Adenylylsulphate Reductase in a Dissimilatory Sulphate-reducing Archaeobacterium

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The recently described extremely thermophilic sulphate-reducing archaeobacterium 'VC-16' has been shown to contain adenylylsulphate reductase (EC 1.8.99.2). The enzyme was purified 26-fold by ammonium sulphate fractionation, DEAE-cellulose chromatography, hydrophobic chromatography and preparative isoelectric focusing. The enzyme preparation gave a single band on analysis by SDS-PAGE. The M_r was 160 000 and the enzyme contained 1 mol FAD, 8 mols non-haem iron and 6 mols labile sulphide per mol enzyme. Ferricyanide was used as electron acceptor. The optimal reaction temperature was 85 °C under the assay conditions used and the pH optimum of the enzyme reaction was 8.0. The K_m values for AMP and ferricyanide were 1 mM and 0.4 mM, respectively. The pI of the archaeobacterial adenylylsulphate reductase was 4.8.

INTRODUCTION

The ability to use sulphate as an external electron acceptor is a common feature of dissimilatory sulphate-reducing bacteria. Until recently, all sulphate-reducing bacteria were assumed to belong to the eubacteria (Fowler *et al.*, 1986); however, an archaeobacterial member of this physiological group was isolated and described by Stetter *et al.* (1987). This new organism, strain 'VC-16' (tentatively called '*Archaeoglobus fulgidus*'), is an extreme thermophile and grows at temperatures between 64 and 92 °C, with an optimum at 83 °C. Phylogenetically, strain VC-16 belongs in all probability to a new branch positioned between the methanogens and the sulphur-dependent, extremely thermophilic archaeobacteria (Stetter *et al.*, 1987; Achenbach-Richter *et al.*, 1987).

For dissimilatory sulphate reduction a common mechanism was proposed by Peck (1962). It involves the enzymes 'ATP sulphurylase' (sulphate adenylyltransferase, EC 2.7.7.4), adenylylsulphate (APS) reductase (EC 1.8.99.2) and bisulphite reductase (sulphite reductase, EC 1.8.99.1). We have obtained experimental evidence for the existence of these three enzymes in strain VC-16. APS reductase was studied first because of its key position in dissimilatory sulphate reduction. In this report we show the purification and partial characterization of the first APS reductase of archaeobacterial origin.

METHODS

Organisms and growth conditions. Packed deep-frozen cells of strain VC-16 were received from K. O. Stetter. The growth medium contained (l⁻¹) 0.34 g KCl, 2.75 g MgCl₂·2H₂O, 3.45 g MgSO₄·7H₂O, 0.25 g NH₄Cl, 0.14 g CaCl₂·2H₂O, 0.14 g K₂HPO₄, 18 g NaCl, 5 g NaHCO₃, 2 mg Fe(NH₄)₂(SO₄)₂·7H₂O, 2 mg Ni(NH₄)₂(SO₄)₂, 1 mg Resazurin, 10 ml trace mineral solution (Balch *et al.*, 1979), 0.5 g yeast extract (Difco), 1 g L(+)-lactate and 0.5 g Na₂S. The pH was adjusted with H₂SO₄ to 6.9. The bacteria were grown anaerobically in batch cultures in 300 l enamel-protected fermenters (HTE Bioengineering) at 85 °C.

Abbreviation: APS, adenylylsulphate

Enzyme assay. APS reductase activity was assayed as described by Trüper & Rogers (1971), with some modifications. The reaction mixture contained 50 mM-Tris/HCl, pH 8.0, 5 mM- $K_3Fe(CN)_6$, 10 mM-EDTA, 2 mM-AMP and 30 mM- Na_2SO_3 . The sulphite solution was always freshly prepared in 50 mM-Tris/HCl, pH 8.0, containing 5 mM-EDTA. The test volume was 1 ml. Absorbance was measured in 1 cm glass cells at 420 nm.

Enzyme purification. All procedures were done at 4 °C. Packed cells were taken up in about twice their volume of 50 mM-Tris/HCl buffer, pH 8.0, and disrupted by ultrasonic treatment. The broken cell mass was then centrifuged at 17000 *g* for 20 min to remove the larger fragments. By subsequent centrifugation of the supernatant at 140000 *g* for 2 h a clear supernatant and a pellet consisting of smaller membrane fragments were obtained.

Ammonium sulphate was added to the supernatant to 50% saturation. The precipitated protein was separated from the supernatant by centrifugation (17000 *g*, 20 min) and discarded. The supernatant was applied to a column of Phenyl-Sepharose CL-4B (1.5 × 20 cm) equilibrated with 50 mM-Tris/HCl, pH 8.0, containing ammonium sulphate at 50% saturation. The enzyme was eluted with a linear gradient of ammonium sulphate between 50% and 0% saturation. The active enzyme fractions were pooled and desalted by a Sephadex G-25 column. The eluate was applied to a column of DEAE-cellulose (5 × 10 cm) equilibrated with 50 mM-Tris/HCl, pH 8.0 and eluted with a gradient of 0–300 mM-NaCl in the same buffer. Active APS reductase fractions were pooled and concentrated by ultrafiltration using an Amicon PM 30 filter. The concentrated protein solution was desalted by a Pharmacia PD-10 column and subjected to preparative isoelectric focusing as described by Winter *et al.* (1975), with minor modification, in an LKB Multiphor 2117. Ultradex (LKB) (4 g) was mixed with 95 ml H_2O and 5 ml Servalyt (pH 4–6) and then poured onto a tray. The gel slurry was evaporated to 65% of its initial weight. A sample (4 ml) was applied to the gel with an applicator. The cathode and the anode electrode strips were soaked in 1 M-NaOH and in 1 M- H_3PO_4 , respectively. The isoelectric focusing was run at 4 °C at 1500 V and 20 mA for 16 h. The focused protein in the gel was visualized by placing a dry filter paper on top of the gel; small amounts of protein were absorbed by the paper and afterwards stained with Coomassie brilliant blue R-250. The part of the gel containing APS reductase was applied to a small column (1.5 × 6 cm) and the protein was eluted with 50 mM-Tris/HCl, pH 8.0.

SDS-PAGE. This was done according to the method of Weber & Osborn (1969) in order to determine the subunit composition of the purified enzyme. The gel was run at pH 7.1 in a 7.5% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS. A low- M_r calibration kit (14000–94000, Pharmacia) was used for standards. The gel was stained for protein with Coomassie brilliant blue R-250.

M_r determination by FPLC. This was done with an analytical FPLC column (Superose 6, Pharmacia) and a flow rate of 24 ml h^{-1} . The column was equilibrated with 50 mM-potassium phosphate buffer, pH 7.5, containing 0.1 M-NaCl. The proteins of Boehringer Combithek 2 were used as references.

Analytical procedures. Flavin was identified by chromatography according to Kilgour *et al.* (1957), with some modifications. The organic phase of the following solvent system was used: n-butanol/acetic acid/water (40:10:50, by vol.). The APS reductase was denatured by heat treatment (15 min, 120 °C), chromatographed on Cellulose MN 300 plates (Macherey & Nagel) and the R_f value of the released flavin component was compared with that for FAD and FMN. The flavins were detected by their fluorescence at 254 nm.

The concentration of the flavin was determined by the method of Rao *et al.* (1967), and non-haem iron by the ferrozine method (Sigma) in 1 cm cells at 560 nm. Labile sulphide was determined according to King & Morris (1966). Absorption spectra were determined in a Beckman M 6 double beam spectrophotometer using 1 cm quartz cells. Reduced spectra were obtained by addition of a few crystals of sodium dithionite to the protein solution.

Protein assay. Protein concentration was measured by the method of Bradford (1976) with bovine serum albumin as a standard.

RESULTS

Purification of the APS reductase

The supernatant of the 50% ammonium sulphate precipitation was loaded on a Phenyl-Sepharose CL-4B column for hydrophobic interaction chromatography (Fig. 1). After pooling and desalting the fractions of the active peak, the solution was applied to a DE-52 column. Fractions containing APS reductase were pooled, concentrated and subjected to preparative isoelectric focusing. The purification procedures are summarized in Table 1.

Properties of the APS reductase

The purified enzyme was analysed by SDS-PAGE. Only one protein band was visible with an apparent M_r of 80 000. The M_r of the native protein, as determined by FPLC, was approximately 160000. The temperature optimum for activity was 85 °C under the assay conditions used. Higher temperatures were inhibitory and no activity was found below 55 °C (Fig. 2). The pH optimum was 8.0.

Fig. 1. Elution profile of APS reductase from strain VC-16 on a Phenyl-Sepharose CL-4B column (1.5×20 cm). Experimental details are given in Methods. The flow rate was 20 ml h^{-1} and 4 ml fractions were collected. $\circ$, APS reductase activity; $\bullet$, protein monitored by recording the absorbance at 280 nm.

Table 1. Purification of APS reductase from the sulphate-reducing archaeobacterium strain VC-16

Activity is expressed in units (U), where 1 U is $1 \mu\text{mol Fe(CN)}_6^{3-}$ reduced min^{-1} . IEF, isoelectric focusing.

Step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Crude extract	60	217.5	2364	0.092	1	100
Supernatant (140000 g)	53	213.5	1654.8	0.129	1.4	98.2
Ammonium sulphate	44	159	822.8	0.193	2.1	73.3
Phenyl-Sepharose	52	143.7	115	1.25	13.6	66.2
DEAE-cellulose	48	134.9	71	1.9	20.7	62.2
Preparative IEF	4	82.8	34.6	2.39	26	38.2

With the purified enzyme, activities were measured by varying the concentrations of the substrates. The K_m values from respective Lineweaver-Burk plots were 1 mM for AMP and 0.4 mM for ferricyanide. However, by varying the concentration of sulphite, a sigmoid substrate saturation curve was obtained and thus no K_m value for sulphite could be calculated.

The nucleotide specificity of APS reductase was determined by replacing AMP with other nucleotides (Table 2). DeoxyAMP and GMP replaced AMP with relative efficiency, while the enzyme showed no activity with UMP.

The absorption spectrum of the purified APS reductase is shown in Fig. 3. The maximum at 390 nm in the oxidized state led us to expect iron-sulphur clusters to be part of the enzyme. The absorption difference in the range from 400 to 500 nm suggested the presence of flavin groups, according to Rao *et al.* (1967). For the spectrophotometric characterization of the flavin moiety, the purified enzyme solution (5 ml) was treated with saturated urea for 48 h at 4°C . The resulting yellow mixture was applied to a Sephadex G-25 column and eluted with 10 mM-Tris/HCl, pH 7.0. One yellow band appeared, which showed characteristic properties of flavins. Maxima occurred at 446, 364 and 280 nm and minima at 404 and 325 nm in the oxidized state. Reduction of the flavin by addition of dithionite resulted in a strong decrease in absorption in the range between 400 and 500 nm. The flavin was identified chromatographically as FAD. The

Fig. 2

Fig. 2. Temperature optimum curve for APS reductase activity from sulphate-reducing archaeobacterium strain VC-16, under the assay conditions used.

Fig. 3

Fig. 3. Absorption spectra of oxidized and reduced APS reductase from sulphate-reducing archaeobacterium strain VC-16. The amount of protein was 1.4 mg. ox, oxidized; red, reduced.

Table 2. Nucleotide specificity of purified APS reductase

Nucleotide	Activity (%)
AMP	100
IMP	41
GMP	68
CMP	24
UMP	0
DeoxyAMP	74
ADP	50
ATP	24

amount of FAD per molecule was determined as described by Rao *et al.* (1967). Non-haem iron and labile sulphide were determined as described above. Each mol of APS reductase from strain VC-16 contained 1 mol FAD, 8 mols non haem iron and 6 mols labile sulphide. The isoelectric point of the enzyme protein (pI) was 4.8, as determined by preparative isoelectric focusing.

DISCUSSION

APS reductase has a key position in dissimilatory sulphur metabolism. The enzyme has been proven to occur in all dissimilatory sulphate-reducing eubacteria, in some thiobacilli and in most phototrophic sulphur bacteria. So far, it has been purified from *Desulfovibrio vulgaris* (the strain used by Peck *et al.*, 1965, does not belong to the species *D. desulfuricans*): *Thiobacillus denitrificans* (Bowen *et al.*, 1966); *Thiobacillus thioparus* (Lyric & Suzuki, 1970); *Thiocapsa roseopersicina* (Trüper & Rogers, 1971); *Chlorobium limicola f. thiosulfatophilum* (Kirchhoff & Trüper, 1974); and *Desulfobulbus propionicus* (Stille & Trüper, 1984). These eubacterial enzymes are iron-sulphur flavoproteins with M_r values between 170000 and 218500, 1 mol FAD, 4–13 mols non-haem iron and 4–12 mols labile sulphide per mol enzyme protein.

The results of the M_r determination and also the molar ratio of the constituents of the archaeobacterial APS reductase reveal strong similarities with the enzymes from eubacteria. Haem groups, as were reported by Trüper & Rogers (1971) for the enzyme from *Thiocapsa roseopersicina*, could not be detected.

The APS reductase from strain VC-16 appeared to be a dimer composed of two equal subunits of M_r 80000. It is not or only slightly bound to the membrane. This agrees with the results reported from eubacterial sulphate reducers. In contrast, Trüper & Peck (1970) found that the enzyme is membrane bound in phototrophic bacteria.

Table 3. *Comparison of the properties of the APS reductases purified so far*
 K_m values (mM) were determined with ferricyanide as electron acceptor. NG, Not given.

Property	<i>Desulfovibrio vulgaris</i> ^a	<i>Thiobacillus denitrificans</i> ^b	<i>Thiobacillus thioparus</i> ^c	<i>Thiocapsa roseopersicina</i> ^d	<i>Chlorobium limicola</i> ^e	<i>Desulfobulbus propionicus</i> ^f	'VC-16'
M_r	220 000	NG	170 000	180 000	210 000	175 000	160 000
Non-haem iron per molecule	6-8	6-11	8-10	4	NG	8	8
FAD per molecule	1	1	1	1	1.4	1	1
Haem per molecule	0	0	0	2	0	0	0
pH optimum	7.4	7.2	7.4	8.0	8.5	7.7	8.0
K_m for sulphite	2	1.5	2.5	1.5	0.91	1.3	NG
K_m for AMP	NG	0.041	0.1	0.073	0.2	0.09	1
K_m for $\text{Fe}(\text{CN})_6^{3-}$	NG	NG	NG	0.13	0.091	0.089	0.4

^aPeck *et al.*, 1965; ^bBowen *et al.*, 1965; ^cLyric & Suzuki, 1970; ^dTrüper & Rogers, 1971; ^eKirchhoff & Trüper, 1974; ^fStille & Trüper, 1984.

An exceptional character of the archaeobacterial APS reductase is its extreme thermophily: eubacterial APS reductases are generally described as mesophilic enzymes. The optimal reaction temperature of the archaeobacterial enzyme (found to be 85 °C under the assay conditions used) is very close to the optimal growth temperature of this organism (83 °C). This high temperature did not allow us to use cytochrome *c* (either from horse heart or from *Candida krusei*) instead of ferricyanide as electron acceptor in the reaction. The use of these cytochromes has been reported by Lyric & Suzuki (1970), Trüper & Rogers (1971), Bramlett & Peck (1975) and Stille & Trüper (1984). A second obvious difference with respect to the eubacterial enzymes is the substrate affinity of the strain VC-16 APS reductase. The K_m values for AMP and ferricyanide are significantly higher than those of all eubacterial enzymes, although one would expect that the reactivity of the substrates is increased at high temperatures. An unusual feature as compared with APS reductases so far described is the sigmoid substrate saturation curve obtained by varying the concentration of sulphite. Further kinetic studies are necessary to explain this effect. An allosteric regulation of the enzyme does not seem to be feasible, as the enzyme reaction proceeds in the opposite (i.e. reductive) direction *in vivo*.

Peck *et al.* (1965) reported that only purine nucleotides could be used as substrates by the APS reductases. This result was confirmed by Bowen *et al.* (1966), Trüper & Rogers (1971) and Stille & Trüper (1984). The archaeobacterial enzyme shows the lowest activities with pyrimidine nucleotides. In contrast, activity with CMP and UMP was shown by Lyric & Suzuki (1970) in *Thiobacillus thioparus*. Surprisingly the strain VC-16 enzyme showed high activity with ADP as a substrate. A probable reason for this is the hydrolysis of the nucleotide at the reaction temperature of 85 °C.

The existence of ATP sulphurylase, bisulphite reductase (unpublished results) and APS reductase in strain VC-16 leads to the conclusion that the pathway for dissimilatory sulphate reduction is identical with that known from eubacteria. It seems that this pathway was formed very early during evolution. However, another explanation may be lateral gene transfer. Further research at the molecular level such as sequence analysis of the different APS reductases is under way to investigate the evolution of this key enzyme of sulphur metabolism.

This work is dedicated to John R. Postgate at the occasion of his 65th birthday, in recognition of his vast contribution to our knowledge of dissimilatory sulphate reduction.

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