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Synthesis and Utilization of a Nonhydrolyzable Phosphoadenosine Phosphosulfate Analog[1]

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3′-Phosphoadenosine 5′-phosphosulfate (PAPS) functions as the high-energy sulfate donor for sulfate ester synthesis in all higher organisms. This activated sulfate, like its adenosine 5′-phosphosulfate precursor, is both chemically labile and vulnerable to sulfohydrolase degradation. These obstacles have limited the utility of the native PAPS in the purification and mechanistic description of the numerous PAPS-utilizing enzymes. This paper describes the synthesis of the 2′- and 3′-isomers of a nonhydrolysable, and thus stable, PAPS analog, β-methylene-PAPS, from the previously described β-methylene-APS (L. Callahan *et al., Anal. Biochem.* 177, 67–71, 1989). The method involves phosphorylation of β-methylene-APS with trimetaphosphate and separation of the resulting mixed 2′(3′)-isomers by ion-pair reverse-phase HPLC. The utilization of this analog as an inhibitor of APS kinase and PAPS translocase, two of the numerous PAPS-utilizing activities, as well as an affinity ligand for purification of APS kinase, is described.

3′-*O*-Phosphoadenosine 5′-*O*-phosphosulfate (3′-PAPS)[3] is the unique donor of sulfate groups required for synthesis of sulfate esters in all higher organisms (2). This molecule is the product of the sequential action of two cytosolic enzymes, ATP-sulfurylase (EC 2.7.7.4) and APS kinase (EC 2.7.1.25), proceeding by way of an unstable "activated" intermediate, adenosine 5′-*O*-phosphosulfate (APS). Once produced, 3′-PAPS is then transferred across the Golgi membrane by a specific PAPS translocase activity (3–7), where it acts as a substrate for membrane-bound sulfotransferases, generating, for example, the chondroitin sulfate proteoglycan of the cartilage extracellular matrix.

Numerous chemical (8–12) and enzymatic (13–22) methods have been devised to produce APS and PAPS; however, the inherent instability of these molecules (due primarily to the phosphosulfur anhydride bond) severely limits their use in the characterization, purification, and kinetic analysis of either sulfate-activating enzyme. In order to provide more useful compounds for these purposes, we undertook the synthesis of a stable analog of the APS intermediate, in which the bridging oxygen of the phosphoanhydride linkage is replaced by a methylene group (1). This analog has recently been utilized for the kinetic analysis of rat chondrosarcoma ATP-sulfurylase (unpublished). A similar description of the copurifying APS kinase reaction necessitated the generation of a stable analog of PAPS. In this paper we report the synthesis of such a PAPS analog, making use of the previously synthesized β-methylene-APS as starting compound. Moreover, we have demonstrated some of the potential uses of the analog as an inhibitor of certain enzyme reactions involving PAPS and as an affinity ligand for the purification of some of the PAPS binding activities. These results suggest that β-methylene-PAPS will be an invaluable tool for the future study of all PAPS-utilizing enzymes, including numerous sulfotransferases, which catalyze the addition of sulfate groups to carbohydrate, protein, and lipid substrates, as well as the PAPS-translocating activities, which have been described only phenomenologically to date.

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[3] Abbreviations used: APS, adenosine 5′-phosphate; 3′-PAPS, 3′-phosphoadenosine 5′-phosphosulfate; 2′-PAPS, 2′-phosphoadenosine 5′-phosphosulfate; 3′,5′-PAP, 3′-phosphoadenosine 5′-phosphate; 2′,5′-PAP, 2′-phosphoadenosine 5′-phosphate, MES, 2-(*N*-morpholino)ethanesulfonic acid; DEAE, diethylaminoethyl; M-APS, β-methylene-APS; M-PAPS, β-methylene-PAPS.

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EXPERIMENTAL PROCEDURES

Materials

[^{35}S]PAPS (1–3 Ci/mmol) was obtained from New England Nuclear. Dowex 50-H^+ was purchased from Bio-Rad Laboratories and DEAE-Sephadex A-25 from Pharmacia P-L Biochemicals. Silica TLC plates (with fluorescent indicator) were purchased from Merck. Sodium trimetaphosphate, tetrametammonium hydroxide, activated charcoal (100–400 mesh), 2′,5′-PAP (Lot A-5638), 3′,5′-PAP (Lot A-5763), and 1,3-diaminopropane (Lot 117F-3536) were obtained from Sigma Chemical Co. High-purity water was obtained from Baxter Health Care Corp. and Sequanol-grade triethylamine from Pierce Chemical Co. All other chemicals used were reagent grade. Mass spectroscopy and nuclear magnetic resonance studies were performed by Dr. Peng-Peng Zhu, Department of Chemistry, University of Chicago.

Methods

APS kinase assay. The assay employed is essentially the method of Sugahara and Schwartz (23) using 50 mM Tris–HCl at pH 8.0 with incubation times of 10 min. Due to tight binding of APS and the potent inhibition of APS kinase by its APS substrate, [^{35}S]APS of extremely high specific activity (908 Ci/mmol) was prepared. This permitted kinetic trials to be conducted in the low nanomolar APS ranges. The standard 25-μl reaction mixture contained 50 nM [^{35}S]APS, 20 mM ATP, pH 7.0, 10 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, and 1.25 μl of the ATP–agarose-purified APS kinase enzyme fraction. In order to allow the use of higher APS concentrations, ammonium sulfate was included in the APS kinase assay, as suggested by the previous studies of Renosto *et al.* (39). The generation of product PAPS was quantitated following isolation by high-voltage paper electrophoresis as previously described (24).

PAPS translocase assay. The translocation of [^{35}S]-PAPS was measured in permeable chondrocytes in which the plasma membrane was disrupted but the subcellular organelles were structurally and functionally intact. Chondrocytes were cultured from the sternum of 14-day-old chick embryos as described (25). After 5 days in culture the chondrocytes were made permeable according to the procedure of Beckers *et al.* (26) without modification. Permeable chondrocytes were pelleted by centrifugation at 1000*g* for 5 min and resuspended in 50 mM Hepes, pH 7.2, 90 mM KCl. Translocation assays were performed by incubating 3–4 $\times$ 10^6 permeable chondrocytes with 0.25 μCi [^{35}S]PAPS for various times at 37°C. Reactions were stopped by adding 10 ml ice-cold buffer (50 mM Hepes, pH 7.2, 90 mM KCl) and placing the tubes on ice. After the cells were washed three times with 10 ml of the same buffer, translocated radioactivity was measured in the final cell pellet by liquid scintillation counting. Controls omitting incubation at 37°C were also performed.

Preparation of chicken epiphyseal APS kinase. The protocol employed is derived from the preparation of rat chondrosarcoma sulfate activation enzymes, as recently reported (24). Enzyme fractions used for these assays were purified through the ATP–agarose affinity column and were enriched approximately 1000-fold in APS kinase activity, yielding a protein concentration of about 5 μg/ml.

Mass spectroscopy. Low-resolution fast-atom-bombardment mass spectra were taken using a VG Analytical 7070 EQ with a PDP 1124 computer and an Ampex disk drive. Samples prepared as the free acid were lyophilized and introduced in a glycerol matrix. The negative ion (Xe) beam was accelerated through a potential of 8 keV (final current, 2 mA); detected ions were accelerated through a potential of 6 keV. The magnet was scanned exponentially down in the current mode. Relative abundances of individual masses from 90 to 518 amu were obtained.

Nuclear magnetic resonance. ^{1}H NMR spectra were recorded at 500 MHz on a GN-Ω-500 instrument built by G. E. NMR Co. with a Sun computer unit operating system. Each sample was dissolved in D_2O and spectra were acquired at room temperature. Positions were assigned using an H_2O reference peak at 4.7 ppm. ^{31}P NMR spectra were obtained on the GN-Ω-300 NMR machine at 121-mHz resonance frequency. Each sample was initially dissolved in D_2O, spectra were obtained at room temperature, and positions were assigned against an inorganic phosphate reference of 0 ppm at pD 7.

Thin-layer chromatography. Analytical thin-layer chromatography was performed on silica TLC aluminum sheets with fluorescent indicator using solvent containing isopropanol (70%):water (20%):concentrated ammonium hydroxide (10%).

High-pressure liquid chromatography. The liquid chromatograph consisted of a Varian 5000 liquid chromatograph system with a UV-100 programmable variable-wavelength detector (Walnut Creek, CA) and a Rheodyne injection valve (Berkeley, CA), Model 7125. Peak areas and retention time were calculated using Hewlett–Packard Reporting Integrator 3390A (Avondale, PA). Separations of nucleotides were performed on an analytical reverse-phase 250 $\times$ 4.6-mm column or a semipreparative reverse-phase 250 $\times$ 10-mm column packed with Apex Octadecyl 5U (Jone Chromatography, Littleton, CO). An RP-18 MPLC guard column (Jone Chromatography) was incorporated in the system between the injector and the column. An aqueous mobile phase of 100 mM triethylamine and 25 mM 1,3-diaminopropane was prepared by adding the reagents to about 90% of the final volume of water and then adjust-

ing the pH to 8.0 using glacial acetic acid. The mobile phase was brought to final volume by addition of water.

RESULTS

Preparation of 2'(3')-O-Phosphoadenosine 5'-O-Phosphomethylenesulfate

The starting compound for the synthesis of the 2'(3')-PAPS analog was adenosine 5'-*O*-phosphomethylenesulfate, or β-methylene-APS, which was previously synthesized in our laboratory (1). The β-methylene-APS is a derivative of the naturally occurring APS, in which the bridging oxygen of the phosphosulfur anhydride linkage is replaced with a more stable methylene group. The *cis*-2',3'-diol of β-methylene-APS is amenable to phosphorylation with trimetaphosphate in aqueous alkaline solution as described by Saffhill (27). Since the methodology for preparation of the PAPS analog has not previously been reported, we present the synthesis in detail.

The disodium salt of β-methylene-APS (473 mg, 1 mmol) and tri(tetramethylammonium)trimetaphosphate (4.59 g, 10 mmol) were dissolved in 1 N sodium hydroxide (10 ml, 10 mmol), and the solution was kept at room temperature. The tri(tetramethylammonium) salt of trimetaphosphate was prepared from sodium trimetaphosphate by passing a 0.1 M solution through a bed of Dowex 50 cation-exchange resin (500 ml) in the tetramethylammonium form (prepared from the hydrogen form by washing with 10 vol of 1 M tetramethylammonium hydroxide) and the resin washed with water (500 ml). The combined filtrate and washings were then evaporated to a small volume under reduced pressure, an equal volume of dioxane was added, and the solution was lyophilized to yield a white solid. At intervals, a sample (1 ml) of the reaction mixture was examined by TLC on silica plate. The spots corresponding to β-methylene-APS (R_f 0.48) and 2'(3')-PAPS analog (R_f 0.20) were scraped, the silica was extracted with water (1 ml), and optical densities were measured. The percentage conversion was 61% after 3 days with no further increase observed thereafter. The reaction solution was next slowly passaged through a column containing 10 g of activated charcoal (100–400 mesh) in water. After washing with 150 ml of water to remove inorganic phosphate, the nucleotide materials were eluted with 50% aqueous pyridine (500 ml). The eluate was evaporated to dryness under reduced pressure with a bath temperature below 40°C. The residue was redissolved in 10 ml of water and the solution lyophilized to remove all traces of pyridine.

Unreacted β-methylene-APS was separated from 2'(3')-*O*-phosphoadenosine 5'-*O*-phosphomethylenesulfate (β-methylene-2'(3')-PAPS) by column chromatography using DEAE-Sephadex A-25 equilibrated in 50 mM NH_4HCO_3 and developed with a gradient of 50–600 mM NH_4HCO_3 (Fig. 1). The starting compound, β-methylene-APS, eluted at approximately 200 mM NH_4HCO_3, while β-methylene-2'(3')-PAPS was found to elute at about 350 mM NH_4HCO_3. Ammonium bicarbonate was removed by repeated evaporation with water under reduced pressure at low bath temperature (<40°C), and the final solution was lyophilized. The ammonium salt of β-methylene-2'(3')-PAPS was finally precipitated from water using 10 vol of absolute ethanol and the product characterized from its negative ion mass spectra m/e = 504.

Separation of 2'-O-Phosphoadenosine 5'-O-Phosphomethylenesulfate and 3'-O-Phosphoadenosine 5'-O-Phosphomethylenesulfate

An isocratic HPLC system was established for the analytical separation of β-methylene-2'-PAPS and β-methylene-3'-PAPS on a reverse-phase column employing a mobile phase consisting of two competitive hetaerons, triethylamine and 1,3-diaminopropane. As shown in Fig. 2, β-methylene-2'-PAPS and β-methylene-3'-PAPS were adequately retained with baseline separation of 3.8 min. For preparative purposes, the system was translated to a semipreparative reverse-phase column, in which milligram quantities of each isomer were obtained per injection over a 30-min period, each with a purity greater than 95%.

The eluant corresponding to each isomer was collected from the semipreparative column after several runs and the pooled material passaged through separate tandem columns of DEAE-Sephadex A-25 in 50 mM NH_4HCO_3. After washing with several column volumes of 50 mM NH_4HCO_3, the adsorbed PAPS analog was eluted with 500 mM NH_4HCO_3 and the eluate monitored with a uv recorder. The uv adsorbing peak was then collected and lyophilized. Prior to the NMR studies, each isomer was again subjected to the purification procedure using the semipreparative column, yielding a purity estimated at greater than 99%. Quantitative chromatography by HPLC showed that the phosphorylation of β-methylene-APS with trimetaphosphate resulted in approximately 60% of the 3',5'-bisphosphate and 40% of the 2',5'-bisphosphate isomers.

Nuclear Magnetic Resonance Studies of the PAPS Analog

The position of phosphorylation was determined from the proton and phosphorous NMR. ^1H NMR spectra were obtained for β-methylene-APS (M-APS) and both β-methylene-PAPS (M-PAPS) isomers in D_2O. A list of peaks is given in Table 1. The peaks were assigned by selectively irradiating an individual peak and obtaining the difference spectrum. In comparison to M-APS, M-PAPS-I displayed a downfield shift of all ring protons, with the largest shift occurring with the 3'-hydrogen. M-PAPS-II also displayed a downfield shift for all

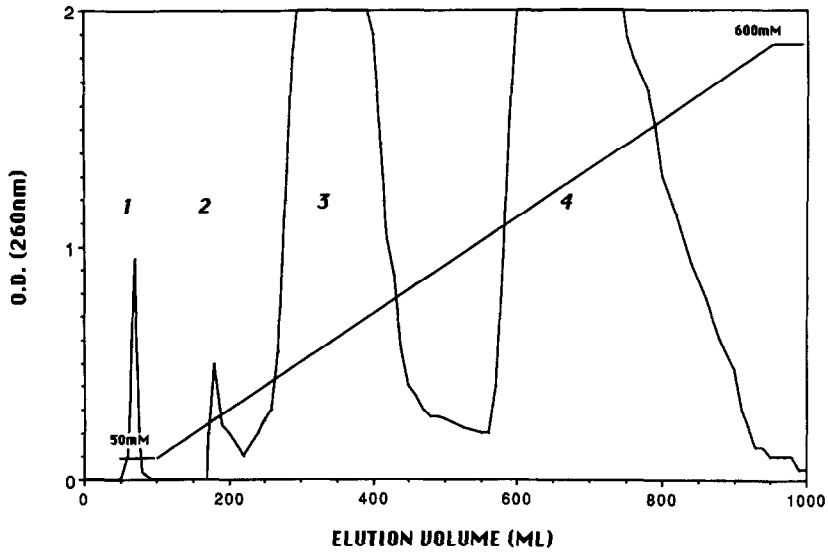

FIG. 1. Purification of β-methylene-2′(3′)-PAPS on a column (3 × 20 cm). Three hundred milligrams of material was loaded on the column of DEAE-Sephadex A-25 in 50 mM NH_4HO_3. After washing with 100 ml of the same buffer, elution was carried out with a linear gradient of 50–500 mM NH_4HCO_3 at a flow rate of 50 ml h^{-1}. The effluent was monitored at 260 nm. The major peak (marked "4") consisted of the mixed isomers of β-methylene-2′(3′)-PAPS. The material found in the peak marked "3" is unreacted β-methylene-APS.

ring protons, with the largest shift now occurring for the 2′-hydrogen. When the other protons are compared, the 1′-hydrogen was shifted furthest downfield in M-PAPS-II, while the 4′-hydrogen was shifted furthest downfield in M-PAPS-I. When the deshielding effects of a phosphate group on both the proximal and the neighboring hydrogen atoms are taken into effect, M-PAPS-I must be 3′-PAPS, given the greater downfield shift of its 3′- and 4′-hydrogen atoms. Therefore, M-PAPS-II can be identified as the 2′-PAPS isomer.

These assignments of phosphorylation were further confirmed by the decoupled ^{31}P spectra. The ^{31}P spectra of 2′,5′-PAP and 3′,5′-PAP showed that the 3′-phosphate phosphorous atom was shifted downfield (higher ppm) relative to the 2′-phosphate phosphorous. A similar pattern was also observed with the two M-PAPS isomers: the isomer first eluted showed a phosphate group phosphorous that was shifted downfield relative to that of the second isomer off the column. The ^1H and ^{31}P NMR spectra taken together with the HPLC purification strategy were thus consistent with the designation of

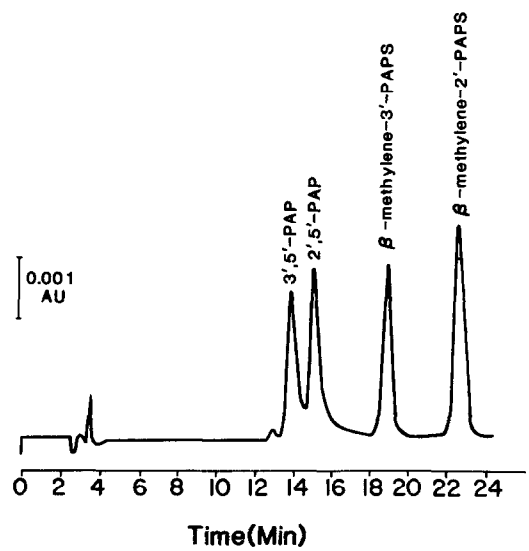

FIG. 2. Separation of 2′(3′),5′-PAP and β-methylene-2′(3′)-PAPS by ion-pair reverse-phase chromatography. The chromatography was conducted under the following conditions: column, Apex Octadecyl 5U (250 × 4.6 mm); mobile phase, 100 mM triethylammonium acetate and 25 mM 1,3-diaminopropane, pH 8.0; flow rate, 1 ml/min; detector, 260-nm fixed wavelength (0.02 aufs); chart speed, 0.5 cm/min; sample size, 1.5 nmol of each component, applied as the triethylammonium salt.

TABLE 1

Proton and Phosphorous Chemical Shifts of Methylene-APS, Methylene-PAPS Isomers, and PAP Isomers

	M-APS	M-PAPS-I[a]	M-PAPS-II	2′,5′-PAP	3′,5′-PAP
1′-H[b]	5.871	6.012	6.156	6.153	6.076
2′-H	4.493	4.732	4.991	4.976	4.740
3′-H	4.294	4.719	4.533	4.549	4.711
4′-H	4.200	4.414	4.282	4.313	4.476
2′,3′-P[c]	—	1.91	1.43	1.36	1.87

[a] PAPS-I refers to the first isomer eluting from the C_{18} column in the presence of 1,3-diaminopropane.

[b] 1′-H refers to the hydrogen atom attached to the C1′ carbon of adenosine. All spectra were recorded in D_2O. HDO was used as an internal standard and given the value 4.647 ppm.

[c] All phosphorous chemical shifts are relative to phosphate (0.0 ppm), which was used as an internal reference.

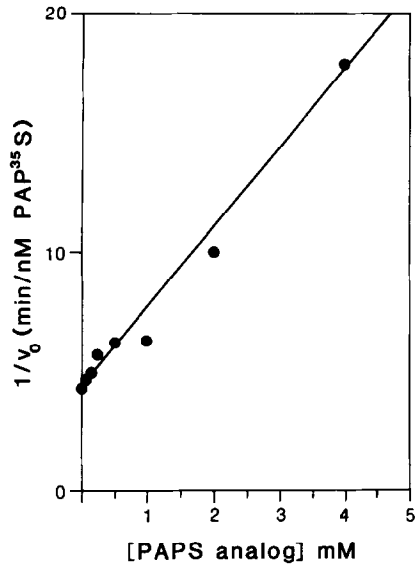

FIG. 3. β-Methylene-PAPS as an inhibitor of APS kinase. APS kinase assay of ATP–agarose-purified chick epiphyseal cartilage fraction in which the APS concentration was fixed at 50 nM, the concentration of ATP held constant at 20 mM, and the PAPS analog varied between 25 μM and 4 mM. Protein concentration of the enzyme solution was approximately 5 μg/ml.

the first eluted isomer (M-PAPS-I) as 3′-*O*-phosphoadenosine 5′-*O*-phosphomethylenesulfate (β-methylene-3′-PAPS) and the second isomer, M-PAPS-II, as 2′-*O*-phosphoadenosine 5′-*O*-phosphomethylenesulfate (β-methylene-2′-PAPS).

Kinetic Applications of the PAPS Analog

In order to test the efficacy of the newly synthesized PAPS analog, some common reactions in which PAPS serves as a substrate or product were analyzed in the presence of the PAPS analog. Figure 3 shows the results of an APS kinase assay in which the analog concentration was varied from 62.5 μM to 4 mM, with the concentrations of the two substrates, APS and ATP, fixed at 50 nM and 20 mM, respectively. The PAPS analog inhibits the kinase reaction in a linear fashion, with greater than 50% inhibition achieved at 1–1.25 mM of the pure 3′-PAPS analog. (It should be noted that the concentration of APS chosen closely approximates the K_m(APS) calculated for rat chondrosarcoma and mouse cartilage (in preparation); however, the 20 mM ATP concentration exceeds the K_m(ATP) observed in these systems.) The ability of the PAPS analog to inhibit PAPS translocation is shown in Fig. 4. Permeable chondrocytes ($3–4 \times 10^6$ cells) were incubated at 37°C with 0.25 μCi [^{35}S]PAPS in the presence or absence of 2 mM PAPS analog. Even after 15 min of incubation, the amount of translocated [^{35}S]PAPS in the presence of the PAPS analog is only 3% of that translocated in the absence of the analog. Work is currently in progress to further define the mechanisms of these and other PAPS-utilizing enzymes through use of the PAPS analog.

PAPS Analog Affinity Chromatography

In order to test the usefulness of the PAPS analog as an immobilized ligand for purifying PAPS-utilizing enzymes, the analog was coupled to Sepharose 4B. The first step was the bromination of β-methylene-PAPS. One millimole of β-methylene-PAPS was dissolved in 20 ml of 2 M sodium acetate, pH 4.0, mixed with 20 ml of 1.0 M sodium acetate, pH 4.0, containing 1.5 mmol of bromine. The reaction vessel was sealed and kept in the dark at room temperature for 24 h. The solution was diluted to 50 mM sodium acetate and the pH adjusted to pH 8 with ammonium hydroxide. The mixture was absorbed to a DEAE-Sephadex A-25 column in 5 mM NH_4HCO_3 and washed with the same buffer. 8-Bromo-β-methylene-PAPS was eluted with 500 mM NH_4HCO_3, dried by rotary evaporation, resuspended in water, and lyophilized. 8-Bromo-β-methylene-PAPS was then purified on a C_{18} reverse-phase HPLC column in 100 mM triethylammonium acetate, pH 8, 10% MeOH, and stored at 4°C. Bromination was approximately 60% complete. This product was then used to synthesize

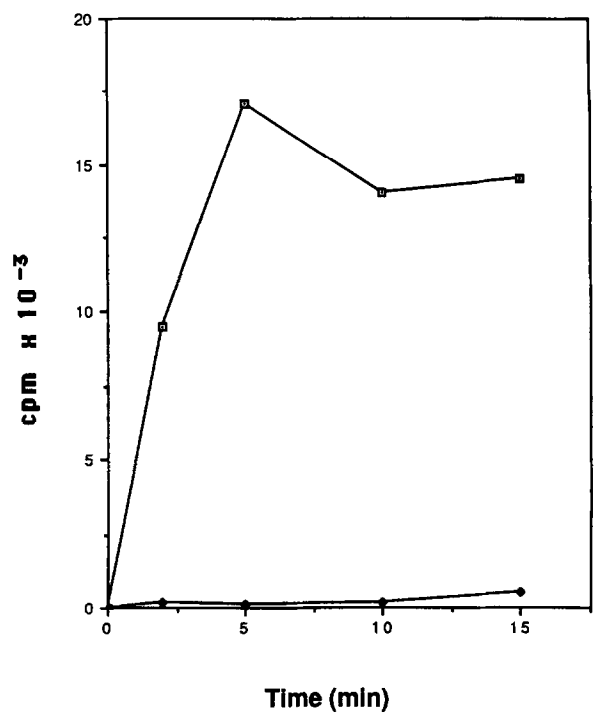

FIG. 4. β-Methylene-PAPS as an inhibitor of PAPS translocase. Permeable chondrocytes were incubated at 37°C with 0.25 mCi [^{35}S]-PAPS in the presence ♦ and absence □ of 2 mM β-methylene-PAPS, and the total transported radioactivity was determined as described.

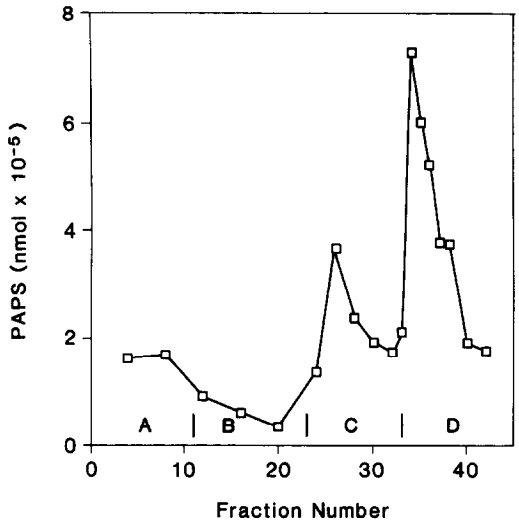

FIG. 5. Elution profile for rat chondrosarcoma. APS kinase was chromatographed on a 5-ml column of PAPS analog coupled to Sepharose 4B. Fraction size was 4.0 ml. The enzyme preparation loaded was partially purified through gel filtration and ion-exchange chromatography as described earlier (24). (A) Enzyme preparation loaded; (B) buffer A wash (0.025 M PO_4 with 10% glycerol); (C) buffer A elution containing 0.05 M KCl; (D) buffer A elution containing 0.25 M KCl. Most activity is found in the elution with 0.25 M KCl (D).

8-(6-aminohexyl)amino-PAPS by modification of a previously described procedure (28–30). One millimole of 8-bromo-PAPS analog and 30 mmol of 1,6-diaminohexane in 50 ml of water were reacted in a sealed tube for 4 h at 75°C. The reaction was >90% complete as judged by analytical C_{18} HPLC chromatography. The reaction mixture was diluted to 200 ml with water, absorbed into a 30-ml AG1 *X* 4 acetate column, and washed with 200 ml of H_2O to remove unreacted diaminohexane. Aminohexyl-PAPS analog was eluted with a linear gradient of H_2O and 1 M acetic acid; in a ratio of 1:1 the eluate was washed and lyophilized twice with H_2O to remove excess acetate.

The free acid of the analog derivative was then coupled to the gel as follows. Twenty milliliters of Sepharose 4B was washed with 5 vol of 0.02 M Na–borate buffer, pH 9.5, and activated with 5 g of CNBr at pH 11.0 at 20°C. The solution was filtered on a sintered glass funnel and washed with 400–500 ml of borate buffer. Ten milliliters of borate buffer containing 20 mg of aminohexyl-PAPS analog was added to the activated Sepharose 4B and stirred on ice at 4°C overnight. The column was washed several times with borate buffer and monitored by uv absorption in order to determine the extent of coupling. Coupling was >95%.

An enzyme preparation of APS kinase from rat chondrosarcoma, partially purified about 100-fold through ammonium sulfate precipitation, Sephacryl S-300 gel filtration, and hydroxylapatite ion-exchange chromatography (24), was applied to the affinity matrix, washed extensively, and eluted with buffer containing salt (Fig. 5). As indicated (Table 2), an approximately 15-fold purification was achieved with about a 52% recovery by chromatography on the β-methylene-PAPS. The specific activity of APS kinase purified through the PAPS analog affinity is higher than that previously obtained in our laboratory.

DISCUSSION

We have had a long-standing interest in the sulfate activation pathway that generates PAPS, the high-energy donor of sulfate groups necessary for the synthesis of sulfate esters in most organisms. To aid our investigations, we recently undertook the chemical synthesis of a stable analog of the phosphosulfate intermediate, APS (1). Our present goal was the production of a similarly stable analog of the PAPS donor molecule. The need for the latter compound became apparent during our attempts to use the APS analog to purify the sulfate-activating enzymes from cartilage. APS is tightly bound by both ATP-sulfurylase and APS kinase; consequently, affinity purification using the APS analog linked to agarose, although advantageous in purifying the two activities approximately 2800- and 2000-fold, respectively, has not been successful in separating these two activities from each other (Geller *et al.*, unpublished). Because PAPS is neither bound as a substrate by nor acts as an inhibitor of the sulfurylase reaction, it was reasoned that an analog of PAPS would prove efficacious in permitting separation of the individual sulfate activation enzymes, if indeed these activities are distinct. Furthermore, the availability of both APS and PAPS analogs was anticipated to facilitate a more complete kinetic investigation of the APS kinase. Additionally, a PAPS analog would be presumed to expedite the purification and characterization of PAPS-utilizing activities such as sulfotransferases and PAPS translocases. Finally, photoaffinity labeling of the individual activities was deemed possible following generation of this compound.

Toward this end several approaches were tried and eventually abandoned, including a *de novo* multistep synthetic scheme (31–34), which produced unstable intermediates, direct phosphosphorylation of the β-methylene-APS analog, which produced an insoluble quaternary amine salt, and synthesis of 2′,3′-cyclic PAPS analog from β-methylene-APS (32), which required enzymatic cleavage. Our present synthetic scheme also employs a cyclic intermediate, based on the method of Saffhill (27), using tri(tetramethylammonium)trimetaphosphate in the selective phosphorylation of the *cis*-2′,3′-diol of unprotected ribonucleotides in the 1 M NaOH solution. For the synthesis to be successful, β-methylene-APS had to be stable in 1 M NaOH over several days at room temperature, since the 2′,3′-cyclic

TABLE 2

APS Kinase Purification through β-Methylene-PAPS Affinity Chromatography

	Volume (ml)	Total protein (mg)	Protein concentration	Specific activity (nmol/min · g)	Total activity (pmol/min)
Enzyme preparation[a] loaded	45	4.23	0.94 mg/ml	3.42	14.5
PAPS analog column pool	28	0.147	0.53 μg/ml	51.3	7.54

[a] Enzyme preparation loaded onto column was partially purified ~100-fold through ammonium sulfate precipitation, Sephacryl S-300 gel filtration, and hydroxylapatite ion-exchange chromatography (24).

PAPS analog intermediate rapidly breaks down, forming a mixture of β-methylene-2′-PAPS and its 3′-isomer. The reaction proceeded to about 60% completion after 3 days, and unreacted β-methylene-APS was conveniently recovered using ion-exchange chromatography.

We were unable to separate the mixed isomers β-methylene-2′(3′)-PAPS by ion-exchange chromatography since the charge density is identical in both molecules. However, recent applications of ion-pairing reverse-phase HPLC techniques have made the separation of a wide range of nucleotides possible (36). Ion-pair separations typically employ amphiphilic, quaternary ammonium as the hetaeron (pairing agent) to increase the relative retention of charged nucleotides on the reverse-phase support and to maximize the selectivity of the separation. Again, several approaches were tried without satisfactory results (37–39).

In the present studies, the mixed isomers of β-methylene-2′(3′)-PAPS were separated by competitive ion-pairing reverse-phase HPLC on an octadecyl silica column using triethylamine as the primary hetaeron and 1,3-diaminopropane as the competing hetaeron. 1,3-Diaminopropane was chosen because it can bind to the two negatively charged phosphate groups of the PAPS analog simultaneously (i.e., the 5′- and the 2′(3′)-phosphate groups). Furthermore it would be expected to bind 3′,5′-phosphate compounds more tightly than their 2′,5′-isomers since the two phosphate groups of the latter compounds are separated further from each other. The less tightly formed ion pair is retained longer by the column (Fig. 2) due to the presence of the more hydrophobic triethylamine, and this effect facilitates the separation of the isomers. The power of the separating system is shown by its separation of 2′,5′-PAP and 3′,5′-PAP, despite the fact that baseline separation was not achieved (Fig. 2).

Use of β-methylene-2′(3′)-PAPS in kinetic trials indicated that it inhibits the forward APS kinase reaction in a linear fashion, as shown in Fig. 3. Given its structural similarity, β-methylene-PAPS would be expected to serve as a dead-end inhibitor of APS kinase. It is interesting to note that the concentration of analog needed to achieve 50% inhibition (~1 mM) is only 25-fold greater than the $[I]_{0.5}$ of PAPS (unpublished results). Investigations are in progress to determine a K_i for the PAPS analog in the forward APS kinase reaction as well as to compare the K_i(PAPS analog) with the K_m(PAPS) in a reverse-direction APS kinase, in which PAPS serves as a substrate. An interesting observation is that β-methylene-2′-PAPS also inhibits the APS kinase reaction; the inhibition is approximately 75% of that obtained with β-methylene-3′-PAPS (data not shown). Renosto *et al.* (39) previously showed that 2′-phospho(iso)-PAPS binds as tightly to fungal APS kinase as does normal PAPS and therefore our results are not incongruent. The inhibition of [^{35}S]PAPS translocation by the PAPS analog (Fig. 4) further demonstrates the potential advantages of this compound in the study of PAPS-utilizing activities including sulfotransferases and translocases. Lastly, the PAPS analog, when coupled to an insoluble matrix, is proving to be a powerful tool for the purification of several PAPS-utilizing activities (unpublished) as well as the APS kinase (shown in Fig. 5).

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