Purification and properties of adenosine 5'-phosphosulphate sulphotransferase from *Euglena*

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Adenosine 5'-phosphosulphate sulphotransferase (APSST) was extracted from *Euglena gracilis* Klebs var. *bacillaris* mutant W₁₀BSmL by freezing and thawing and was purified about 10000-fold (to homogeneity) with 10.5 % recovery by (NH₄)₂SO₄ precipitation, Sephadex G-100 chromatography, Reactive Blue-agarose, Reactive Dye-agarose, DEAE-cellulose, preparative isoelectric focusing and non-inactivating SDS/PAGE. The active APSST, with a molecular mass of 102 kDa and multiple forms from pI 5.0 to 5.5, is a tetramer held together by covalent (probably disulphide) bonds. An apparent *K_m* of the purified enzyme for adenosine 5'-phosphosulphate (APS) of 0.1 μM is obtained when dithiothreitol is used as the thiol. The enzyme is stimulated by Mg²⁺, Ca²⁺ or Ba²⁺, and uses almost any thiol; dithiothreitol and dithioerythritol give the highest activity. In the absence of APS, the enzyme is inactivated (and is rendered monomeric) by thiols but is protected from thiol inactivation by AMP, adenosine 5'-phosphoramidate (APA) or adenosine 5'-monosulphate (AMS), which also inhibit APSST activity somewhat. The enzyme resists inactivation by SDS in the absence of thiols; SDS stimulates APSST activity at low concentration, but high concentrations are inhibitory.

INTRODUCTION

Adenosine 5'-phosphosulphate sulphotransferase (APSST) is the first enzyme in the 'APS pathway' of sulphate reduction and utilizes APS formed from ATP and sulphate via ATP sulphurylase. Although APSST is widely distributed in organisms with the APS pathway of sulphate reduction, including most O₂-evolving photosynthesizers (all eukaryotic algae, some prokaryotic blue-green algae and all higher plants tested [1,2]), it has not been purified to homogeneity or studied in any detail, because of the limited amounts in cells and its easy inactivation during purification. In *Euglena gracilis* var. *bacillaris* the APSST is located on the outside of the inner membrane of mitochondria [3]. We have found that this APSST can be extracted from cells by freezing and thawing without inactivation and that the enzyme can be stabilized in Hepes/KOH buffer without thiols. The present paper reports the purification to homogeneity and the properties of this APSST from *Euglena*. A brief abstract of this work has appeared [4].

MATERIALS AND METHODS

Enzyme purification

All steps were performed at 4 °C unless otherwise indicated.

Preparation of crude extracts. A bleached mutant (W₁₀BSmL) of *Euglena gracilis* Klebs var. *bacillaris* Cori lacking plastids [5] was cultured at 26 °C in darkness for 5 days in Hutner's pH 3.5 medium [6] with vitamin B-12 concentration decreased to 50 ng/l; the culture was harvested at 1500 g for 3 min. The combined cell pellets obtained from 10 litres of cell culture (about 50 g wet wt.) were resuspended and washed with about 200 ml of buffer I (50 mM-potassium phosphate buffer, pH 7.4, containing 0.3 M-sucrose) by centrifugation, and then resuspended in 83 ml of the same buffer. A 400 mg portion of crude trypsin (Sigma no.

T-8128) was added to the cell suspension which was then incubated at 4 °C for 1 h with gentle stirring. The cells were pelleted at 1500 g for 3 min, washed twice with buffer II (25 mM-Hepes/KOH buffer, pH 7.4, containing 5 mM-EDTA and 0.3 M-sucrose) by centrifugation and then resuspended in 100 ml of standard buffer (10 mM-Hepes/KOH buffer, pH 7.4, containing 1 mM-EDTA). The cell suspension was passed through a Yeda Press at 6.9 MPa (1000 lbf/in²) of N₂ twice, and was then frozen in liquid N₂ for 5 min and thawed below 10 °C in a cool water bath. The freezing and thawing was repeated three times and the resulting mixture was centrifuged at 1500 g for 3 min. The supernatant fluid was re-centrifuged at 20000 g for 20 min and the resulting supernatant fraction was used as the crude cell extract for APSST purification.

(NH₄)₂SO₄ precipitation and Sephadex G-100 chromatography. Solid (NH₄)₂SO₄ (291 g) was slowly added to 1 litre of the cell crude extract to reach 50 % saturation. The mixture was centrifuged at 20000 g for 10 min. Solid (NH₄)₂SO₄ (185 g) was then added to the supernatant fluid to reach 75 % saturation, and the APSST activity was pelleted by centrifugation at 20000 g for 10 min. The precipitated protein was dissolved in 10 ml of standard buffer and centrifuged at 20000 g for 10 min. The supernatant fluid was applied to a Sephadex G-100 gel-filtration column (2.8 cm × 110 cm) followed by standard buffer; 10 ml fractions were collected at 20 ml/h. The standard molecular-mass markers used were as follows: BSA, 66 kDa and 132 kDa; carbonic anhydrase, 30 kDa; cytochrome c, 13 kDa.

Ion-exchange chromatography. The fractions containing APSST from Sephadex G-100 were pooled and loaded on to a Reactive Blue-agarose column (2 cm × 35 cm) that had been equilibrated with standard buffer. Some 80–90 % of the enzyme passed through the column. This unadsorbed fraction was then loaded on to a Reactive Dye-agarose column (2.0 × 30 cm) equilibrated in standard buffer. The Reactive Dye-agarose

Abbreviations used: AMS, adenosine 5'-monosulphate; APA, adenosine 5'-phosphoramidate; APS, adenosine 5'-phosphosulphate (adenylyl sulphate); APSST, adenosine 5'-phosphosulphate sulphotransferase; IEF, isoelectrofocusing.

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column was composed of a mixture of equal volumes of Reactive red-, Reactive Green- and Reactive Yellow-agarose. The enzyme does not bind to this column either, and the unadsorbed fraction was then applied to a DEAE-cellulose column (1.2 cm $\times$ 40 cm), previously equilibrated in standard buffer, followed by 300 ml of a linear gradient of 0–250 mM-NaCl in standard buffer; 5 ml fractions were collected. The active fractions were pooled, concentrated and desalted to a final volume of 3 ml with an Amicon P10 membrane.

Preparative IEF and SDS/PAGE. The preparative IEF was performed in a granular gel bed containing Ampholine, described previously [7,8]. A 4 g portion of Sephadex G-75 for IEF (superfine grade) (Sigma Chemical Co.) was slowly added to 100 ml of gel buffer containing 5.0 ml of pH 5–7 Bio-lyte (Bio-Rad Laboratories). The concentrated and desalted APSST sample from the DEAE-cellulose column was loaded after 37 % of the water in the gel had evaporated. Then 10 μ l of pI standard markers (Bio-Rad laboratories) was applied to one side of the gel bed. NaOH (1.0 M) was used for the anode and H_3PO_4 (1.0 M) for the cathode. Electrofocusing took place at 300 V for 20 h at 4 °C. After IEF was complete, the gel was divided into 30 fractions, each of which was placed in a tube containing 2 ml of standard buffer. A 10 μ l sample from each fraction was used to assay APSST activity. The active fractions were pooled and passed through a small Sephadex G-50 column followed by standard buffer to recover the enzyme. The enzyme solution was then concentrated to 2–3 ml at a pressure of 0.28 MPa (40 lbf/in²) of N_2 in an Amicon model 12 filtration apparatus with an Amicon P10 (10 kDa cut-off) membrane.

Preparative non-inactivating SDS/PAGE was performed as described below for fully denaturing SDS/PAGE except that during sample treatment no thiol was present in the sample buffer, heating was omitted, and SDS concentration was reduced to 0.25 % (w/v). The term 'non-inactivating SDS/PAGE' is used throughout this paper when APSST is separated by SDS/PAGE under these modified conditions. Equal volumes of the enzyme and the sample buffer [125 mM-Tris/HCl buffer, pH 7.0, containing 0.5 % (w/v) SDS and 20 % (v/v) glycerol] were mixed and left to stand at 4 °C for 30 min before being applied to the gel. Electrophoresis was conducted at 150 V for 50 h at 4 °C. After electrophoresis was complete, two strips were cut from the gel. One was stained for protein (see below) and one was sliced into 0.5 cm pieces, which were used to assay APSST as acid-volatile radioactivity. The active band was then cut from the rest of the gel and sliced into small pieces. APSST was eluted into dialysis tubing from the gel by disc electrophoresis at 50 V for 8 h or overnight at 4 °C. The enzyme solution was then concentrated by microconcentrator or Amicon P10 membrane.

APSST assay

APSST activity was determined by measuring acid-volatile radioactivity. Each complete assay mixture contained, in a total of 0.2 ml in an Eppendorf 1.5 ml microcentrifuge tube without a cap: 20 μ mol of Tris/HCl buffer, pH 9.0, 0.4 nmol of APS (0.4 μ Ci/nmol), 6 μ mol of dithiothreitol, 6 μ mol of MgCl_2 and an appropriate amount of APSST. The reaction was started by addition of the enzyme or dithiothreitol, not by addition of APS (see the Results section). Incubation was carried out at 25 °C for 2.0 min unless otherwise indicated. Acid-volatile radioactivity [9] was assayed by using modifications kindly provided by Dr. Christian Brunold (personal communication). After the incubation, 10 μ l of 0.5 M-sodium thiosulphate was added to the reaction mixture, which was vortex-mixed, and the Eppendorf tube was placed in a scintillation-counting vial containing 1.0 ml of 1.0 M-triethanolamine. The vial was capped immediately after

the addition of 200 μ l of 1 M- H_2SO_4 to the reaction mixture and was left to stand overnight, for complete transfer of acid-volatile radioactivity to the triethanolamine, or for 1 h, for a quick assay to locate enzyme activity on columns during purification. The Eppendorf tube was then removed and the trapped radioactivity was determined after addition of 5.0 ml of BCS scintillation fluid (Amersham Corp.). All calculations of APS conversion into acid-volatile radioactivity in this paper are made on the basis that 1 μ mol of APS is converted into 1 μ mol of SO_2 , which is absorbed completely by the triethanolamine trap. Separate experiments (results not shown) verify complete transfer of $^{35}\text{SO}_2$ to the triethanolamine overnight.

Preparation of ^{35}S -labelled APS

^{35}S -labelled APS was formed in the reaction of ATP and carrier-free $^{35}\text{SO}_4^{2-}$ catalysed by ATP sulphurylase partially purified from mitochondria of *Euglena gracilis* Klebs var. *bacillaris* Cori mutant W₁₀BSmL [3]. The reaction mixture (100 ml) contained 10 mM-Tris/HCl buffer, pH 8.5, 1 mM-ATP, 1 mM- MgCl_2 , 1 mCi of carrier-free $\text{H}_2^{35}\text{SO}_4$, 3 units of ATP sulphurylase and 5 units of inorganic pyrophosphatase (Sigma Chemical Co.). The reaction mixture was incubated at 30 °C overnight with gentle shaking, and the reaction was stopped by heating at 100 °C for 3 min. After the solution had cooled to 37 °C, 6 g of D-glucose, 100 units of hexokinase (Sigma Chemical Co.) and 100 units of myokinase (Sigma Chemical Co.) were added and the mixture was incubated at 37 °C for 4 h [10]. The mixture was then loaded onto a DEAE-Sephadex A-25 column (1.0 cm $\times$ 60 cm) that had been previously equilibrated with 50 mM-triethylammonium bicarbonate buffer, pH 7.6 [10]. Elution was conducted with 500 ml of a linear gradient of 0.25–1.0 M-triethylammonium bicarbonate buffer. Fractions containing radioactive APS were pooled, and triethylammonium bicarbonate buffer was removed at reduced pressure. The APS preparation prepared showed only one quenching spot (at 254 nm), which was coincident with the only radioactive spot after paper electrophoresis at pH 5.8 in 20 mM-citrate buffer.

Analytical electrophoresis

Fully denaturing SDS/PAGE was performed according to previous methods [11,12], with some modifications. Slab gels containing 10.5 % (w/v) acrylamide in the resolving gel (14 cm $\times$ 20 cm $\times$ 1.5 mm) were cast at room temperature with 3 μ l of 10 % (w/v) ammonium persulphate and 0.7 μ l of *NNN'*-tetramethylethylenediamine per ml of gel solution. The stacking gel containing 4 % (w/v) acrylamide was also cast at room temperature. Samples in solubilization buffer [62.5 mM-Tris/HCl buffer, pH 6.8, containing 2.5 % (w/v) SDS, 10 % (v/v) glycerol, 2.5 % (v/v) 2-mercaptoethanol and 0.01 % (w/v) Bromophenol Blue] were incubated for 3 min in a boiling-water bath before application to the gel. The upper and lower reservoir buffer contained 192 mM-glycine, 25 mM-Tris and 0.1 % (w/v) SDS, pH 8.4. Electrophoresis of sample though the stacking gel was performed at 75 V for 1 h and electrophoresis through the resolving gel was at 150 V overnight at 4 °C.

In order to measure the enzyme activity in the SDS/PAGE gel, non-inactivating SDS/PAGE was performed as described above.

IEF-PAGE was performed according to previous methods [13] with modifications. A slab gel (14 cm $\times$ 20 cm $\times$ 1.5 mm) containing 6 % (w/v) acrylamide and 2 % (v/v) Ampholine (pH 3–10) was polymerized chemically at room temperature. Samples in the standard buffer containing 1 % (v/v) Ampholine were applied after subjecting the gel to 200 V for 1 h. The anode buffer was 10 mM-NaOH and the cathode buffer was 20 mM- H_3PO_3 . IEF was conducted at 300 V for 30 h. IEF standards from Bio-Rad Laboratories were (pI values in parentheses) phyco-

Table 1. Summary of APSST purification from *Euglena*

APSST activity was measured as described in the Materials and methods section. Total activity was measured as [³⁵S]APS converted into acid-volatile radioactivity.

Step	Protein (mg)	Total activity (pmol/min)	Specific activity (pmol/min per mg)	Recovery (%)	Purification (fold)
Crude extract	5945	80470	13.5	100	1.0
(NH ₄) ₂ SO ₄	993	60353	60.8	75	4.5
Sephadex G-100	323	47075	146	59	11
Reactive Blue-agarose	63	32993	525	41	39
Reactive Dye-agarose	24	28084	1155	35	86
DEAE-cellulose	3.6	16818	4622	21	342
Preparative IEF	0.61	11829	19410	15	1438
SDS/PAGE*	0.059	8288	139752	10	10352

* Non-inactivating (no thiol or heat).

cyanin (4.65), lactoglobulin B (5.1), bovine carbonic anhydrase (6.0), human carbonic anhydrase (6.5), horse myoglobin (7.0), lentil lectin (8.2, 8.4, 8.6) and cytochrome *c* (9.6).

APSST activity in the gel of IEF-PAGE or non-inactivating SDS/PAGE was measured as acid-volatile radioactivity. The gel was sliced into 0.5 cm pieces and each slice was incubated in 0.4 ml of reaction mixture at 25 °C for 10 min. The acid-volatile radioactivity trapped in a vial containing triethanolamine was determined as described above.

The SDS/PAGE gels were stained in a solution containing 40 % (v/v) methanol, 10 % (v/v) acetic acid and 0.25 % (w/v) Coomassie Brilliant Blue R for 1 h or overnight. Destaining of the gels was accomplished by incubating the gel in several 200 ml changes of a solution containing 40 % (v/v) methanol and 10 % (v/v) acetic acid. IEF-PAGE gels were stained in a solution containing 10 % (v/v) acetic acid, 27 % (v/v) propan-2-ol, 0.5 % (w/v) CuSO₄ and 0.04 % Coomassie Brilliant Blue R. Destaining of the IEF gels was accomplished in a solution containing 7 % (v/v) acetic acid, 12 % (v/v) propan-2-ol and 0.5 % (w/v) CuSO₄.

Paper electrophoresis was performed at 1800 V (36 V/cm) for 50 min with 20 mM-sodium citrate buffer, pH 5.8, on Whatman 3MM paper, as previously described [3]. Nucleotides (APS, ATP etc.) were located as quenching spots under 254 nm light. Radioactive areas were located by exposure of the electrophoretograms to Kodak X-ray film for 2–7 days.

Protein determination

Protein was determined by the method of Bradford [14].

RESULTS AND DISCUSSION

Optimum assay conditions

A number of experiments (results not shown) were conducted to optimize the conditions for the APSST reaction such as the influence of pH, buffer system, amount of enzyme, incubation time and concentration of dithiothreitol, APS or MgCl₂ on the activity of APSST purified through to the DEAE-cellulose step (see Table 1). Activity increases with APS concentration up to 2 μM, but 5 μM and above is inhibitory. APSST requires a high pH to produce acid-volatile radioactivity, and Tris/HCl buffer gives higher activity than Tricine/KOH buffer or Hepes/KOH buffer. The optimized assay conditions for forming acid-volatile radioactivity by APSST purified through to the DEAE-cellulose column step are given in Table 2; dithiothreitol and APS are indispensable for activity, and MgCl₂ is stimulatory. It should be

Table 2. Optimum conditions for the assay of *Euglena* APSST

APSST activity was determined by measuring acid-volatile radioactivity trapped in triethanolamine as described in the Materials and methods section. Each complete assay mixture contained, in a total of 0.2 ml, 100 mM-Tris/HCl buffer, pH 9.0, 2.0 μM-APS (0.4 μCi/nmol of APS), 30 mM-dithiothreitol, 30 mM-MgCl₂ and 5 μg of APSST purified through to the DEAE-cellulose stage. Incubation was carried out at 25 °C for 2 min.

	APSST activity	
	(pmol of [³⁵ S]APS converted into acid-volatile radioactivity)	(%)
Complete mixture	46.6	100
– MgCl ₂	17.8	38
– Dithiothreitol	0.1	0.2
– APSST (or enzyme heated at 100 °C for 3.0 min)	0	0

emphasized that the APSST reaction does not show a linear relation with time on prolonged incubation, as discussed below. However, with initial rates over 2 min the assay is linear with enzyme (protein) concentration.

Purification

APSST was released from cells of *Euglena* mutant W₁₀BSmL into Hepes/KOH buffer by freezing and thawing. The enzyme was very stable in Hepes/KOH buffer, pH 7.4, but was inactivated slowly over a prolonged period in a Tris/HCl buffer system. Thiols such as dithiothreitol or 2-mercaptoethanol were not added during the purification of APSST, because they strongly inactivated the enzyme. The overall enzyme purification is summarized in Table 1. The SDS/PAGE step (without thiols and heat during sample preparation) resulted in a homogeneous active preparation of APSST. The homogeneous APSST represented a purification of 10000-fold and a recovery of about 10 % over the original crude cell extracts.

Properties

Physical properties. The APSST activity peak is eluted from a pre-calibrated Sephadex G-100 column at a position corresponding to a molecular mass of 120 kDa (results not shown). The

Fig. 1. Completely purified APSST (as shown in Table 1) on non-inactivating SDS/PAGE

Lane 1, molecular-mass standard markers: myosin, 200 kDa; β -galactosidase, 116 kDa; phosphorylase *b*, 97 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; bovine trypsinogen, 24 kDa; soya-bean trypsin inhibitor, 20 kDa; α -lactalbumin 14 kDa. Lane 2, APSST purified to homogeneity as shown in Table 1. The arrows indicate protein bands. The relative APSST activity in gel slices is shown to the right. Peak activity = 65 pmol of APS converted into acid-volatile radioactivity/min.

Fig. 2. Completely purified APSST (as shown in Table 1) on non-inactivating SDS/PAGE after treatment with thiols or sulphite

Lanes 1, 2 and 4, purified APSST (5 μ g) treated with dithiothreitol (1.0 M), 2-mercaptoethanol (1.0 M) and Na_2SO_3 (0.5 M) respectively. Lane 3, molecular-mass standard markers as given in Fig. 1. The arrows indicate APSST subunit.

enzyme purified to homogeneity separates into two protein bands on non-inactivating SDS/PAGE (Fig. 1). The major one with a molecular mass of 102 kDa shows APSST activity but the minor band with a molecular mass of 25 kDa is inactive (Fig. 1), which is consistent with the single active peak obtained on Sephadex G-100 gel filtration. On fully denaturing SDS/PAGE (by using 2-mercaptoethanol and heat), the upper band disappears completely and an increase in the lower band is seen (Fig. 2, lane 2). Therefore the active form of APSST forming acid-volatile radioactivity from [^{35}S]APS and dithiothreitol appears to be a tetramer held together by covalent bonds, probably disulphide bridges. Further evidence in favour of the disulphide bonds is obtained from non-inactivating SDS/PAGE after APSST is treated with reagents that cleave disulphide bonds such as sulphite (0.5 M) or thiols (1.0 M) such as dithiothreitol or 2-mercaptoethanol (Fig. 2); KCN (0.5 M) produces the same result, as expected (results not shown). The band after treatment with sulphite is broader, is more diffuse, stains more lightly and has a slightly greater mobility compared with thiol-treated enzyme (Fig. 2). This may be because cleavage of disulphide bonds with sulphite yields equimolar amounts of $-\text{S}-\text{SO}_3^-$ and $-\text{S}^-$ ($-\text{SH}$) groups whereas thiols yield all $-\text{S}^-$ ($-\text{SH}$) groups.

On IEF-PAGE or preparative IEF, APSST activity can be obtained from about four protein bands which span a relatively broad pH region from 5.0 to 5.5 (results not shown). This suggests that the APSST may exist in multiple forms having different pI values; however, they all give the same band on non-inactivating SDS/PAGE (Fig. 1). The pI values also explain why the APSST enzyme does not appreciably bind to cation-exchangers such as Reactive Blue-agarose or other Reactive Dye-agarose columns, for in the Hepes/KOH, pH 7.4, standard buffer the enzyme has a net negative charge.

On a double-reciprocal plot ($1/[S]$ versus $1/v$), linearity and an apparent K_m of 0.1 μM -APS are obtained when a saturating amount of dithiothreitol is used as the thiol (results not shown), indicating that the enzyme has a very high affinity for its APS substrate.

Effect of bivalent cations. Addition of MgCl_2 , CaCl_2 and BaCl_2 (at 30 mM) stimulate APSST activity as much as 3-fold. CoCl_2 (2 mM), ZnCl_2 (2 mM), FeCl_2 (2 mM), HgCl_2 (0.1 mM) or CuCl_2 (0.1 mM) in the reaction mixture causes precipitation and inhibits APSST activity by about 50%. EDTA (2.0 mM) stimulates enzyme activity about 1.7-fold, probably because it prevents enzyme inactivation by trace amounts of heavy-metal ions; the stimulation by EDTA also indicates that Mg^{2+} , Ca^{2+} and Ba^{2+} are not bound to the enzyme to begin with and are therefore truly stimulatory rather than indispensable.

Thiols as artificial substrates. APSST is thought to catalyse the transfer of the sulpho group ($-\text{SO}_3^-$) from APS to a thiol carrier [1]. Glutathione was the physiological compound most active with the partially purified *Chlorella* enzyme [15]. Like the *Chlorella* enzyme [16], the *Euglena* enzyme can use various organic thiols as artificial substrates (Table 3). When dithiothreitol or glutathione (30 mM) is used as the thiol, the product of the reaction catalysed by APSST from *Euglena* is sulphite (as with the partially purified *Chlorella* enzyme [16]), which was confirmed by pH 5.8 citrate paper electrophoresis [16] and by conversion of the sulphite spot into acid-volatile radioactivity (results not shown).

Inactivation and protection by various compounds. Table 4 shows the effects of three types of compounds on APSST activity. These include products of sulphate reduction such as cysteine and methionine, possible metabolic intermediates such as

Table 3. Thiols as substrate for purified APSST from *Euglena*

APSST activity was determined by measuring acid-volatile radioactivity trapped in triethanolamine; additions were as given in Table 2 except that the type of thiol (30 mM in all cases) was varied. Relative activity: 1000 = 24 pmol of APS converted into acid-volatile radioactivity per min. APSST was purified through to the DEAE-cellulose step.

Thiol	Relative activity
Dithioerythritol	1000
Dithiothreitol	976
Cysteamine hydrochloride	441
2-Mercaptoethanol	370
2,3-Dimercaptopropan-1-ol	348
L-Homocysteine	318
L-Glutathione	228
L-Cysteine	216
Sodium thioglycollate	192
Thioacetamide	11
Sodium thiosulphate	0

Table 4. Effects of various compounds on APSST activity

The incubation mixture was as given in Table 2 with the additions shown below. APSST was purified through to the DEAE-cellulose step. Incubation was for 2 or 20 min.

Addition	Concentration (mM)	APSST activity (% of control)		Activity difference (%) (20 min - 2 min)
		2 min	20 min	
None (control)		100*	100†	0
L-Cysteine	2.0	73	85	12
L-Cysteinesulphinic acid	2.0	75	79	4
L-Cysteic acid	2.0	92	81	-11
L-S-Sulphocysteine	2.0	99	81	-18
L-Methionine	2.0	103	82	-21
L-Methionine sulphoxide	2.0	91	85	-6
L-Methionine sulphone	2.0	107	76	-31
Na ₂ SO ₄	2.0	89	79	-10
AMS	2.0	62	170	108
AMP	2.0	24	133	109
Cyclic AMP	2.0	80	90	10
APA	0.5	46	147	101
ATP	2.0	87	92	5
ADP	2.0	90	96	6

* 100% = 47.5 pmol of APS converted into acid-volatile radioactivity.

† 100% = 105 pmol of APS converted into acid-volatile radioactivity.

S-sulphocysteine, and compounds expected to bind at or near the active site of APSST such as AMP, APA and AMS. The first two categories of compounds seem to have little effect on APSST activity. With the third group, the results varied with the analogue used and the length of time the enzyme was incubated. In short incubations (2 min), these compounds inhibited APSST activity; in longer incubations (20 min), APSST activity was increased compared with the control. (ATP or ADP, unlike AMP, show little effect on the APSST reaction.) This phenomenon could be the result of progressive inactivation of APSST during the normal reaction and protection from inactivation by APA, AMP or AMS. To demonstrate inactivation and determine its cause,

Fig. 3. Preincubation of APSST with dithiothreitol

After preincubation of APSST in the reaction mixture of Table 2 without APS, with (●) or without (○) 0.5 mM-APA as shown, APS as in Table 2 was added to start the reaction. The preincubation was at 25 °C for the time shown; the incubation was at 25 °C for 2.0 min. 100% = 24.0 pmol of APS converted with 0.5 mM-APA; 100% = 48.8 pmol of APS converted without APA.

Fig. 4. Effect of APA on kinetics of APSST reaction

The incubation mixture was as given in Table 2 except that 2 µg of enzyme was added to start the reaction and APA was added at concentrations of 2 mM (▼) or 0.5 mM (▽) or not at all (○). Acid-volatile radioactivity was assayed as before.

an experiment employing preincubation of APSST with APS, dithiothreitol or MgCl₂ was conducted. Preincubation with APS or MgCl₂ had no effect (results not shown). However, as may be seen in Fig. 3, preincubation with dithiothreitol causes enzyme inactivation. Further preincubation experiments showed that all thiols (at 30 mM), including dithiothreitol, dithioerythritol, glutathione, 2-mercaptoethanol, cysteine and homocysteine, inactivate the enzyme. The inactivation of APSST by thiols is irreversible. For this reason thiols are not added during enzyme purification. To minimize inactivation the assay reaction for APSST should be started by adding the enzyme or dithiothreitol, not by adding APS.

Preincubation of APSST with dithiothreitol (30 mM) inactivated the enzyme strongly, but this inactivation was overcome to a significant extent in the presence of 0.5 mM-APA (Fig. 3). Kinetic studies with APA (Fig. 4) during the normal reaction showed that APA has a dual effect. On the one hand, it inhibits APSST activity by decreasing the initial velocity of the reaction as APA concentration is increased. On the other hand, it protects

Fig. 5. Effect of final SDS concentration in the reaction mixture on APSST activity

The APSST activity was measured as given in Table 2 except that SDS was added to the final concentration shown.

the enzyme from progressive inactivation by thiol substrates. AMP and AMS act in the same way as APA (results not shown); this explains why APSST activity in the presence of AMP, APA or AMS is much less than that of the control at 2 min of incubation and is much higher at 20 min of incubation (Table 4). It also explains why the reaction is not linear with time (beyond 2 min) even though the substrates are at saturation (Fig. 4). The inhibition of APSST by AMP, APA or AMS is reversible on dilution (results not shown) and is concentration-dependent (see, for example, Fig. 4). APS may protect the enzyme from thiol inactivation as well, but there seems to be no experimental way of determining this at present.

The fact that APSST is a tetramer held together by disulphide bonds may explain why thiols such as 2-mercaptoethanol or dithiothreitol and reagents such as KCN or sulphite inactivate the enzyme and why AMP, APA or AMS can protect APSST from thiol inactivation. Dithiothreitol, 2-mercaptoethanol, KCN or Na_2SO_3 would be expected to break the disulphide bridges that hold the subunits together and thereby convert the enzyme from the tetramer into monomers, whereas APS, APA, AMP or AMS are expected to bind to the enzyme and might

thereby prevent the approach of thiols, KCN or sulphate to the disulphide bridges.

SDS effect. APSST is unusual in resisting denaturation by SDS. In fact, SDS can stimulate or inhibit APSST activity (measured as acid-volatile radioactivity), depending on the final concentration in the assay mixture (Fig. 5). SDS inhibition is reversible; even if the enzyme is pretreated with a high SDS concentration (up to 1.5%, w/v), APSST still shows full activity, as long as the final SDS concentration in the assay mixture is lower than 0.01%. This has permitted the use of non-inactivating SDS/PAGE (in the absence of thiols and heat) to purify the enzyme as described in the Materials and methods section.

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