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Adenylylsulfate Reductase of *Chlorobium limicola*

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Abstract. Adenylylsulfate reductase was purified from *Chlorobium limicola.* The most important properties of the enzyme were compared with those of APS reductases from *Thiocapsa*, thiobacilli and sulfate-reducing bacteria.

Key words: Chlorobium — Adenylyl Sulfate Reductase — Sulfur Metabolism.

The presence of adenylylsulfate (APS) reductase activity n green phototrophic sulfur bacteria was first demonstrated by Thiele (1968). The formation of the reaction product, APS, was shown by Trüper and Peck (1970), who studied additional strains and proved that the possession of APS reductase appears to be a common property of Chlorobiaceae and Chromatiaceae, not, however, of the Rhodospirillaceae. So far, the only APS reductase purified and characterized from a phototrophic bacterium, is the enzyme of *Thiocapsa roseopersicina* (Trüper and Rogers, 1971). This enzyme in many respects resembles those isolated from *Desulfovibrio vulgaris* (Peck *et al.*, 1965), *Thiobacillus denitrificans* (Bowen *et al.*, 1966), and *T. thioparus* (Lyric and Suzuki, 1970), but — in contrast to these — contains two heme groups per molecule. The aim of the present study was to purify the enzyme from *Chlorobium limicola* and compare its properties with those of the *Thiocapsa* enzyme.

Methods

Chlorobium limicola neotype strain DSM 245 (Pfennig and Trüper, 1971) of the German Collection of Microorganisms, Göttingen, was used. For cultivation of the bacteria, preparation of cell free extracts, flavin and protein determinations, enzyme assays, as well as origin of biochemicals *cf.* Trüper and Rogers (1971).

Results and Discussion

Attempts to purify the enzyme following the method developed for *Thiocapsa* APS reductase (Trüper and Rogers, 1971) resulted in considerable losses of activity, even after changing elution gradients. Finally, the following purification procedure proved to be most suitable: The soluble protein of the cells was subjected to $(NH_4)_2SO_4$ fractionation. The precipitate between 50 and 70% saturation was dissolved in 0.01 M Tris-HCl,

Table 1. Efficiency of purification of APS reductase from *Chlorobium limicola*

	Total protein (mg)	Total activity[a]	Specific activity[a] (mg^{-1})	Purification factor	Yield (%)
Supernatant fluid of 105000×g	1072	431	0.40	1.0	100.0
50—70% $(NH_4)_2SO_4$ precipitate	46.3	128.5	2.78	6.9	29.8
Sephadex G-200 eluate	13.6	78.2	5.75	14.3	18.2

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

pH 8.5 and applied to a Sephadex G-200 column (90 cm, 1.5 cm ⌀). Elution from the column with the same buffer regularly showed 2 protein maxima the second of which always containing all of the APS reductase activity. The active fractions were united and concentrated by Diaflo-Ultrafiltration The efficiency of a typical purification procedure is shown in Table 1.

The specific activity in the soluble fraction before purification was more than twice as high than in *Thiocapsa* extracts (Trüper and Rogers, 1971), the maximum purification factor obtained was about 15. The preparation was much less stable than the enzyme of *Thiocapsa*, therefore the purification procedure demanded rapid handling. More than 30% of the activity were lost after 2 hrs at +22°C or after more than 2 days at +4°C. Only when kept at −20°C the enzyme was stable for several weeks. It lost 30—50% of its activity, however, by repeated thawing and freezing.

An incubation at +70°C for 2 min led to a 50% decrease in activity. Heating at +60°C for longer periods resulted in protein precipitation with simultaneous activity losses. Thus, heat steps are not useful for further purification.

The molecular weight of the enzyme was determined by column chromatography on Sephadex G-200 using ferritin, aldolase, albumin and chymotrypsinogen as reference proteins (Fig. 1). The preparation had a molecular weight of $2.1 \cdot 10^5$ Daltons. This value is in the same range as the molecular weights of other APS reductases (Table 3).

The absorption spectrum of the purified enzyme between 350 and 600 nm showed little differentiation (Fig. 2). In the oxidizing state a maximum at 412 nm and a shoulder at 550 nm may be seen. The reduced spectrum showed a maximum at 414 nm and shoulders at 523 and

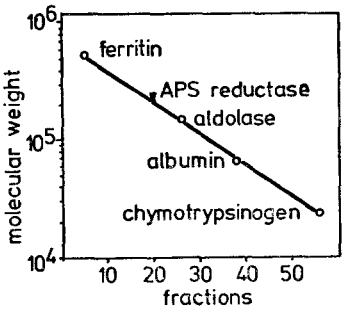

Fig. 1. Determination of the molecular weight of purified *Chlorobium limicola* APS reductase. *Method: cf.* Andrews (1964); reference proteins were from a Boehringer Combithek II of Boehringer Mannheim GmbH. Sephadex G-200 column (diameter 1.5 cm, length 90 cm) elution with 0.01 m Tris-HCl buffer, pH 8.5; volume of single fractions: 2.5 ml

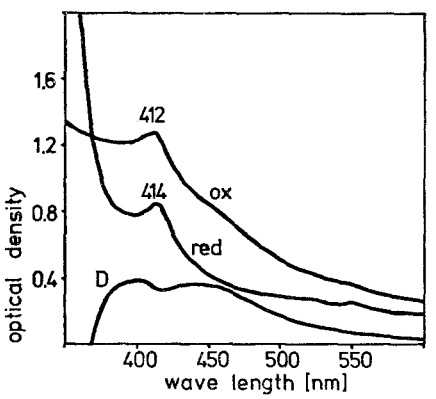

Fig. 2. Absorption spectra of purified APS reductase from *Chlorobium limicola*. *ox* oxidized enzyme; *red* enzyme reduced with 1 mg NaS_2O_2 per ml; *D* differential spectrum (oxidized minus reduced)

550 nm. Contrary to the findings of Trüper and Rogers (1971) with APS reductase of *Thiocapsa* the enzyme of *Chlorobium limicola* does obviously not contain heme groups, although the presence of non heme iron may be expected. The differential spectrum shows a maximum at 445 nm indicating the presence of flavin groups. These have been found in all APS reductases studied so far. From the differential coefficient for flavin adenine dinucleotide, an amount of about 1.4 moles FAD/mole enzyme was calculated.

In the ferricyanide coupled assay system for APS reductase all components proved to be essential (Table 2). Not so in the cytochrome *c* (of *Candida krusei*) coupled system, where an optical density increase

Table 2. Assay requirements of *Chlorobium limicola* APS reductase in the ferricyanide coupled system

Reaction mixture	Δ O.D. $\frac{1\text{ cm}}{420\text{ nm}}$ / min
Complete	0.105
Minus AMP	0.004
Minus sulfite	0.000
Minus enzyme	0.000

Table 3. Comparison of some properties of APS reductases thus far purified

	Desulfovibrio vulgaris[a]	*Thiobacillus denitrificans*[b]	*Thiobacillus thioparus*[c]	*Thiocapsa roseopersicina*[d]	*Chlorobium limicola*
Molecular weight	$2.2 \cdot 10^5$	n	$1.7 \cdot 10^5$	$1.8 \cdot 10^5$	$2.1 \cdot 10^5$
Flavin per molecule	1	1	1	1	1
Heme per molecule	0	0	0	2	0
Nonheme Fe	+	+	+	+	+
Spec. activity	7.3	8.5	6.4	8.7	5.8
pH optimum	7.4	7.2	7.4	8.0	8.7
K_m for sulfite	$2 \cdot 10^{-3}$	$1.5 \cdot 10^{-3}$	$2.5 \cdot 10^{-3}$	$1.5 \cdot 10^{-3}$	$9.1 \cdot 10^{-4}$
K_m for AMP	n	$4.1 \cdot 10^{-5}$	$10 \cdot 10^{-5}$	$7.3 \cdot 10^{-5}$	$2 \cdot 10^{-4}$
K_m for $Fe(CN)_6^{3-}$	n	n	n	$1.3 \cdot 10^{-4}$	$9.1 \cdot 10^{-5}$
Cytochrome *c* utilized as e^- acceptor	n	n	+	+	−

Specific activity, pH optimum and K_m values for ferricyanide assay.
[a] Data from Peck *et al.* (1965).
[b] Data from Bowen *et al.* (1966).
[c] Data from Lyric and Suzuki (1970).
[d] Data from Trüper and Rogers (1971).
n = not given.

was recorded only in the absence of AMP or sulfite, *i.e.*, the enzyme protein itself somehow reacted with cytochrome *c*. Horse heart cytochrome *c* was ineffective. The inability of *C. limicola* APS reductase to use cytochrome *c* as the electron acceptor was surprising, since APS reductase of *Thiobacillus thioparus* as well as that of *Thiocapsa* reacted readily with cytochrome *c* (Lyric and Suzuki, 1970; Trüper and Rogers, 1971).

The optimum pH of *C. limicola* APS reductase in the ferricyanide assay was found to be 8.7, and thus was higher than those reported from other APS reductases (*cf.* Table 3). This finding was surprising, since *Chlorobium* species prefer pH 6.8 for growth. With the purified enzyme, activities were measured by varying the concentrations of the reactants, one at a time. From the respective Lineweaver-Burk-plots the K_m values were calculated as $9.1 \cdot 10^{-4}$ M for sulfite, $2 \cdot 10^{-4}$ M for AMP and $9.1 \cdot 10^{-5}$ M for ferricyanide. As may be seen from Table 3 the affinity for sulfite and ferricyanide is higher in the *C. limicola* enzyme than in the *Thiocapsa* enzyme, while that for AMP is lower.

Substrate inhibition was found at AMP concentrations higher than 1.5 μmoles AMP/ml, while sulfite and ferricyanide were not inhibitory at higher concentrations tested.

Although the information on APS reductase from *C. limicola* we have collected so far is still limited, a comparison with the other APS reductases purified shows significant differences (Table 3). The most striking difference between the APS reductase of *Thiocapsa* and *C. limicola* is that the former contains two heme groups per molecule, the latter does not. The reason for this difference could be sought in the entirely different fine structure of the photosynthetic apparatus in Chromatiaceae and Chlorobiaceae, which might have implications in the molecular structure of the enzyme.

The fact that *C. limicola* APS reductase did not react with *Candida krusei* cytochrome *c* as the electron acceptor might point towards a higher acceptor specificity of the enzyme as compared with *Thiocapsa* APS reductase. Test with isolated genuine *Chlorobium* cytochromes will be done to explain this question.

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