Kinetic mechanism of adenosine 5'-phosphosulphate kinase from rat chondrosarcoma

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Biosynthesis of the activated sulphate donor adenosine 3'-phosphate 5'-phosphosulphate (PAPS) involves the sequential action of two enzyme activities. ATP-sulphurylase catalyses the formation of APS (adenosine 5'-phosphosulphate) from ATP and free sulphate, and APS is then phosphorylated by APS kinase to produce PAPS. Initial-velocity patterns for rat chondrosarcoma APS kinase indicate a single-displacement formal mechanism with K_m^{APS} 76 nM and $K_m^{ATP} = 24 \mu\text{M}$. Inhibition studies using analogues of substrates and products were carried out to determine the reaction mechanism. An analogue of PAPS, adenosine 3'-phosphate 5'-[β -methylene]phosphosulphate, ex-

hibited competitive inhibition with APS and non-competitive inhibition with ATP. An analogue of APS, adenosine 5'-[β -methylene]phosphosulphate was also competitive with APS and non-competitive with ATP. Adenosine 5'-[β -imido]triphosphate showed competitive inhibition with respect to ATP and produced mixed-type inhibition, with a pronounced intercept effect and a small slope effect, with respect to APS. These results are in accord with the formulation of the predominant pathway as a steady-state ordered mechanism with APS as the leading substrate and PAPS as the final product released.

INTRODUCTION

Adenosine 3'-phosphate 5'-phosphosulphate (PAPS) is the sulphate donor used to generate sulphate esters in mammalian tissues. Adenosine 5'-phosphosulphate (APS) kinase (ATP-adenylylsulphate 3'-phosphotransferase, EC 2.7.1.25) catalyses the second step in the sulphate-activation pathway which produces PAPS. In this reaction the APS intermediate, produced from ATP and inorganic sulphate by ATP-sulphurylase (ATP-sulphate adenylyltransferase, EC 2.7.7.4), is phosphorylated at the 3'-position of the ribose moiety to form the stable PAPS product.

APS + ATP → ADP + PAPS

As the equilibrium constant for the ATP-sulphurylase is rather unfavourable (10^{-8}) in the physiological direction [1], APS kinase plays the important role of continually removing APS, thus driving the overall sulphate-activation pathway. PAPS is also structurally more stable than APS, which is subject to spontaneous degradation under physiological conditions. Although it has been suggested that these two enzymes are coupled, enzyme-titration experiments have indicated that no 'substrate channelling' occurs in *Penicillium chrysogenum* [2]. However, our earlier findings in rat chondrosarcoma [3] suggest significant structural differences between rat chondrosarcoma and *P. chrysogenum* enzymes. In addition, the kinetic mechanism of ATP-sulphurylase reported in the preceding paper [4] suggests that there may be important functional differences as well.

Although APS kinase has been described in yeast extracts [5] and a variety of other organisms [6–11], a thorough kinetic investigation has been carried out only for the fungus, *P. chrysogenum* [2,8]. Therefore in order to gain a thorough understanding of the sulphate-activating pathway in mammalian tissues, and to find reasons for the dual defect exhibited by the brachymorphic mouse [12,13], studies of the individual kinase

reaction were extended to complete a steady-state kinetic analysis of the formal mechanism of rat chondrosarcoma APS kinase.

EXPERIMENTAL

Materials

The radiolabelled compounds, [^{35}S]PAPS (> 400 Ci/mmol) and $\text{Na}_4^{32}\text{P}_2\text{O}_7$ (1–20 Ci/mmol), were purchased from New England Nuclear (Boston, MA, U.S.A.). Biogel P-2 and Bio-Rad dye were obtained from Bio-Rad Laboratories (Mellville, NY, U.S.A.). APS, ATP, adenosine 5'-[β -methylene]triphosphate, adenosine 5'-[β -imido]triphosphate (p[NH]ppA) and dithiothreitol were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.). Whatman 3 MM chromatography sheets (46 cm × 57 cm) and anhydrous pyridine from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) were used in high-voltage paper electrophoresis. Aqueous counting scintillant from Amersham (Arlington Heights, IL, U.S.A.) was used for scintillation counting. MgCl_2 , NaF, $\text{Na}_4\text{P}_2\text{O}_7$, NaH_2PO_4 , K_2HPO_4 , $(\text{NH}_4)_2\text{SO}_4$, Tris/HCl, EDTA, imidazole and KOH were all reagent grade. Ultrapure PAPS was purchased from Dr Sanford Singer, University of Dayton, OH, U.S.A. All other chemicals were reagent grade. Adenosine 5'-[β -methylene]phosphosulphate (Me-APS) and adenosine 3'-phosphate 5'-[β -methylene]phosphosulphate (Me-PAPS) were synthesized in our laboratory as described [14,15]. [^{35}S]APS was prepared from [^{35}S]PAPS as described by Geller et al. [3]. Recovery of [^{35}S]APS was routinely 90–95%.

Preparation of enzymes

The rat chondrosarcoma enzyme preparation used in all kinetic assays was purified from rat chondrosarcoma by $(\text{NH}_4)_2\text{SO}_4$ precipitation, and Sephacryl S-300, hydroxyapatite and ATP-

Abbreviations used: PAPS, adenosine 3'-phosphate 5'-phosphosulphate; APS, adenosine 5'-phosphosulphate; Me-APS, adenosine 5'-[β -methylene]phosphosulphate; Me-PAPS, adenosine 3'-phosphate 5'-[β -methylene]phosphosulphate; p[NH]ppA, adenosine 5'-[β -imido]triphosphate.

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Figure 1 ATP and APS initial-velocity and secondary patterns for rat chondrosarcoma APS kinase

(a) Initial-velocity pattern for the rat chondrosarcoma APS kinase reaction with ATP varied at the following concentrations of APS: $\blacktriangle$, 250 nM; $\square$, 150 nM; $\bullet$, 100 nM; $\triangle$, 50 nM; $\blacksquare$, 25 nM; $\circ$, 10 nM. (b) Secondary plot of primary intercepts (min $\cdot$ g of protein/nmol of PAPS) versus reciprocal APS concentration. (c) Secondary plot of primary slopes (min $\cdot$ g of protein $\cdot$ mM ATP/nmol of PAPS) versus reciprocal APS concentration. Lines shown are modelled using the rate equation and the kinetic constants to provide the best fit for the whole data set.

Table 1 Kinetic constants for rat chondrosarcoma APS kinase

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

Constant	Value
K_m^{APS}	76 ± 14 nM
K_m^{ATP}	24 ± 6 μ M
V_{max}	4.5 ± 0.3 nmol/min $\cdot$ g
K_s	2.0 ± 0.7 μ M ²
$K_i^{Me-PAPS}$	560 ± 70 μ M
K_i^{Me-APS}	40 ± 2 μ M
$K_i^{D(NH)ppA}$	2.2 ± 0.5 mM

affinity column chromatography as described previously [3]. Protein concentrations were determined by the Bio-Rad assay with bovine γ -globulin as the standard and Bio-Rad dye as the stain.

Enzyme assay

The APS kinase assay was modified from that previously described [3]. The 25 ml reaction mixture contained 5 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 12.5 ml [^{35}S]APS (diluted to the appropriate concentration from 1 mM stock), 5 ml of ATP (diluted to the appropriate concentration from 100 mM stock), 2.25 ml of buffer

Figure 2 APS kinase inhibition by Me-PAPS with respect to APS

(a) The inhibition pattern for the rat chondrosarcoma APS kinase reaction in the presence of the following concentrations of Me-PAPS: $\bullet$, uninhibited; $\triangle$, 0.5 mM; $\blacksquare$, 1 mM; $\circ$, 2 mM. (b) Secondary plot of primary intercepts (min $\cdot$ g of protein/nmol of PAPS) versus Me-PAPS concentration. (c) Secondary plot of primary slopes (min $\cdot$ g of protein $\cdot$ μ M APS/nmol of PAPS) versus Me-PAPS concentration.

A [4] and 1.5 ml of enzyme preparation, purified approximately 2000-fold [3]. The concentrations of APS and ATP were adjusted to suit experimental conditions. The reaction time was 10 min. Product accumulation was linear with time, and steady-state velocities were linear with enzyme concentration under the conditions employed.

Data processing

Kinetic data were processed by the method described in the preceding paper [4]. Deviations that appear in the linear double-reciprocal plots at low substrate concentrations are not represented as significantly poor fits in untransformed hyperbolic plots.

RESULTS

In order to evaluate the APS kinase reaction mechanism individually, the following experiments were carried out. Initial-velocity patterns of bisubstrate-variation experiments (Figure 1) indicate a single-displacement (also known as sequential) mechanism. The level of APS useful for kinetic studies was restricted by APS substrate inhibition at high APS concentrations (results not shown), as previously noted [8,16], but the kinetic coefficients were evaluated according to the rate equation:

$$1/V_0 = \{K_m^{APS}/(V_{max}[APS])\} + \{K_m^{ATP}/V_{max}[ATP]\} + \{K_s/(V_{max}[ATP][APS])\} + 1/V_{max} \quad (1)$$

and are shown in Table 1.

Figure 3 APS kinase inhibition by Me-PAPS with respect to ATP

(a) The inhibition pattern for the rat chondrosarcoma APS kinase reaction in the presence of the following concentrations of Me-PAPS: ●, uninhibited; △, 0.5 mM; ■, 1 mM; ○, 2.5 mM. (b) Secondary plot of primary intercepts (min · g of protein/nmol of PAPS) versus Me-PAPS concentration. (c) Secondary plot of primary slopes (min · g of protein · mM ATP/nmol of PAPS) versus Me-PAPS concentration.

Inhibition experiments were employed to investigate the order of substrate entry and product discharge from the enzyme. Me-PAPS and Me-APS were used as dead-end inhibitors. Me-PAPS was used as an inhibitor of the kinase reaction with APS (Figure 2) and ATP (Figure 3) as the varied substrates. The initial-velocity pattern with respect to APS showed a marked slope effect with no significant change in the intercepts, indicating competitive inhibition. As indicated by the pattern of inhibition in Figure 3, Me-PAPS was non-competitive with respect to ATP. If PAPS was the last product discharged in the mechanism with APS as the leading substrate (see Scheme 1), Me-PAPS would be competitive with APS and non-competitive with respect to ATP. On the other hand, if PAPS was the first product released, it would be non-competitive with respect to both substrates. The data presented in Figures 2 and 3 clearly show that the pattern of inhibition is consistent with PAPS as the second product discharged.

The results obtained with Me-APS as a dead-end inhibitor suggest that APS binds to the enzyme first. Experiments using Me-APS with varying APS concentration generated initial-velocity patterns consistent with competitive inhibition (results not shown) but, because of substrate inhibition, higher APS concentrations did not help distinguish the primary intercepts from the origin. Me-APS produced a clear slope and intercept effect with respect to ATP, indicating non-competitive inhibition (Figure 4). If ATP was bound first in an ordered mechanism, Me-APS would be expected to show uncompetitive inhibition with respect to ATP instead of the observed non-competitive be-

Figure 4 APS kinase inhibition by Me-APS with respect to ATP

(a) The inhibition pattern for the rat chondrosarcoma APS kinase reaction in the presence of the following concentrations of Me-APS: ●, uninhibited; △, 50 mM; ■, 100 mM; ○, 200 mM. (b) Secondary plot of primary intercepts (min · g of protein/nmol of PAPS) versus Me-APS concentration. (c) Secondary plot of primary slopes (min · g of protein · mM ATP/nmol of PAPS) versus Me-APS concentration.

haviour. However, the non-competitive inhibition with respect to ATP shown by both Me-APS and Me-PAPS could also be explained by a random or partially random order of addition of the substrates.

In order to determine the order of addition or the randomness of addition of the substrates APS and ATP, an inhibitor competitive with respect to ATP was sought. Of those tested, adenosine 5'-[β,γ -methylene]triphosphate inhibition was not linear with the concentration of the inhibitor. Because of its structural similarity to both ATP and APS, ADP did not produce clear inhibition patterns. p[NH]ppA proved to be a more reliable inhibitor of the reaction, but it did not show a simple pattern of inhibition with respect to the substrates. With respect to ATP, this ATP analogue did show a competitive inhibition pattern (Figure 5), i.e. there were no significant differences among the values of the primary intercepts. However, this analogue of ATP produced mixed-type inhibition with respect to APS. If APS binds to the enzyme first, p[NH]ppA would be uncompetitive with respect to APS. As shown in Figure 6, the inhibitor did cause a small slope effect as well as a significant intercept effect. One of the major problems in studying the mechanism of an enzyme such as APS kinase which binds nucleotide derivatives is the structural similarity of the substrates and products. It is possible and quite likely that p[NH]ppA interacts with more than one form of the enzyme and/or enzyme-substrate complexes. The pattern generated by varying APS might be explained if p[NH]ppA bound to free enzyme as well as the enzyme-APS complex in a mechanism in which APS is the leading substrate as shown in Scheme 1. However, solely on the basis of the foregoing evidence, it is still possible that the isolated kinase mechanism

Figure 5 APS kinase inhibition by p[NH]ppA with respect to ATP

(a) The inhibition pattern for the rat chondrosarcoma APS kinase reaction in the presence of the following concentrations of p[NH]ppA: ▲, uninhibited; □, 0.5 mM; ●, 2 mM; △, 4 mM. (b) Secondary plot of primary intercepts (min · g of protein/nmol of PAPS) versus p[NH]ppA concentration. (c) Secondary plot of primary slopes (min · g of protein · mM ATP/nmol of PAPS) versus p[NH]ppA concentration.

Figure 6 APS kinase inhibition by p[NH]ppA with respect to APS

(a) The inhibition pattern for the rat chondrosarcoma APS kinase reaction in the presence of the following concentrations of p[NH]ppA: ●, uninhibited; △, 0.5 mM; ■, 1 mM; ○, 2.5 mM. (b) Secondary plot of primary intercepts (min · g of protein/nmol of PAPS) versus p[NH]ppA concentration. (c) Secondary plot of primary slopes (min · g of protein · μM APS/nmol of PAPS) versus p[NH]ppA concentration.

Scheme 1 APS kinase mechanism

has a random or partially random order of addition. The results of these inhibition studies accord best with a steady-state ordered mechanism, with APS as the leading substrate and PAPS as the final product released (Scheme 1).

DISCUSSION

To gain a better understanding of the interactions of the sulphate-

activating enzymes, detailed kinetic analysis of each activity individually is crucial. The complete reaction mechanism for ATP-sulphurylase is reported in the preceding paper [4] and the mechanism of the reaction catalysed by APS kinase from rat chondrosarcoma is described in Figures 1–6. The initial-velocity pattern shown in Figure 1 indicates a single-displacement (sequential) mechanism. This form of mechanism is consistent with previous studies of this enzyme from other sources [8,16–18].

However, the order of substrate addition and product release differs significantly. ATP binds before APS, and PAPS leaves before ADP in both *P. chrysogenum* [8] and *Escherichia coli* [18]. Although the data obtained using inhibitors of the rat chondrosarcoma kinase enzyme do not definitively rule out the possibility of a random or partially random order of addition, they strongly suggest that APS binds first and PAPS leaves last in rat chondrosarcoma. The difficulty caused by the structural similarity between products and substrates, as well as the impossibility of studying this enzyme in a totally isolated system without the presence of the sulphurylase enzyme, leads to problems in unambiguously elucidating the reaction mechanism. However, in the preceding paper [4], we clearly show that APS is the last product released from rat chondrosarcoma ATP-sulphurylase, which also contrasts with the *P. chrysogenum* ATP-sulphurylase studies [19]. Thus it appears that the sulphate-activating enzymes from rat chondrosarcoma utilize different reaction mechanisms from those used by the fungal enzymes.

The data in Figure 1 also allow determination of K_m values for APS (76 nM) and ATP (24 μ M). The value for ATP is similar to that obtained for APS kinase in *Chlamydomonas reinhardtii* [16] but much lower than that obtained for *P. chrysogenum* [8] and *Micrococcus denitrificans* [17], whereas the value for APS is uniformly lower (up to tenfold) than in these other organisms. As in many of these previous studies, APS substrate inhibition at high concentrations affects the APS kinase from rat chondrosarcoma, but has been omitted from the initial-velocity patterns for clarity.

The results of these kinetic investigations reveal marked differences between the rat chondrosarcoma enzymes and those of lower organisms. As demonstrated by the manifestation of the defect in the brachymorphic mouse, sufficient quantities of PAPS are required for normal physiology in higher organisms. Yet there are severe obstacles to the synthesis of PAPS through this pathway. The equilibrium constant for the sulphurylase reaction favours the physiologically reverse direction and the APS intermediate is chemically unstable (spontaneously degrades) as well as biochemically labile (degradation by sulphohydrolases). As APS is the last product to be released from the individual sulphurylase reaction, and it appears likely that it is the first substrate to bind to the kinase, it is possible that the enzymes may transfer the APS intermediate between the active sites. This type of 'channelling', described in a number of pathways [20–22], would be a useful mechanism for preserving APS and allowing efficient conversion into PAPS. It is also possible that APS remains bound to the same active site for the phosphorylation

step. Further biochemical and structural characterization studies are needed to determine the existence of a physical or functional interaction between ATP-sulphurylase and APS kinase. However, our results suggest that sulphate activation in higher eukaryotes has evolved into a more efficient pathway than in lower organisms, utilizing a specific order of substrate addition and product release in order to overcome the obstacles of this important process.

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REFERENCES

- 1 Farooqui, A. A. (1980) *Int. J. Biochem.* **12**, 529–536
- 2 Renosto, F., Martin, R. L. and Segel, I. H. (1989) *J. Biol. Chem.* **264**, 9433–9437
- 3 Geller, D. H., Henry, J. G., Belch, J. and Schwartz, N. B. (1987) *J. Biol. Chem.* **262**, 7374–7382
- 4 Lyle, S., Geller, D. H., Ng, K., Westley, J. and Schwartz, N. B. (1994) *Biochem. J.* **301**, 349–354
- 5 Robbins, P. W. and Lipmann, F. (1958) *J. Biol. Chem.* **233**, 686–690
- 6 Burnell, J. N. and Anderson, J. W. (1973) *Biochem. J.* **134**, 565–579
- 7 Schwenn, J. D. and Jender, H. G. (1981) *Phytochemistry* **20**, 601–604
- 8 Renosto, F., Seubert, P. A. and Segel, I. H. (1984) *J. Biol. Chem.* **259**, 2113–2123
- 9 Schriek, U. and Schwenn, J. D. (1986) *Arch. Microbiol.* **145**, 32–38
- 10 Hommes, F. A., Moss, L. and Touchton, J. (1987) *Biochim. Biophys. Acta* **924**, 270–275
- 11 Matsuo, K., Moss, L. and Hommes, F. A. (1987) *Neurochem. Res.* **12**, 345–349
- 12 Sugahara, K. and Schwartz, N. B. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 6615–6618
- 13 Sugahara, K. and Schwartz, N. B. (1982) *Arch. Biochem. Biophys.* **214**, 589–601
- 14 Callahan, L., Ng, K., Geller, D. H., Agarwal, K. and Schwartz, N. B. (1989) *Anal. Biochem.* **177**, 67–71
- 15 Ng, K., D'Souza, M., Callahan, L., Geller, D. H., Kearns, A. E., Lyle, S., and Schwartz, N. B. (1991) *Anal. Biochem.* **198**, 60–67
- 16 Jender, H. G. and Schwenn, J. D. (1984) *Arch. Microbiol.* **138**, 9–14
- 17 Burnell, J. N. and Whatley, F. R. (1975) *Anal. Biochem.* **68**, 281–288
- 18 Satischandran, C. and Markham, G. D. (1989) *J. Biol. Chem.* **264**, 15012–15021
- 19 Seubert, P. A., Hoang, L., Renosto, F. and Segel, I. H. (1984) *Arch. Biochem. Biophys.* **225**, 679–691
- 20 Fahien, L. A., MacDonald, M. J., Teller, J. K., Fibich, B. and Fahien, C. M. (1989) *J. Biol. Chem.* **264**, 12303–12312
- 21 Fukushima, T., Deckers, R. V., Anderson, W. M. and Spivey, H. O. (1989) *J. Biol. Chem.* **264**, 16483–16488
- 22 Srivastana, D. K., Smolen, P., Betts, G. F., Fukushima, T., Spivey, H. O. and Bernhard, S. A. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6464–6468