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A SIMPLE SENSITIVE FLUORIMETRIC ASSAY OF APS-KINASE ACTIVITY

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Abstract—Adenosine phosphosulphokinase (APS-kinase or ATP:adenylylsulphate 3′-phosphotransferase; EC 2.7.1.25) catalyses the formation of 3′-phosphoadenosine-5′-phosphosulfate (PAPS). Its activity in various tissues was measured by transferring the sulphate from PAPS, a product of APS-kinase reaction, to 4-methylumbelliferone (4-MU) to form 4-MU-sulphate (4-MUS) using phenolsulphotransferase (PST) extracted from rat liver. Desalting with Sephadex G-25, together with the addition of EDTA effectively removed the Mg^{2+} ions from the rat liver extract and thereby inhibited the APS-kinase activity therein in the subsequent PST reaction. 4-MUS formed was measured indirectly by a decrease in the fluorescence of 4-MU by a continuous fluorimetric assay. Kinetic data showed that the substrate, APS, at concentrations at and above 132 μM inhibited the APS kinase reaction. Pyrophosphate (PP) also inhibited the reaction. The apparent K_m for APS was 14 μM. Two apparent K_m values of 0.12 mM and 1.06 mM were obtained for ATP, while that for Mg^{2+} was 0.09 mM.

3′-Phosphoadenosine-5′-phosphosulfate (PAPS)* is the universal sulphate donor in almost all biological systems. Its role in post-translational modification is particularly impressive by the large number of proteins which can be sulphated in their tyrosine residues [1, 2]. In addition, the discovery of PAPS as a phosphate donor in protein phosphorylation [3] has conferred on this "active sulphate" the status of an "active phosphate" as well. In view of the important and encompassing role of PAPS in diverse cellular functions, it is timely to examine its synthesis, which would regulate sulphate conjugation and phosphorylation.

The biosynthesis of PAPS from inorganic sulphate involves a sequence of two enzymatic reactions, with the formation of an intermediate, APS, which is further phosphorylated to produce PAPS by APS-kinase:

$$ATP + SO_4 \overset{\text{ATP-sulfurylase}}{\rightleftharpoons} APS + PP. \quad (1)$$

$$APS + ATP \overset{\text{APS-kinase}}{\rightleftharpoons} PAPS + ADP. \quad (2)$$

Although the latter reaction is highly favourable thermodynamically [4] there is little information on this reaction in mammalian systems possibly due to the fact that it is not easy to separate it from ATP-sulfurylase [5]. There is suggestive evidence that both ATP-sulfurylase and APS-kinase exist as a single entity as they appeared to be eluted at a MW of 68,000 [6]. The activity of APS-kinase was first measured by Robbins and Lipmann [5] by coupling ADP formed to NADH oxidation, using pyruvate kinase and lactic dehydrogenase. Its activity in plant extracts was determined in a similar manner [7]. PAPS production from the APS-kinase reaction could also be followed by the catalytic PAP–PAPS assay [8] or by the disappearance of nitrophenol [9]. In this paper, a relatively simple and sensitive assay is presented for its measurement and some values were determined for the different fractions of hepatic tissues of the rat and mouse.

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* Abbreviations: PAPS, 3′-phosphoadenosine-5′-phosphosulfate; 4-MU, 4-methylumbelliferone; 4-MUS, 4-methylumbelliferone-sulphate; PST, phenosulphotransferase; PP, pyrophosphate; APS, adenosine phosphosulphate; DTT, dithiothreitol.

MATERIALS AND METHODS

Chemicals. APS, ATP, DTT, 4-MU and sulfatase (Type V from limpets) were from Sigma Chemical Co. (St. Louis, MO). PAP^{35}S of specific radioactivity of 33.3 GBq/mmol (or 0.9 Ci/mmol) was purchased from New England Nuclear (Boston, MA).

Preparation of APS-kinase from various tissues. Homogenates (20%) were prepared from freshly excised liver, kidney and small intestine in cold 0.15 M KCl–30 mM DTT solution using the polytron. This was sequentially centrifuged at 15,000 *g* for 15 mins and 108,000 *g* for 30 min. These fractions were designated the 15,000 *g* supernatant and 108,000 *g* cytosolic fractions, respectively. All extracts including the crude homogenates were stored at −80° in small aliquots until the assays were performed.

Removal of Mg^{2+} *ions by gel-filtration.* Desalting was carried out by passing 2.5 ml of the 108,000 *g* cytosolic fraction of rat liver through a prepacked column containing Sephadex G-25 (PD-10, from Pharmacia, Uppsala, Sweden) according to the instructions of the supplier. The column was equilibrated and eluted with the same extracting solution (0.15 M KCl–30 mM DTT). After discarding the effluent and the first 2.5 ml, the next 1.5 ml was

collected and stored in small aliquots at −80°. This fraction was termed "desalted PST extract" to be used in the PST assay.

Assay of APS-kinase. The assay incubate contained the following with final concentrations given in parentheses: 30 μl of 1 mM APS (100 μM), 20 μl of 140 mM ATP (9.3 mM), 15 μl of 140 mM Mg^{2+} (7 mM) and 50 mM phosphate buffer of pH 7.6 in a total volume of 270 μl. The reaction was started by the addition of 30 μl of the various enzyme preparations: the crude homogenate, 108,000 *g* cytosol or 15,000 *g* supernatant. At 10 min, the APS-kinase reaction was terminated by boiling in a water bath for 1 min. After centrifugation to remove the precipitated proteins, 0.2 ml of the supernatant was removed for the determination of PAPS formed by the subsequent PST reaction with 4-MU as acceptor:

$$\text{PAPS} + \text{4-MU} \xrightarrow{\text{PST}} \text{4-MUS} + \text{PAP}.$$

Measurement of PAPS by the PST reaction. An assay mixture of the following composition was prepared with their final concentrations given in parentheses: 0.05 ml of a 0.1 mM 4-MU (0.29 μM) and 0.4 ml of 0.1 M EDTA (2.35 mM) adjusted to pH 7.2 and made up to a total volume of 17 ml with 50 mM phosphate buffer, pH 7.2. To 1.7 ml of this assay mixture was added 0.2 ml of the reacted incubate from the APS-kinase reaction above. The PST reaction was started by the addition of 0.1 ml of a mixture containing 1:1:1 ratio, by volume, of "desalted PST extract", 0.1 M EDTA adjusted to pH 7.2 and 0.1 M PP. It is important to carry out a control to ensure that both ATP-sulfurylase and APS-kinase present in the "desalted PST extract" are completely inactivated, failing which PAPS may be generated in this second step from the unreacted ATP and APS (and possibly inorganic sulphate which is a degradation product of APS). The employment of EDTA [10] and PP had been shown to be effective in this respect [11]. A suitable control would be one with boiled enzyme extract together with a full complement of the reactants employed in the APS-kinase step.

The formation of 4-MUS was monitored continuously in a cuvette placed in a thermostatically-controlled cell-holder maintained at 37°. This temperature was achieved by circulating water from an external water bath kept at 58–60°, depending on the ambient temperature which may vary between 25–27°. Circulation was by means of a peristaltic pump (Pharmacia, P-1) operating at full speed of 80 ml/hr. The decrease in the fluorescence of 4-MU, on the addition of a mixture of "desalted enzyme extract", EDTA and PP was continuously monitored for 6 min at excitation, 319 nm and emission, 447 nm. These values were established on the LS-5 luminescence spectrophotometer (Perkin-Elmer) using the prescan mode. The initial and final relative fluorescence units (RFU) were recorded and the slope of the plot was used to calculate the rate of reaction. As the cell-holder can accommodate four cuvettes, two other cuvettes were each filled with the requisite volume of assay mixture and left in the holder to equilibrate at 37°.

The overall sulphate conjugation of 4-MU. To establish the concentration of 4-MU that is suitable for the PST reaction described above, the overall three-step sulphate conjugation of 4-MU was carried out. Although this involves multistep enzymatic reactions, 4-MU is the substrate of only the PST reaction. The assay mixture was prepared in a final volume made up to 20 ml with 50 mM phosphate buffer at pH 7.2 containing the following with their final concentrations in parentheses: 1 ml of 10 mM sodium sulphate (0.5 mM), 0.5 ml of 140 mM ATP (3.5 mM), 1 ml of 140 mM Mg^{2+} (7 mM). To an aliquot of 1.9 ml of this assay mixture was added 50 μl 4-MU varying from 7.82 to 62.5 nM in final concentration. The reaction was started as above with 50 μl of the 108,000 *g* cytosolic fraction of rat liver. The decrease in fluorescence was monitored as described above for the APS-kinase/PST assay.

Identification of 4-MUS in the PST reaction. To ensure that the decrease in fluorescence of 4-MU was indeed due to the formation of 4-MUS, its positive identification was deemed necessary. This was carried out as follows:

(a) Synthesis of 4-MUS: identical conditions to the standard assay procedure for the overall sulphate conjugation of 4-MU were employed to synthesize 4-MUS, but the reaction was terminated by adding 50 μl 5% zinc sulphate and 50 μl 0.3 M barium hydroxide. Controls were carried out without the addition of either APS, ATP or Mg^{2+}. After centrifugation, the supernatant was passed through a Millex-filter (Millipore) of 0.45 μm pore size and 25 μl was injected for HPLC analysis of the unreacted 4-MU.

(b) Separation of 4-MU and 4-MUS: 4-MUS formed *in vitro* was then separated from the residual 4-MU by the procedure of van Kempen and Jansen [12], using Dowex-50 W, hydrogen form (100–200 mesh). Elution was carried out with 2 ml of water. The effluent and eluate contained 4-MUS while 4-MU was retained on the column [12].

(c) Hydrolysis of 4-MUS by HCl and sulfatase: the combined fraction of effluent and eluate in a total volume of 4 ml was divided into four equal aliquots. To one was added 2 N HCl to pH 1 for acid hydrolysis in a boiling water bath for 30 min. Another fraction was adjusted to pH 5.5 with 0.5 M sodium acetate buffer, and 0.1 ml of a solution of sulfatase (10 units) was introduced, followed by incubation overnight at 37°. Control tubes, with the omission of HCl and sulfatase, were similarly treated. The hydrolysed and unhydrolysed samples, after adjusting to pH 5.5 were filtered and analysed by HPLC-fluorimetry for identification of 4-MU.

(d) HPLC-fluorimetric analysis of 4-MU in hydrolysate: the high-performance liquid chromatograph (Waters) consists of a Model 100A solvent delivery system, a model U6K injector, a guard column packed with Whatman pellicular ODS CSK 1 and a microbore column (100 × 2.1 mm) packed with Hypersil ODS of 5 μm particle size. The mobile phase consisted of methanol:water (60:40) and the flow rate was 0.3 ml/min. The detection of 4-MU that remained in the reaction tube and after the hydrolysis of 4-MUS by HCl and sulfatase, was by means of the HP-1046A (Hewlett-Packard) fluorescence detector, set at excitation, 319 nm and emission, 447 nm, with the PMT gain set at 9. The

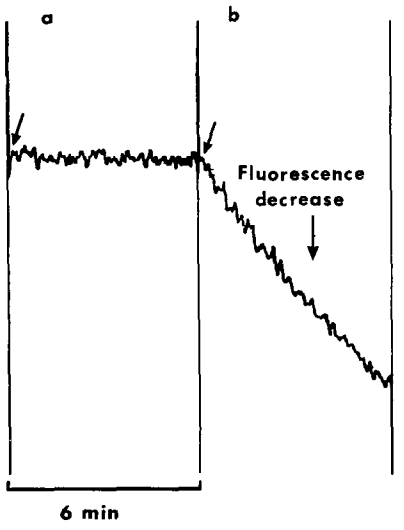

Fig. 1. Typical traces showing the time course of (a) control in which ATP, Mg^{2+} or sodium sulphate was omitted from the assay mixture and (b) a standard assay mixture containing 3.5 mM ATP, 7 mM Mg^{2+}, 0.5 mM sodium sulphate and 0.25 μM 4-MU. At the arrow, the enzyme extract was added and the fluorescence of 4-MU was monitored at 447 nm emission. Similar profiles of tracings were obtained for the APS-kinase/PST assay.

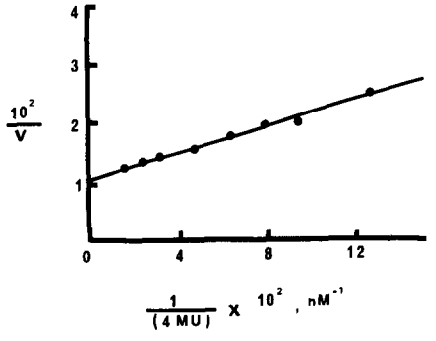

Fig. 2. Lineweaver–Burk plot of the overall sulphate conjugation of 4-MU, as analysed by the computer programme, ENZPACK [14] where v, velocity was expressed in degrees of slope of tracing, which corresponded to a decrease in RFU of 4-MU/min, in the presence of 7.82–62.5 nM 4-MU.

signal from the detector was recorded on the HP3390 integrator.

Protein determination. This was carried out by the procedure of Lowry [13] with bovine serum albumin as standard.

RESULTS

Kinetics of the overall sulphate conjugation of 4-MU

A typical assay showed a decrease in the fluorescence of 4-MU within 6 min of incubation in contrast to controls where there was no change in RFU (Fig. 1). The reaction occurred optimally at pH 7.2–7.4 and linearly for enzyme protein up to 150 μg/assay incubate. Four plots of analysis on the computer programme, ENZPACK [14] gave an apparent K_m of 10.1 to 10.4 nM for 4-MU; of these, the Lineweaver–Burk plot [15] is shown in Fig. 2. Maximum velocity was attained at sodium sulphate from 0.5 to 7 mM. Analysis by HPLC showed that there was a decrease in 4-MU (retention time = 1.7 min) in the reacted incubate and the demonstration of 4-MU in the hydrolysates. Hydrolysis of 4-MUS isolated by Dowex chromatography with acid or sulfatase liberated 4-MU; however, complete hydrolysis was observed only with HCl.

Kinetic data of APS-kinase reaction as measured by coupling PAPS formed to 4-MU sulphation

The optimum pH for the APS-kinase reaction was 7.6 in 50 mM phosphate buffer. Under the standard assay conditions, there was no formation of 4-MUS when ATP or APS was omitted or when boiled enzyme was used. It must be noted that both ATP and APS were present in the control with boiled enzyme. As there was no evidence for the production of 4-MUS in the last control, it could be concluded that the "desalted PST extract" did not exhibit APS-kinase activity. This indeed is a prerequisite for the APS-kinase assay presented in this paper.

With APS concentrations between 1.65 and 100 μM, the APS-kinase reaction showed typical classical kinetics with an apparent K_m of 14 μM as analysed by four different plots using the "ENZPACK" programme [14]. Only the Lineweaver–Burk plot [15] is shown in Fig. 3a. Substrate inhibition by APS was observed from 132 to 264 μM APS concentration; these points are however not included in Fig. 3a.

An apparent K_m value of 0.24 mM was obtained for ATP by the Lineweaver–Burk plot (Fig. 3b) measured between ATP concentration of 0.23 and 14 mM. However, from this plot, there was suggestive evidence of two K_m values as indicated by the dotted lines in Fig. 3b. An apparent K_m value of 0.12 mM was obtained when plotted for low ATP concentrations between 0.23 and 2.3 mM and another apparent K_m of 1.06 mM when data obtained from higher concentrations of 2.3 to 14 mM ATP were analysed. No inhibition of APS-kinase was observed even at 14 mM ATP concentration.

Exogenous Mg^{2+} added to the incubate between 46 μM to 7 mM did not stimulate the APS-kinase reaction compared to controls without Mg^{2+}. It thus appeared that Mg^{2+} ions present in the enzyme preparation may suffice. Inhibition at high concentration of Mg^{2+} from 9.3 to 18.7 mM was however observed with about 50% inhibition at the highest concentration tested. When the APS-kinase reaction was carried out with the desalted enzyme preparation, the dependence on Mg^{2+} ions was evident. Based on data obtained with the "desalted PST extract", an apparent K_m of 0.09 mM for Mg^{2+} was established (Fig. 3c). As this enzyme extract had less protein content, the APS-kinase activity in this set of data was relatively lower.

Effects of phosphorylated compounds on the APS-kinase reaction

Four compounds, namely pyrophosphate (PP), ADP, 3′-phosphoadenosine-5′-phosphate (PAP) and phosphonoacetic acid were introduced into the APS-kinase assay mixture at final concentrations of 0.1 and 1 mM. The first three are end products of ATP sulfurylase, APS-kinase and PST respectively,

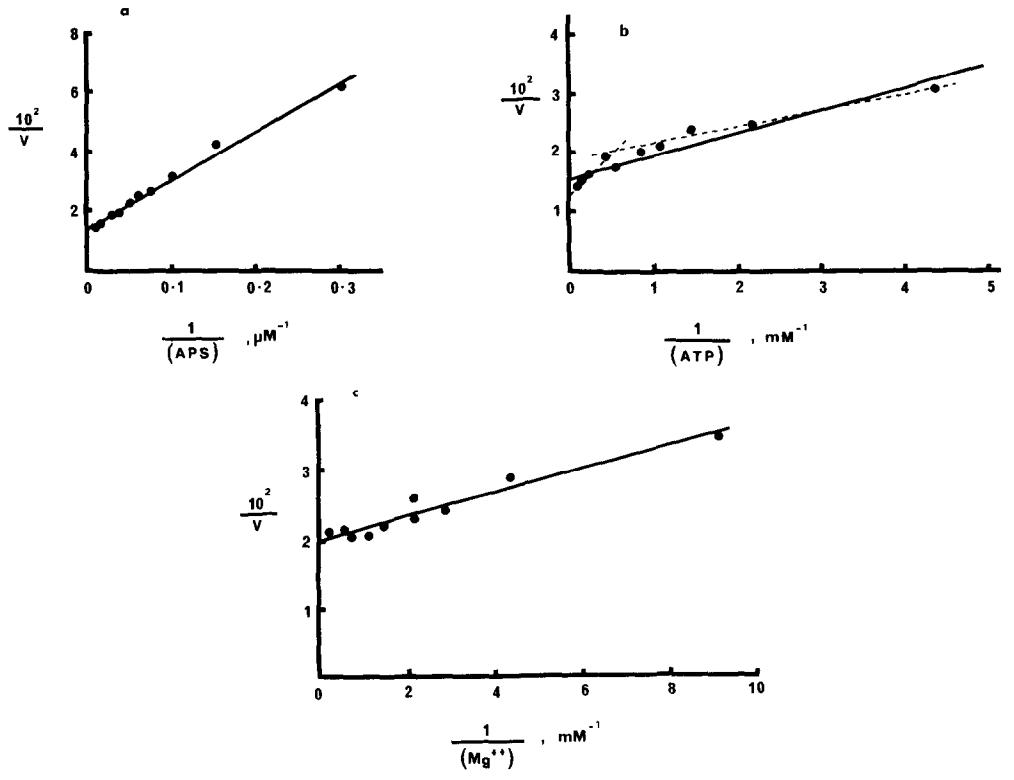

Fig. 3. The Lineweaver–Burk plots, generated by the computer programme, ENZPACK, [14] for the APS-kinase reaction where v, velocity was expressed in degrees of slope (which corresponded to a decrease in RFU of 4-MU/time) against the concentrations of (a) 3.3–100 μM APS; (b) 0.23–14 mM ATP; and (c) 0.11–4.66 mM Mg^{2+}; this last set of assays was carried out with the "desalted PST extract".

while phosphonoacetic acid was reported to protect the degradation of PAPS [16]. Of these, ADP and phosphonoacetic acid had no effects at both concentrations on the APS-kinase coupled to PST assay system. PAP, however showed a 46% inhibition at 0.1 mM and 89% inhibition at 1 mM, while PP at 1 mM inhibited APS-kinase by about 27% inhibition. These compounds also inhibited the overall sulphate conjugation of 4-MU. In fact, complete inhibition was observed at both 0.1 mM and 1 mM PAP, while the inhibitory effect of PP between 0.33 and 16.7 mM was dose-dependent, varying from 19 to 55%.

Specific activity of APS-kinase in tissues of rat and mouse

This was quantitated by comparing the rate of decrease in fluorescence of 4-MU monitored by the PST reaction with those carried out with added known amounts of standard PAPS. Since labelled $PAP^{35}S$ was available during the course of this study, it was employed although nonlabelled PAPS should be equally suitable. A typical standard set of traces is shown in Fig. 4a using 220–1760 pmoles of $PAP^{35}S$. For calculation, the angle formed with the horizontal axis was measured in degrees to represent the velocity of the reaction corresponding to the amount of PAPS added or generated in the APS-kinase reaction. The corresponding standard curve so derived for PAPS is shown in Fig. 4b, from which the specific activities of various enzyme preparations were extrapolated. It was noted that the crude homogenates as well as the partially fractionated preparations of liver, kidney and small intestine had measurable APS-kinase activity (Table 1).

DISCUSSION

The sensitivity of the APS-kinase assay described in this paper is attributed to (i) the high intensity of fluorescence of 4-MU and (ii) the high affinity of PST for 4-MU with an apparent K_m value of 10 nM (Fig. 2). Thus from stoichiometric considerations, nanomolar to micromolar concentrations of PAPS produced in the APS-kinase reaction should be measurable by coupling it to 4-MU sulphation. This is indeed the kind of concentration of PAPS that would be generated *in vitro* by APS-kinase as the reaction is known to be inhibited at low concentrations of APS, e.g. at 10 μM in the yeast [5] and in other systems [6]. This substrate inhibition by APS was also observed in the rat liver extract. As a corollary, any assay of APS-kinase, based on the coupling of PAPS to an acceptor molecule requires that the PST has a high affinity for it. 4-MU appears to satisfy this stringent requirement with the added advantage of possessing intense native fluorescence. Previous spectrophotometric assays of APS-kinase [5, 8, 9] seemed to lack sensitivity of this order of magnitude.

The disappearance of 4-MU was used to monitor indirectly the formation of 4-MUS. The nature of

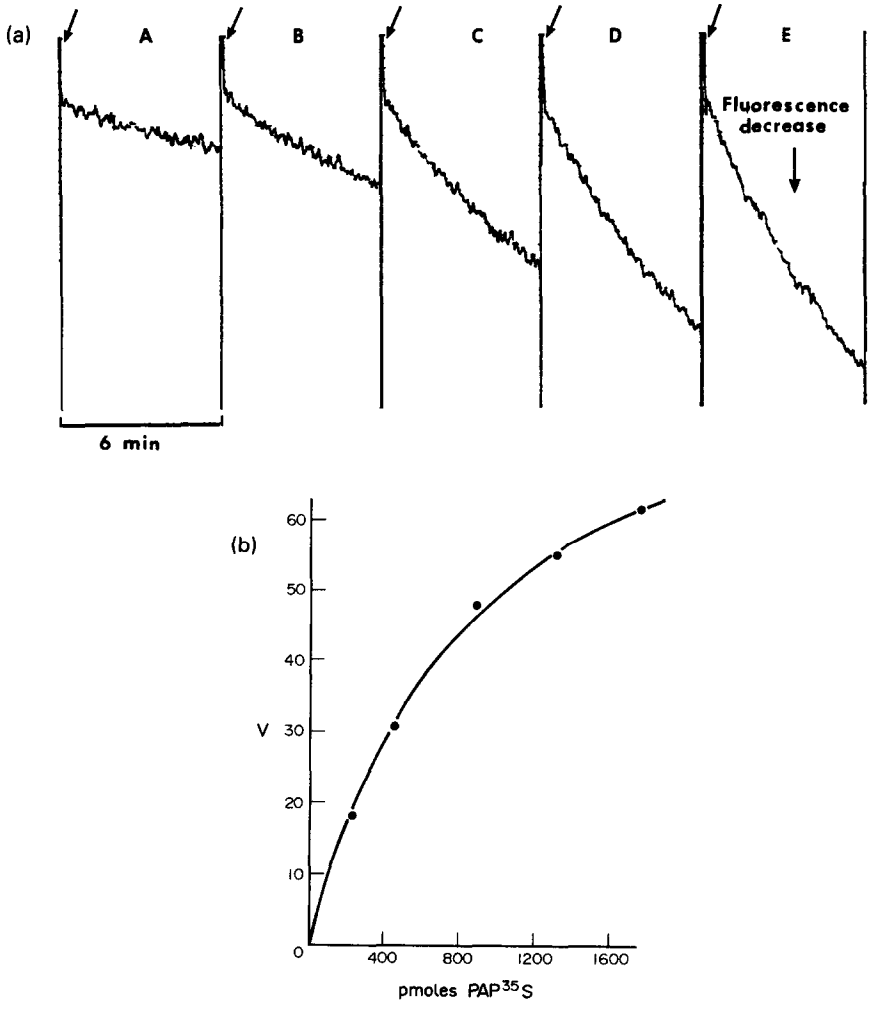

Fig. 4. A typical standard set of tracings (a) obtained by adding 220–1760 pmoles $PAP^{35}S$ to the PST reaction, in steps of 220 pmoles, from A to E. The standard curve (b) was generated from the above figures by measuring the slope, expressed in degrees, which represented the velocity, v, corresponding to a decrease in RFU of 4-MU/time.

Table 1. Specific activity of APS-kinase in various extracts of rat and mouse

Tissue	Enzyme fractions	pmoles PAPS/min/mg protein* Rat	Mouse
Liver	108,000 *g*	813 (0.48)	327 (0.45)
	15,000 *g*	650 (0.54)	265 (0.47)
	homogenate	73 (1.23)	86 (0.96)
Kidney	108,000 *g*	116 (0.35)	477 (0.33)
	15,000 *g*	57 (0.38)	307 (0.39)
	homogenate	NM (0.72)	100 (0.90)
Small intestine	108,000 *g*	129 (0.21)	585 (0.15)
	15,000 *g*	118 (0.26)	523 (0.17)
	homogenate	61 (0.42)	253 (0.26)

* Values are averages of triplicate assays.

Values in brackets represent the amount of protein (in mg) present in the assay incubate of the APS-kinase reaction.

NM, not measurable.

the sulphate conjugate was established by HPLC-fluorimetry. It is conceivable that the assay procedure described in this paper is also applicable, with some modifications, to the measurement of PAPS generation from ATP and inorganic sulphate.

The apparent K_m for APS was 14 μM. At higher concentrations of APS (132–264 μM) there was substrate inhibition which was also observed in other systems [5, 6, 17]. This could represent a self-regulatory mechanism whereby excess APS would switch off the APS-kinase reaction unless it is utilized to form PAPS or degraded. For parsimony, this appears to be ideal for an enzyme which exhibits extremely favourable kinetics [4, 5].

Two apparent K_m values of 0.12 and 1.06 mM were established for ATP. These values are one and two orders of magnitude higher than that for APS. It has been reported that at least 4 moles of ATP must be bound to the enzyme for maximal activity of APS-kinase suggesting that it acts as an allosteric activator [6]. However, under the conditions of our assay with enzyme extracts which had not been subjected to any purification, there was no evidence of such an activation. In contrast to the inhibitory effect of high concentration of ATP on the overall sulphate conjugation assayed with different substrates [18–22], ATP up to 14 mM did not inhibit the APS-kinase reaction.

There was minimal requirement for exogenous Mg^{2+} in the APS-kinase reaction. The amount present in the enzyme extract seemed adequate. Data obtained using the "desalted PST extract" showed an apparent K_m for Mg^{2+} of 0.09 mM, which is similar in magnitude to the low K_m value obtained for ATP.

In all the enzyme fractions examined, highest APS-kinase activity was observed in the 108,000 *g* cytosolic fraction. The activities in the kidney and small intestine of the mouse were higher than the hepatic value (Table 1). This correlates with the higher PAPS generation *in vitro* observed previously [23, 24]. Likewise, the higher APS-kinase activity in rat liver compared to mouse liver was similarly reflected in PAPS generation *in vitro* [24, 25]. The APS-kinase activity in the crude homogenates could also be measured readily. There was little or no quenching of 4-MU fluorescence as indicated by the similar initial RFU values with all the different enzyme preparations. This was probably due to the removal of proteins by boiling and centrifugation in the first step of the APS-kinase/PST assay procedure.

At present, little is known about the control of APS-kinase activity. Being an enzyme which catalyses a highly favourable reaction, it is most likely to be regulated in a negative manner. Its control may be responsible for the low intracellular concentration of PAPS in various tissues [25]. Two compounds, PP and PAP when tested *in vitro* showed inhibition of the APS-kinase assay system. As the APS-kinase assay was coupled to the PST reaction, it was not possible to delineate its site of action. However, PP had been shown to have no effect on the PST reaction [11]. Therefore, it can be concluded that it inhibits APS-kinase. PAP, on the other hand is a known potent inhibitor of PST [26]. In view of this, its site of action could only be resolved by the separation of APS-kinase from PST.

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