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PURIFICATION AND PROPERTIES OF ATP-SULPHURYLASE FROM *NITROBACTER AGILIS*

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SUMMARY

1. As in other bacteria, SO_4^{2-} in *Nitrobacter agilis* is first activated by ATP, before it is reduced. Both ^{35}S-labelled adenosine 5'-phosphosulphate ([^{35}S]APS) and ^{35}S-labelled 3'-phosphoadenosine 5'-phosphosulphate ([^{35}S]PAPS) are produced from $^{35}SO_4^{2-}$ and ATP in cell-free extracts of this bacterium. The enzymes catalysing these reactions are found in the supernatant fractions left after centrifuging cell extracts at 144 000 $\times$ *g* for 4 h.

2. In addition to the ^{35}S radioassay technique, the bioluminescence method of the firefly *Photinus pyralis* (luciferin–luciferase) was used to follow ATP production from APS and PP_i during the purification of the enzyme.

3. ATP-sulphurylase ATP- sulphate adenyltransferase, EC 2.7.7.4), which catalyzes the formation of APS, was purified about 820-fold by DEAE-cellulose, DEAE-Sephadex and Sephadex G-200 column chromatography, followed by the sucrose density gradient. A starch-gel electrophoresis of the purified enzyme produced a single protein band.

4. K_m values for the purified enzyme are as follows: ATP, $1.4 \cdot 10^{-3}$ M; APS, $2.5 \cdot 10^{-5}$ M; PP_i, $1.2 \cdot 10^{-4}$ M; $MgCl_2$ $3.5 \cdot 10^{-4}$ M.

5. The molecular weight of the enzyme is about 700 000 and the sedimentation coefficient is 12.8 S. The pH optimum is 7.4.

6. The enzyme was inhibited by group IV anions. The following thiol reagents stimulated activity: GSH, L-cysteine, β-mercaptoethanol and Cleland's reagent (dithiothreitol). *p*-Chloromercuribenzoate was a strong inhibitor and this effect was reversed by thiol compounds.

Abbreviations: APS, adenosine 5'-phosphosulphate; PAPS, 3'-phosphoadenosine 5'-phosphosulphate; PCMB, *p*-chloromercuribenzoate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-(5-phenyloxazolyl-2)-benzene; Cleland's reagent, dithiothreitol; NEM, N-ethylmaleimide.

INTRODUCTION

ATP-sulphurylase (ATP: sulphate adenylyltransferase, EC 2.7.7.4) catalyses the activation of sulphate by ATP forming adenosine 5'-phosphosulphate (APS) and liberating PP_i. This reaction, first reported in yeast[1] and liver[3], is the first step in the metabolism of sulphate in both assimilatory and dissimilatory sulphate-reducing systems. A second enzyme, APS-kinase (ATP: adenylyl sulphate 3'-phosphotransferase, EC 2.7.1.25) further activates APS to 3'-phosphoadenosine 5'-phosphosulphate (PAPS) in the assimilatory system. Although ATP-sulphurylase and APS-kinase from yeast have been characterized by ROBBINS AND LIPMANN[4,5] and WILSON AND BANDURSKI[6], comparatively little is known about sulphate reduction in the nitrifying bacteria. We have previously studied the incorporation of $^{35}SO_4^{2-}$ into APS and PAPS in the cell-free extracts of Nitrosomonas[7,8]. In this paper results are presented for the sulphate-activating enzyme in Nitrobacter. Thus, ATP-sulphurylase has been purified and characterized with the aid of the luciferin–luciferase system of the firefly for determining ATP production from APS and PP_i.

MATERIALS AND METHODS

Chemicals

1,4-Bis-(5-phenyloxazolyl-2)benzene (POPOP) and 2,5-diphenyloxazole (PPO) were obtained from Packard Instrument Co., Australia. Carrier-free $^{35}SO_4^{2-}$ (specific activity 14 mC/mmole) was purchased from the Radiochemical Centre, Amersham, England. Yeast inorganic pyrophosphatase (800 units/mg protein; 1 unit will liberate 1 μmole of PP_i per min) and creatine phosphokinase (disodium salt) (1 mg will transfer 15–50 μmoles phosphate from phosphocreatine to ADP) were products of Nutritional Biochemical Corp., Cleveland, U.S.A. AMP, ADP, ATP, bovine serum albumin, firefly lanterns, EDTA (sodium salt) and *p*-chloromercuribenzoate (PCMB) were purchased from Sigma Chemical Co., Mo., U.S.A. Sephadex products were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and from Balston Ltd., England, and DEAE- and CM-celluloses from Whatman Co., England. Cytochrome *c* (horse heart Type III) and the following crystalline enzymes; alcohol dehydrogenase, aldolase, catalase, fumarase, glyceraldehyde-3-phosphate dehydrogenase, urease and lactate dehydrogenase, were purchased from Calbiochem, Calif., U.S.A. Pyridine sulphur trioxide was obtained from Aldrich Chemical Co. Inc., Wisc., U.S.A. All other chemicals were of analytical grade.

Preparation of APS

APS was prepared by the method of BADDILEY *et al.*[9] from pyridine sulphur trioxide and AMP. The reaction mixture containing 40 mg AMP and 1.5 mg pyridine sulphur trioxide was incubated for 15 min at 21°. APS was isolated from this reaction mixture by high-voltage electrophoresis on Whatman 3 MM paper in 0.1 M sodium citrate buffer (pH 5.1) at 1.5 kV for 90 min. APS was eluted in distilled water (20 ml) and concentrated to 2 ml in a rotary evaporator at 28° for 4 h.

Preparation of PAPS

This was prepared from ATP and carrier-free $^{35}SO_4^{2-}$ using a sulphate-activat-

ing enzyme complex (Fraction III) from baker's yeast[10]. About 165 μmoles of [^{35}S]-PAPS, formed after a 9-h incubation at 37°, was isolated from the reaction mixture by passing through a Dowex column, as described by HODSON AND SCHIFF[11].

Source of enzyme

The nitrifying bacteria used were *Nitrosomonas europaea* and *Nitrobacter agilis*, kindly supplied by Dr. Jane Meiklejohn, Rothamsted Experimental Station, Harpenden, England, and by Dr. M. I. H. Aleem, University of Kentucky, Lexington, U.S.A., respectively. Pure cultures of Nitrosomonas were grown in a pH-stat unit at pH 7.7, as described by LOVELOCK AND NICHOLAS[12], and Nitrobacter in batch cultures at pH 7.8 (ref. 13). The cells, harvested at 35 000 × *g* in a Sorvall RC-2 refrigerated centrifuge fitted with a continuous flow head, were washed 3 times in cold 0.05 M phosphate buffer (Na^+–K^+, pH 7.4) before use.

Methods for disrupting cells

Full details are given in RESULTS, but a brief description of the equipment used is given here. An MSE ultrasonic titanium probe (20kcycles/sec) was used to disrupt the cells. A sample (cells 1 g wet weight per 5 vol. of 0.05 M Tris–HCl buffer (pH 7.4)) was placed in a double-walled glass container through which ice-cold water was continuously circulated. An Aminco French pressure cell (40 ml capacity), fitted with a rotor-driven press and set at 25 000 lb/inch^2 and 4°, was also used to crush the cells. Other aliquots of the cell suspension were shaken for 30 sec with Ballitini glass beads (size 16, Sigma Chemical Co.) in a Mickle disintegrator. The cell homogenates were then centrifuged first at 20 000 × *g* in a Sorvall SS1 unit and then at 144 000 × *g* in a Spinco Model L (rotor No. 50) ultracentrifuge.

Sucrose density gradient

Density gradient centrifugation was carried out in the swingout rotor (SW-39L) in a Spinco Model L ultracentrifuge, according to the method of MARTIN AND AMES[14]. Linear gradients of sucrose solutions were prepared in cellulosenitrate tubes (from 10 to 50% or from 35 to 45% (w/v)). Gradients were stored at 4° for 8 h prior to use. The sample (1 ml) was layered on the gradient and, after centrifuging for 16 h at 150 000 × *g*, the fractions were collected by puncturing the bottom of the cellulosenitrate tube. The absorption of these portions was determined at 280 and 418 nm in a Schimadzu spectrophotometer. Purified beef liver catalase (EC 1.11.1.6) was used as an internal marker for determining sedimentation coefficients of the ATP-sulphyrylase. The sedimentation coefficient for catalase is 11.3 S (ref. 14).

Starch-gel electrophoresis

Starch gel was prepared as described by KOHN[15], using 0.1 M Tris–citrate buffer (pH 8.1). The electrophoresis was carried out at 2° at a constant current of 20 mA (350 V) for 3 h. After electrophoresis the starch gel was cut horizontally into three slices. The top and bottom layers were stained with 0.1% (w/v) nigrosin in methanol–distilled water–glacial acetic acid (1:1:1, by vol.). The middle slice was cut vertically into 1-cm portions starting from the origin. These were homogenized in 0.05 M Tris–HCl (pH 7.4) buffer and tested individually for enzyme activity.

Methods for concentrating protein fractions

The dilute enzyme fractions pooled after the column chromatography were rapidly concentrated under N_2 by the use of ultrafiltration apparatus (Amicon Corp., Mass. U.S.A.), employing a Diaflo membrane (100 Å).

Molecular weight

The molecular weight of the enzyme was determined by the molecular sieve procedure of ANDREWS[16] and DETERMAN[17]. Sephadex G-200 was allowed to swell for 15 days in 0.02 M Tris–HCl buffer (pH 7.4) containing 0.05 M KCl and was then packed into a column (2.5 cm × 90 cm; bed volume, 310 ml). The column was washed with 5 l of equilibrating buffer (0.05 M Tris–HCl and 0.15 M KCl (pH 7.4)) and then calibrated with cytochrome *c* (horse heart Type III), bovine serum albumin, lactate dehydrogenase, catalase, aldolase, fumarase, urease, α-crystallin and blue dextran. The purified ATP-sulphurylase, dissolved in 2.5 ml of buffer, was applied to the column and eluted with the same buffer. The elution volume of the enzyme was then determined.

DEAE-cellulose and Sephadex G-200 column chromatography

DEAE-cellulose-32, after precycling with 0.5 M NaOH and then 0.5 M HCl, was equilibrated with 0.02 M Tris–HCl buffer (pH 7.4) before packing into columns. A cellulose to protein ratio of 1 ml:20 mg was used.

Crystalline bovine serum albumin, 4 g dissolved in 5 ml of 0.05 Tris–HCl buffer (pH 7.4), was applied to Sephadex G-200 columns that had been previously equilibrated with 0.05 M Tris–HCl buffer containing 2 mM EDTA (sodium salt). Elution with the same buffer was continued until the effluent no longer absorbed at 280 nm. Recovery of the enzyme from the columns was considerably improved by this pretreatment.

Assay for enzymic activity of the enzyme ATP sulphurylase

(1) Enzyme activity was determined by following the production of [^{35}S]APS and [^{35}S]PAPS from ATP and $^{35}SO_4^{2-}$. The reaction was measured in the direction of APS synthesis by including pyrophosphatase, which hydrolyzes the PP_i formed to P_i (refs. 6, 7). The complete reaction mixture in 0.5 ml contained (μmoles): 100 Tris–HCl (pH 7.4); 20 $MgCl_2$; 0.2 EDTA (sodium salt); 20 ATP; 10 Na_2SO_4; 50 creatine phosphate; and 0.15 enzyme unit of creatine phosphokinase (disodium salt); 0.10 enzyme unit of pyrophosphatase (from yeast, 800 units/mg protein); 5 μC $^{35}SO_4^{2-}$ and a crude extract of cells of either Nitrobacter, Nitrosomonas or yeast in 0.05 M Tris–HCl (pH 7.4). Purified enzyme from Nitrobacter was assayed in the same way.

The mixture was incubated at 37° for 40 min, when the reaction was terminated by immersing first in boiling water for 90 sec and then in ice–salt for 5 min. Protein was removed by centrifuging at 2000 × *g* for 15 min and 0.05-ml aliquots of the supernatant fraction were applied to a Whatman 3MM chromatography paper. Electrophoresis in 0.1 M sodium–citrate buffer (pH 5.1) was continued for 90 min at 1.5 kV (ref. 18). [^{35}S]APS, [^{35}S]PAPS, $^{35}SO_4^{2-}$ were detected by cutting the dried paper into sections (1.5 cm × 2.5 cm) and counting in a toluene solution (5 ml) of PPO (3.0 g/l) and POPOP (0.3 g/l) in a Packard Tri-Carb liquid scintillation spectrometer (Model 3375).

(2) Enzyme activity of purified fractions was also determined by following the production of ATP from APS and PP_i (refs. 23, 24). The luciferin–luciferase system of the firefly *Photinus pyralis* was used to determine the ATP produced in the assay. The light emitted was measured in an EKCO scintillation unit (EKCO Electronics Ltd., England). The 3-ml reaction mixture in the vial was made up as follows (ml): 1.76, 0.1 M sodium phosphate (pH 7.4); 1.0, 0.5 M arsenate buffer (pH 7.2); 0.1, 0.40 M Tris–HCl (pH 7.4); 0.05, 0.006 M $MgCl_2$. PP_i (sodium salt) (0.02 ml, 50 nmoles) and APS (0.05 ml, 0.3 μmole) were added to the reaction mixture, which was then equilibrated at 20° in a water bath for 15 min. The luciferin–luciferase extract (0.02 ml), prepared by the method of STANLEY AND WILLIAMS[19], was then added to initiate the reaction.

The vial was shaken and quickly placed in the well of the EKCO liquid scintillation spectrometer. Counting was started immediately, recording 20 counts each of 0.1 min; then the purified ATP-sulphurylase (amount used depending on the activity of the enzyme) was added and a further 20 counts, each of 0.1 min, were recorded. A standard curve for ATP was made with each new batch of firefly enzyme.

Protein determination

Protein in crude extracts was determined by the method of LOWRY *et al.*[20]. However, in more purified fractions the method of WARBURG AND CHRISTIAN[21] was used.

Spectral studies

Absolute spectra of purified fractions of ATP-sulphurylase were determined in a Unicam SP800 spectrophotometer, fitted with an expansion recorder (Servoscribe type RE 511) at 20 mV.

RESULTS

Distribution of APS and PAPS in cell-free extracts

The sulphate-activating enzymes were found mainly in the supernatant fraction (Table III, Fraction III, S144) left after centrifuging the cell extract (Fraction II, S20) for 4 h, as shown in Table I. Enzymic activity was retained in Fraction III (S144) after dialysis for 20 h against 0.05 M Tris–HCl buffer (2 l) containing 1 μmole of β-mercaptoethanol.

The extracts prepared by either the lysozyme techniques[22], French pressure cell or ultrasonic treatment (20 kcycles/sec) were extremely viscous, indicating that the cell nucleoplasm had not been disrupted. These extracts were, however, readily clarified by adding deoxyribonuclease. The 15-min ultrasonic treatment (20 kcycles/sec) followed by treatment in an Aminco French pressure cell resulted in an extract that had the highest enzymic activity. Thus, in all subsequent experiments the Nitrobacter cells were disrupted by this method.

Comparison of [^{35}S]APS and [^{35}S]PAPS in Nitrosomonas, Nitrobacter and baker's yeast

The cell-free extract of Nitrosomonas was prepared by the ultrasonic treatment of the cells suspended in 0.05 M Tris–HCl buffer (pH 7.4), as described earlier[8]. The Nitrobacter cells were disrupted as described above and those of baker's yeast by the

TABLE I

LOCALIZATION OF THE SULPHATE-ACTIVATING ENZYME

Washed cells (5.0 g wet wt., 42 mg protein) suspended in 0.05 M Tris–HCl buffer (pH 7.4), containing EDTA (sodium salt) (1 mM) and GSH (0.1 mM), were treated with an ultrasonic probe (20 kcycles/sec) for 15 min, followed by disruption in a French pressure cell (see MATERIALS AND METHODS). The homogenate thus obtained was centrifuged at 10 000 × *g* for 30 min and the pellet resuspended in 2 ml 0.05 M Tris–HCl buffer (pH 7.4), containing 1 μg deoxyribonuclease (P10) for 1 h. Supernatant fraction (S10) was centrifuged at 144 000 × *g* for 4 h (S144) and the pellet portion resuspended in the Tris–HCl buffer (P144). A portion of the S144 fraction was dialyzed against the Tris–HCl buffer containing 1 μmole β-mercaptoethanol for 16 h. Enzyme assays are detailed in MATERIALS AND METHODS.

Fraction	*Specific activity (nmoles [35S]APS and [35S]PAPS produced in 40 min per mg protein)*	
	APS	*PAPS*
Supernatant (S10/30 min)	8.6	16.7
Supernatant (S144/4 h)	11.3	24.3
Pellet (P144/4 h)	2.0	3.1
Supernatant (S144/4 h) dialyzed for 20 h against Tris–HCl buffer	12.9	26.5

TABLE II

COMPARISON OF [35S]APS AND [35S]PAPS IN *Nitrosomonas europaea*, *Nitrobacter agilis* AND BAKER'S YEAST

Extracts were prepared from washed cells of Nitrosomonas, Nitrobacter and yeast, respectively, in 0.05 M Tris–HCl buffer (pH 7.4), containing EDTA (sodium salt) (1 mM) and GSH (0.1 mM), as described in MATERIALS AND METHODS. ^{35}S-labelled nucleotides were determined in the supernatant fraction left after centrifuging the extracts at 20 000 × *g* for 30 min. Aliquots of the supernatant fraction were incubated with $^{35}SO_4^{2-}$, and the products separated by high-voltage electrophoresis were determined in a liquid scintillation spectrometer (see MATERIALS AND METHODS).

Source	*[35S]APS produced (nmoles/0.5 ml)*	*[35S]PAPS produced (nmoles/0.5 ml)*	*Specific activity of ATP-sulphurylase (nmoles [35S]APS produced/40 min per mg protein)*
I. *Nitrosomonas europaea*, 5 g wet wt. cells per 25 ml Tris–HCl buffer, disrupted by the ultrasonic probe (20 kcycles/sec) for 10 min followed by treatment in French pressure cell	4.4	7.1	0.9
II. *Nitrobacter agilis*, 5 g wet wt., cells extracted prepared as in I	9.3	16.9	3.7
III. Yeast cells, 5 g wet wt. suspended in 25 ml of the Tris–HCl buffer and broken in an Aminco French pressure cell*	25.6	46.4	50

* For details see ref. 10.

French pressure cell method[10]. Supernatant fractions from the three microorganisms, prepared by centrifuging the homogenates at 20 000 × *g* for 1 h, were incubated in reaction mixtures containing ATP, $^{35}SO_4^{2-}$ and pyrophosphatase, as in the radioassay procedure. Both [^{35}S]APS and [^{35}S]PAPS were detected in all extracts; the highest values were found in baker's yeast (25.6 nmoles APS and 49.9 nmoles PAPS per mg protein), followed by those of Nitrobacter (9.3 nmoles APS and 16.9 PAPS per mg protein), and the lowest amounts were recorded in Nitrosomonas (4.4 nmoles APS and 7.1 nmoles PAPS per mg protein) (Table II). In view of the very low activity in the cell-free extracts of Nitrosomonas, enzyme purification was attempted for *Nitrobacter agilis*.

Assay of ATP from APS and PP_i

The bioluminescent method of BALHARRY AND NICHOLAS[23,24] was used to determine ATP formation from APS and PP_i by ATP sulphurylase. The firefly assay

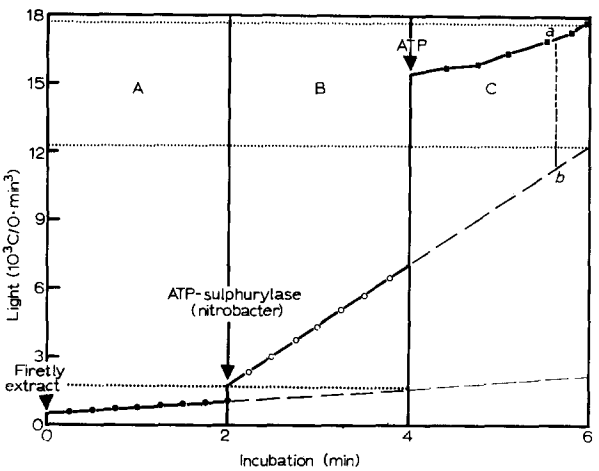

Fig. 1. Determination of ATP-sulphurylase by the bioluminescence technique. The reaction vial contained (in μmoles): sodium phosphate buffer (pH 7.5), 176; sodium arsenate buffer (pH 7.2), 500; Tris–HCl buffer (pH 7.4), 40; $MgCl_2$, 0.3; as well as PP_i (sodium salt), 50 nmoles; and APS, 0.3 μmole; in 3 ml. The reaction was initiated by adding 0.02 ml firefly extract at 20°. Nitrobacter ATP-sulphurylase (0.05 ml) and ATP (100 pmoles) were added as indicated. Light flashes were recorded in an EKCO liquid scintillation spectrometer. An enzyme unit is the amount producing 1 nmole ATP/min per mg protein. (1) Segment A: ●—●, ATP-sulphurylase activity of the firefly extract. The line fitted to these points has been extrapolated into Segments B and C. (2) Segment B: ○—○, the combined ATP-sulphurylase activities of the firefly and Nitrobacter. The line fitted to these points has been extrapolated into Segment C. (3) Segment C: ■—■, a cubic function, with the inflexion at 6 min, is fitted to the data so that the light produced per 0.1 min by the ATP internal standard at 5.5 min can be determined *(a minus b)*.

method is illustrated in Fig. 1. The light reaction, initiated by adding 0.02 ml of the firefly extract to the reaction mixture containing only APS and PP_i (Segment A), resulted from a low ATP-sulphurylase activity in the firefly extract. The Nitrobacter enzyme fraction (Table III, Fraction III, 0.05 ml) was added at 2 min (Segment B); thus, the rate of light production in this segment is a measure of the ATP-sulphurylase of the firefly *plus* that of the cell-free extract of Nitrobacter. An internal standard of ATP (100 pmoles) was added at 4 min (Segment C), resulting in an increase in light

TABLE III

PURIFICATION OF ATP-SULPHURYLASE

Washed cells (50 g wet wt., 2050 mg protein) were extracted with 0.05 M Tris–HCl (pH 7.4), by ultrasonication followed by treatment in a French pressure cell. ATP formation from APS and PP_i was measured by the firefly assay (see MATERIALS AND METHODS). One unit of enzyme activity is the amount producing 1 nmole ATP/min per mg protein.

Fraction		*Total protein (mg)*	*Total activity (units)**	*Specific activity*** *(units/mg protein)*	*Recovery (%)*
I.	Homogenate in 0.05 M Tris–HCl (pH 7.4)	2050	410.0	0.16	100
II.	Supernatant fraction left after centrifuging (I) at 20 000 × *g* (S20)	1446	39.4	0.38	95.2
III.	Supernatant fraction left after centrifuging (II) at 144 000 × *g* (S144) and dialyzed	1070	363.8	0.85	88.7
IV.	Fraction (S144), eluted by a linear gradient of Tris–HCl (pH 7.4) from DEAE-cellulose column	165	306.9	11.6	74.8
V.	Fraction IV, eluted by a linear gradient of Tris–HCl (pH 7.4) (0.1–0.2 M) from DEAE-cellulose column	111	257.5	21.1	62.8
VI.	(V), eluted with 0.075 M Tris–HCl (pH 7.4) from Sephadex A50 column	61	209.8	56.4	51.1
VII.	(VI), eluted with 0.05 M Tris–HCl (pH 7.5) from Sephadex G-200 column	28	96.9	123.2	23.6
VIII.	(VII), eluted with 0.05 M Tris–HCl (pH 7.4) from Sephadex G-200 column	22	68.7	131.2	16.7

* Enzyme unit: nmoles ATP/min (firefly assay).
** Specifici activity: nmoles ATP/min per mg protein.

emission. The straight lines (X–Y–Z) were fitted to the data of Segments A, B and C, so that the enzyme activities of the bacterial extracts were readily determined. The percentage error was usually less than 5%.

The enzyme activities of Fractions I and II (Table III) were also determined by following the incorporation of $^{35}SO_4^{2-}$ into [^{35}S]APS and [^{35}S]PAPS (Fig. 2).

Purification of the enzyme

Results for the enzyme purification are summarized in Table III. Cell extracts (Fraction II, S20) (50 g wet weight suspended in 250 ml of 0.05 M Tris–HCl buffer (pH 7.4) were prepared by ultrasonic treatment followed by further breakage in a French pressure cell (see MATERIALS AND METHODS). The extract was then centriguged at 144 000 × *g* to obtain Fraction III (S144). The dialyzed enzyme (Fraction III, S144) was applied onto a DEAE-cellulose-32 column (8.2 cm × 59 cm) previously equilibrated with 0.02 M Tris–HCl buffer (pH 7.4). After washing the column with 4 l of 0.05 M Tris–HCl buffer (pH 7.4), the enzyme was eluted with a linear gradient of 0.05–0.25 M Tris–HCl buffer (pH 7.4) (Fig. 3). The most active fractions at 0.14 M buffer were pooled (240 ml) and concentrated to 20 ml by pressure dialysis (see MATERIALS AND METHODS) and then dialyzed for 6 h against 0.075 M Tris–HCl buffer (pH 7.4) (Fraction IV). This preparation was loaded onto another DEAE-cellulose-32 column (2.1cm × 36 cm) equilibrated with 0.05M Tris–HCl buffer (pH 7.4). The column

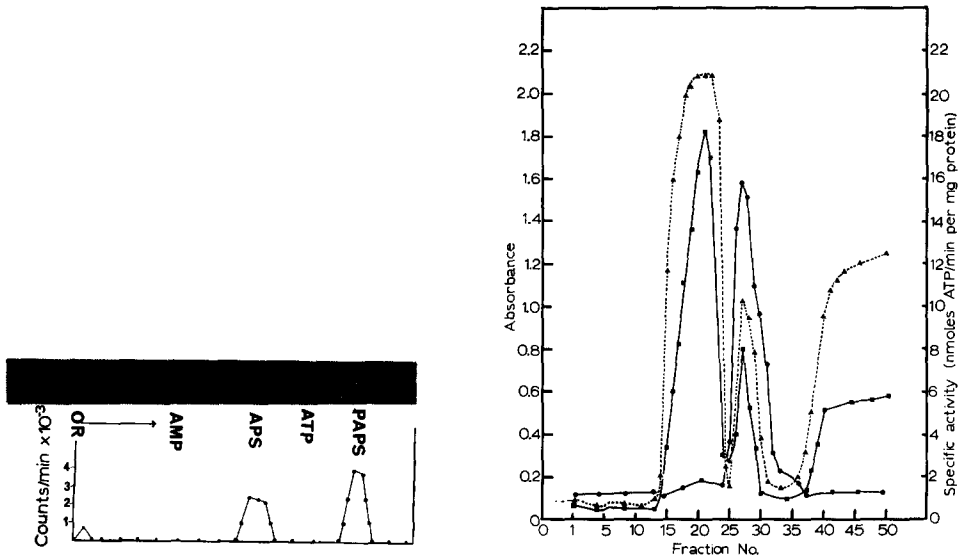

Fig. 2. An electrophoretogram of ^{35}S-labelled nucleotides [^{35}S]APS and [^{35}S]PAPS prepared from $^{35}SO_4^{2-}$ and ATP using ATP-sulphurylase enzyme from Nitrobacter. The left-hand side shows an ultraviolet print, with ATP and AMP as markers. The right-hand side shows the counts of [^{35}S]APS and [^{35}S]PAPS assayed in a liquid scintillation spectrometer. OR is the point of application of the sample.

Fig. 3. Elution pattern of ATP-sulphurylase from DEAE-cellulose-32 column. The column (8.2 cm × 59 cm) was packed as described in MATERIALS AND METHODS. ATP-sulphurylase activity represents ATP formation from APS and PP_i measured by the firefly luciferin–luciferase method (see MATERIALS AND METHODS). ▲— — —▲, $A_{280\ nm}$; ■—■, $A_{418\ nm}$; ●—●, enzymic activity.

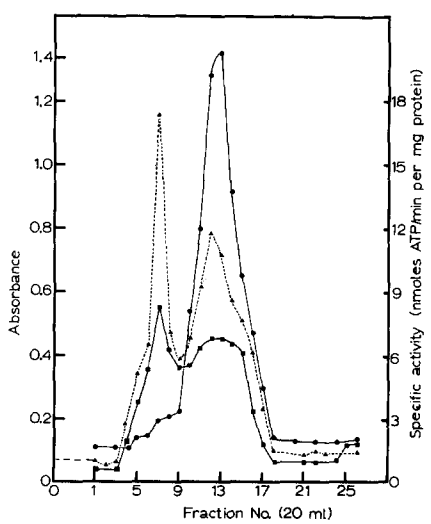

Fig. 4. Elution pattern of ATP-sulphurylase from DEAE-Sephadex-A50 column (3.5 cm × 40 cm). The enzyme was eluted with 0.075 M Tris–HCl buffer (pH 7.4). ▲— — —▲, $A_{280\ nm}$; ■—■, $A_{418\ nm}$; ●—●, enzymic activity.

was eluted by a linear gradient (0.1–0.2 M Tris–HCl buffer (pH 7.4)); active fractions appeared in 0.15 M buffer.

Fraction V was concentrated and dialysed as before and then loaded onto a DEAE-Sephadex-A50 column (3.5 cm × 40 cm), previously equilibrated with 0.04 M Tris–HCl buffer (pH 7.4). A step-wise elution was made with 250 ml of 0.075 and 0.1 M Tris–HCl buffers, respectively (Fig. 4).

The concentrated and dialyzed enzyme fraction (Fraction VI, 7.5 ml) was applied to a pre-equilibrated Sephadex G-200 column (3.5 cm × 40 cm). The enzyme was eluted with 0.05 M Tris–HCl buffer containing 2 mM EDTA (sodium salt) (Fig. 5A). The most active fractions were pooled, reconcentrated and then dialyzed

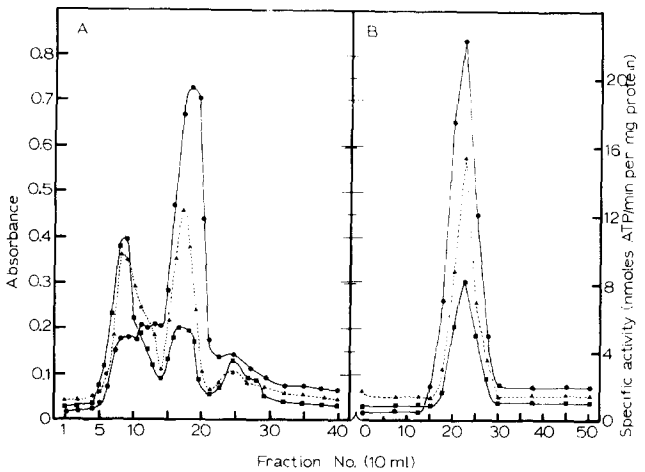

Fig. 5. Elution pattern of ATP-sulphurylase from Sephadex G-200. Enzymic activity represents the ATP production from APS and PP_i (see MATERIALS AND METHODS). ▲—▲, $A_{280\ nm}$; ■—■, $A_{418\ nm}$; ●—●, enzymic activity. A. First Sephadex G-200. B. Second Sephadex G-200.

against 0.02 M Tris–HCl buffer containing 0.2 mM EDTA (sodium salt) for 6 h. The enzyme thus obtained (5 ml, Fraction VII) was again loaded onto a similarly equilibrated Sephadex G-200 column (1.5 cm × 70 cm). The elution pattern for the enzyme is shown in Fig. 5B. The enzyme (Fraction VIII) could not be purified further either by chromatography on CM-cellulose, CM-Sephadex, hydroxyapatite columns or fractionation with either calcium phosphate gel, protamine sulphate or $(NH_4)_2SO_4$.

Purity of enzyme

The enzyme obtained from the Sephadex G-200 column (Fig. 5B, Fraction VIII) was invariably associated with a cytochrome. On reduction with $Na_2S_2O_4$ the following bands were formed: 551 nm (α); 521.5 nm (β) and 418 nm (γ). The ratio of the cytochrome to protein in Fraction VIII was found to be around 1:6, based on the extinction coefficient of $27 \cdot 10^3$ (ref. 25).

The enzyme preparation separated into two components, a major protein band (S) and a minor one containing the cytochrome (cyt) (Fig. 6a) after electrophoresis (see MATERIALS AND METHODS). The band S (Fig. 6a) which contained about 70–80% of the total protein, had ATP-sulphurylase activity only; at pH 8.1, the mobility of

this protein was $5.3 \cdot 10^{-6}$ $cm^2 \cdot v^{-1} \cdot min^{-1}$. These two protein bands were eluted separately from the starch gel in 0.1 M Tris–HCl buffer (pH 7.4); component S had a marked absorption at 280 nm only, whereas the band with cytochrome (reduced with $Na_2S_2O_4$) had maxima at 418 and 550 nm.

The other vertical portion of the gel was stained with 0.5% (w/v) benzidine in 70% ethanol (pH 5.2), containing 0.003% (v/v) H_2O_2, which detects haem compounds The cyt band only reacted with this reagent (Fig. 6b). The enzyme (1 ml, Fraction VIII, Table III) was resolved into two components, a minor band at 20%(Fig. 7A, cyt) and a larger one at 40% (Fig. 7A, S), on a sucrose density gradient (10–50% (w/v) sucrose). The former contains a cytochrome and the latter ATP-sulphurylase.

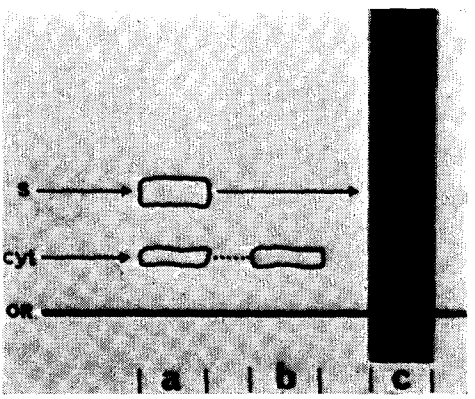

Fig. 6. Electrophoretic patterns of the purified enzyme on starch-gel strip. The electrophoresis was carried out in 0.1 M Tris–citrate buffer (pH 5.1) at 2° and 20 mA (350 V) for 3 h. Protein bands were stained with 0.1% (w/v) nigrosin in methanol–distilled water–glacial acetic acid (1:1:1, by vol.). OR is the point of application of the sample; cyt is the cytochrome band; S is ATP-sulphurylase. a, Fraction VIII (Table III) was eluted from Sephadex G-200 column; b, cytochrome component, separated in a sucrose density gradient (10–50%); c, ATP-sulphurylase from sucrose density gradient (35–45%).

The bioluminescent technique (Assay 2) showed that enzyme activity was in fraction S only. This protein was dialyzed for 24 h (with three changes) against 0.04 M Tris–HCl buffer (pH 7.4). The dialyzed enzyme, together with marker beef liver catalase (0.2 ml containing 1 mg protein), was layered onto a 4.0-ml sucrose gradient from 30 to 45% (w/v) and centrifuged for 20 h at 2°. Ten fractions (each 0.5 ml) were collected and assayed for enzyme activity. In this fractionation, only one protein peak associated with ATP-sulphurylase activity ('S', Fig. 7B) was detected. This fraction 'S', which contained a single protein band when separated by starch-gel electrophoresis (Fig. 6C), had a sedimentation coefficient of 12.8 S. Its molecular weight, determined by the method of ANDREWS[16], was found to be around 700 000.

pH optimum and time-course of the reaction

The production of [^{35}S]APS was maximal between pH 7.3 and 7.5 and was linear over a 70-min incubation period. Thus, a 40-min incubation period was used routinely to assay the enzyme.

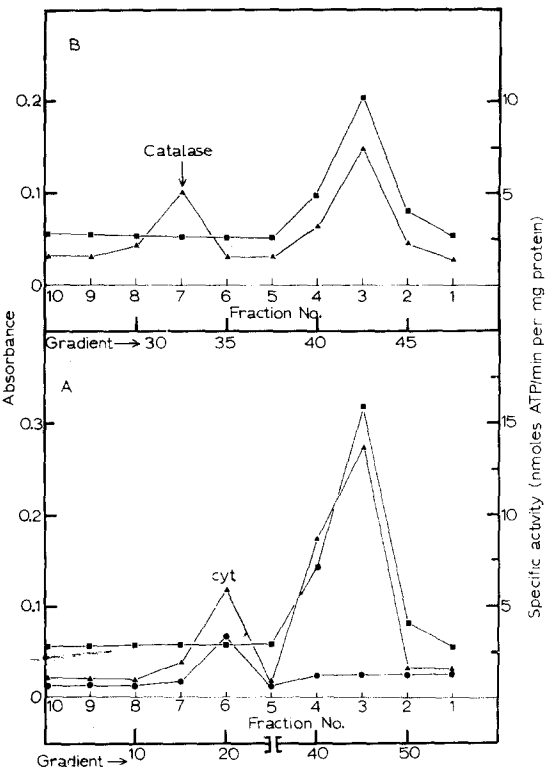

Fig. 7. A. Sucrose density gradient centrifugation of partially purified ATP-sulphurylase (Fraction VII, Table III). 1.0 ml of a solution containing 2.0 mg of enzyme was layered onto the sucrose gradient (10–50%) and centrifuged at 250 000 × *g* for 16 h. ▲—▲, $A_{280\ nm}$; ●—●, $A_{418\ nm}$; ■—■, ATP-sulphurylase activity. B. Sucrose density gradient centrifugation of purified ATP-sulphurylase (Fraction VII, Table III). 1.0 ml of a solution containing 1.6 mg of purified enzyme and about 10.0 μg of crystalline catalase in 0.05 M Tris–HCl buffer (pH 7.4) was layered onto the sucrose gradient and centrifuged as in ▲—▲, $A_{280\ nm}$; ■—■, ATP-sulphurylase activity.

Effect of protein concentration and heat stability

There was a linear relationship between protein concentration (20–200 μg) and activity. In most experiments, 50 μg protein were used. The enzyme activity showed a linear increase with temperature up to 40° under the assay conditions (Assay 2), followed by a sharp decline in activity above 55°.

Effect of ATP concentration on production of APS

The K_m value for ATP was $1.4 \cdot 10^{-3}$ M. Amounts of ATP above 12 μmoles/0.5 ml reaction mixture inhibited the enzyme.

Metabolism of [^{35}S]APS by purified ATP-sulphurylase

The results in Table IV show that about 86% of the [^{35}S]APS is converted to $^{35}SO_4^{2-}$ and ATP after a 40-min incubation at 37°. The omission of any of the substrates APS, PP_i or $MgCl_2$ greatly reduced enzyme activity.

TABLE IV

PRODUCTION OF $^{35}SO_4^{2-}$ FROM [^{35}S]APS FROM PURIFIED ENZYME ATP-SULPHURYLASE (FRACTION 8, TABLE III)

The reaction mixture contained (in μmoles/0.5 ml reaction mixture): 100 Tris–HCl; 10 $MgCl_2$; 0.3 EDTA (sodium salt); 10 Na_2SO_4; 0.30 [^{35}S]APS (5000 counts/min); 10 PP_i; and enzyme preparation (0.15 ml containing 0.02 mg protein). The reaction mixture was incubated at 37° for 40 min and 0.025-ml aliquots were subjected to high-voltage electrophoresis and the products thus separated counted in a liquid scintillation spectrometer as in the radioassay of ATP-sulphurylase (MATERIALS AND METHODS).

Reaction mixture	*$^{35}SO_4^{2-}$ produced (nmoles/mg protein)*	*% [^{35}S]APS utilized*
1. Complete	270.1	86.0
2. Omit PP_i	41.1	16.1
3. Omit [^{35}S]APS	32.0	11.9
4. Omit $MgCl_2$	59.1	18.2

Effect of substrate concentration on enzyme activity

The rate of production of ATP was measured at various concentrations of APS, PP_i and $MgCl_2$ using a partially purified ATP-sulphurylase (Fraction VIII, Table III). The results show normal Michaelis–Menten kinetics and in each case there is a linear relation between ATP formed and the substrates. The calculated K_m values are $1.2 \cdot 10^{-4}$ M for APS, $2.5 \cdot 10^{-5}$ M for PP_i and $3.4 \cdot 10^{-3}$ M for $MgCl_2$.

Stability of the enzyme and enzyme storage

The enzyme (Fraction VIII) lost its activity after a 4-week storage at −15°,

TABLE V

EFFECT OF GROUP VI ANIONS ON THE PRODUCTION OF APS FROM $^{35}SO_4^{2-}$

The complete reaction mixture is given in MATERIALS AND METHODS, except that molybdate, chromate, tungstate and selenate were added as indicated.

Inhibitor	*Thiol concn. (M)*	*Total activity of [^{35}S]APS (nmoles/mg protein)*	*Inhibition (%)*
None		8.01	0
Molybdate	$3 \cdot 10^{-4}$	2.10	74
	$3 \cdot 10^{-5}$	2.41	70
	$3 \cdot 10^{-6}$	2.98	60
Chromate	$3 \cdot 10^{-4}$	2.42	71
	$3 \cdot 10^{-5}$	4.00	50
	$3 \cdot 10^{-6}$	4.19	47
Tungstate	$3 \cdot 10^{-4}$	1.99	79
	$3 \cdot 10^{-5}$	3.61	56
	$3 \cdot 10^{-6}$	4.22	48
Selenate	$3 \cdot 10^{-4}$	3.21	64
	$3 \cdot 10^{-5}$	4.01	50
	$3 \cdot 10^{-6}$	5.53	32

and dialysis of this preparation against phosphate buffer (pH 7.4, 0.05 M, Na^+–K^+), did not restore the enzyme. However, dialysis against the barbiturate buffer, Tris–phosphate buffer or Tris–sulphate buffer delayed the denaturation of the enzyme. In other experiments, cells were extracted in 0.1 M phosphate buffer (pH 7.0) and the entire purification steps were done in phosphate buffer (pH 7.4), but this procedure did not improve the stability of the enzyme. It lost 95% of activity at 60° for 10 min.

Activators and inhibitors

Effect of group VI anions. The effect of molybdate, chromate, tungstate and selenate, each at three concentrations ($3 \cdot 10^{-4}$, $3 \cdot 10^{-5}$ and $3 \cdot 10^{-6}$ M), on the purified enzyme was examined (Table V). All the anions inhibited the incorporation of $^{35}SO_4^{2-}$ into [^{35}S]APS.

Thiol inhibitors. Of the six thiol compounds tested, β-mercaptoethanol, Cleland's reagent, cysteine and GSH greatly activated ATP-sulphurylase, whereas methionine and *N*-ethylmaleimide were slightly inhibitory at higher concentrations

TABLE VI

EFFECT OF VARIOUS THIOL-BINDING COMPOUNDS ON ATP-SULPHURYLASE ACTIVITY

The reaction mixture is as described in MATERIALS AND METHODS, except that thiol compounds were added as indicated.

Compounds added to reaction mixture	*Amount (μmoles)*	*ATP formed (nmoles/min per mg protein)*	*ATP-sulphurylase activity (% of control)*
None	—	36	100
GSH	0.5	67	186
	1.0	71	197
	2.0	67	186
	4.0	48	133
L-Cysteine	0.5	41	114
	1.0	44	122
	2.0	33	92
	4.0	34	94
L-Methionine	0.5	36	100
	1.0	34	94
	2.0	33	92
	4.0	29	80
Cleland's reagent	0.5	37	103
	1.0	37	103
	2.0	52	140
	4.0	39	108
N-Ethylmaleimide	0.5	29	81
	1.0	34	94
	2.0	33	92
	4.0	29	81
β-Mercaptoethanol	0.5	42	116
	1.0	53	141
	2.0	36	100
	4.0	29	81

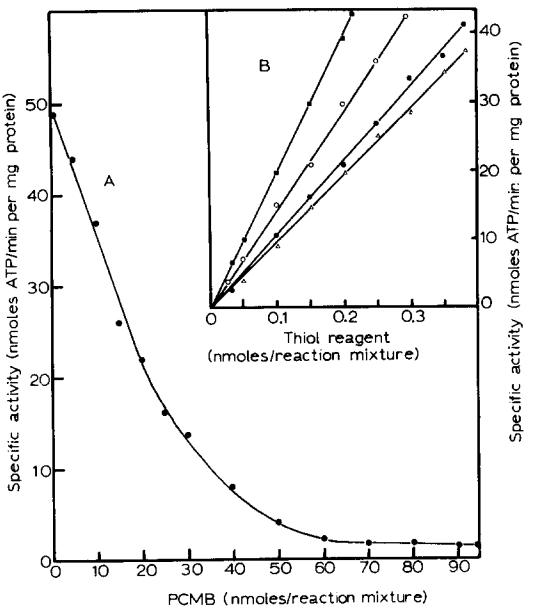

Fig. 8. A. Inhibitory effect of PCMB on ATP-sulphurylase (Assay 2 in MATERIALS AND METHODS). PCMB had no effect on the luciferin–luciferase enzyme system. B. Reversal of the PCMB-inhibited enzyme (Assay 2 in MATERIALS AND METHODS). Reactivation of the PCMB-inhibited enzyme was determined after adding (0.1–0.4 μmole) GSH, dithiothreitol, cysteine and β-mercaptoethanol, respectively. ■—■, L-cysteine; ○—○, dithiothreitol; ●—●, GSH; △—△, β-mercaptoethanol.

(Table VI). In contrast, PCMB inhibited the enzyme at low concentrations (Fig. 8A), and this effect was reversed by either L-cysteine, Cleland's reagent, GSH or β-mercaptoethanol (Fig. 8B), but not by methionine or *N*-ethylmaleimide.

DISCUSSION

The present investigation indicates that "sulphate activation" in Nitrobacter proceeds *via* Reactions 1 and 2, as in yeast[26].

$$ATP + SO_4^{2-} \rightleftharpoons APS + PP_i \quad (1)$$

$$ATP + SO_4^{2-} \rightleftharpoons PAPS + ADP \quad (2)$$

The "sulphate-activating" enzymes (ATP:sulphate adenylyltransferase, EC 2.7.7.4, and ATP:adenylyl sulphate 3'-phosphotransferase, EC 2.7.1.25) are located in the soluble fraction of the cells, as in *Escherichia coli*[27], yeast[3–5] and Nitrosomonas[8]. The ATP-sulphurylase activities in the two nitrifying microorganisms (Nitrosomonas and Nitrobacter) are very low compared with that of yeast.

Since the activity of ATP-sulphurylase is low in cell-free extracts (18 nmoles APS per mg protein per 40 min), as determined by the radioassay method, a more rapid and sensitive bioluminescence technique has been used to determine ATP formed from APS and PP_i by the enzyme[23,24]. The low K_m values for APS ($2.5 \cdot 10^{-5}$ M) and PP_i ($1.4 \cdot 10^{-4}$ M) and the comparatively high K_m values for ATP ($1.4 \cdot 10^{-3}$ M)

determined for the highly purified enzyme indicate the relatively high affinity of ATP-sulphurylase for APS or PP_i.

BANDURSKI *et al.*[28] found that ATP-sulphurylase from yeast is relatively non-specific with respect to the anion which participates in the reaction; thus, all the group VI anions substitute for SO_4^{2-}. In experiments reported herein, however, molybdate, chromate, tungstate and selenate inhibited the incorporation of $^{35}SO_4^{2-}$ into APS and PAPS in extracts of Nitrobacter. Similar results have been reported for the enzyme in higher plants[29,30].

The activation of ATP production from APS and PP_i by the purified enzyme, in the presence of GSH, L-cysteine, β-mercaptoethanol and Cleland's reagent, and its inhibition by PCMB may be compared with similar effects reported for APS-reductase[30-32] from Desulphovibrio and ATP-sulphurylase from bacteria[2] and animal tissues[35]. Thus, sulphydryl groups appear to be required for ATP-sulphurylase from Nitrobacter, in contrast to the lack of inhibition by PCMB for ATP-sulphurylases[30,33] from higher plants and APS-reductase[34] from Desulphovibrio.

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