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Adenosine 5′-Triphosphate Sulphurylase from *Saccharomyces cerevisiae*

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1. ATP sulphurylase from *Saccharomyces cerevisiae* was purified 140-fold by using heat treatment, DEAE-cellulose chromatography and Sepharose 6B gel filtration. 2. The enzyme was stable at −15°C, optimum reaction velocity was between pH 7.0 and 9.0, and the activation energy was 62kJ/mol (14.7kcal/mol). 3. The substrate was shown to be the $MgATP^{2-}$ complex, free ATP being inhibitory. 4. Double-reciprocal plots from initial-velocity studies were intersecting and the K_m of each substrate was determined at infinite concentration of the other (K_m $MgATP^{2-}$, 0.07mM; MoO_4^{2-}, 0.17mM). 5. Radioisotopic exchange between the substrate pairs, adenosine 5′-[^{35}S]sulphatophosphate and SO_4^{2-}, $^{35}SO_4^{2-}$ and adenosine 5′-sulphatophosphate, occurred only in the presence of either $MgATP^{2-}$ or PP_i. This suggests, along with the initial-velocity data, a sequential reaction mechanism in which both substrates bind before any product is released. 6. The enzyme reaction was specific for ATP and was not inhibited by L-cysteine, L-methionine, SO_3^{2-}, $S_2O_3^{2-}$ (all 2mM) nor by *p*-chloromercuribenzoate (1mM). 7. Competitive inhibition of the enzyme with respect to MoO_4^{2-} was produced by SO_4^{2-} ($K_i = 2.0$mM) and non-competitive inhibition by sulphide ($K_i = 3.4$mM). 8. Adenosine 5′-sulphatophosphate inhibited strongly and concentrations as low as 0.02mM altered the normal hyperbolic velocity–substrate curves with both $MgATP^{2-}$ and MoO_4^{2-} to sigmoidal forms.

ATP sulphurylase (EC 2.7.7.4, ATP–sulphate adenylyltransferase) synthesizes adenosine 5′-sulphatophosphate from ATP and inorganic SO_4^{2-}. This is the first reaction of a two-step sequence in the formation of 'active sulphate', adenosine 3′-phosphate 5′-sulphatophosphate, which is a sulphate donor for a wide variety of compounds and is also involved in the reduction of sulphate.

Robbins & Lipmann (1958), who first characterized the yeast enzyme, found that the standard free-energy change of the reaction was approx. +46kJ/mol (+11kcal/mol); thus at equilibrium the amount of adenosine 5′-sulphatophosphate is difficult to measure. The discovery by Wilson & Bandurski (1958) that yeast ATP sulphurylase liberated PP_i on incubation with ATP and MoO_4^{2-} led to the use of this anion in an assay system for the enzyme.

ATP sulphurylase is widespread in Nature and has been prepared from a number of sources including micro-organisms, plants and animals, but few detailed studies of the enzyme reaction have been published. A sequential mechanism has been proposed by Tweedie & Segel (1971*b*) for a homogeneous enzyme from *Penicillium chrysogenum* and by Shoyab & Marx (1972) for ATP sulphurylase purified approx. 500-fold from Furth mouse mastocytoma. On the other hand, Levi & Wolf (1969), who used a 1000-fold purified enzyme from rat liver, suggested that the mechanism is Ping Pong.

In the present paper, purification and some properties of yeast ATP sulphurylase are described. A sequential reaction mechanism is suggested for the enzyme on the basis of initial-velocity studies by using the molybdolysis assay and radioisotope-exchange reactions.

Experimental

Materials

AMP, ADP, ATP, CTP, UTP, *p*-chloromercuribenzoic acid, bovine serum albumin (fraction V), yeast inorganic pyrophosphatase (type III, 500 units/mg) and Tris were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); GTP, dTTP, L-cysteine hydrochloride and L-methionine were from Calbiochem (Australia) (Carlingford, N.S.W., Australia); starch was from Connaught Medical Research Laboratories (Toronto, Canada); Whatman DE-32 DEAE-cellulose and 3MM chromatography paper were from H. Reeve Angel and Co. Ltd. (London E.C.4, U.K.); Sepharose 6B was from Bio-Rad Laboratories (Richmond, Calif., U.S.A.); $Na_2{}^{35}SO_3$ (solid) and $^{35}SO_4^{2-}$ (aqueous solution, pH 6–8, carrier-free) were from The Radiochemical Centre, Amersham, Bucks., U.K. 2,5-Diphenyloxazole, 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and standard [^{14}C]toluene were obtained from

Packard Instrument Co. (Chicago, Ill., U.S.A.); Blue Dextran was from Pharmacia (Uppsala, Sweden); all other reagents were of best purity available.

Adenosine 5'-sulphatophosphate and adenosine 5'-[^{35}S]sulphatophosphate were synthesized from AMP, sulphite and ferricyanide in the presence of adenosine 5'-sulphatophosphate reductase by the method of Adams *et al.* (1971). Nucleotide solutions were titrated to neutral pH and their concentrations determined spectrophotometrically by using the molar extinction coefficient values of Burton (1969).

Sodium sulphide solutions were prepared immediately before use from $Na_2S,9H_2O$ crystals dissolved in O_2-free twice-distilled water (King & Morris, 1967).

Baker's yeast (*Saccharomyces cerevisiae*) was obtained as active dried yeast (Dryco) from Mauri Bros. and Thompson (Adelaide, S. Austral., Australia) and stored in a cool dry place.

The 0.1M-Tris–HCl, 0.1M-Tris–maleate–NaOH, and 0.1M-citric acid–sodium citrate buffers were prepared by the method of Gomori (1955). The pH of Tris–HCl was adjusted according to temperature (Sigma Technical Bulletin 106B, 1967).

Methods

Enzyme extraction and purification. All procedures were carried out at 0–2°C in 0.05M-Tris–HCl buffer, pH7.5, containing 1mM-EDTA (sodium salt). Dried yeast (100g) was suspended in 200ml of the buffer and frozen at −15°C for 24h. The frozen block was sheared on a grinding wheel (Leis & Ralph, 1960), and the suspension obtained was re-frozen and ground again. After being adjusted to pH7.5 with 1M-NaOH, the extract was centrifuged at 27000*g* for 1h in a Sorvall SS-3 Automatic Superspeed Centrifuge (SS-34 rotor). The supernatant (fraction I) was then centrifuged at 60000*g* for 30min (Spinco model L, rotor type 50Ti). Enzyme activity was entirely located in the supernatant (fraction II), which was adjusted to pH7.5.

Fraction II was quickly warmed to 50°C and maintained at this temperature for 5min with constant stirring. It was then centrifuged at 12000*g*/15min (Sorvall SS-34 rotor).

The supernatant (fraction III) was loaded on a DEAE-32 cellulose column (28.0cm×2.5cm), prepared according to the manufacturer's instructions (Whatman Technical Bulletin IE2) and previously equilibrated with the Tris buffer. A linear gradient generated from 500ml of the Tris buffer and 500ml of 0.5M-NaCl in the same buffer was used to develop the column (Fig. 1), and 5.5ml fractions were collected by using an automatic fraction collector (Patons Industries, Beaumont, S. Austral., Australia), the flow rate being 80–100ml/h. Active fractions were pooled and concentrated (approx. 20-fold) by ultrafiltration under N_2 by using a PM-10 membrane and ultrafiltration cell model 52 (Amicon, Lexington, Mass., U.S.A.), thus obtaining fraction IV.

Fraction IV was loaded on a Sepharose 6B column (45.0cm×1.7cm; void volume 26.5ml, determined by elution of Blue Dextran) prepared as described by Reiland (1971) and eluted with the above Tris buffer (flow rate 18–20ml/h). The active fractions were concentrated (about 10-fold) as described above to obtain fraction V.

Molybdolysis assay. A modification of the method of Bandurski *et al.* (1956) was used. The reaction mixture contained (total vol. 0.5ml): Tris–HCl buffer, pH8.0, 50μmol; $MgCl_2$, 1.5μmol; ATP, 1μmol; Na_2MoO_4, 2μmol; inorganic pyrophosphatase, 2 units; enzyme, 0.01ml. Controls without MoO_4^{2-} and with boiled enzyme were included. The tubes were equilibrated at 30°C for 0.5min before the reaction was started by adding the enzyme. Incubation was for 5min at 30°C in a reciprocating water bath, and 0.5ml of ice-cold trichloroacetic acid (10%, w/v) was added to stop the reaction. The tubes were placed in ice to minimize acid-catalysed breakdown of ATP. Phosphate (P_i) was determined immediately in the reaction mixture. When crude enzyme preparations were used, the resultant protein precipitate was centrifuged at 2000*g* for 10min in an MSE bench centrifuge at 2°C and a sample (0.5ml) of the supernatant was used for the phosphate assay.

Determination of phosphate. A modification of the method of Fiske & SubbaRow (1925) was used. The reaction mixture was diluted to 2.0ml with water, and then 0.5ml of $(NH_4)_6Mo_7O_{24},4H_2O$ [2.5% (w/v) in 2.5M-H_2SO_4] and 0.1ml of the test reagent were quickly mixed into it. After the mixture had been standing for 20min the E_{750} was determined in a Shimadzu QV 50 spectrophotometer (1cm light-path cell). A standard series for P_i (0–0.4μmol of P_i) was determined each time.

The test reagent was prepared as follows: 1g of 1-amino-2-naphthol-4-sulphonic acid, 3g of Na_2SO_3,-$7H_2O$ and 6g of $Na_2S_2O_5$ were mixed in a mortar and stored at 2°C in the dark; 0.25g of this mixture in 10ml of water was prepared fresh daily.

Sulphide inhibition. To minimize loss from the oxidation of sulphide, the reaction tubes were sealed with Suba-Seals (W. Freeman, Barnsley, Yorks., U.K.) after addition of sulphide to the reaction mixture and incubated for 2min. Standards for P_i were determined in the presence of each sulphide concentration used, since sulphide increased the colour intensity.

Protein assay. The micro biuret method of Itzhaki & Gill (1964) was used with dry bovine serum albumin as a standard.

Starch-gel electrophoresis. Electrophoresis was carried out in a starch gel (12%, w/v), prepared as described by Brewer (1970), by using the apparatus of Graham (1963). Samples of fraction V (0.01ml),

loaded on to Whatman 3MM chromatography paper (0.4cm×0.2cm), were inserted 1cm apart in the set gel. An ice pack was placed over the gel and a voltage gradient of 30V/cm applied for 1.5h. The buffer used for gel preparation and in the electrode compartments was 0.1M-Tris–citrate, pH8.1 (obtained on dilution of 0.76M-Tris adjusted to pH8.4 with solid citric acid). The gel was sliced horizontally and one-half stained for protein with Nigrosine–Amido Black [1% (w/v) Nigrosine, 1% (w/v) Amido Black in methanol–acetic acid–water (5:1:5, by vol.)]. Each lane in the other half was cut transversely into 1.0cm×0.5cm sections, which were macerated in 0.1M-Tris–HCl buffer, pH8.0, and assayed for ATP sulphurylase by the molybdolysis assay (30min incubation). Thus the location of enzyme activity and protein bands could be correlated.

High-voltage paper electrophoresis. Nucleotides and sulphur oxyanions were separated at room temperature on Whatman 3MM chromatography paper by using 0.1M-sodium citrate buffer, pH5.0, and the apparatus of Tate (1968). A voltage gradient of 30V/cm was applied for 1 or 1.5h (SO_4^{2-} runs off the paper after 1.5h).

Nucleotides were detected on the dried paper by u.v. absorption by a hand-held monitor, and radioactivity was detected either by passing the paper through a Packard 7201 radiochromatogram scanner, or by liquid-scintillation counting for quantitative determination.

Liquid-scintillation counting. Sections of paper (2.5cm×2.0cm) cut from electrophoretograms were placed in glass vials containing 1ml of scintillation solvent and counted for radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer model 3375. Scintillation solvent contained 5.0g of 2,5-diphenyloxazole and 0.3g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/l of toluene. Channel ratios were used to determine the counting efficiency (usually about 80%) in comparison with an $^{35}SO_4^{2-}$ standard also spotted on paper. This standard had been previously calibrated against a standard [^{14}C]-toluene sample (5.73×10^5d.p.m./g), assuming equal efficiency of detection of ^{14}C and ^{35}S.

Isotope-exchange studies. (*a*) Adenosine 5′-sulphatophosphate–$^{35}SO_4^{2-}$. Reaction mixtures (total vol. 0.1ml) contained: Tris–HCl buffer, pH8.0, 10μmol; adenosine 5′-sulphatophosphate, 0.05μmol; $^{35}SO_4^{2-}$ (carrier-free), 0.01ml (2.47×10^6d.p.m.); enzyme (fraction V), 0.01ml. When $MgCl_2$, ATP and inorganic pyrophosphatase were used, the amounts were 0.6μmol, 0.5μmol and 2 units respectively. Boiled-enzyme controls were included for each reaction mixture. The reactions were stopped after incubation for 30, 60, 90 and 120min respectively at 30°C by placing the tubes in a boiling-water bath for 2min and then in ice. Portions (0.025ml) of the reaction mixture were spotted 2.5cm apart, 12cm from the end of Whatman 3MM chromatography paper (15.5cm×57cm) for high-voltage electrophoresis. Standard nucleotide solutions (AMP, ADP, adenosine 5′-sulphatophosphate and ATP) were also spotted on the paper. Lanes 2.5cm wide corresponding to the original points of sample application were cut into 2.0cm sections and counted for radioactivity in vials in the liquid-scintillation spectrometer.

(*b*) Adenosine 5′-[^{35}S]sulphatophosphate–SO_4^{2-}. The reaction mixtures (0.1ml) contained: Tris–HCl buffer, pH8.0, 10μmol; adenosine 5′-[^{35}S]sulphatophosphate, 0.1μmol (2.0×10^5d.p.m.); Na_2SO_4, 0.5μmol; enzyme (fraction V), 0.01ml. Magnesium chloride (0.6μmol) and ATP and PP_i (each 0.5μmol) were used in the appropriate reaction mixtures. Boiled-enzyme controls were included. The reactions were carried out and the radioactivity in adenosine 5′-sulphatophosphate and SO_4^{2-} was determined as described above.

Statistical treatment. A computer program (written by Mrs. M. Atkinson of this Department) was used to treat the data from Michaelis–Menten velocity–substrate plots by the method of Wilkinson (1961), thus determining K_m, $V_{max.}$ and their associated standard errors. In the inhibitor studies, the standard errors of $1/V_{max.}$ were calculated [S.E.M. of $1/V_{max.}$ = (S.E.M. of $V_{max.}$)/$V_{max.}^2$] to determine whether the lines produced in the double-reciprocal plots intersected to the left of, or on, the vertical axis. Thus the type of inhibition was determined to be non-competitive or competitive respectively according to the nomenclature of Cleland (1970).

Results

Enzyme activity

Purification of the enzyme. ATP sulphurylase was purified about 100-fold from the crude yeast extract (Table 1). The elution profiles from DEAE-cellulose and Sepharose 6B are shown in Figs. 1 and 2. Fraction V was resolved by starch-gel electrophoresis into three protein bands, which migrated towards the anode. The fastest-moving band was only lightly stained by Nigrosine–Amido Black, and ATP sulphurylase activity was located in the faster of the two darker bands which were of approximately equal intensity.

All enzyme studies were made with fraction V, which was free of adenosine triphosphatase, inorganic pyrophosphatase and adenylyl sulphate kinase. This fraction retained 90% of its activity over 2 months at −15°C.

Other methods for determining enzyme activity. To check the validity of the molybdolysis assay for the enzyme, a radiochemical assay was used. Adenosine

Table 1. *Preparation of ATP sulphurylase*

The molybdolysis assay was used to determine enzyme activity. Methods used are described in the Experimental section.

Fraction	Volume (ml)	Total activity (μmol of $MgATP^{2-}$ used/min)	Total protein (mg)	Specific activity (μmol of $MgATP^{2-}$ used/min per mg)	Relative purity	Recovery (%)
I 27000*g* supernatant of ground yeast extract	117	48	9480	0.005	1	100
II 60000*g* supernatant	105	60	9030	0.007	1.4	125
III 50°C treatment for 5min	60	56	3960	0.014	2.8	116
IV Gradient elution from DEAE-cellulose	7	34	133	0.256	51	71
V Elution from Sepharose 6B	2	25	36	0.69	139	52

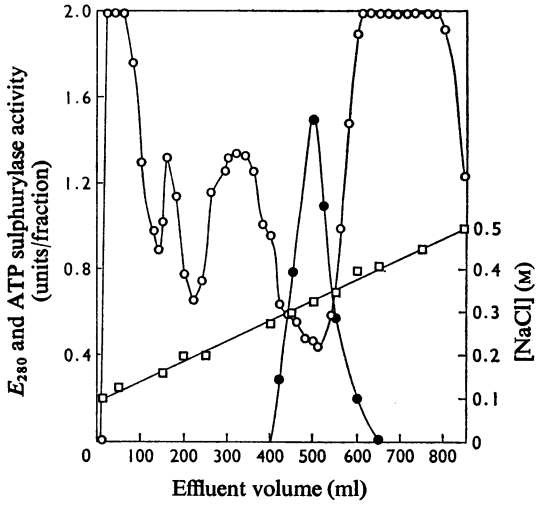

Fig. 1. *Elution of ATP sulphurylase from DEAE-cellulose*

After being warmed at 50°C, the yeast extract (fraction III) was loaded on a DEAE-cellulose column and eluted with a linear gradient of 0–0.5M-NaCl in 0.05M-Tris–HCl buffer, pH7.5, containing 1mM-EDTA (sodium salt) as described in the Experimental section. ●, Units of enzyme activity (μmol of $MgATP^{2-}$ used/min)/fraction; ○, E_{280} (1cm cell); □, concn. of NaCl determined by refractive-index measurements calibrated against prepared standard NaCl solutions (0–0.5M). Fraction IV was obtained by concentrating the effluent collected from 450 to 575ml.

5′-[^{35}S]sulphatophosphate was produced on incubating fraction V with Mg^{2+}, ATP and $^{35}SO_4^{2-}$ (Fig. 3). Its electrophoretic mobility was identical with that of a standard sample of adenosine 5′-sulphatophosphate prepared by the method of Adams *et al.* (1971). In addition, the activity of fraction V was also assayed in the reverse direction and the ATP synthesized was determined by the firefly luciferin–luciferase method (Balharry & Nicholas, 1970). The activity found, 0.62μmol/min per mg, was equivalent to that determined by the molybdolysis assay (Table 1).

Effects of incubation time, enzyme concentration, temperature and pH. At 30°C, activity was constant for 4min at all substrate concentrations used, and for at least 10min at saturating substrate concentrations when 30% of the ATP was utilized. The enzyme concentrations and incubation times used throughout this work were adjusted to ensure that the amount of product formed was always linear with time, i.e. initial velocities were being measured (Dixon & Webb, 1964).

Enzyme activity was proportional to protein concentration over the range 10–62μg of protein per reaction mixture (0.5ml). The enzyme is stable for at least 10min at 50°C, although heating at 60°C rapidly inactivates it. An Arrhenius plot of initial velocities determined over the temperature range 11–40°C was linear, and the activation energy was calculated to be 62 kJ/mol (14.7kcal/mol).

The maximum velocity (μmol of $MgATP^{2-}$ used/min per mg) increased threefold from pH5.0 to 7.0 (0.1M-Tris–maleate–NaOH buffer). There was no further change between pH7.0 and 9.0 (0.1M-Tris–HCl) and activity was similar in the two buffers. In this study, inorganic pyrophosphatase concentrations

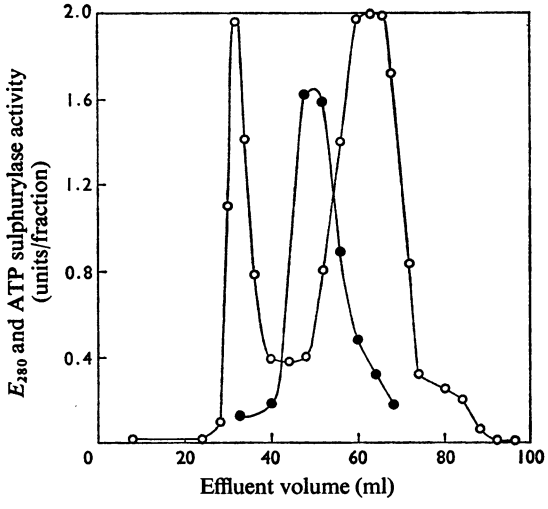

Fig. 2. *Elution of ATP sulphurylase from Sepharose 6B*

Fraction IV obtained by DEAE-cellulose chromatography was loaded on a Sepharose 6B column as described in the Experimental section and eluted with 0.05M-Tris–HCl buffer, pH7.5, containing 1mM-EDTA (sodium salt). ●, Units of enzyme activity (μmol of $MgATP^{2-}$ used/min)/fraction; ○, E_{280} (1cm cell). Fraction V was obtained by concentrating the effluent collected between 42 and 62ml.

were adjusted to ensure that there was sufficient activity of this enzyme at each pH. ATP sulphurylase was assayed as a routine in 0.1M-Tris–HCl buffer (pH8.0, 30°C).

Role of Mg^{2+}

Mg^{2+} is essential for ATP sulphurylase activity, and Fig. 4(*a*) shows that maximum activity was obtained when the concentrations of Mg^{2+} and ATP were equal. This suggests that the substrate of the enzyme is the complex $MgATP^{2-}$. As the ATP concentration is increased above that of Mg^{2+}, thus increasing the amount of free unbound ATP, the enzyme reaction is inhibited (Fig. 4*a*). Excess of Mg^{2+} (up to 7.5mM), however, did not alter the activity.

The concentration of $MgATP^{2-}$ can be calculated from the concentrations of the free ions by using the stability constant (log $K = 4.3$ in 0.1M-Tris–HCl at pH8.0, 30°C; O'Sullivan, 1969). In Fig. 4(*b*), $MgATP^{2-}$ was kept constant at 0.4mM throughout, and the free ATP concentration was varied at two concentrations of molybdate. This Dixon plot of the results shows that free ATP is a non-competitive inhibitor of the enzyme with respect to MoO_4^{2-} (Dixon & Webb, 1964); but the K_i value cannot be estimated because the results are not sufficiently precise.

In subsequent studies with the enzyme, the concentration of $MgCl_2$ was maintained at 1.0mM above that of ATP. Thus the concentration of free Mg^{2+} was approx. 1mM throughout, whereas that of the inhibitor, free ATP, was minimal (Cleland, 1970).

Determination of K_m for $MgATP^{2-}$ and MoO_4^{2-}

The initial-velocity patterns obtained were a series of straight lines intersecting to the left of the vertical axis (Fig. 5). The slopes of the lines were altered as the concentration of the fixed substrate was increased, indicating that there is a reversible connexion between the points of combination of substrates $MgATP^{2-}$ and MoO_4^{2-} with the enzyme (Cleland, 1970). This suggests that both substrates bind to the enzyme before any product is released, i.e. a sequential mechanism.

Double-reciprocal plots of the vertical intercepts obtained from the initial-velocity plots (Fig. 5) versus each corresponding fixed substrate concentration were linear (Fig. 6). The Michaelis constant of each substrate and the maximum velocity of the reaction were determined from these plots: K_m (MoO_4^{2-}) = 0.17±0.01mM; K_m ($MgATP^{2-}$) = 0.07±0.01mM; $V_{max.}$ = 2.11±0.06μmol of $MgATP^{2-}$ used/min per mg of protein.

Radioisotope-exchange reactions

To differentiate further between a mechanism in which both substrates are added before any product is released, and a Ping Pong mechanism where product release occurs before the addition of the second substrate, radioisotope exchange was measured between adenosine 5′-[^{35}S]sulphatophosphate and SO_4^{2-} and between adenosine 5′-sulphatophosphate and $^{35}SO_4^{2-}$. The Ping Pong mechanism predicts that ^{35}S should exchange between adenosine 5′-sulphatophosphate and SO_4^{2-} in the absence of any other substrate; however, there should be no exchange under these conditions if the mechanism is sequential. The results in Figs. 7 and 8 show that ^{35}S exchange between adenosine 5′-sulphatophosphate and SO_4^{2-} was only found when either $MgATP^{2-}$ or PP_i was present in the reaction mixture. Thus the mechanism appears to be sequential. The observation that only little $^{35}SO_4^{2-}$ is incorporated into adenosine 5′-sulphatophosphate (Fig. 7) is probably due to the strong inhibitory effect exerted by the latter compound on the enzyme.

Effects of nucleotide triphosphates, sulphur compounds and p-chloromercuribenzoate

When equivalent amounts of CTP, GTP, ITP, dTTP and UTP were substituted for ATP in the molybdolysis assay, the activity was less than 1% of that

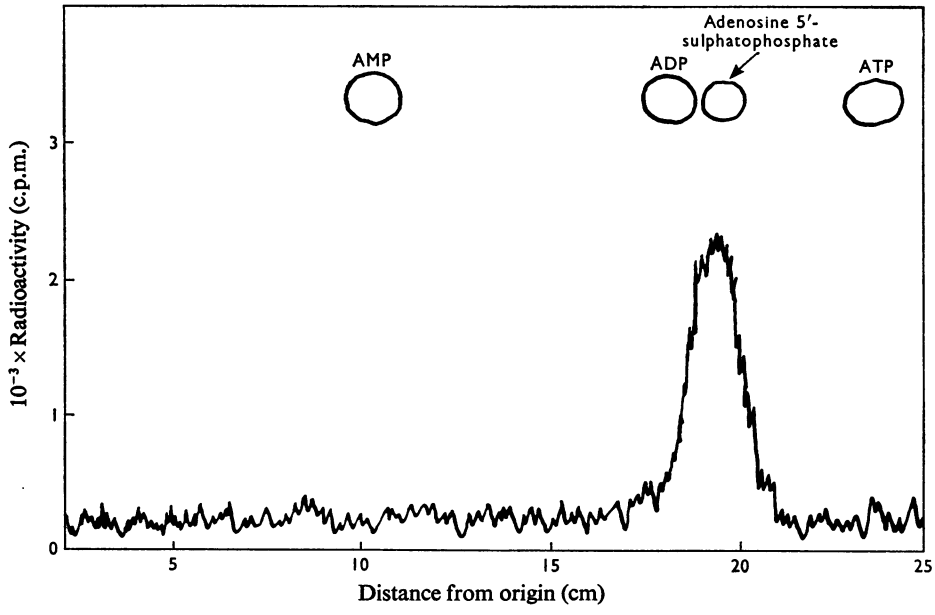

Fig. 3. *Production of adenosine 5'-[^{35}S]sulphatophosphate from ATP and SO_4^{2-}*

The reaction mixture (total vol. 0.1 ml) contained: Tris–HCl buffer, pH 8.0, 10 μmol; ATP, 0.5 μmol; $MgCl_2$, 1.5 μmol; $^{35}SO_4^{2-}$ (carrier-free), 5.1×10^4 d.p.m.; fraction V, 0.01 ml; the mixture was incubated at 30°C for 30 min. The reaction was stopped by placing the tube in boiling water for 2 min, and then immediately placing it in ice. Boiled-enzyme controls were included. Samples (0.025 ml) were spotted on chromatography paper, subjected to high-voltage electrophoresis for 1.5 h and scanned for radioactivity as described in the Experimental section. After 1.5 h, SO_4^{2-} runs off the paper. The nucleotide spots shown were standard samples detected by u.v. absorption. No significant radioactivity above background was detected in the controls.

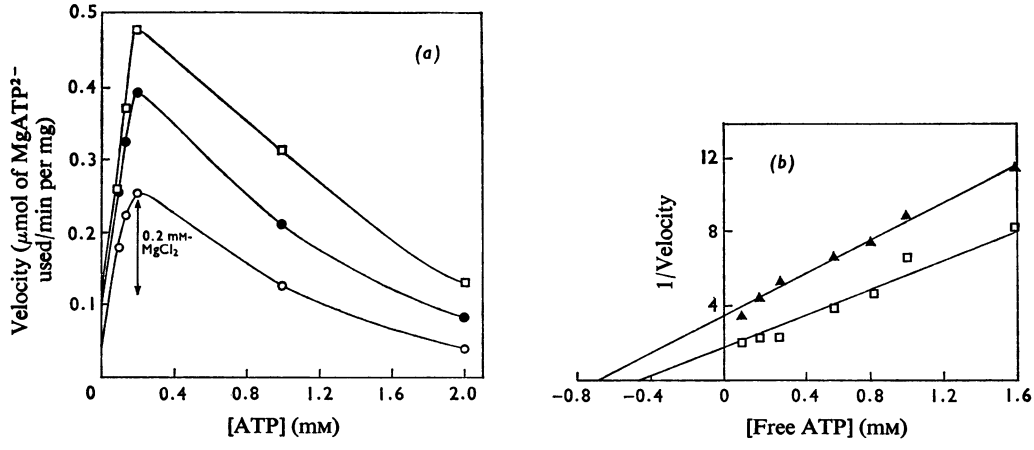

Fig. 4. *Free ATP as an inhibitor of the enzyme*

(*a*) The ATP concentration was increased from 0.1 to 2.0 mM while $MgCl_2$ was kept constant at 0.2 mM. Activity was assayed by the molybdolysis method as described in the Experimental section. Concentrations of MoO_4^{2-} used (mM): ○, 0.4; ●, 1.0; □, 2.0. (*b*) The $MgATP^{2-}$ concentration was kept at 0.4 mM and free ATP, the inhibitor, was varied from 0.1 to 1.6 mM. A Dixon plot is shown of the data obtained at two concentrations of MoO_4^{2-} (mM): ▲, 0.5; □, 2.0.

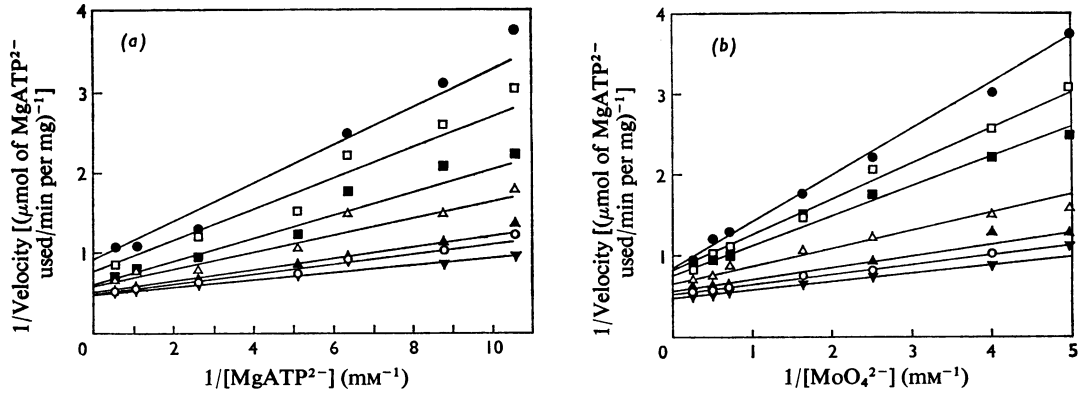

Fig. 5. *Double-reciprocal plots of velocity versus varied substrate concentration at different fixed concentrations of the second substrate*

(*a*) Plot of reciprocal velocity versus increasing $MgATP^{2-}$ concentrations at several fixed concentrations of MoO_4^{2-}. The molybdolysis assay of ATP sulphurylase was used as described in the Experimental section. Concentrations of MoO_4^{2-} used (mM): ●, 0.2; □, 0.25; ■, 0.4; △, 0.6; ▲, 1.4; ○, 2.0; ▼, 4.0. (*b*) Reciprocal velocity plotted versus concentration of MoO_4^{2-} at several $MgATP^{2-}$ concentrations. Concentrations of $MgATP^{2-}$ used (mM): ●, 0.095; □, 0.114; ■, 0.157; △, 0.196; ▲, 0.381; ○, 0.955; ▼, 1.91. The lines shown are those calculated from the statistical treatment of Wilkinson (1961).

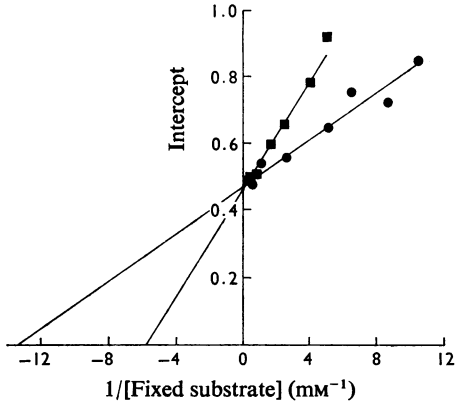

Fig. 6. *Determination of K_m for MoO_4^{2-} and $MgATP^{2-}$*

The vertical intercepts from Figs. 5(*a*) and 5(*b*) were plotted against each corresponding concentration of fixed substrate; ■, MoO_4^{2-}; ●, $MgATP^{2-}$. The negative reciprocal of the intercept on the horizontal axis gives the K_m (mM) (MoO_4^{2-}, 0.17±0.01; $MgATP^{2-}$, 0.07±0.01).

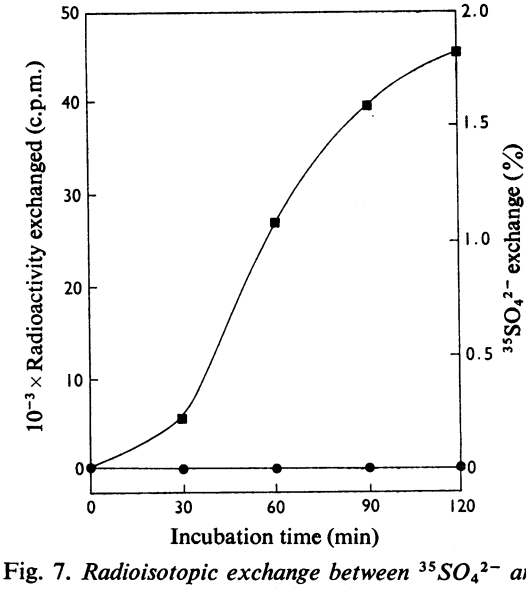

Fig. 7. *Radioisotopic exchange between $^{35}SO_4^{2-}$ and adenosine 5′-sulphatophosphate*

Reaction mixtures (see the Experimental section) containing $^{35}SO_4^{2-}$ (2.47×10^6 d.p.m., carrier-free) and adenosine 5′-sulphatophosphate (0.5mM) with (■) or without (●) 5mM-$MgATP^{2-}$ were incubated for the times shown and the amount of radioactivity incorporated into adenosine 5′-sulphatophosphate was determined by high-voltage paper electrophoresis for 1h and liquid-scintillation counting as described in the Experimental section.

with ATP. Thiosulphate, sulphite (both sodium salts), L-methionine and L-cysteine (all 2mM) had no effect on the enzyme, but significant inhibition was produced by SO_4^{2-}, sulphide (both sodium salts) and adenosine 5′-sulphatophosphate. The nature of the inhibition produced by sulphate and sulphide was

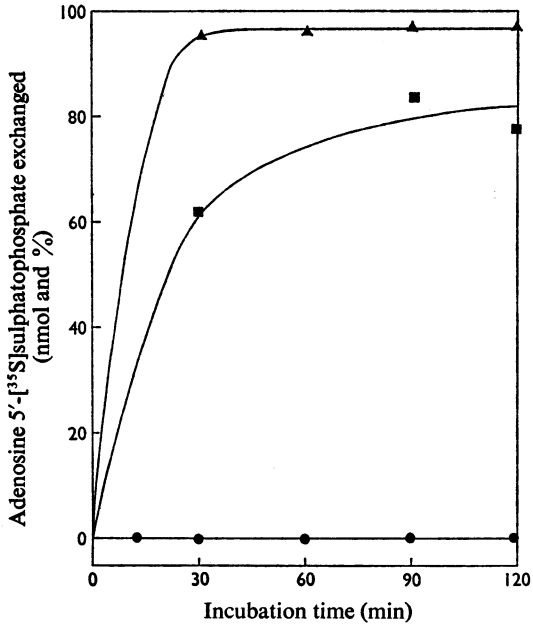

Fig. 8. *Radioisotopic exchange between adenosine 5'-[^{35}S]sulphatophosphate and SO_4^{2-}*

The reaction mixtures contained adenosine 5'-[^{35}S]-sulphatophosphate (2.0×10^5 d.p.m.) and 5mM-SO_4^{2-} with no addition (●), 5mM-$MgATP^{2-}$ added (■) or 5mM-PP_i added (▲). Samples of the reaction mixtures were subjected to high-voltage electrophoresis for 1h and the radioactivity incorporated into SO_4^{2-} was determined by liquid-scintillation counting. Any $^{35}SO_4^{2-}$ produced by degradation of adenosine 5'-[^{35}S]sulphatophosphate was accounted for.

determined from double-reciprocal velocity–substrate plots as described under 'Methods'. SO_4^{2-} was found to inhibit the enzyme in a competitive manner with respect to MoO_4^{2-}, and sulphide exhibited non-competitive inhibition with respect to this substrate. Re-plots of the vertical intercepts or slopes obtained from these double-reciprocal plots versus inhibitor concentration were linear, indicating linear inhibition in both cases (Cleland, 1970). The K_i values for each inhibitor were also obtained from these plots: SO_4^{2-}, 2.0mM; sulphide, 3.4mM.

Low amounts of adenosine 5'-sulphatophosphate produced marked changes in the shape of the velocity–substrate curves (Fig. 9), since the normal Michaelis–Menten hyperbolic form was altered to a sigmoidal curve. *p*-Chloromercuribenzoate (1mM) did not affect enzyme activity, indicating that the enzyme does not have readily available thiol groups.

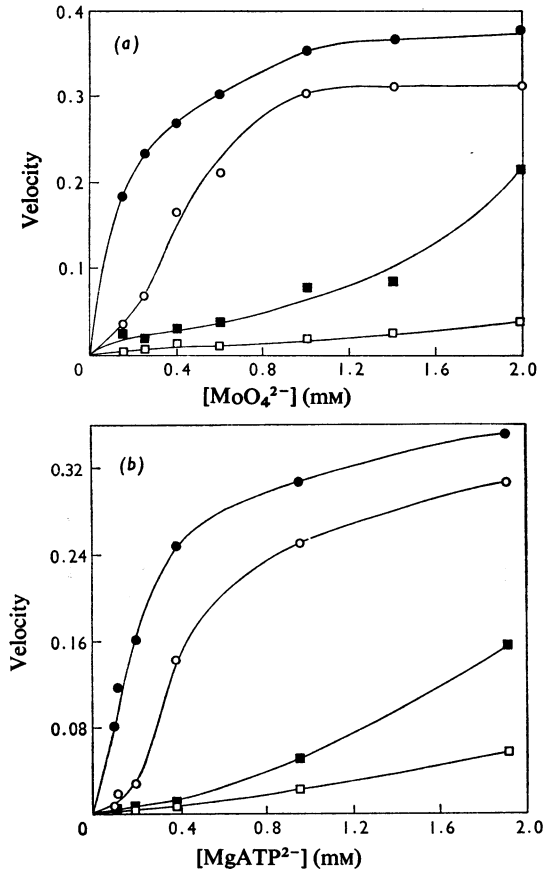

Fig. 9. *Adenosine 5'-sulphatophosphate as an inhibitor of ATP sulphurylase*

(*a*) Velocity (μmol of $MgATP^{2-}$ used/min per mg) was measured by the molybdolysis assay versus increasing MoO_4^{2-} concentrations in the presence of several adenosine 5'-sulphatophosphate concentrations. The $MgATP^{2-}$ concentration was kept constant at 1.91mM. Concentrations of inhibitor (mM): ●, 0; ○, 0.02; ■, 0.04; □, 0.08. (*b*) The MoO_4^{2-} concentration was kept constant at 2.0mM and the velocity measured at several different concentrations of adenosine 5'-sulphatophosphate, while $MgATP^{2-}$ concentration was increased. Concentrations of inhibitor (mM): ●, 0; ○, 0.02; ■, 0.04; □, 0.06.

Discussion

The molybdolysis assay is based on the finding of Bandurski *et al.* (1956) that ATP sulphurylase produces AMP and PP_i from ATP in the presence of MoO_4^{2-}. By analogy with the physiological activity of the enzyme, it is assumed that an extremely unstable anhydride, AMP–MoO_4, is produced. That the

breakdown of this complex to AMP and PP_i is not rate-limiting is indicated by the proportional increase in product formation with enzyme concentration. The methods adopted in the present work took into account the various objections raised to this assay by other workers (Levi & Wolf, 1969; Roy & Trudinger, 1970; Shaw & Anderson, 1971). Thus any interference by adenosine triphosphatase activity was measured in control tubes in the absence of MoO_4^{2-}. Bandurski & Wilson (1957) point out that MoO_4^{2-} inhibits adenosine triphosphatase; thus erroneously high control values are obtained, which adversely affect calculation of ATP sulphurylase activity. This factor may account for the increase in total enzyme activity from fraction I to fractions II and III during purification (Table 1). In all the experiments, however, fraction V, which was free from adenosine triphosphatase and inorganic pyrophosphatase, was used. Boiled-enzyme controls monitored any breakdown of ATP by trichloroacetic acid or MoO_4^{2-} and also non-specific release of P_i from proteins (Wheldrake & Pasternak, 1965) by MoO_4^{2-}. Although MoO_4^{2-} is not the physiological substrate, fraction V catalysed both the production of adenosine 5′-[^{35}S]-sulphatophosphate from $MgATP^{2-}$ and $^{35}SO_4^{2-}$, and of ATP from adenosine 5′-sulphatophosphate and PP_i. In addition, SO_4^{2-} competitively inhibited the molybdolysis reaction.

In agreement with our results, previous work with ATP sulphurylase has shown either an absolute requirement for, or activation by, Mg^{2+}. That the actual substrate is the $MgATP^{2-}$ complex was also suggested by Tweedie & Segel (1971*b*) for the *P. chrysogenum* enzyme.

Although the enzyme has been prepared from several animal and plant sources and from a variety of micro-organisms, detailed kinetic studies have been published by only a few authors and conflicting reaction mechanisms have been proposed. The intersecting double-reciprocal plots (Fig. 5) obtained for the yeast enzyme indicate that both substrates bind before either product is released. Complete initial-velocity patterns, where each substrate is in turn varied at different fixed concentrations of the other, have only been previously determined by Tweedie & Segel (1971*b*), using an homogeneous enzyme from *P. chrysogenum*, and their results suggest a similar mechanism. Product-inhibition studies are required to determine whether the substrates bind in an ordered or random manner (Cleland, 1963*a*,*b*,*c*). The products of the ATP sulphurylase molybdate reaction are PP_i and the unstable AMP–MoO_4 complex. The assay method precluded the use of PP_i as a product inhibitor, since the resultant P_i produced on incubation with inorganic pyrophosphatase prevented measurement of the small amounts of P_i produced by ATP sulphurylase (less than 0.4μmol/reaction mixture). The effects of AMP and MoO_4^{2-}, produced by breakdown of the complex, AMP–MoO_4, were also studied. MoO_4^{2-} (up to 60mM) did not inhibit the enzyme, whereas AMP was a competitive inhibitor with respect to both $MgATP^{2-}$ ($K_i = 2.0$mM) and MoO_4^{2-} ($K_i = 1.7$mM). That this inhibition pattern does not fit any of those predicted by Cleland (1963*a*) may be due to the fact that the true reaction product is the AMP–MoO_4 complex.

The ^{35}S-exchange experiments (Figs. 7 and 8), where no exchange between adenosine 5′-sulphatophosphate and SO_4^{2-} was observed in the absence of either $MgATP^{2-}$ or PP_i, suggest, as do the initial-velocity studies, that the reaction mechanism is sequential. ATP sulphurylase also catalyses $^{32}PP_i$–ATP exchange, which was shown by Segal (1956), Tweedie & Segel (1971*b*) and Shaw & Anderson (1971, 1972) to be entirely dependent on SO_4^{2-}; thus their results also support a sequential mechanism. Levi & Wolf (1969), however, found that the enzyme from rat liver catalysed $^{32}PP_i$–ATP exchange in the absence of SO_4^{2-}, and suggested a Ping Pong mechanism in which ATP is bound first to the enzyme and PP_i is released before SO_4^{2-} binds. Sequential addition of substrates to the enzyme was also proposed by Shoyab & Marx (1972), who isolated enzyme–substrate complexes of ATP sulphurylase from Furth mouse mastocytoma.

L-Cysteine and L-methionine, the end products of sulphate activation and reduction, in which ATP sulphurylase is the first enzyme of the reaction sequence, did not inhibit the yeast enzyme. Sulphide, however, is an inhibitor. At pH8.0, sulphide exists mainly as SH^- ($pK_1 = 7.04$, $pK_2 = 11.96$; *Handbook of Chemistry and Physics*, 1969–70); thus it is probably this entity that is acting as a feedback inhibitor of the enzyme.

Adenosine 5′-sulphatophosphate is a strong inhibitor, and its effect on the velocity–substrate curves (Fig. 9) suggests that it is an allosteric inhibitor (Monod *et al.*, 1965). These effects of sulphide and adenosine 5′-sulphatophosphate on the yeast enzyme are in contrast with those found by Tweedie & Segel (1971*b*) for the *P. chrysogenum* enzyme, where sulphide was an allosteric inhibitor and inhibition by adenosine 5′-sulphatophosphate was non-competitive with respect to MoO_4^{2-}.

The lack of sensitivity of the yeast enzyme to *p*-chloromercuribenzoate is in agreement with the results for ATP sulphurylase from higher plants (Adams & Johnson, 1968; Shaw & Anderson, 1972). The enzyme from *Desulphovibrio desulphuricans* (Baliga *et al.*, 1961) was neither inhibited by iodoacetate nor stimulated by thiol compounds. In contrast, the enzyme from rat liver (Levi & Wolf, 1969) was inhibited by *p*-chloromercuribenzoate, and thiol reagents alleviated the effect. The mouse mastocytoma enzyme (Shoyab *et al.*, 1972), however, was not affected by thiol compounds.

The broad activity response to pH over the range 7.0–9.0 is in general agreement with previous studies on the enzyme from yeast (Robbins, 1962), from spinach leaves (Balharry & Nicholas, 1970; Shaw & Anderson, 1972) and from *P. chrysogenum* (Tweedie & Segel, 1971*a*). Similarly, the lability of the enzyme at temperatures higher than 50°C and its specificity for ATP are in agreement with previous work (Wilson & Bandurski, 1958; Tweedie & Segel, 1971*a*).

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