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The Enzymology of Adenosine Triphosphate Sulphurylase from Spinach Leaf Tissue

KINETIC STUDIES AND A PROPOSED REACTION MECHANISM

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1. Sulphate-dependent PP_i–ATP exchange, catalysed by purified spinach leaf ATP sulphurylase, was correlated with the concentration of $MgATP^{2-}$ and $MgP_2O_7^{2-}$; ATP sulphurylase activity was not correlated with the concentration of free Mg^{2+}. 2. Sulphate-dependent PP_i–ATP exchange was independent of PP_i concentration, but dependent on the concentration of ATP and sulphate. The rate of sulphate-dependent PP_i–ATP exchange was quantitatively defined by the rate equation applicable to the initial rate of a bireactant sequential mechanism under steady-state conditions. 3. Chlorate, nitrate and ADP inhibited the exchange reaction. The inhibition by chlorate and nitrate was uncompetitive with respect to ATP and competitive with respect to sulphate. The inhibition by ADP was competitive with respect to ATP and non-competitive with respect to sulphate. 4. ATP sulphurylase catalysed the synthesis of [^{32}P]ATP from [^{32}P]PP_i and adenosine 5′-sulphatophosphate in the absence of sulphate; some properties of the reaction are described. Enzyme activity was dependent on the concentration of PP_i and adenosine 5′-sulphatophosphate. 5. The synthesis of ATP from PP_i and adenosine 5′-sulphatophosphate was inhibited by sulphate and ATP. The inhibition by sulphate was non-competitive with respect to PP_i and adenosine 5′-sulphatophosphate; the inhibition by ATP was competitive with respect to adenosine 5′-sulphatophosphate and non-competitive with respect to PP_i. It was concluded that the reaction catalysed by spinach leaf ATP sulphurylase was ordered; expressing the order in the forward direction, $MgATP^{2-}$ was the first product to react with the enzyme and $MgP_2O_7^{2-}$ was the first product released. 6. The expected exchange reaction between sulphate and adenosine 5′-sulphatophosphate could not be demonstrated.

ATP sulphurylase (ATP–sulphate adenylyltransferase, EC 2.7.7.4) catalyses the reaction:

$$ATP + SO_4^{2-} \underset{}{\overset{Mg^{2+}}{\rightleftharpoons}} \text{adenosine 5′-sulphatophosphate} + PP_i$$

The free energy change of the reaction is approx. +46 kJ/mol (Roy & Trudinger, 1970). The enzyme has been highly purified from yeast (Robbins & Lipmann, 1958), rat liver (Levi & Wolf, 1969), *Penicillium chrysogenum* (Tweedie & Segel, 1971) and spinach leaf (Shaw & Anderson, 1972). The properties of the yeast, *Penicillium* and spinach enzymes are similar, though the enzyme from *Penicillium* differs from the other two in having a higher molecular weight and in being sensitive to thiol-group reagents. The enzyme from rat liver has little in common with the enzymes from the other three sources; Levi & Wolf (1969) reported that purified rat liver enzyme catalysed PP_i–ATP exchange in the absence of sulphate and that the initial rate of PP_i–ATP exchange in the absence of sulphate was greater than the rate of synthesis of adenosine 5′-sulphatophosphate. They postulated that an adenylated form of the enzyme, synthesized from ATP, was an intermediate in the reaction. By using the terminology of Cleland (1963*a*), the reaction mechanism proposed by Levi & Wolf (1969) for rat liver ATP sulphurylase was Ping Pong. Roy & Trudinger (1970), in reviewing the enzymology of the ATP sulphurylases, showed that Levi & Wolf (1969) did not report whether the K_m values cited for each substrate of the rat liver enzyme were independent of the other substrate; K_m values for an enzyme with a Ping Pong mechanism are meaningless, since the affinity of the enzyme for one substrate is dependent on the concentration of the other substrate. However, Tweedie & Segel (1971) reported that the affinities of the *Penicillium* ATP sulphurylase for ATP and molybdate (an alternative substrate to sulphate) are independent of each other. Further, the ATP sulphurylases from *Penicillium*

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and from spinach both catalyse sulphate-dependent PP_i–ATP exchange; these enzymes do not catalyse PP_i–ATP exchange in the absence of sulphate (Tweedie & Segel, 1971; Shaw & Anderson, 1972). These observations are inconsistent with the mechanism proposed by Levi & Wolf (1969) for the rat liver enzyme.

Shaw & Anderson (1971) have described a method for the study of ATP sulphurylase involving sulphate-dependent $[^{32}P]PP_i$–ATP exchange. The kinetic and mechanistic theory of exchange reactions, described by Boyer (1959), has been applied to some amino acid-dependent PP_i–ATP exchange reactions catalysed by aminoacyl-tRNA synthetases (Rouget & Chapeville, 1968; Cole & Schimmel, 1970*a*; Santi *et al.*, 1971). The present paper reports a study of the kinetics of purified spinach leaf ATP sulphurylase by using the sulphate-dependent $[^{32}P]PP_i$–ATP exchange assay and the synthesis of $[^{32}P]$ATP from $[^{32}P]PP_i$ and adenosine 5′-sulphatophosphate; the kinetics of the reactions are consistent with an ordered reaction mechanism.

Experimental

Chemicals

Adenosine 5′-sulphatophosphate was synthesized by the method of Cherniak & Davidson (1964). After the synthesis, nucleotides were precipitated with diethyl ether and dissolved in aq. 0.5M-NH_3. The solution was adjusted to pH6 with aq. NH_3 and applied to chromatograms of acid-washed Whatman 3MM paper. The chromatograms were developed in solvent I for 24h; adenosine 5′-sulphatophosphate was detected by fluorescence quenching of u.v. light and eluted with water. Some contaminating nucleotides were eluted with adenosine 5′-sulphatophosphate; these were removed by chromatography as described by Adams *et al.* (1971). Chromatography in solvents I and II and electrophoresis in system I showed the product to be identical with adenosine 5′-sulphatophosphate. The concentration of adenosine 5′-sulphatophosphate was estimated from the extinction coefficient reported by Baddiley *et al.* (1957). The concentration was confirmed enzymically by the formation of ATP in the presence of 3 units of ATP sulphurylase (method 2). Adenosine 5′-[^{35}S]-sulphatophosphate was synthesized enzymically as described by Shaw & Anderson (1972).

[$\beta\gamma$-^{32}P]ATP was prepared as described by Burnell & Anderson (1973) except that 5 units of ATP sulphurylase were used; the specific radioactivity of the product was approx. 1Ci/mol. The product was confirmed as ATP by chromatography in solvent II and electrophoresis in system I.

All other chemicals were obtained from the sources described by Shaw & Anderson (1972).

Methods

Purification and assay of ATP sulphurylase. ATP sulphurylase was purified from spinach leaf tissue as described by Shaw & Anderson (1972); purified enzyme did not catalyse PP_i–ATP exchange in the absence of sulphate. Sulphate-dependent PP_i–ATP exchange is expressed as nmol of PP_i exchanged/min (ATP sulphurylase units). The specific activity of purified enzyme in the standard sulphate-dependent PP_i–ATP exchange assay was 3000–4000 units/mg of protein; the concentration of purified enzyme was 30–40 units/ml.

Sulphate-dependent PP_i–ATP exchange (method 1) was measured as described by Shaw & Anderson (1972) except that the $[^{32}P]$ATP synthesized was counted for radioactivity in a gas-flow planchet counter. Standard assays contained 2mM-$[^{32}P]PP_i$, 2mM-ATP, 40mM-K_2SO_4, 10mM-$MgCl_2$ and 100mM-Tris–HCl buffer, pH8, and approx. 3 units of ATP sulphurylase in a total volume of 1ml.

ATP sulphurylase activity was also measured by the incorporation of $[^{32}P]PP_i$ into ATP in the presence of adenosine 5′-sulphatophosphate (method 2). Reaction mixtures were incubated at 35°C for 15min and contained 1mM-$MgCl_2$, 0.4mM-$[^{32}P]PP_i$ (approx. 0.5μCi), 0.1mM-adenosine 5′-sulphatophosphate and approx. 0.002 unit of enzyme in a final volume of 1ml; adenosine 5′-sulphatophosphate was omitted from control assays. Reactions were terminated by the addition of 2ml of 7.5% (w/v) trichloroacetic acid. $[^{32}P]$ATP was separated from $[^{32}P]PP_i$ as described by Shaw & Anderson (1971) and counted for radioactivity in a gas-flow planchet counter. ATP sulphurylase activity, measured by method 2, is expressed as the adenosine 5′-sulphatophosphate-dependent synthesis of ATP in nmol/min (ATP sulphurylase units). The ^{32}P-labelled compound synthesised in assay method 2 was confirmed as $[^{32}P]$ATP by the procedures described by Shaw & Anderson (1971) and by electrophoresis in system I. The ratio of activity measured by method 1 : activity measured by method 2 was approx. 0.01.

Chromatography and electrophoresis. Descending paper chromatograms were run on acid-washed Whatman 3MM paper. The following solvents were used: I, propan-1-ol–aq. NH_3 (sp.gr. 0.88)–water (6:3:1, by vol.); II, isobutyric acid–aq. NH_3 (sp.gr. 0.88)–water (66:1:33, by vol.). Electrophoresis in system I was conducted on acid-washed Whatman 3MM paper for 2h at 11V/cm in 0.1M-citrate buffer, pH8 (Balharry & Nicholas, 1970). Labelled compounds on chromatograms and electrophoretograms were detected with a gas-flow radiochromatogram scanner.

All other methods were as described by Shaw & Anderson (1972).

Analysis of kinetic data. K_m values were calculated

as described by Anderson & Fowden (1970). All double-reciprocal plots of kinetic data were linear.

Results

Attainment of chemical and isotopic equilibrium in the standard sulphate-dependent PP_i–ATP exchange assay

PP_i–ATP exchange was proportional to enzyme concentration up to 0.005mg of protein/ml (approx. 15 units); purified ATP sulphurylase did not catalyse PP_i–ATP exchange in the absence of sulphate. Reaction mixtures approached isotopic equilibrium (Duffield & Calvin, 1946) irrespective of the concentration of ATP and PP_i used. The time taken to attain 50% of isotopic equilibrium, however, was dependent on the concentration of ATP and PP_i. Standard reaction mixtures containing 3 units of ATP sulphurylase attained 50% of isotopic equilibrium after approx. 2h. The rate of PP_i–ATP exchange of incubation mixtures, in which the label was supplied as [$\beta\gamma$-^{32}P]ATP, was approximately the same as the rate of exchange in reaction mixtures in which the label was supplied as [^{32}P]PP_i. The rate of sulphate-dependent PP_i–ATP exchange, calculated by the method of Davie *et al.* (1956), was constant at any specified enzyme concentration for 3h under a variety of substrate concentrations, but with some permutations of substrate concentrations isotopic equilibrium was attained in less than 3h. Further, neither adenosine 5′-sulphatophosphate, ADP nor AMP was detected after 3h in incubation mixtures containing any of the permutations of substrate concentrations tested. This implies that the substrate concentration of ATP was virtually constant. Taken collectively, our results indicate that these incubation mixtures come to chemical equilibrium almost instantaneously.

Role of Mg^{2+} in reactions catalysed by ATP sulphurylase

The sulphate-dependent PP_i–ATP exchange reaction catalysed by spinach leaf ATP sulphurylase is magnesium dependent (Shaw & Anderson, 1971, 1972); exchange is also dependent on ATP concentration, but essentially independent of PP_i concentrations greater than 0.125mM. The forms of magnesium in reaction mixtures containing 0.125–2mM-Na_4ATP, 0.125–2mM-$Na_4P_2O_7$, 1.25–40mM-Na_2SO_4, 100mM-Tris–HCl buffer, pH8, and 0.06–20mM-$MgCl_2$ were calculated by the method of Perrin & Sayce (1967) and studied with respect to PP_i–ATP exchange; the stability constants used to calculate the various magnesium complexes are summarized in Table 1. In reaction mixtures containing 10–20mM-$MgCl_2$ almost all of the ATP and PP_i occurred as $MgATP^{2-}$ and $MgP_2O_7^{2-}$ irrespective of the concentration of ATP and/or PP_i. The concentration of the $MgATP^{2-}$ form was correlated with sulphate-dependent PP_i–ATP exchange. This principle is illustrated for reaction mixtures containing the amounts of ATP and PP_i used in a standard PP_i–ATP exchange assay (Fig. 1). The concentration of $MgP_2O_7^{2-}$ also correlated with sulphate-dependent PP_i–ATP exchange, but the affinity of the enzyme for PP_i in the sulphate-dependent PP_i–ATP exchange assay is extremely high and essentially independent of PP_i concentration. It is therefore not possible to establish the active species of PP_i from this analysis. At very high concentrations of $MgCl_2$, however, the sulphate-dependent PP_i–ATP exchange is inhibited; the inhibition was correlated with a decrease in the concentration of $MgP_2O_7^{2-}$ as insoluble $Mg_2P_2O_7$ was formed. In incubation mixtures containing more than 10mM-$MgCl_2$ or less than 2mM-PP_i, less than 40mM-Na_2SO_4 or less than 2mM-ATP, the proportion of ATP and PP_i in the $MgP_2O_7^{2-}$ and $MgATP^{2-}$ forms was even greater than shown for the conditions illustrated in Fig. 1.

The concentration of free Mg^{2+} did not correlate with ATP sulphurylase activity. We conclude that Mg^{2+} is required for the sulphate-dependent PP_i–ATP exchange reaction to form $MgATP^{2-}$ and $MgP_2O_7^{2-}$ complexes, which are the active species of ATP and PP_i respectively. Cole & Schimmel (1970*b*) and Tweedie & Segel (1971) reached a similar conclusion with respect to the isoleucyl-tRNA synthetase of *Escherichia coli* and the ATP sulphurylase from *P. chrysogenum* respectively, though Santi *et al.* (1971) concluded that Mg^{2+} itself was the active form of magnesium for the phenylalanyl-tRNA synthetase of *E. coli*. The qualitative effect of $MgCl_2$ concentration on phenylalanine-dependent PP_i–ATP exchange, however, was different from that observed for sulphate-dependent PP_i–ATP exchange catalysed by ATP sulphurylase (Shaw & Anderson, 1972).

Table 1. *Cumulative dissociation constants used to compute the concentration of species of PP_i, ATP, ADP, sulphate and free Mg^{2+} in incubation mixtures at pH8*

Species	Cumulative pK	Reference
$HATP^{3-}$	6.95	Alberty (1969)
$MgATP^{2-}$	4.0	Alberty (1969)
$MgHATP^{-}$	8.44	Alberty (1969)
$NaATP^{3-}$	1.06	Cole & Schimmel (1970*b*)
$HP_2O_7^{3-}$	8.95	Alberty (1969)
$H_2P_2O_7^{2-}$	15.07	Alberty (1969)
$MgP_2O_7^{2-}$	5.41	Alberty (1969)
$MgHP_2O_7^{-}$	12.01	Alberty (1969)
$Mg_2P_2O_7$	7.75	Alberty (1969)
$NaP_2O_7^{3-}$	1.0	Cole & Schimmel (1970*b*)
$MgSO_4$	2.4	Dawson *et al.* (1969)
HSO_4^{-}	2.0	Aylward & Findlay (1966)
$MgADP^{-}$	3.01	Alberty (1969)
$NaADP^{2-}$	0.65	Dawson *et al.* (1969)
$HADP^{2-}$	6.88	Alberty (1969)
MgHADP	8.33	Alberty (1969)

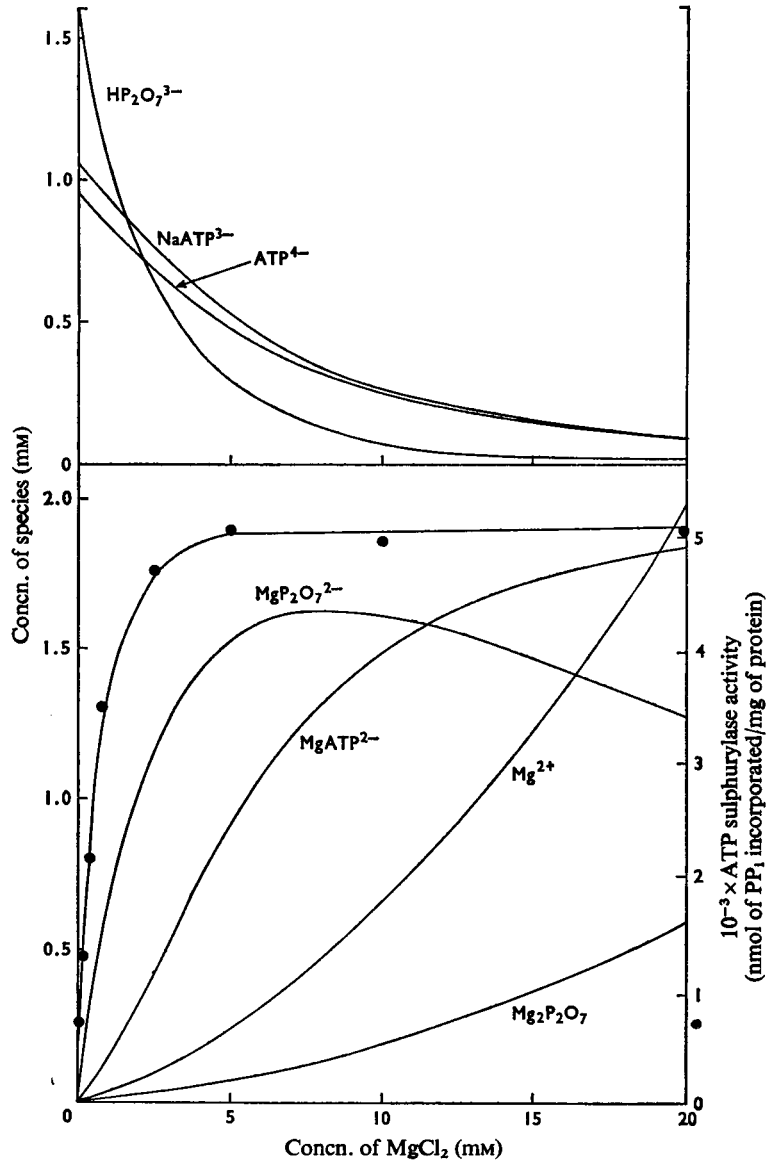


Fig. 1. *Effect of concentration of $MgCl_2$ on the forms of PP_i and ATP and on ATP sulphurylase activity*

Reaction mixtures contained 2μmol of $Na_4P_2O_7$, 2μmol of Na_4ATP, 40μmol of Na_2SO_4, 100μmol of Tris–HCl, pH 8, 8.4 units of enzyme and the amounts of $MgCl_2$ specified, in a final volume of 1 ml. ATP sulphurylase activity was measured by PP_i–ATP exchange (method 1). Since the concentration of individual species of ATP and PP_i varied with the concentration of Mg^{2+}, the exchange equation of Davie *et al.* (1956) could not be applied. Therefore ATP sulphurylase activity is expressed as sulphate-dependent incorporation of PP_i into ATP. ●, ATP sulphurylase activity. The forms of PP_i and ATP were calculated as described in the text. Only major species of PP_i and ATP are shown. The concentration of free Mg^{2+} is also shown.

In studies of ATP sulphurylase by method 2, the optimum concentration of $MgCl_2$ was approx. 0.25–1.0mM; higher concentrations of $MgCl_2$ (4–10mM) were inhibitory. The method of Perrin & Sayce (1967) was used to calculate the forms of PP_i at various concentrations of $MgCl_2$ in the incubation mixtures used in method 2. At 0.25–1.0mM-$MgCl_2$, approx. 80% of the PP_i was present as $MgP_2O_7^{2-}$;

at 4–10mM-$MgCl_2$, approx. 45–70% of the PP_i was present as $Mg_2P_2O_7$. This indicates that $MgP_2O_7^{2-}$ is the form of PP_i used in the back reaction.

Substrate-concentration studies of sulphate-dependent PP_i–ATP exchange

The effect of ATP concentration (0.125–2mM) and sulphate concentration (1.25–40mM) on sulphate-dependent PP_i–ATP exchange in the presence of 10mM-$MgCl_2$ was studied in a factorial experiment; ATP and PP_i were present predominantly as the $MgATP^{2-}$ and $MgP_2O_7^{2-}$ forms (Fig. 1). PP_i–ATP exchange was dependent on the concentration of both ATP and sulphate and both substrates were required for exchange to occur; this rules out the possibility of a non-sequential mechanism as has been proposed for the rat liver ATP sulphurylase (Levi & Wolf, 1969). Double-reciprocal plots showed that the reaction was not inhibited by sulphate or ATP and the intercepts and slopes for each concentration of ATP and sulphate were linear functions of $[ATP]^{-1}$ and $[sulphate]^{-1}$. PP_i–ATP exchange was independent of the concentration of PP_i at the concentrations of PP_i, ATP and sulphate examined.

Under steady-state conditions, the initial velocity of a bireactant sequential reaction can be described by the equation:

$$v = \frac{V[A][B]}{K_{ia}+K_a[B]+K_b[A]+[A][B]} \quad (1)$$

where A and B are the first and second substrates respectively and K_{ia}, K_a and K_b are kinetic constants (Cleland, 1970). Kinetic constants analogous to K_{ia}, K_a and K_b were calculated for the PP_i–ATP exchange from secondary plots of the slopes and intercepts of double-reciprocal plots by using the procedures applicable to the calculation of the constants of a bireactant sequential mechanism (Cleland, 1970). The values of the constants analogous to K_{ia}, K_a and K_b, assuming ATP is the first substrate, were 0.85, 0.33 and 3.5mM respectively and 8.6, 3.5 and 0.33mM assuming that sulphate was the first substrate. Eqn. (1) quantitatively accounted for the PP_i–ATP exchange data when the experimentally determined constants were substituted into the equation in lieu of the theoretical constants irrespective of whether sulphate or ATP was considered as the first substrate. This agreement suggests that the kinetics of initial-velocity studies under steady-state conditions also apply to the equilibrium conditions of sulphate-dependent PP_i–ATP exchange, as has been assumed by Cleland (1967) for deriving theoretical rate equations for exchange reactions. The data also suggest that the mechanism of the reaction catalysed by spinach ATP sulphurylase is inconsistent with a Ping Pong mechanism.

Inhibition studies of sulphate-dependent PP_i–ATP exchange

Some nucleotides and anions were surveyed as possible inhibitors of sulphate-dependent PP_i–ATP exchange; 0.5mM-chlorate and 1mM-ADP were powerful inhibitors, effecting 51% and 54% inhibition of exchange in standard assays. Nitrate was a less effective inhibitor.

The kinetics of the inhibition of sulphate-dependent PP_i–ATP exchange in the presence of 0.25–1.0mM-$KClO_3$, 0.4–2mM-ADP and 5–20mM-KNO_3 were studied in factorial experiments containing 1.25–40mM-K_2SO_4 and 1.25–2mM-ATP in the presence of 2mM-PP_i and 10mM-$MgCl_2$. The inhibition of exchange by chlorate was uncompetitive with respect to ATP (Fig. 2) but competitive with respect to sulphate. Qualitatively similar results were obtained with nitrate. The data suggest that chlorate and nitrate combine with the enzyme form usually occupied by sulphate or with a closely related form in equilibrium with the sulphate-accepting form. The inhibition of exchange by ADP, however, was competitive with respect to ATP and non-competitive with respect to sulphate (Fig. 3). ATP, PP_i and ADP in the presence of 10mM-$MgCl_2$ were present as $MgATP^{2-}$, $MgP_2O_7^{2-}$ and $MgADP^-$ forms; the inhibition of exchange by ADP could not be accounted for by a decrease in the concentration of $MgATP^{2-}$ or $MgP_2O_7^{2-}$ forms.

It is very unlikely that chlorate, nitrate and ADP

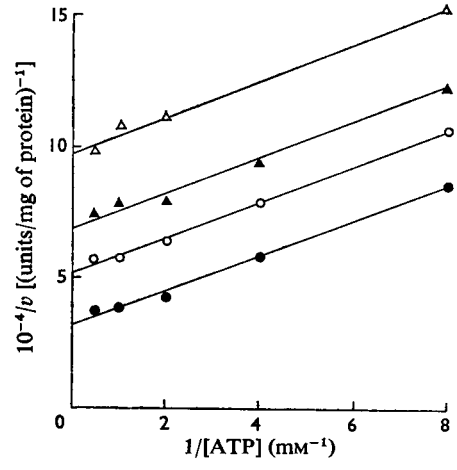


Fig. 2. *Double-reciprocal plot of the effect of concentration of ATP on ATP sulphurylase activity with and without potassium chlorate*

ATP sulphurylase activity was measured by PP_i–ATP exchange (method 1) except that the standard amount of ATP was replaced with the amounts specified. ●, No $KClO_3$; ○, 0.25mM-$KClO_3$; ▲, 0.5mM-$KClO_3$; △, 1mM-$KClO_3$.

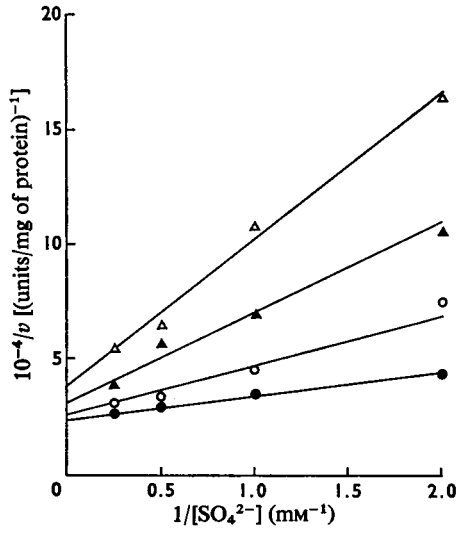
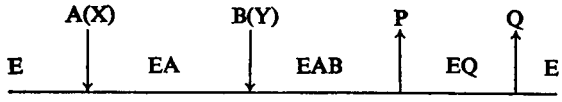


Fig. 3. *Double-reciprocal plot of the effect of concentration of sulphate on ATP sulphurylase with and without ADP*

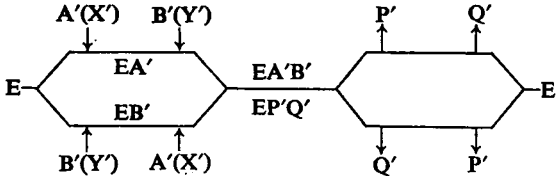
ATP sulphurylase activity was measured by PP_i–ATP exchange (method 1) except that the standard amount of sulphate was replaced with the amounts of sulphate specified. ●, No ADP; ○, 0.4mM-ADP; ▲, 1mM-ADP; △, 2mM-ADP.

are converted into products, since ATP sulphurylase does not catalyse PP_i–ATP exchange when sulphate is replaced with chlorate or nitrate nor when ATP (or sulphate) is replaced with ADP. We presume that ADP and chlorate are dead-end inhibitors.

Cleland (1963*b*,*c*) has described the inhibition patterns for an ordered sequential reaction mechanism under steady-state conditions as follows:



where E is the enzyme, A and B are the first and second substrates respectively, P and Q are products, and X and Y are dead-end inhibitors of A and B respectively. The inhibition of activity by X would be competitive with respect to A and non-competitive with respect to B. The inhibition of activity by Y would be competitive with respect to B and uncompetitive with respect to A. For the random reaction mechanism (Scheme 1):



in which X′ and Y′ are dead-end inhibitors of A′ and B′ respectively, the inhibition of activity by X′ is competitive with respect to both A′ and B′; the inhibition of activity by Y′ is also competitive with respect to A′ and B′. It is noted that Cleland (1963*b*,*c*) was referring to initial velocities under steady conditions. The theory, however, has been applied to enzyme systems at chemical equilibrium (Rouget & Chapeville, 1968; Santi *et al.*, 1971). The patterns of inhibition of ATP sulphurylase activity by chlorate, nitrate and ADP with respect to sulphate and ATP suggest that the reaction catalysed by spinach leaf ATP sulphurylase is ordered and that ATP combines with the enzyme first; the inhibition patterns are inconsistent with a random mechanism.

Studies of the formation of ATP by using PP_i and adenosine 5′-sulphatophosphate as substrates

Balharry & Nicholas (1970) described the synthesis of ATP from adenosine 5′-sulphatophosphate and PP_i by using the luciferin–luciferase assay to measure the amount of ATP synthesized. Purified ATP sulphurylase, in our experiments, catalysed the synthesis of [^{32}P]ATP from [^{32}P]PP_i and adenosine 5′-sulphatophosphate in the presence of Mg^{2+}, confirming the results of Balharry & Nicholas (1970); the synthesis of [^{32}P]ATP was demonstrated by chromatography and by electrophoresis in system I as described by Shaw & Anderson (1971). Some properties of the reaction were studied; $K_m(PP_i)$ was 16μM and K_m (adenosine 5′-sulphatophosphate) was 3.5μM. The role of $MgCl_2$ in the reaction has been discussed above. The synthesis of ATP in reaction mixtures lacking $MgCl_2$ was large; trace amounts of Mg^{2+} in some substrates (up to 5μM in the incubation mixture) together with the high affinity of the enzyme for PP_i (presumably as $MgP_2O_7^{2-}$) was sufficient to account for the enzyme activity in the absence of $MgCl_2$. Addition of EDTA (0.5μmol) abolished the synthesis of ATP in controls without Mg^{2+}; EDTA itself was not inhibitory. These properties are in general agreement with the properties described by Balharry & Nicholas (1970).

The effects of concentration of adenosine 5′-sulphatophosphate (2–8μM) and PP_i (6–100μM) on the activity of the reverse reaction was studied in a factorial experiment in the presence of 1mM-$MgCl_2$. The slopes and intercepts of double-reciprocal plots of activity against concentration of adenosine 5′-sulphatophosphate were dependent on the concentration of PP_i. Similarly the slopes and intercepts of double-reciprocal plots of activity against concentration of PP_i were dependent on the concentration of adenosine 5′-sulphatophosphate. These studies indicated that the reaction mechanism for the reverse reaction was sequential.

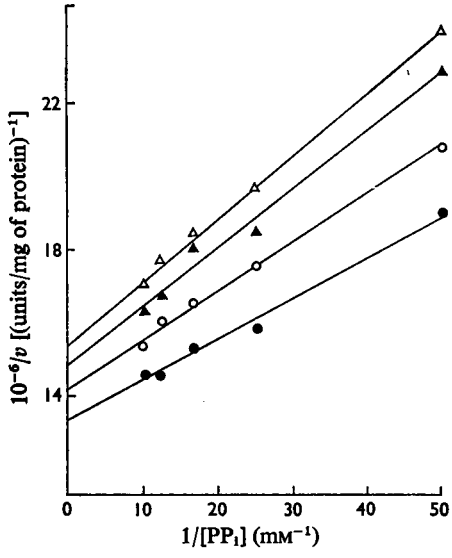


Fig. 4. *Double-reciprocal plot of the effect of concentration of PP_i on ATP sulphurylase activity with and without ATP*

ATP sulphurylase activity was measured by the formation of ATP (method 2) except that the standard amount of PP_i was replaced with the amounts specified and the amount of adenosine 5'-sulphatophosphate was decreased to 8nmol. ●, No ATP; ○, 0.125mM-ATP; ▲, 0.25mM-ATP; △, 0.5mM-ATP.

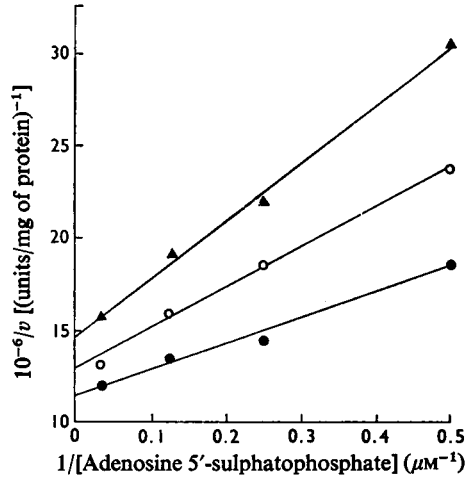
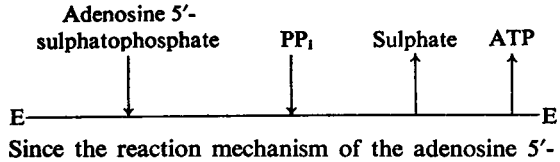


Fig. 5. *Double-reciprocal plot of the effect of concentration of adenosine 5'-sulphatophosphate on ATP sulphurylase activity with and without Na_2SO_4*

ATP sulphurylase activity was measured by the formation of ATP (method 2) except that the standard amount of adenosine 5'-sulphatophosphate was replaced with the amounts specified and the amount of PP_i was decreased to 0.1μmol. ●, No Na_2SO_4; ○, 10mM-Na_2SO_4; ▲, 20mM-Na_2SO_4.

ATP and sulphate, end products of the back reaction, inhibited the synthesis of ATP from PP_i and adenosine 5'-sulphatophosphate. The inhibition of activity by ATP was competitive with respect to adenosine 5'-sulphatophosphate and non-competitive with respect to PP_i (Fig. 4). The inhibition of activity by sulphate was non-competitive with respect to PP_i and non-competitive with respect to adenosine 5'-sulphatophosphate (Fig. 5). The kinetics of the inhibition of the reverse reaction by ATP and sulphate are consistent with the kinetics of an ordered sequential mechanism (Cleland, 1963*b*,*c*). The inhibition kinetics indicate that the order of addition of the substrates and release of the products for the reverse reaction is as follows:



Since the reaction mechanism of the adenosine 5'-sulphatophosphate-dependent synthesis of ATP is sequential and adenosine 5'-sulphatophosphate is the first substrate to react with the enzyme, it is possible to calculate the kinetic constants applicable to eqn. (1) (Cleland, 1970). The constants K_a, K_b and K_{ia} can be designated K(adenosine 5'-sulphatophosphate), $K(PP_i)$ and K (enzyme–adenosine 5'-sulphatophosphate) respectively; empirically derived values for these constants were 3.5μM, 16μM and 2.8μM respectively.

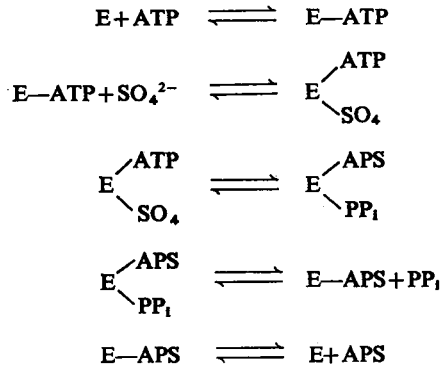
Studies of sulphate–adenosine 5'-sulphatophosphate exchange

Isotopic exchange between [^{35}S]sulphate and adenosine 5'-sulphatophosphate could not be demonstrated over a wide range of reaction conditions; the conditions included 5–40mM-$K_2{}^{35}SO_4$ (up to 100μCi), 10–200μM-adenosine 5'-sulphatophosphate, 0.125–2mM-ATP and 1–20 units of ATP sulphurylase with and without 4μM-PP_i in reactions running from 15min to 2h. The procedures used to isolate adenosine 5'-[^{35}S]sulphatophosphate included paper chromatography (Shaw & Anderson, 1972), electrophoresis in system I and a combination of both methods; the synthesis of adenosine 5'-[^{35}S]sulphatophosphate could be readily demonstrated when Mg^{2+}-dependent pyrophosphatase was added (Shaw & Anderson, 1972). Similarly, isotopic exchange between adenosine 5'-[^{35}S]sulphatophosphate and sulphate could not be demonstrated; the conditions included 10 and 50μM-adenosine 5'-[^{35}S]sulphatophosphate, 20mM-

Na_2SO_4 and 1mM-ATP incubated with or without 4μM-PP_i for 15min and 1h.

Discussion

The kinetics of substrate interaction and product-inhibition studies of the reverse reaction catalysed by ATP sulphurylase indicate that the reaction mechanism is sequential. Expressing the order in the forward direction, $MgATP^{2-}$ is the first substrate to react with the enzyme and $MgP_2O_7^{2-}$ is the first product released. The forms of sulphate and adenosine 5'-sulphatophosphate (APS) in the following reaction sequence are unknown:



This mechanism predicts that sulphate-dependent PP_i–ATP exchange could proceed in the absence of exogenous adenosine 5'-sulphatophosphate. PP_i–ATP exchange need only involve the first four partial reactions and chemical equilibrium could be reached after a single turnover, depending on incubation conditions, to form the enzyme–adenosine 5'-sulphatophosphate complex; a similar proposal has been advanced by Cole & Schimmel (1970*a*) for the PP_i–ATP exchange reaction catalysed by isoleucyl-tRNA synthetase. The rapidity with which sulphate-dependent PP_i–ATP exchange comes to chemical equilibrium and the failure to detect the synthesis of free adenosine 5'-sulphatophosphate in single enzyme experiments is consistent with the proposal that exchange need only involve the first four partial reactions, although the failure to detect free adenosine 5'-sulphatophosphate could be a function of the free-energy change of the reaction. The kinetics of the inhibition of exchange by chlorate, nitrate and ADP is also consistent with the proposed mechanism.

One consequence of the proposed mechanism is that it should be possible to demonstrate both PP_i–ATP exchange and sulphate–adenosine 5'-sulphatophosphate exchange. Exchange was readily demonstrated between [^{32}P]PP_i and ATP and [^{32}P]ATP and PP_i over a wide range of incubation conditions including low concentrations of PP_i (125μM). The expected exchange between [^{35}S]sulphate and unlabelled adenosine 5'-sulphatophosphate and adenosine 5'-[^{35}S]sulphatophosphate and unlabelled sulphate under essentially analogous conditions employed in the study of PP_i–ATP exchange could not be demonstrated. Levi & Wolf (1969) and Tweedie & Segel (1971) were also unable to demonstrate sulphate–adenosine 5'-sulphatophosphate exchange.

Another consequence of the proposed reaction mechanism is that sulphate should inhibit sulphate-dependent PP_i–ATP exchange (Santi *et al.*, 1971). Our inability to detect inhibition of exchange by the proposed second substrate is analogous to the results of Rouget & Chapeville (1968) and Cole & Schimmel (1970*a*) for other PP_i–ATP exchange reactions.

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