprocal apparent molecular weight ($1/M_{app}$, multiplied by 10^6) plotted against protein concentration. The molecular weight is determined by extrapolation of the protein concentration to zero. Temperature: 5 C; equilibrium centrifugation at 6,800 rev/min.*

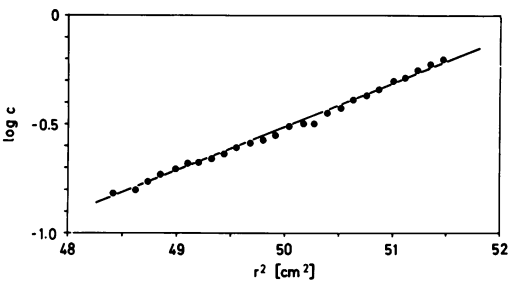

FIG. 5. *Sedimentation equilibrium of purified adenylyl sulfate reductase. c, Protein concentration (optical density at 280 nm); r, distance of sample from the axis of rotation.*

colorless fractions, was yellowish green and showed a spectrum typical for flavines with absorption maxima at 365, 440, and 280 nm and minima at 315 and 400 nm. The absorption in the range below 300 nm indicated the presence of a peptide moiety. Reduction of the flavine by addition of dithionite resulted in a strong decrease in absorption in the range between 400 and 500 nm.

Based on the differential extinction coefficient for FAD at 450 nm, 9.8×10^3 liters $\times$ mole$^{-1} \times$ cm^{-1} (19), the concentration of the flavine was determined in the following way. A 1.0-ml amount of the purified enzyme (containing 1.09 mg of protein) was measured at 450 nm against a blank of distilled water in 1.0-cm cells. A 1.0-mg amount of solid sodium dithionite was then

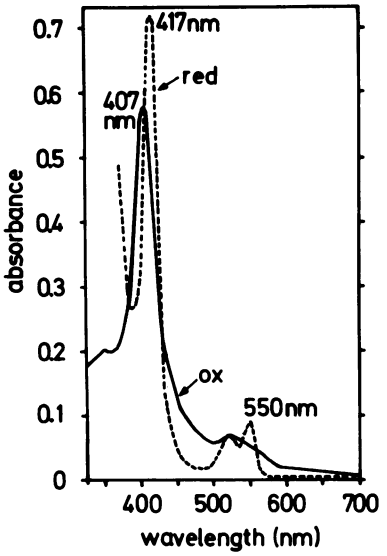

FIG. 6. *Absorption spectra of the purified adenylyl sulfate reductase. ox, Oxidized enzyme; red, enzyme reduced with 1 mg of dithionite/ml.*

added and, after mixing, the solution was measured again. From the difference in absorbancy of 0.065, a concentration of 6.064 nmoles of FAD/mg of protein was calculated. This value, however, is subject to error, since *c*-type cytochromes also show absorbance changes at 450 nm, although to a lesser degree. As no detailed studies on the heme moiety of APS reductase have been carried out, a correction does not appear feasible.

Determination of nonheme iron and labile sulfide. The APS reductases of *D. vulgaris* (18) and of thiobacilli (2, 10) contain nonheme iron as well as acid-labile sulfide groups. By using the bathophenanthrolin method, we found that the enzyme of *T. roseopersicina* contains 23.21 nmoles of Fe/mg of protein (average of two determinations).

Labile sulfide was determined by the methylene blue method (22; Pachmayr, Ph.D. thesis, Univ. of Munich, Munich, Germany, 1960). The purified enzyme contains 32.11 nmoles of labile sulfide per mg of protein (average of two determinations).

Molar ratio of the enzyme constituents. From the results reported above, it becomes clear that flavine, nonheme iron, and labile sulfide groups are constituents of all APS reductases so far known from nonphototrophic and phototrophic bacteria. The additional occurrence of cytochrome *c* in the molecule of the enzyme from *T. roseopersicina*, however, is new.

Based on the molecular weight of the enzyme of 180,000, the following ratio between the constituents was found. Each molecule of enzyme APS reductase from *T. roseopersicina* contains one flavine group, two heme groups (cytochrome *c*), four atoms of nonheme iron, and six labile sulfide groups (Table 2). Thus the enzyme appears to possess a relatively complex structure. It cannot consist of several identical subunits since it contains only one flavine group.

The APS reductases from *D. vulgaris*, *T. thioparus*, and *T. denitrificans* have been found to contain 1 mole of FAD per mole of enzyme and 6 to 8, 8 to 10, or 6 to 11 g atoms of nonheme iron, respectively, per mole of enzyme (2, 10, 12)

Assay requirements. For the reduction of ferricyanide in the APS reductase assay with the purified enzyme, the presence of all reactants, i.e., enzyme, sulfite, and AMP, was necessary. Sulfite cannot be replaced by thiosulfate. In the assay system with cytochrome *c* as the electron acceptor, the presence of all of these components was also necessary. Cytochrome *c* from *C. krusei* was active; horse heart cytochrome *c* was not.

The reaction product, APS, was identified by paper chromatography and autoradiography as previously reported for crude cell fractions (21).

Electron acceptors. Like the APS reductase from *T. thioparus* (10), the enzyme from *T. roseopersicina* functions with ferricyanide as well as with microbial cytochrome *c* as the electron acceptor. Peck et al. (18) and Bowen et al. (2) were not able to show any reactivity of the enzymes from *D. vulgaris* and *T. denitrificans* with cytochrome *c*. However, because mammalian cytochrome *c* was used in both cases, their results cannot be considered as final as long as they have not been confirmed with microbial cytochrome *c*.

Nucleotide specificity. As has been shown with crude extracts (21), a number of nucleotides are able to react with APS reductase instead of AMP. Table 3 shows the relative nucleotide specificity of the enzyme in the ferricyanide- and cytochrome *c*-coupled assay systems. In the ferricyanide test, AMP, deoxy-adenosine 5'-monophosphate (dAMP), inosine 5'-monophosphate (IMP), and guanosine 5'-monophosphate

TABLE 2. *Molar ratio of enzyme constituents*

Enzyme	Protein (nmoles/mg)	Approximate ratio (moles/mole of enzyme protein)
APS reductase	5.55	1
Flavine	6.06	1
Heme (cytochrome *c*)	13.26	2
Nonheme iron	23.21	4
Labile sulfide	32.11	6

(GMP) were active, whereas, in the cytochrome *c* test, only AMP, dAMP, and IMP showed measurable activities. The fact that AMP had the highest reactivity in the cytochrome *c* assay suggests that it is the natural acceptor of sulfite in vivo. For GMP, Lyric and Suzuki (10) found the comparatively much higher reactivity of 91% with the APS reductase of *T. thioparus*.

Influence of pH on enzyme activity. A series of tests at different *p*H values under otherwise constant conditions revealed a *p*H optimum of 8.0 in the ferricyanide-coupled test system and 9.0 in the cytochrome *c*-coupled assay (Fig. 7). Peck et al. (18), Bowen et al. (2), and Lyric and Suzuki (10) found values of 7.4, 7.2, and 7.0, respectively, in the ferricyanide-coupled test. In the cytochrome *c*-coupled test, Lyric and Suzuki (10) found a *p*H optimum of 9.5. Thus the values for the enzyme of *T. roseopersicina* are much closer together than those for the APS reductase of *T. thioparus*. According to Lyric and Suzuki (11), the high *p*H optimum of the enzyme (with cytochrome *c*) of the latter organism is of regulatory importance, since this organism contains sulfite oxidase (9) as a second sulfite-oxidizing enzyme.

TABLE 3. *Nucleotide specificity of purified adenylyl sulfate reductase*

Nucleotide[a]	Activity (%)	
	With $Fe(CN)_6^{3-}$	With cytochrome *c*
AMP	100	100
IMP	90	62
GMP	51	9
CMP	8	7
UMP	8	9
dAMP	128	80
ADP	0	0
ATP	0	0
3',5'-cyclic AMP	0	0

[a] Abbreviations: AMP, adenosine 5'-monophosphate; IMP, inosine 5'-monophosphate; GMP, guanosine 5'-monophosphate; CMP, cytidine 5'-monophosphate; UMP, uridine 5'-monophosphate; dAMP, deoxy-adenosine 5'-monophosphate; ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate.

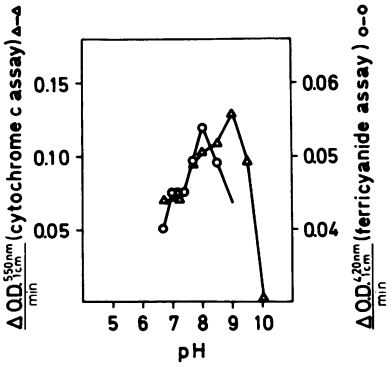

FIG. 7. *Effect of pH on the activity of purified adenylyl sulfate reductase. Tris buffer as described in Materials and Methods.*

Influence of substrate concentration on enzyme activity. With the purified enzyme, activities were measured by varying the concentrations of the reactants, one at a time. Figures 8 and 9 show the dependence of enzyme activity upon sulfite concentration in the ferricyanide and cytochrome *c* assays, respectively. In the ferricyanide assay, substrate inhibition by AMP occurred at concentrations higher than 0.5 μmole of AMP/ml, whereas ferricyanide and sulfite were not inhibitory at higher concentrations tested. In the cytochrome *c* assay, AMP became inhibitory at 0.08

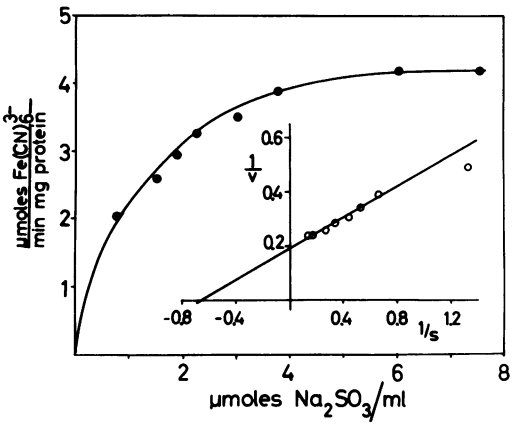

FIG. 8. *Effect of sulfite concentration on ferricyanide-coupled adenylyl sulfate reductase activity.*

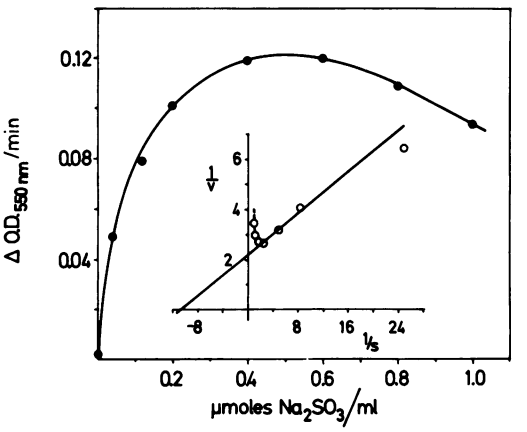

FIG. 9. *Effect of sulfite concentration on cytochrome c-coupled adenylyl sulfate reductase activity.*

TABLE 4. *K_m values for sulfite, adenosine 5'-monophosphate (AMP), ferricyanide, and cytochrome c*

Substrate	Test with	
	Cytochrome *c*	Ferricyanide
Sulfite	9.3×10^{-5} M	1.5×10^{-3} M
AMP	5.0×10^{-5} M	7.3×10^{-5} M
$Fe(CN)_6^{3-}$		1.3×10^{-4} M
Cytochrome *c*	3.3×10^{-5} M	

TABLE 5. *Effect of inhibitors on the activity of purified adenylyl sulfate reductase*

Inhibitor	Final concn	Time of preincubation (min)	Activity (%) of control	
			$Fe(CN)_6^{3-}$ assay	Cytochrome *c* assay
*p*CMB[a]	1×10^{-3}		77	0
	1×10^{-3}	10	72	0
	5×10^{-4}		78	1
	1×10^{-4}		84	47
Iodoacetamide	1×10^{-2}		78	81
	1×10^{-2}	10	33	35
	5×10^{-3}		53	92
	5×10^{-3}	10	50	
	1×10^{-3}		93	96
N-ethylmaleimide	1×10^{-2}		55	0
	1×10^{-2}	10	48	0
	5×10^{-3}		71	0
	5×10^{-3}	10	48	
	1×10^{-3}		93	30
	1×10^{-3}	10	100	
	5×10^{-4}		100	100
	5×10^{-4}	10	100	
Sodium arsenite	1×10^{-2}		100	12
	1×10^{-2}	10	100	
	1×10^{-3}		100	58
	1×10^{-4}			61
NaN_3	1×10^{-2}		100	48
	1×10^{-3}		100	80
	2×10^{-4}	10	100	
	1×10^{-4}		100	80
	1×10^{-4}	10	100	
KCN	2×10^{-4}	10	100	
	1×10^{-4}	10	100	

[a] *p*CMB, *p*-Chloromercuribenzoate.

μmole/ml, sulfite at 0.5 μmole/ml, and cytochrome *c* at 0.07 μmole/ml. A similar pattern of substrate inhibition was reported for the enzyme purified from *T. thioparus* (10).

The K_m values for sulfite, AMP, ferricyanide, and cytochrome *c* were calculated from the Lineweaver-Burk plots (Table 4). The much higher affinity of the enzyme for sulfite in the cytochrome *c*-coupled test as compared with the ferricyanide-coupled test speaks in favor of cytochrome *c* as the in vivo electron acceptor (Table 4).

Inhibitor experiments. The effects of several inhibitors upon the activity of APS reductase are shown in Table 5. Although the purified enzyme proved to be quite susceptible to numerous inhibitors, it was not markedly affected in crude extracts of *T. roseopersicina* (21) and whole cells of *C. vinosum* (7).

The sulfhydryl inhibitors *p*-chloromercuribenzoate and *N*-ethylmaleimide were more effective in the cytochrome *c*-coupled tests than in the ferricyanide-coupled assays; iodoacetamide inhibited equally in both assays. The inhibitory effects of these three reagents were prevented completely by simultaneous addition of β-mercaptoethanol at a final concentration of 5.6×10^{-3} M.

With the enzyme of *T. thioparus*, sulfhydryl inhibitors acted more strongly in the ferricyanide test system than in the cytochrome *c*-coupled assay (10). For the *D. vulgaris* enzyme, Peck et al. (18) found strong inhibition by sulfhydryl inhibitors, but Bowen et al. (2) did not find complete inhibition by these reagents for the *T. denitrificans* enzyme.

Like the *T. thioparus* enzyme, that of *T. roseopersicina* is inhibited by arsenite only in the cytochrome *c*-coupled assay system. Lyric and Suzuki (10) therefore suggested that, in the binding of cytochrome *c*, a dithiol group is involved.

The APS reductase of *T. roseopersicina* is not inhibited by azide or cyanide in the ferricyanide-coupled test system. Experiments with azide in the cytochrome *c* assay, however, resulted in inhibition of the enzyme. This might be due to blocking of the enzyme-bound heme groups or perhaps to inactivation of the substrate cytochrome *c*. No inhibition by EDTA was noted.

DISCUSSION

The basic difference between the APS reductases so far known from thiobacilli and sulfate-reducing bacteria and the APS reductase from the phototrophic sulfur bacterium *T. roseopersicina* is that the latter contains enzyme-bound heme groups. This might be explained by the fact that, in the red phototrophic sulfur bacteria, APS reductase in vivo is membrane bound (21), i.e., located in a system with a high degree of order, whereas in *Desulfovibrio* and *Thiobacillus*

species, the enzyme is contained in the soluble protein fraction, i.e., in a system with a lower degree of order.

The electron flow within the enzyme molecule might be drawn as shown in Fig. 10. From the enzyme-bound heme groups the electrons would lead over into the noncyclic electron flow of photosynthesis.

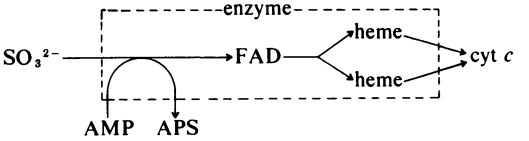

FIG. 10. *Electron flow within the enzyme molecule from Thiocapsa roseopersicina.*

Bartsch et al. (1) isolated a flavine-containing cytochrome from *C. vinosum* strain D, which they named cytochrome *c*-552. They also isolated comparable cytochromes from *C. minus* (cytochrome *c*-552) and *Chlorobium limicola* f. *thiosulfatophilum* (cytochrome *c*-553). Olson and Chance (14) suggested that *Chromatium* cytochrome *c*-552 is reduced via an electron transport chain starting with inorganic electron donors (sulfide, elemental sulfur, thiosulfate). Morita et al. (13) found that cytochrome *c*-552 remained in the oxidized state (in whole cells) as long as the cells were kept anaerobic without electron donors. It was reduced immediately upon addition of molecular hydrogen, sulfide, or thiosulfate. Cusanovich et al. (5) showed that cytochrome *c*-552 is also present in succinate-grown cells of *C. vinosum* strain D and that it remains oxidized at low light intensities. The purified cytochrome *c*-552 is not reduced by the following tested substrates in 10-fold molar excess under anaerobic conditions: thiosulfate, succinate, malate, lactate, pyruvate, reduced nicotinamide adenine dinucleotide, reduced nicotinamide adenine dinucleotide phosphate, or molecular hydrogen (1). The molecular weight of *Chromatium* cytochrome *c*-552 was determined as 72,000; the cytochrome contains two heme groups and one flavine group per molecule (1).

It is questionable whether cytochrome *c*-552 is a constituent of the APS reductase of phototrophic bacteria. More likely, it may act as the acceptor for the electrons coming from the heme groups of APS reductase. The flavine moiety of the cytochrome *c*-552 could have, as indicated by Hind and Olson (6), a donor function in an electron transfer to nicotinamide adenine dinucleotide driven by energy-rich products of photophosphorylation (~I). On the basis of light-induced absorption changes in chromatophores of *C. vinosum* strain D, Cusanovich et al. (5) identified a heme component ("component 4") which, in the electron-transport chain, is located between elemental sulfur and cytochrome *c*-552. It seems possible that in this case the authors measured spectral changes of APS reductase in vivo.

The best-known enzyme containing heme as well as flavine groups is lactate dehydrogenase of yeasts, which is also termed cytochrome b_2 (L-lactate: ferricytochrome *c* oxidoreductase, EC.1.1.2.3). Cytochrome b_2 is similar to the APS reductase purified from *T. roseopersicina* with respect to the absorption spectrum as well as to spectral changes occurring upon substrate additions. Also in this case, an electron flow from substrate (lactate) to enzyme-bound flavine and then to enzyme-bound heme is assumed (11). The similarity of the reaction mechanisms, however, is restricted to the electron flow between reduced flavine and heme, since the substrates of the enzymes are of quite different nature.

Table 6 shows a comparison of the APS reductases from *D. vulgaris* (18), *T. denitrificans* (2), *T. thioparus* (10) and *T. roseopersicina* which thus far have been purified and characterized.

To date, APS reductase has been found only in bacteria living on the basis of a dissimilatory mass transformation of sulfur compounds (15, 21). The presence of the enzyme has been definitely proven for the sulfate-reducing bacteria *D. desulfuricans* (15), *D. vulgaris* (18), *D. africanus* (4), *Desulfotomaculum orientis* (M. R. Lanigan and H. D. Peck, Jr., Bacteriol. Proc. 1963, p. 123), and *D. nigrificans* (15). From these data, it might be generalized that all sulfate-reducing bacteria contain APS reductase. In the thiobacilli, definite evidence has been obtained only for *T. denitrificans* (2) and *T. thioparus* (10). In this group of microorganisms, there exists another sulfite-oxidizing enzyme, sulfite oxidase, which neither needs AMP nor produces APS.

Within strains of the phototrophic bacteria tested thus far, APS reductase has been found only in representatives of the phototrophic sulfur bacteria (21). Because these organisms are strict anaerobes, a sulfite oxidase is a priori not expected. Indeed, Thiele (20) was unable to demonstrate this enzyme in phototrophic sulfur bacteria. The occurrence of APS reductase only in the three groups of bacteria mentioned above leads to the question of possible evolutionary connections. The evolution of microorganisms has been comprehensively reviewed by Broda (3) from a bioenergetic point of view. Broda came to the conclusion that sulfate-reducing bacteria and phototrophic sulfur bacteria developed independently from "anaerobic phototrophs," and the

TABLE 6. *Comparison of the properties of the adenylyl sulfate reductases thus far purified*

Property[a]	*Desulfovibrio vulgaris* (references 16, 24)	*Thiobacillus denitrificans* (reference 2)	*T. thioparus* (reference 13)	*Thiocapsa roseopersicina*
Molecular wt	2.2×10^5	n[b]	1.7×10^5	1.8×10^5
Sedimentation constant	10.88×10^{-13}	n	n	9.7×10^{-13}
Nonheme iron per molecule	6–8	6–11	8–10	4
FAD per molecule	1	1	1	1
Heme per molecule	0	0	0	2
Specific activity (units/mg); $Fe(CN)_6^{3-}$ assay	7.3	8.5	6.4	8.7
*p*H optimum; $Fe(CN)_6^{3-}$ assay	7.4	7.2	7.4	8.0
K_m for sulfite; $Fe(CN)_6^{3-}$ assay	2×10^{-3}	1.5×10^{-3}	2.5×10^{-3}	1.5×10^{-3}
K_m for AMP; $Fe(CN)_6^{3-}$ assay	n	4.1×10^{-5}	10×10^{-5}	7.3×10^{-5}
K_m for $Fe(CN)_6^{3-}$	n	n	n	1.3×10^{-4}
K_m for cytochrome *c*	n	n	1.4×10^{-5}	3.3×10^{-5}
K_m for sulfite; cytochrome *c* assay	n	n	1.7×10^{-5}	9.3×10^{-5}
K_m for AMP; cytochrome *c* assay	n	n	2.5×10^{-6}	5.9×10^{-5}
*p*H optimum; cytochrome *c* assay	n	n	9.5	9.0

[a] FAD, Flavine adenine dinucleotide; AMP, adenosine 5'-monophosphate.
[b] n, Not given.

thiobacilli then developed from the phototrophic sulfur bacteria. On the other hand, the considerations of Peck (16, 17) concerning the evolution of sulfur metabolism lead to the conclusion that the thiobacilli developed from sulfate reducers. One may therefore assume that the common ancestors of these three groups already possessed APS reductase and that the enzyme lost its binding to the particle fraction together with its heme portion during the change from the phototrophic to the chemoorganotrophic or chemolithotrophic way of life. Within the group of the thiobacilli, the enzyme was lost completely by several species and functionally replaced by sulfite oxidase during the change to aerobiosis.

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