Kinetic mechanism of ATP-sulphurylase from rat chondrosarcoma

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ATP-sulphurylase catalyses the production of adenosine 5'-phosphosulphate (APS) from ATP and free sulphate with the release of PP_i. APS kinase phosphorylates the APS intermediate to produce adenosine 3'-phosphate 5'-phosphosulphate (PAPS). The kinetic mechanism of rat chondrosarcoma ATP-sulphurylase was investigated by steady-state methods in the physiologically forward direction as well as the reverse direction. The sulphurylase activity was coupled to APS kinase activity in order to overcome the thermodynamic constraints of the sulphurylase reaction in the forward direction. Double-reciprocal initial-velocity plots for the forward sulphurylase intersect to the left of the ordinate for this reaction. K_m^{ATP} and $K_m^{sulphate}$ were found to be 200 and 97 μ M respectively. Chlorate, a competitive inhibitor with respect to sulphate, showed uncompetitive inhibition with

respect to ATP with an apparent K_i of 1.97 mM. Steady-state data from experiments in the physiologically reverse direction also yielded double-reciprocal initial-velocity patterns that intersect to the left of the ordinate axis, with a K_m^{APS} of 39 μ M and a $K_m^{pyrophosphate}$ of 18 μ M. The results of steady-state experiments in which Mg²⁺ was varied indicated that the true substrate is the MgPP_i complex. An analogue of APS, adenosine 5'-[β -methylene]phosphosulphate, was a linear inhibitor competitive with APS and non-competitive with respect to MgPP_i. The simplest formal mechanism that agrees with all the data is an ordered steady-state single displacement with MgATP as the leading substrate in the forward direction and APS as the leading substrate in the reverse direction.

INTRODUCTION

Sulphate activation in mammalian tissues requires the sequential action of the cytosolic enzymes ATP-sulphurylase (ATP-sulphate adenylyltransferase, EC 2.7.7.4) and adenosine 5'-phosphosulphate (APS) kinase (ATP-adenylylsulphate 3'-phosphotransferase, EC 2.7.1.25). The product adenosine 3'-phosphate 5'-phosphosulphate (PAPS) is then translocated into the Golgi, where reactions catalysed by membrane-bound sulphotransferases generate a variety of sulphate esters. Although the sulphate-activation pathway has been described for several organisms [1], a thorough kinetic analysis of the reactions catalysed by these two enzymes in a single species has been achieved only for the fungus *Penicillium* [2–7]. The ATP-sulphurylase reaction has been particularly difficult to study because of its unfavourable equilibrium constant (10^{-8}) in the physiological direction, the chemical and biochemical lability of APS and potent inhibition of the enzyme by APS itself.

Our previous studies of ATP-sulphurylase and APS kinase from a transplantable rat chondrosarcoma showed that the activities possessed similar optimal pH and temperature profiles and were fractionated together through an extensive purification procedure [8], thus raising the question of the physical relationship between the two enzymes. Furthermore, we have discovered an intriguing dual defect affecting both enzymes of sulphate activation [9,10] which has led to a search for ways in which a single abnormality might alter both enzymes. In addition to biochemical and structural characterization currently in progress, kinetic analysis of the two enzymes, individually and in coupled reactions, is a powerful approach to elucidating their relationship. Thus the studies in this and the following paper [11]

describe the complex kinetic behaviour of the overall sulphate-activation pathway using experiments designed appropriately to study the two activities both individually and as a coupled system. Steady-state kinetic studies of the reaction catalysed by the ATP-sulphurylase of rat chondrosarcoma, which describe the initial-velocity patterns and the inhibition by products and dead-end inhibitors, including adenosine 5'-[β -methylene]phosphosulphate (Me-APS) [12], are presented in the present paper. These results differ significantly from previously reported data obtained for the enzymes from lower organisms, suggesting an important evolutionary change in the action of this system.

EXPERIMENTAL

Materials

Na₄³²P₂O₇ (1–60 Ci/mmol) and H₂³⁵SO₄ (10 mCi/ml, carrier-free) were obtained from New England Nuclear (Boston, MA, U.S.A.). Dithiothreitol (DTT), APS, ATP and alkaline phosphatase were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The APS used for sulphurylase kinetic analyses yielded a single spot on t.l.c. on silica in propan-2-ol/water/30% NH₄OH (7:2:1, by vol.) and a single sharp peak on h.p.l.c. analysis with a Mg²⁺-binding permutation of reversed-phase ion pairing. All other chemicals used were reagent grade. Me-APS, in which the phosphosulphate anhydride linkage is replaced by a more stable methylene bridge, was prepared as previously described [12]. Before use, the purity and chemical structure of this compound were verified by both n.m.r. spectroscopy and m.s.

ATP-sulphurylase was prepared from extracts of Swarm rat chondrosarcoma tissue by the method previously described [8];

Abbreviations used: PAPS, adenosine 3'-phosphate 5'-phosphosulphate; APS, adenosine 5'-phosphosulphate; Me-APS, adenosine 5'-[β -methylene]phosphosulphate; DTT, dithiothreitol.

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these preparations also contained APS kinase activity. Purified *Penicillium chrysogenum* APS kinase (from Dr. Irwin Segel, University of California at Davis, CA, U.S.A.) was stored frozen in 40 mM Tris/HCl, pH 4.0, and diluted into buffer A (25 mM NaHPO₄/KH₂PO₄, pH 7.8, 10% glycerol, 1 mM DTT, 1 mM EDTA) before use.

Kinetic assay

The forward sulphurylase assay was modified from Sugahara and Schwartz [10]. The standard reaction mixture was 25 μ l containing 20 mM MgCl₂, 14 mM Tris/HCl (pH 8.0), 6.5 μ l of buffer A and 1 μ l of enzyme preparation, purified through an ATP-affinity column [8]. Concentrations of ATP and H₂³⁵SO₄ (400–1000 Ci/mol) were varied according to experimental conditions. The reaction was carried out for 10 min and stopped with ice-cold ethanol and freezing in solid CO₂. Products were separated and measured as described previously [10]. Product accumulation was found to be linear with time, and steady-state velocities were linear with enzyme concentration under the conditions employed.

The reverse assay is a modification of that described by Burnell and Roy [13], which measures the velocity of the reaction in the direction of ATP formation. Unless otherwise specified, a standard 1 ml incubation mixture contained the following components at the indicated final concentrations: 50 mM Na₂HPO₄/KH₂PO₄ buffer (pH 7.8), 12 mM MgCl₂, 0.5 mM DTT and 5 mM NaF. Concentrations of Na₄P₂O₇, containing 6.66 μ Ci of ³²P, and of APS, from a 5 mM stock kept in 50% ethanol at –20 °C to minimize decomposition, were varied according to experimental conditions. Reactions were started by the addition of 50 μ l of enzyme solution, purified as far as the hydroxyapatite step [8]. The reaction time was 20 min, and product formation was measured as described previously [8]. Product accumulation was found to be linear with time, and steady-state velocities (concentration of ATP per unit time) were linear with enzyme concentration under the conditions employed.

Data processing

Kinetic data were processed by unweighted iterative non-linear least-squares fitting of initial velocities as a function of substrate concentration. The kinetic parameters obtained by fitting the data points to rectangular hyperbolic plots were then used to plot the initial-velocity pattern in linear double-reciprocal form as shown in Figures 1–6. Kinetic constants were derived from secondary plots of the best-fit parameters from the primary data pattern. Figure 3 was additionally analysed by using the kinetic constants with the appropriate rate equation to model the overall initial-velocity pattern for comparison with the data set as described in the Results section. The confidence limits shown in the secondary plots of kinetic figures represent standard error values derived from the non-linear least-squares fitting of the primary data values. Inhibition types were assigned by considering the statistical significance of the difference between intercept and/or slope values of double-reciprocal plots from experiments in the presence and absence of the inhibiting agent. Thus an inhibitor was assigned as competitive when the data pattern showed a significant difference in the slope values and no variation in the intercept values, uncompetitive when there was a significant change in the intercepts and no difference in the slopes, and non-competitive when there was a significant change in both the intercepts and slopes.

RESULTS

Forward sulphurylase reaction

The forward ATP-sulphurylase reaction is measured in the presence of APS kinase activity which removes the APS and thus helps drive the forward reaction. To ensure that the velocity of the sulphurylase reaction was the rate being measured and that the kinase reaction was not rate-limiting, purified APS kinase from *P. chrysogenum* was added to the reaction mixture. At high substrate concentrations (2 mM ATP and 1 mM SO₄²⁻), the velocity increased about 3-fold, whereas at lower concentrations (1 mM ATP and 0.25 mM SO₄²⁻), the velocity did not increase at all. Thus it appears that the kinase reaction becomes rate-limiting at high substrate concentrations, an effect that is relieved by the added kinase enzyme, whereas at lower concentrations the sulphurylase reaction is rate-limiting and the additional kinase activity has no effect.

The kinetic mechanism of the reaction catalysed by ATP-sulphurylase in the physiologically forward direction of APS synthesis was investigated through bisubstrate variation experiments. *P. chrysogenum* APS kinase (0.16 pmol/min as measured by the kinase assay [8], at 200 μ M ATP and 40 nM [³⁵S]APS) was added to the forward sulphurylase assay to ensure that the sulphurylase reaction was rate-limiting at all concentrations. Initial-velocity patterns are shown in Figure 1. The primary pattern presented in this Figure indicates that the mechanism is

Figure 1 SO₄²⁻ and ATP initial-velocity and secondary patterns for ATP-sulphurylase

(a) Initial-velocity pattern for the rat chondrosarcoma ATP-sulphurylase reaction with ATP varied at the following concentrations of sulphate: ○, 2 mM; ▲, 1 mM; □, 0.5 mM; ●, 0.15 mM. (b) Secondary plot of primary intercepts (min · mg of protein/nmol of APS) versus reciprocal ATP concentration. (c) Secondary plot of primary slopes (min · mg · mM ATP/nmol of APS) versus reciprocal ATP concentration.

Figure 2 ATP-sulphurylase inhibition by chlorate with respect to ATP

(a) The inhibition pattern for the rat chondrosarcoma ATP-sulphurylase forward reaction in the presence of the following concentrations of chlorate: $\circ$, uninhibited; $\blacksquare$, 1 mM; $\triangle$, 2.5 mM; $\bullet$, 5 mM. (b) Secondary plot of primary intercepts (min · mg of protein/nmol of APS) versus chlorate concentration. (c) Secondary plot of primary slopes (min · mg · mM ATP/nmol of APS) versus chlorate concentration. The sulphate concentration was 0.5 mM.

Table 1 Kinetic constants of rat chondrosarcoma ATP-sulphurylase

Values are derived from the non-linear least-squares fits of the slope and intercept replots.

Constant	Value
K_m (ATP)	$200 \pm 80 \mu\text{M}$
K_m (SO_4^{2-})	$97 \pm 4 \mu\text{M}$
K_x (forward)	$(91 \pm 2) \times 10^3 \mu\text{M}^2$
V_i	$3.35.0.05 \text{ nmol/min} \cdot \text{mg}$
App. K_i (ClO_4^{2-})	$1.97 \pm 0.4 \text{ mM}$
K_m (APS)	$39 \pm 4 \mu\text{M}$
K_m (PP_i)	$18 \pm 7 \mu\text{M}$
V_r^*	$160 \pm 15 \text{ nmol/min} \cdot \text{mg}$
K_x (reverse) [†]	$94 \pm 19 \mu\text{M}^2$
App. K_i (Me-APS)	$12 \pm 2 \mu\text{M}$

* Value normalized to account for difference in enzyme preparations of the forward and reverse assays.

[†] $K_x = [k_{+1}/(k_{-2} + k_{+3})]/[K_i/k_{+2}(k_{+3} + k_{+4})]$. K_x is evaluated as the ratio of the slope-change term [the cross term in eqn. (1)] to the concentration-independent term ($1/k_{+3} + 1/k_{+4}$).

single displacement (also known as a ternary complex or sequential mechanism).

Potassium perchlorate, which exhibited competitive inhibition with respect to sulphate (results not shown), was used as a dead-end inhibitor to determine the order of addition of the substrates

Figure 3 APS and PP_i initial-velocity and secondary patterns for ATP-sulphurylase in the physiologically reverse direction

(a) Initial-velocity pattern for the rat chondrosarcoma ATP-sulphurylase reaction with PP_i varied at the following concentrations of APS: $\circ$, 3.45 μM ; $+$, 2.5 μM ; $\triangle$, 1.25 μM ; $\bullet$, 0.8 μM ; $\square$, 0.5 μM ; $\blacktriangle$, 0.35 μM ; $\blacksquare$, 0.25 μM ; $\times$, 0.13 μM . The lines shown represent computer-modelled plots superimposed on the experimental points. This computer model was generated from eqn. (1) using the following kinetic constants: K_m (APS), 41 μM ; K_m (PP_i), 33 μM ; K_x , $8.9 \times 10^{-11} \text{ M}^2$; V_{max} , 99 nM s^{-1} . (b) Secondary plot of primary intercepts (s/nM ATP) versus reciprocal APS concentration. (c) Secondary plot of primary slopes (s · $\mu\text{M PP}_i$ /nM ATP) versus reciprocal APS concentration. The points in (b) and (c) represent the slopes and intercepts of primary plots individually fitted by non-linear least-squares procedures as described in the Experimental section.

for the forward reaction. Chlorate showed a pattern characteristic of uncompetitive inhibition with respect to ATP (Figure 2). This pattern indicates that ATP-sulphurylase binds ATP first and that chlorate competes with sulphate for the enzyme-ATP complex as a dead-end inhibitor. Table 1 presents the values for the kinetic coefficients derived from the experiments illustrated in Figures 1 and 2.

Reverse sulphurylase reaction

Initial-velocity patterns for the reaction catalysed by ATP-sulphurylase in the direction of ATP synthesis are shown in Figure 3. The data were modelled according to eqn. (1):

$$1/v_0 = \{K_m^{\text{APS}}/(V_{\text{max}}[\text{APS}])\} + \{K_m^{\text{PP}_i}/(V_{\text{max}}[\text{PP}_i])\} + \{K_x/(V_{\text{max}}[\text{PP}_i][\text{APS}])\} + 1/V_{\text{max}} \quad (1)$$

The close fit of the modelled lines to the data supports the use of this equation. The concentration of Mg^{2+} was sufficiently high that essentially all of the PP_i was present as MgPP_i at this pH and ionic strength [14]. The primary pattern in Figure 3 indicates that the mechanism is sequential. At higher APS concentrations

Figure 4 $MgCl_2$ as an activator for ATP-sulphurylase in the reverse direction

(a) Initial-velocity pattern for the rat chondrosarcoma ATP-sulphurylase reaction with PP_i varied at the following concentrations of $MgCl_2$: $\square$, 580 μ M; $\blacklozenge$, 290 μ M; $\blacksquare$, 120 μ M; $\diamond$, 46 μ M. (b) The secondary plot of primary slopes (s $\cdot$ mM Mg^{2+} / μ M ATP) versus inverse Mg^{2+} concentration.

(results not shown), there is pronounced substrate inhibition, especially at low $MgPP_i$ concentrations. This competitive substrate inhibition has constituted a severe constraint on the APS concentration range useful for kinetic studies in the present work.

The results of studies in which the concentration of Mg^{2+} ion was varied at several PP_i concentrations are presented in Figure 4. The data pattern produced was that of 'competitive activation', which would be expected if the role of Mg^{2+} is to form the true substrate $MgPP_i$. Similar studies with APS as the varied substrate at different Mg^{2+} concentrations have yielded patterns that do not intersect on the ordinate axis but are insufficiently precise to provide a definitive assignment as to the slope effect of Mg^{2+} . This is unfortunate because an unambiguous assignment as an 'uncompetitive activator' with respect to APS would be compelling evidence for a strictly ordered sequence with APS as the leading substrate, whereas an activator for the leading substrate would show different behaviour [15].

Confirmation of the order of substrate entry and assignment of the order of product discharge were sought in inhibition experiments with the Me-APS as a dead-end inhibitor. As shown in Figure 5, the Me-APS was non-competitive with respect to $MgPP_i$. The data given in Figure 6 were consistent with the expected competitive inhibition by the Me-APS with respect to APS, but APS substrate inhibition did not allow experimentation in a substrate concentration range suitable for critical examination of any intercept effects.

The simplest formal mechanism for ATP-sulphurylase that

Figure 5 Reverse ATP-sulphurylase inhibition by Me-APS with PP_i as varied substrate

(a) Inhibition pattern for rat chondrosarcoma ATP-sulphurylase reaction in the presence of the following concentrations of Me-APS: $\square$, 0 μ M; $\bullet$, 10 μ M; $\triangle$, 25 μ M; $\blacksquare$, 50 μ M; $\circ$, 100 μ M. (b) Secondary plot of primary intercepts (s/nM ATP) versus Me-APS concentrations. (c) Secondary plot of primary slopes (s $\cdot$ μ M PP_i / nM ATP) versus Me-APS concentration.

agrees with all the data is an ordered steady-state single displacement with $MgAT$ as the leading substrate in the forward direction and APS as the leading substrate in the reverse direction (Scheme 1).

DISCUSSION

Previous data on the sulphate-activating enzymes from rat chondrosarcoma and normal and mutant mouse cartilage all suggested a special relationship between ATP-sulphurylase and APS kinase from these tissues [8–10]. Although the formal mechanisms of the individually analysed reactions do not fully describe this complex pathway, the activities can be characterized independently as a means of defining the nature of their interaction. The sulphurylase reaction can be characterized in its physiologically reverse, but chemically favoured, direction by supplying APS and PP_i . It can also be studied in the physiologically forward direction by the common device of supplying a sufficient excess of a coupling enzyme to remove the product. As mentioned, the purified rat chondrosarcoma enzyme preparation contained APS kinase activity. However, at high substrate concentrations, the kinase reaction becomes rate-limiting. This may be due to product inhibition by APS which is not completely removed by the amount of APS kinase activity that co-purifies with the sulphurylase. At lower substrate concentrations the velocity of the sulphurylase reaction is low enough to allow for adequate conversion of APS into PAPS and alleviate this effect. Therefore excess purified *P. chrysogenum* kinase was included in

Figure 6 Reverse ATP-sulphurylase inhibition by Me-APS with APS as varied substrate

(a) Inhibition pattern for the rat chondrosarcoma ATP-sulphurylase reaction in the presence of the following concentrations of Me-APS: ●, 0 μM ; □, 10 μM ; △, 25 μM ; ○, 50 μM ; ◇, 100 μM . (b) Secondary plot of primary slopes ($\text{s} \cdot \mu\text{M APS/nM ATP}$) versus Me-APS concentration.

the forward assay to ensure that the sulphurylase reaction was the rate-limiting step at all concentrations. The addition of pyrophosphatase to the reaction mixture appeared to have no

effect on the forward assay (results not shown) and was left out in the kinetic studies.

The single-displacement (ternary complex) mechanism described here for rat chondrosarcoma ATP-sulphurylase is in general agreement with previous studies of this enzyme from various sources [7,13,16–18]. The kinetic parameters determined by these steady-state methods are also similar to those previously obtained for ATP-sulphurylase from other sources [2,3,13,19–23]. However, the present results establish the assignment of ATP as the leading substrate in an ordered mechanism for the forward direction, in contrast with the enzyme from *P. chrysogenum* which has a random order of substrate addition [3].

Chlorate has previously been shown to be an inhibitor competitive with sulphate and uncompetitive with respect to ATP for the sulphurylase from rat liver [23] and spinach leaf [17]. In the present work, potassium perchlorate was also shown to be competitive with sulphate, as the primary intercepts of the inhibited lines did not differ significantly from each other or from the intercept of the uninhibited line (results not shown). Chlorate was uncompetitive with respect to ATP (Figure 2) for the enzyme from rat chondrosarcoma. This is clear evidence that the enzyme binds ATP first in an ordered mechanism, and chlorate acts as a simple dead-end inhibitor competing with sulphate for the enzyme-ATP complex (Scheme 1). $\text{Na}_2\text{S}_2\text{O}_3$ and KNO_3 were also examined but were less effective inhibitors of the sulphurylase reaction.

Most of the kinetic work reported here was carried out with Mg^{2+} concentrations sufficiently high that essentially all of the pyrophosphate was present as the Mg^{2+} complex. However, some important results were obtained by varying the concentration of Mg^{2+} ion as an activator, always at large stoichiometric excess relative to PP_i . The results in Figure 4 indicate that Mg^{2+} combines reversibly with PP_i to form the true substrate for the reverse reaction. This 'competitive activation' in Figure 4 is (inversely) analogous to the competitive inhibition seen when an inhibitor combines reversibly with the substrate rather than with the enzyme [24]. In contrast, similar tests of the Mg^{2+} -activating effect with APS as the varied substrate did not yield a common ordinate intercept (results not shown), thus eliminating the possibility of a rapid equilibrium-ordered mechanism with MgPP_i as the leading substrate, but were inconclusive with regard to the possibility of a slope effect. An ordered mechanism with MgPP_i as the leading substrate is also discounted by the findings in Figures 5 and 6, which show that a compound that binds as an analogue of APS is non-competitive with respect to MgPP_i . All the data are in accord with an ordered steady-state mechanism (for the physiologically reverse reaction) with APS as the leading substrate; however, some contribution to the velocity by an

Scheme 1 ATP-sulphurylase mechanism

opposite-order pathway in a partially random mechanism cannot be conclusively ruled out by these data for the reverse reaction.

The simplest formal mechanism given in Scheme 1 is consistent with all the data for the reverse sulphurylase reaction with the corresponding steady-state rate equation. Eqn. (1) was used with the kinetic coefficients in Table 1 as starting values to model the initial-velocity pattern and secondary plots shown in Figure 3. The lines in Figure 3 are the modelled pattern, demonstrating that the proposed mechanism can generate the observed behaviour.

It is significant to note that the rat chondrosarcoma enzyme differs from that of *P. chrysogenum*. Fungal ATP-sulphurylase and APS kinase have been separated and purified to homogeneity [4,16] whereas these enzymes from rat chondrosarcoma have been co-purified through extensive separation techniques [8]. Clearly, there are physical differences between the enzymes of mammals and those of lower organisms. The results of this study indicate that there are functional differences as well. In order to facilitate the transfer of the intermediate APS between the sulphurylase and kinase in higher organisms, it is possible that some co-operation between the ATP sulphurylase and APS kinase enzymes may occur. APS, as the last product released rather than the first, may play an important role in mammals where a more interactive and highly ordered pathway may be crucial. This finding is also consistent with studies presented in the following paper [11] which have elucidated the reaction mechanism of rat chondrosarcoma APS kinase.

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