Molecular Cloning, Expression, and Characterization of Human Bifunctional 3’-Phosphoadenosine 5’-Phosphosulfate Synthase and Its Functional Domains*

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The universal sulfonate donor, 3’-phosphoadenosine 5’-phosphosulfate (PAPS), is synthesized by the concerted action of ATP sulfurylase and adenosine 5’-phosphosulfate (APS) kinase, which in animals are fused into a bifunctional protein. The cDNA for human PAPS synthase (hPAPSS) along with polymerase chain reaction products corresponding to several NH2- and COOH-terminal fragments were cloned and expressed in COS-1 cells. A 1–268-amino acid fragment expressed APS kinase activity, whereas a 220–623 fragment evinced ATP sulfurylase activity. The 1–268 fragment and full-length hPAPSS (1–623) exhibited hyperbolic responses against APS substrate with equivalent Km values (0.6 and 0.4 μM, respectively). The 1–268 fragment demonstrated Michaelis-Menten kinetics against ATP as substrate (Km = 0.26 mM); however, full-length hPAPSS exhibited a sigmoidal response (apparent Km = 1.5 mM) suggesting cooperative binding. Catalytic efficiency (Vmax/Km) of the 1–268 fragment was 64-fold higher than full-length hPAPSS for ATP. The kinetic data suggest that the COOH-terminal domain of hPAPSS exerts a regulatory role over APS kinase activity located in the NH2-terminal domain of this bifunctional protein. In addition, the 1–268 fragment and full-length hPAPSS were overexpressed in Escherichia coli and column purified. Purified full-length hPAPSS, in contrast to the COS-1 cell-expressed cDNA construct, exhibited a hyperbolic response curve against ATP suggesting that hPAPSS is perhaps modified in vivo.

The sulfonation of endobiotics and xenobiotics is a fundamental metabolic process of major importance. The sulfoconjugation of biomolecules occurs widely, involves compounds with molecular weights ranging in size from <103 to >106 and results in a dramatic change in the physicochemical property of the sulfonated compounds (1). Sulfonated macromolecules such as glycosaminoglycans and proteoglycans are involved in cell surface structure and connective tissue. The sulfonation of tyrosine residues has been established as a widespread post-translational modification for many secretory and membrane proteins. Sulfolipids such as sphingolipids and galactolipids are concentrated in the brain, peripheral nerves, and reproductive tissues. Additionally, sulfoconjugation is important in the biotransformation of many endogenous low molecular weight compounds such as neurotransmitters and hormones, e.g. catecholamines, isothiocyanates, and steroids. The sulfonation of drugs and xenobiotics functions primarily to inactivate and clear these generally hydrophobic compounds from the body, although there are examples where the active form of a drug is sulfonated.

In the course of sulfonation, inorganic sulfate must be activated prior to being transferred to an acceptor molecule (2). In mammalian metabolism, 3’-phosphoadenosine 5’-phosphosulfate (PAPS) has been identified as the activated sulfate molecule (3) and represents the universal sulfonate (SO3–) donor for all sulfotransferase reactions (4). The activation of inorganic sulfate to form PAPS results from the concerted action of two enzymes (3, 5, 6). The first step is catalyzed by ATP-sulfurylase (ATP:sulfate adenylyltransferase, EC 2.7.7.4) and involves the reaction of inorganic sulfate with ATP to form adenosine-5’-phosphosulfate (APS) and inorganic pyrophosphate (PPi). This reaction results in the formation of a high energy phosphoric-sulfuric acid anhydride bond that is the chemical basis for sulfate activation (8). In the direction of APS formation the enzyme has an unfavorable equilibrium (Keq ~ 10–7 M) (9); it has been suggested that the reaction is driven in the physiologic direction by the hydrolysis of PPi, by the action of a ubiquitous inorganic pyrophosphatase (9). The second step is catalyzed by APS kinase (ATP:adenylylsulfate 3’-phosphotransferase, EC 2.7.1.26) and involves the reaction of APS with ATP to form PAPS and ADP. Unlike ATP sulfurylase, APS kinase is not involved in the activation of sulfate, and its raison d’être is not known (8).

ATP sulfurylase and APS kinase cloned from bacteria (10–13), fungi (14, 15), yeast (16, 17), and plants (18, 19) are found on separate polypeptide chains. In these species the molecular mass of ATP sulfurylase ranges from 35.2 to 63.9 kDa, whereas the molecular mass of APS kinase ranges from 22.3 to 29.7 kDa. In contrast to what is found in bacteria, fungi, yeast, and plants, however, ATP sulfurylase and APS kinase isolated from mammalian tissues, e.g. rat chondrosarcoma (20) and guinea pig adrenal (21) are physically linked on bifunctional proteins of Mr 56,000 and 55,000, respectively. Confirmation of this important finding was obtained with the cloning of cDNAs from the marine worm (21) and mouse (22) that expressed a protein containing both ATP sulfurylase and APS kinase activity (PAPS synthase). The dual nature of worm and mouse PAPS synthase indicates that APS kinase and ATP sulfurylase, lo-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) 153477.

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1 The abbreviations used are: PAPS, 3’-phosphoadenosine 5’-phosphosulfate; hPAPS, human PAPS; APS, adenosine-5’-phosphosulfate; PCR, polymerase chain reaction; Fmoc, N-(9-fluorenylmethoxycarbonyl); CMV, cytomegalovirus.

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cated on separate polypeptide chains in lower life forms, have been fused into a single bifunctional protein in higher organisms. Using an expressed sequence tag from a directionally cloned human brain cDNA library that shares 60% identity to yeast APS kinase (23), we have cloned the cDNA for human PAPS synthase using fetal brain RNA. To identify functional domains, various NH2- and COOH-terminal constructs of hPAPS synthase were expressed in COS-1 cells, assayed for catalytic activity, subjected to kinetic analysis, and the results compared with those obtained with full-length hPAPS synthase. Additionally, the full-length PAPS synthase protein and the enzymatically active NH2-terminal peptide fragment were overexpressed in bacteria, purified, and analyzed kinetically for comparison to the COS-1 cell-expressed recombinant DNA constructs.

### EXPERIMENTAL PROCEDURES

#### Materials

Radionucleotides (α-32P)ATP for DNA sequencing and [35S]PAPS for enzyme assays were purchased from NEN Life Science Products. Oligonucleotides were obtained from Cruachem (Dulles, VA). Taq polymerase and reagents for PCR were obtained from Perkin-Elmer. Human fetal brain poly(A)−RNA was obtained from CLONTECH (Palo Alto, CA). TA cloning kit was purchased from Invitrogen (San Diego, CA). Version-2 sequencing kit was obtained from U. S. Biochemical Corp. Reverse transcription-PCR kit was purchased from Life Technologies, Inc. Agarose was purchased from FMC (Rockland, ME) and thin-layer chromatography plates came from E. Merck (Darmstadt, Germany).

#### Construction of Full-length NH2- and COOH-terminal Fragments of hPAPS Synthase for COS-1 Cell Expression

All PCR primers used to generate the various constructs were designed based on the DNA sequence of hPAPS synthase and are indicated in Fig. 1. The depicted sequences given for PCR primers contain an overhang region identified by underlining.

Reverse transcription-PCR was performed using human fetal brain poly(A)+ RNA and the antisense primer AS-4 (5′-CTAAAGGAACGAHTGTCGACACCCAC-3′) which corresponds to a region in the 3′-untranslated region. Reverse transcription and cDNA purification were performed according to instructions provided by the manufacturer (Life Technologies, Inc.). First strand cDNA was synthesized using 1 μg of human fetal brain poly(A)+ RNA and Superscript II reverse transcriptase. RNA in the presence of the primer was denatured for 7 min at 75°C and then chilled on ice for 1 min; transcription was carried out at 42°C for 30 min. The reaction was stopped by incubation at 72°C for 15 min. The RNA was degraded with RNase H at 52°C, and the cDNA was purified using a GlassMAX spin cartridge (Life Technologies, Inc.). An aliquot of the first strand cDNA was used as a template for subsequent amplification using the antisense primer AS-1 and the sense primer S-3 (5′-9-AATTTAATATAACCGCGTACGTAGGAGATCCCGGG-3′). The S-1 primer extends from the 5′-untranslated region into the coding region and contains a variation of the Kozak sequence (24).

Thermal cycle phases consisted of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s. The PCR product was analyzed on a 1% low melting agarose gel; the expected product (1.98 kilobase pairs) was sliced out of the gel and purified with a spin cartridge (Superrec-01) according to the manufacturer’s instructions (Panvera, Madison, WI). The 1.98-kilobase pair PCR product (contains the complete coding region) was directly ligated into a PCR 3.1 bidirectional mammalian TA cloning-expression vector, the ligated plasmid construct transformed into F-10 competent cells and recombinant clones were selected. Recombinant bacteria were mini-prepped (Promega, Madison, WI), and the resulting plasmid was verified for the presence of the PAPS synthase insert and the CMV promoter in the proper orientation by DNA sequencing using T7 and PCR 3.1 reverse primer provided by Invitrogen.

The full-length hPAPS synthase PCR product served as a template for constructing NH2-terminal (amino acids 1–174, 1–209, 1–268) and COOH-terminal (amino acids 220–623, and 264–623) fragments (cf. Fig. 1). Procedures for performing PCR, isolation of PCR products, insertion into the TA cloning vector, transformation, and sequencing were the same as those used for the full-length construct. The amino acid 1–174 PCR product was obtained using the sense primer S-1 and the antisense primer AS-1 (5′-CTATTAGATGATCTCCCTTTGACACCCTCTCT-3′). The amino acid 1–268 PCR product was obtained using the sense primer S-1 and the antisense primer AS-2 (5′-CTATACATCAAGGACGTCTGTGCTAGCCAA-3′). The amino acid 1–268 PCR product was obtained using the sense primer S-1 and the antisense primer AS-3 (5′-CTACTGACCCCTCAGCATC-3′). All antisense primers contain a CTA overhang which generates a TAG stop codon on the sense strand. The amniotic fluid 260–623 PCR product was obtained using the sense primer S-2 (5′-3′GAAGCCTGATGTGCTTGA-3′) and the antisense primer AS-4. The amino acid 264–623 PCR product was obtained using the sense primer S-3 (5′-9-AATTTAATATAACCGGTACGGAGATCCCGGG-3′) and the antisense primer AS-4. The overhang of the S-3 primer along with a mutation of the C following the ATG start codon to a G (cf. Fig. 1) creates the same Kozak sequence variation as is present for the full-length construct (the mutation converts the normal glutamine residue to a glutamate). The NH2–COOH-terminal constructs were cloned into the PCR 3.1 expression vector, similar to the full-length construct; the plasmids were verified for proper orientation with respect to the CMV promoter by DNA sequencing. Controls consisting of an empty vector and a vector with the full-length construct inserted in the reverse orientation relative to the DNA extracted with phenol-chloroform. The DNA preparation was diluted into appropriate exchange buffer and concentrated by Centricon-100 centrifugation (also removes small digestion fragments). Two pET-19b vectors, containing a proprietary 122-base pair Ncol-NdeI cassette (Veritas, Potomad, MC) encoding the calmodulin-binding site of calciumin followed by a histidine tag and enterokinase cleavage site, were similarly digested with BamHI and purified. Amplified PCR products were ligated into the two vectors. The NH2- and COOH-terminal constructs were isolated and grown in bulk to obtain large quantities of DNA using a mega-prepare kit as described by the manufacturer (Qiagen, Chatsworth, CA). The purified plasmids were used for transfection of mammalian COS-1 cells.

#### Overexpression of Full-length PAPS Synthase and 1–268 NH2-terminal Peptide Expression Vector Construction—The full-length PAPS synthase and 1–268 NH2-terminal PCR 3.1 constructs were amplified by PCR using primers designed to contain BamHI restriction sites: full-length PAPS synthase primers; forward primer, 5′-TAAGGATCATGGAATCCCTCCGGG-3′; reverse primer, 5′-TGAGGATCCAGCTGAGCTGAC-3′; 1–268 NH2-terminal peptide primers: forward primer, 5′-TAAGGATCCATGGAGATCCCGGGG-3′; reverse primer, 5′-TAAGGATCCTACTGACACCC-3′.

Briefly, PCR conditions were optimized (annealing 52/48°C, 35 cycles) to give sufficient amounts of product for cloning. After preparative PCR ~0.5 μg of product was excised, eluted from agarose gels, and digested with BamHI after which the restriction enzyme was inactivated and the DNA extracted with phenol-chloroform. The DNA preparation was diluted into appropriate exchange buffer and concentrated by Centricon-100 centrifugation (also removes small digestion fragments). Two pET-19b vectors, containing a proprietary 122-base pair Ncol-NdeI cassette (Veritas, Potomad, MC) encoding the calmodulin-binding site of calciumin followed by a histidine tag and enterokinase cleavage site, were similarly digested with BamHI and purified. Amplified PCR products were ligated into the two vectors. The NH2- and COOH-terminal constructs were isolated and grown in bulk to obtain large quantities of DNA using a mega-prepare kit as described by the manufacturer (Qiagen, Chatsworth, CA). The purified plasmids were used for transfection of mammalian COS-1 cells.

#### Purification and Characterization of Histidine Fusion Protein—Briefly, colonies are grown in ampicillin medium to an OD of 0.6, at which time 1 mm isopropyl-1-thio-β-D-galactopyranoside is added to induce expression. Cells are collected by centrifugation and extracted with TE buffer plus 1 mg/ml lysozyme (extracts are frozen at −70°C). Extracts are thawed, homogenized with tube and pestle to reduce viscosity, and clarified by centrifugation; the supernatants are adjusted with 0.25 M NaCl and adsorbed to nickel columns (nitrilotriacetic acidagarose). The columns are washed with buffer A (20 mm Tris-HCl, pH 7.8, plus 0.25 M NaCl and eluted with 0.5 μM imidazole, pH 7.0. Column eluates are dialyzed against 10 mm Tris-HCl, pH 7.6, 1 mM EDTA, and 1 mm dithiothreitol in 20% sucrose. The eluted material is analyzed by SDS-polyacrylamide gel electrophoresis to demonstrate the isolated ~70 kDa full-length PAPS synthase or ~30 kDa 1–268 NH2-terminal peptide by silver staining and immunoblot. A preparation of His-fusion protein is carried out using 1 liter of cells, which have endogenous APS kinase “repressed” by adding cysteine and methionine to the growth medium.
Expression of Full-length, NH2-domain, and COOH-domain Constructs of hPAPS Synthase

COS-1 cells (ATCC, Rockville, MD) were grown in 75-cm$^2$ flasks in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin in an atmosphere of 5% CO$_2$, 95% air at 37 °C. When cells reached 50–60% confluency they were placed in serum- and antibiotic-free medium and the incubations continued for an additional 15 h. The transfection medium was removed and cells were placed in fresh complete medium containing serum and antibiotics was added and the incubations continued for an additional 15 h. The transfection medium was removed and cells were placed in fresh complete medium and incubated for another 48 h, after which cells were washed three times with phosphate-buffered saline, harvested by scraping, centrifuged briefly at 1000 g, and resuspended in lysis buffer (20 mM Tris-HCl (pH 7.5), 20% sucrose, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 175 µg/ml aprotinin and soybean trypsin inhibitor, 1 mM EDTA, 1 mM 4-methylumbelliferyl-$b$-D-galactoside). The prepared cellular extracts were assayed for enzymatic activity.

Enzyme Assays

The substrate $[^{35}S]$APS was prepared from $[^{35}S]$PAPS by a modification of the procedure outlined by Satishchandran et al. (25). Briefly, $[^{35}S]$PAPS was treated with mung bean 3'-nuclease (Sigma), which yields a mixture of 3'-phosphate and 5'-phosphate. The preparation of $[^{35}S]$APS was subjected to gel filtration using a 1-ml Sephadex G-25 mini-column (Amersham Pharmacia Biotech), and the purified radioactive $[^{35}S]$APS was treated with mung bean 3'-phosphatase (Sigma), which generates $[^{35}S]$APS. The substrate $[^{35}S]$APS was prepared from $[^{35}S]$PAPS by a modification of the procedure outlined by Satishchandran et al. (25). Briefly, $[^{35}S]$PAPS was treated with mung bean 3'-nuclease (Sigma), which yields a mixture of 3'-phosphate and 5'-phosphate. The preparation of $[^{35}S]$APS was subjected to gel filtration using a 1-ml Sephadex G-25 mini-column (Amersham Pharmacia Biotech), and the purified radioactive $[^{35}S]$APS was treated with mung bean 3'-phosphatase (Sigma), which generates $[^{35}S]$APS. The substrate $[^{35}S]$APS was prepared from $[^{35}S]$PAPS by a modification of the procedure outlined by Satishchandran et al. (25). Briefly, $[^{35}S]$PAPS was treated with mung bean 3'-nuclease (Sigma), which yields a mixture of 3'-phosphate and 5'-phosphate. The preparation of $[^{35}S]$APS was subjected to gel filtration using a 1-ml Sephadex G-25 mini-column (Amersham Pharmacia Biotech), and the purified radioactive $[^{35}S]$APS was treated with mung bean 3'-phosphatase (Sigma), which generates $[^{35}S]$APS.

The APS kinase assay was performed in a total volume of 20 µl and consisted of 6 µl of reaction buffer (50 mM potassium phosphate (pH 7.5), 12 mM MgCl$_2$, 45 mM dithiothreitol, 5 mM NaF), 2 µl enzyme preparation, 4 µl of 40 mM PPi, 4 µl of 100–1000 µM ATP, and 4 µl of H$_2$O. Reactions were carried out at 37 °C for 15 min for the APS kinase assay and 5 min for the ATP sulfurylase assay and stopped by placing reaction tubes in boiling water for 5 min. 1-µl aliquots were transferred to polyethyleneimine-cellulose thin-layer chromatography plates and chromatographed using 0.9 M LiCl as the solvent system. Following chromatography the thin-layer chromatography plates were dried and exposed overnight to x-ray film (Eastman Kodak Co.). The respective spots for PAPS, APS, and sulfate were cut out, and the radioactivity determined by liquid scintillation. For the overexpressed and purified NH$_2$-terminal fragment of hPAPS synthase and the full-length hPAPS synthase protein, samples containing 1 µg of protein were assayed.

$\beta$-Galactosidase Assay—Cellular extracts (100 µl) were diluted with 175 µl of water and 225 µl of 2X assay buffer (Promega). Samples were mixed and incubated at 37 °C for 90 min, and the reaction was stopped by adding 50 µl of 1 M NaCO$_3$. The reaction product was assayed by OD at 420 nm, and the $\beta$-galactosidase activity (in units) was determined using a standard curve.

Peptide Synthesis and Antisera Production—All peptides based on human PAPS synthase were prepared by Peptide Technologies Corp. (Gaithersburg, MD). Briefly, NH$_2$-terminal-specific peptide (LDGD-NIRGQLNKLGS, residues 86–102) and COOH-terminal-specific peptide (RGTRRPRVLLHHPLGKTDDDDPVMRMKQH, residues 442–471) were synthesized by solid-phase methods coupling Fmoc butyl-Ser Wang resin; the final peptide was 98.4% pure. The COOH-terminal-specific peptide also with Cys at the NH$_2$ terminus was synthesized starting with Fmoc butyl-ber Wang resin; the final peptide was 98.4% pure. The NH$_2$-terminal-specific peptide was used for the enzyme assays.

The substrate $[^{35}S]$APS was prepared from $[^{35}S]$PAPS by a modification of the procedure outlined by Satishchandran et al. (25). Briefly, $[^{35}S]$PAPS was treated with mung bean 3'-nuclease (Sigma), which yields a mixture of 3'-phosphate and 5'-phosphate. The preparation of $[^{35}S]$APS was subjected to gel filtration using a 1-ml Sephadex G-25 mini-column (Amersham Pharmacia Biotech), and the purified radioactive $[^{35}S]$APS was treated with mung bean 3'-phosphatase (Sigma), which generates $[^{35}S]$APS.

The APS kinase assay was performed in a total volume of 20 µl and consisted of 6 µl of reaction buffer (50 mM potassium phosphate (pH 7.5), 12 mM MgCl$_2$, 45 mM dithiothreitol, 5 mM NaF), 2 µl enzyme preparation, 4 µl of 40 mM PPi, 4 µl of 100–1000 µM ATP, and 4 µl of H$_2$O. Reactions were carried out at 37 °C for 15 min for the APS kinase assay and 5 min for the ATP sulfurylase assay and stopped by placing reaction tubes in boiling water for 5 min. 1-µl aliquots were transferred to polyethyleneimine-cellulose thin-layer chromatography plates and chromatographed using 0.9 M LiCl as the solvent system. Following chromatography the thin-layer chromatography plates were dried and exposed overnight to x-ray film (Eastman Kodak Co.). The respective spots for PAPS, APS, and sulfate were cut out, and the radioactivity determined by liquid scintillation. For the overexpressed and purified NH$_2$-terminal fragment of hPAPS synthase and the full-length hPAPS synthase protein, samples containing 1 µg of protein were assayed.

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Functional Domains of Human PAPS Synthase

The NH₂-terminal Domain of hPAPS Synthase—The 1–268 amino acid NH₂-terminal fragment of hPAPS synthase is significantly homologous to APS kinase cloned from other species (Table I), specifically yeast Saccharomyces cerevisiae (GenBank™ accession no. S17244), the filamentous fungus Penicillium chrysogenum (GenBank™ accession no. U39393), Escherichia coli cys D gene product (GenBank™ accession no. A44200), symbiotic nodulating bacteria Rhizobium melliloti (GenBank™ accession no. M68858), and Azospirillum brasilense (GenBank™ accession no. M94886) nod Q gene products, and the plant Arabidopsis thaliana (GenBank™ accession no. S47640). Sequence alignment (Fig. 2) reveals 51 identities (25%) and 88 similarities (44%). Furthermore, there are three conserved nucleotide binding motifs (indicated by bold boxes in Fig. 2): a P-loop nucleotide binding motif, a GTP interactive motif, and a PAPS binding motif. The P-loop motif is variously represented as GXXXXGK (29, 30), GXXXGK(TS) (31), GXXXXGK(S/T) (32), GXXXGK(T/S) (33), and GAXGXXGK (34). It is the latter form that is present in hPAPS synthase.

The GTP interactive motif consists of DXXG (30, 32), and the PAPS binding motif consists of K/A(0)GXXGHXXN/E/XX(0–1)FT (35). Additionally, a FISP motif, present between the P-loop and DXXG motifs, is also conserved (cf. Fig. 2). It has been suggested that the serine in the FISP motif serves as a phosphorylation intermediate in the phosphorylation of APS to form PAPS (35). The sequence alignment strongly suggests that the NH₂-terminal region of hPAPS synthase represents the APS kinase portion of this bifunctional protein.

When the sequences depicted in Fig. 2 were analyzed for spacing between the conserved motifs, a remarkable consistency was noted. The spacing between the P-loop motif and the GTP binding motif consists of 101 amino acid residues in 4/7 cases, 103 residues in 2/7 cases, and 110 residues in one case. The spacing between the GTP interactive motif and the PAPS binding motif is 3 amino acid residues in 7/7 instances. The conservation of motifs and their spacing is suggestive of some functional importance, although the significance of that remains to be elucidated.

The COOH-terminal Domain of hPAPS Synthase—In contrast to the substantial homology of the 1–268 amino acid NH₂-terminal fragment of hPAPS synthase with APS kinase of bacteria, fungi, yeast, and plants, the 404 amino acid COOH-terminal fragment (residues 220–623) and ATP sulfurylase of bacteria, fungi, and yeast are considerably less homologous. Interestingly, however, plants are an exception to the rule in that ATP sulfurylase of Solanum tuberosum and A. thaliana are 63% and 62% homologous to the COOH-terminal fragment of hPAPS synthase, respectively (Table I). Alignment of the COOH-terminal fragment of hPAPS synthase with ATP sulfurylase of the filamentous fungi P. chrysogenum (GenBank™ accession no. U70353) and A. nidulans (GenBank™ accession no. M68858), the plant A. thaliana (GenBank™ accession no. X82541), the plant A. thaliana (GenBank™ accession no. U05218), yeast S. cerevisiae (GenBank™ accession no. 08536), nod Q gene products of the symbiotic bacteria A. brasilense (GenBank™ accession no. M74586), and nod Q gene products of the symbiotic bacteria A. brasilense (GenBank™ accession no. M94886), and R. meliloti (GenBank™ accession no. M68858) and the cys N gene product of E. coli (GenBank™ accession no. M74586) reveals only 5 identities (1%) and 35 similarities (8%). If, however, bacteria are removed from the alignment, identities increase to 61 (15%) and similarities to 141 (35%) (Fig. 3). The alignment problem with bacteria relates to the fact that ATP sulfurylase is encoded by two genes: nod P and nod Q genes for the nodulating bacteria and cys D and cys N genes for E. coli; however, it should be noted that the nod P and cys D gene products are in themselves highly homologous. The same is true for the ATP sulfurylase portion of the nod Q gene products of A. brasilense.

### Table I

| Protein                | Identities | Homology |
|------------------------|-----------|----------|
| **NH₂-terminal domain**| 56        | 65       |
| S. cerevisiae (202)    | 54        | 62       |
| P. chrysogenum (211)   | 42        | 49       |
| A. thaliana (276)      | 51        | 60       |
| E. coli cys C (201)    | 48        | 56       |
| R. meliloti nod P Q KIN (204) | 48 | 55 |
| A. brasilense nod Q KIN (197) | 39 | 44 |
| A. nidulans SUL COOH-terminal (179) | 39 | 44 |
| P. chrysogenum SUL COOH-terminal (178) | 38 | 45 |
| **COOH-terminal domain** | 58 | 63 |
| S. tuberosum (424)    | 57        | 62       |
| A. thaliana (463)      | 27        | 31       |
| P. chrysogenum (572)   | 25        | 28       |
| A. nidulans (574)      | 24        | 28       |
| S. cerevisiae (521)    | 23        | 26       |
| R. meliloti nod P (299) | 20    | 25       |
| A. brasilense nod P (301) | 16 | 20  |
| A. brasilense nod Q SUL (423) | 15 | 19  |
| E. coli cys D (302)    | 15        | 19       |
| R. meliloti nod Q SUL (437) | 15 | 19  |
| A. thaliana (457)      | 15        | 19       |

* Amino acid residues 1–268.
* KIN indicates APS kinase.
* SUL indicates ATP sulfurylase.
* *A* Amino acid residues 220–623.
and *R. meliloti* and the *cys N* gene product of *E. coli*. In filamentous fungi, yeast and plants ATP sulfurylase is encoded by a single gene. The complexity in sequence analysis, notwithstanding the homology noted for the COOH-terminal fragment of hPAPS synthase with ATP sulfurylase of fungi, yeast, and, particularly, plants suggests that ATP sulfurylase activity resides within the COOH-terminal domain of this bifunctional protein.

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### Functional Domains of Human PAPS Synthase

| hN-domain | R. meliloti nod Q KIN | A. brasilense nod Q KIN | A. brasilense nod Q KIN | P. chrysogenum |
|-----------|----------------------|------------------------|------------------------|----------------|
| hN-domain | R. meliloti nod Q KIN | A. brasilense nod Q KIN | P. chrysogenum | |
| hN-domain | R. meliloti nod Q KIN | A. brasilense nod Q KIN | P. chrysogenum | |
| hN-domain | R. meliloti nod Q KIN | A. brasilense nod Q KIN | P. chrysogenum | |
| hN-domain | R. meliloti nod Q KIN | A. brasilense nod Q KIN | P. chrysogenum | |

**FIG. 2. Amino acid sequence alignment of cloned APS kinase.** Sequences from several species were compared with the 1–268 amino acid sequence for the NH₂-terminal domain of hPAPS synthase (hN-domain). Identities and similarities are boxed. Sequences within bold boxes representing nucleotide binding motifs and a FISP sequence denoted by asterisks are described under “Results.” *R. meliloti* nod Q gene product; *A. brasilense* nod Q gene product.

**FIG. 3. Amino acid sequence alignment of cloned ATP sulfurylase.** Sequences from several species were compared with the 220–263 amino acid sequence for the COOH-terminal domain of hPAPS synthase (hC-domain).
Co-transfections were achieved using LipofectAMINE® and enzyme assays using 10,000 rpm supernatants obtained following cell lysis were performed as described under “Experimental Procedures.” Histograms represent the mean of three replicates; error bars are indicated. Control represents cellular extract from full-length hPAPS synthase construct inserted in the reverse orientation. Amino acid residues contained within each segmental construct are indicated. * , p = 0.023; #, p = 0.0023; §, p = 0.0003; $, p = 0.01; †, p = 0.002 compared with control.

**Catalytic Activity and Kinetic Analyses**

Three NH2-terminal fragments of hPAPS synthase (residues 1–174, 1–209, and 1–268) were constructed based on the APS kinase sequence homology information. The 1–174 amino acid construct lacks the PAPS binding motif, whereas the 1–209 and 1–268 amino acid constructs contain all the nucleotide binding motifs described above for APS kinase of other species (cf. Fig. 2). Two COOH-terminal fragments of hPAPS synthase (residues 220–623 and 264–623) were also constructed, and with the exception of *S. tuberosum* and *A. thaliana*, these fragments contain minimal homology to ATP sulfurylase of fungi, yeast, and bacteria. All constructs were co-transfected into COS-1 cells along with β-galactosidase and cellular extracts assayed for APS kinase, ATP sulfurylase and β-galactosidase activities, as well as the presence of either NH2-terminal-specific or COOH-terminal-specific immunoreactive peptides.

**APS Kinase Activity**—Cells transfected with the 1–268 amino acid NH2-terminal fragment of hPAPS synthase demonstrated a 7-fold increase in APS kinase activity over cells transfected with a vector containing full-length hPAPS synthase in the reverse orientation with regard to the CMV promoter (Fig. 4, panel A). Furthermore, cytosolic extracts of cells transfected with this construct demonstrated an NH2-terminal-specific immunoreactive protein band of the appropriate size (~30 kDa) (Fig. 5). Cells transfected with the full-length hPAPS synthase cDNA also exhibited a significant increase in APS kinase activity, although the activity was only about one-third that obtained with the 268 amino acid NH2-terminal domain construct (Fig. 4, panel A). In contrast, cells transfected with the other two NH2-terminal constructs (residues 1–174 and 1–209) and the two COOH-terminal constructs (residues 220–623 and 264–623) did not exhibit APS kinase activity above that seen for the control cells (Fig. 4, panel A) and failed to display an NH2-terminal-specific immunoreactive protein band (Fig. 5).

The COS-1 cell-expressed 1–268 amino acid NH2-terminal fragment of hPAPS synthase displayed Michaelis-Menten kinetics against both APS (Fig. 6A) and ATP (Fig. 7A) substrates. Double-reciprocal transformations revealed a 

\[ K_m = 0.6 \mu M \]

for APS and 0.23 mM for ATP. For comparison, kinetic analyses for the bacterial overexpressed and purified 1–268 amino acid NH2-terminal fragment of hPAPS synthase were carried out. The overexpressed and purified peptide fragment had a 

\[ K_m = 2.3 \mu M \]

(Fig. 6B), a value somewhat higher than that for the COS-1 cell-expressed 1–268 amino acid NH2-terminal fragment (Fig. 6A). On the other hand, the overexpressed and purified 1–268 amino acid NH2-terminal fragment had a 

\[ K_m = 0.26 \mu M \]

for ATP (Fig. 7B), a value essentially the same as that for the COS-1 cell-expressed 1–268 amino acid NH2-terminal fragment (Fig. 7A).

COS-1 cell-expressed full-length hPAPS synthase, likewise, demonstrated Michaelis-Menten kinetics when tested against the APS substrate (Fig. 8A); a Lineweaver-Burk plot yielded a 

\[ K_m = 0.4 \mu M \]

When full-length hPAPS synthase was tested...
against the ATP co-substrate, however, a sigmoidal dose-response curve was obtained indicating cooperative substrate binding (Fig. 9A, solid curve); an empirically derived $K_m$ was 1.5 mM. The latter finding suggests that the COOH-terminal domain of hPAPS synthase plays a regulatory role on APS kinase activity located in the NH2-terminal domain. Because control COS-1 cells demonstrate endogenous catalytic activity (cf. Fig. 4) and exhibit high endogenous PAPS synthase protein on Western analysis (cf. Fig. 5), an APS kinase kinetic analysis against ATP substrate was performed on nontransfected COS-1 cells (Fig. 9A, broken curve) to compare with the results obtained with the transfected COS-1 cells (Fig. 9A, solid curve). Importantly, both curves were similarly sigmoidal; in addition, the curve for the transfected cells is clearly shifted upward indicating an increase in the concentration of hPAPS synthase protein as a result of the transfection; furthermore, the empirically derived $K_m$ values were essentially the same. As with the 1–268 amino acid NH2-terminal fragment of hPAPS synthase, kinetic analyses were performed using the bacterial overexpressed and purified full-length hPAPS synthase for comparison with the COS-1 cell-transfected protein. The overexpressed and purified protein had a $K_m$ for APS of 2.6 μM (Fig. 8B), a value again somewhat higher than that for the COS-1 cell-expressed full-length protein (Fig. 8A). Interestingly, in contrast to endogenous and COS-1 cell-expressed PAPS synthase, overexpressed and purified hPAPS synthase demonstrated a hyperbolic curve against ATP as substrate (Fig. 9B) with a $K_m$ for ATP of 0.45 mM.

The APS $K_m$ values of 0.6 and 0.4 μM for the NH2-terminal domain and full-length constructs of hPAPS synthase, respectively, are similar to the APS $K_m$ value for APS kinase of purified guinea pig PAPS synthase. The APS $V_{max}$ for the NH2-terminal domain was 667 pmol/min/mg protein, a value significantly higher than the 222 pmol/min/mg protein $V_{max}$ for full-length hPAPS synthase. Analogously, the ATP $V_{max}$ for the NH2-terminal domain was 426 pmol/min/mg, a value significantly higher than the $V_{max}$ for the expressed full-length hPAPS synthase of 50 pmol/min/mg protein (difference between transfected and endogenous values; cf. Fig. 9A).

Although $V_{max}$ is a function of the amount of enzyme present, which in turn could be influenced by transfection efficiency, the differential in the $V_{max}$ values between the NH2-terminal domain and full-length hPAPS synthase is considered credible because the observations were consistent for multiple transfections. The APS $K_m$ value for the NH2-terminal domain (0.6 μM) is similar to that for full-length hPAPS synthase (0.4 μM). On the other hand, the empirically derived ATP $K_m$ value of 1.5 mM for the full-length hPAPS synthase construct is 7-fold higher than the $K_m$ value of 0.2 mM for the NH2-terminal domain. Regarding catalytic efficiency, APS $V_{max}/K_m$ for the NH2-ter-
The NH$_2$-terminal domain is 2-fold higher than for full-length hPAPS synthase reflecting primarily an increase in $V_{\text{max}}$; however, ATP $V_{\text{max}}/K_m$ for the NH$_2$-terminal domain is a marked 64-fold higher than for full-length hPAPS synthase reflecting increased catalytic efficiency of the NH$_2$-terminal domain. A similar analytical evaluation with the overexpressed and purified proteins resulted in a comparable conclusion. That is, the $V_{\text{max}}$ value for PAPS formation was severalfold higher for the NH$_2$-terminal fragment compared with the full-length protein. A summary of the kinetic data for COS-1 cell-expressed NH$_2$-terminal fragment of hPAPS synthase and the full-length hPAPS synthase protein is presented in Table II.

**DISCUSSION**

The data presented clearly indicate that APS kinase activity resides within the NH$_2$-terminal segment of bifunctional hPAPS synthase, whereas ATP sulfurylase activity is located in the COOH-terminal region of the protein: the 1–268 amino acid NH$_2$-terminal segment exhibited catalytically efficient APS kinase activity, and the 220–623 amino acid COOH-terminal portion exhibited ATP sulfurylase activity. Since PAPS synthase is a highly conserved protein from the marine worm (21) to the mouse (22), to the guinea pig, and to the human being (this work), this conclusion would apply to all PAPS synthase fusion proteins. Although the data presented in Figs. 4 and 5 are from a single experiment, additional multiple experiments were performed with essentially identical results, i.e. the NH$_2$-terminal 1–268 and COOH-terminal 220–623 constructs were consistently expressed, whereas the other NH$_2$- and COOH-terminal constructs were not expressed. The reason for the failure of the 1–174 and 1–209 NH$_2$-terminal and the 264–623 COOH-terminal constructs of hPAPS synthase to be expressed by the COS-1 cells is not readily understood. All PCR products were prepared and inserted into the same vector in a functional domain is 2-fold higher than for full-length hPAPS synthase reflecting primarily an increase in $V_{\text{max}}$; however, ATP $V_{\text{max}}/K_m$ for the NH$_2$-terminal domain is a marked 64-fold higher than for full-length hPAPS synthase reflecting increased catalytic efficiency of the NH$_2$-terminal domain. A similar analytical evaluation with the overexpressed and purified proteins resulted in a comparable conclusion. That is, the $V_{\text{max}}$ value for PAPS formation was severalfold higher for the NH$_2$-terminal fragment compared with the full-length protein. A summary of the kinetic data for COS-1 cell-expressed NH$_2$-terminal fragment of hPAPS synthase and the full-length hPAPS synthase protein is presented in Table II.

**ATP Sulfurylase Activity**—Cells transfected with the full-length hPAPS synthase cDNA demonstrated a significant increase in ATP sulfurylase activity over cells transfected with a vector containing full-length hPAPS synthase in the reverse orientation with regard to the CMV promoter (Fig. 4, panel B). Cells transfected with the 405 amino acid COOH-terminal segment (residues 220–623) of hPAPS synthase exhibited a significant increase in ATP sulfurylase activity that was higher than that found for the full-length preparation (Fig. 4, panel B). Additionally, these cells also demonstrated a COOH-terminal-specific immunoreactive protein band of the appropriate size (47 kDa) (Fig. 5). On the other hand, cells transfected with the three NH$_2$-terminal constructs and the 360 amino acid COOH-terminal construct (residues 264–623) failed to exhibit an increase in ATP sulfurylase activity (Fig. 4, panel B) and also failed to manifest a COOH-terminal-specific immunoreactive protein band (Fig. 5).

**FIG. 8.** APS kinase kinetic analysis of full-length hPAPS synthase against APS as substrate. Extract of COS-1 cells after transfection with full-length hPAPS synthase (A) and bacterial overexpressed and purified full-length hPAPS synthase (B) were assayed for APS kinase against various concentrations of APS as described under “Experimental Procedures.” Data points represent the average of three independent experiments. Double-reciprocal transformations are shown in the inset.

**FIG. 9.** APS kinase kinetic analysis of PAPS synthase against ATP as substrate. Extracts of COS-1 cells transfected with either an empty vector (○) or a vector containing full-length hPAPS synthase (●) (A) and bacterial overexpressed and purified full-length hPAPS synthase (●) (B) were assayed for APS kinase against various concentrations of ATP as described under “Experimental Procedures.” Data points represent the average of three independent experiments. A double-reciprocal transformation is shown in the inset of B.
similar fashion and verified by sequencing. Plasmids were prepared, and transfections carried out similarly for all constructs. Based on the fact that expression of β-galactosidase was constant for all co-transfections (Fig. 4, panel C), it seems likely that the transfection efficiency would also have been relatively constant for all of the constructs. It is conceivable that shortening the NH2-terminal domain from the carboxyl end, i.e. from amino acid 268 to 209 and amino acid 268 to 174, hindered proper folding of the proteins thus subjecting them to rapid degradation by cellular proteases. Likewise, expression of the 360 amino acid COOH-terminal fragment of hPAPS synthase was not detectable, presumably for the same reason. That is, critical amino acid residues are missing from the NH2-terminal region of the shorter COOH-terminal fragment resulting in improper folding of the protein, exposing it to cellular proteases that caused rapid degradation. It is of some interest to note that there is a common 49 amino acid overlapping region for the two active constructs (residues 220–268) indicating some obstacles to the synthesis of activated sulfate and production of a more efficient pathway that is able to overcome inherent obstacles to the synthesis of activated sulfate and production of a multifunctional enzyme system could represent an evolutionary step creating a接轨于共同氨基酸序列领域的共同氨基酸序列区域。这表明分离的NH2-terminal domain from the rest of the protein removes an inhibitory influence that the COOH-terminal domain appears to have on the APS kinase activity exhibited by the 1–268 NH2-terminal domain. This conclusion is based on the kinetic analyses, which revealed that, in contrast to the 1–268 amino acid NH2-terminal domain which manifested a hyperbolic response to ATP as substrate (COS-1 cell-expressed and bacterial overexpressed and purified proteins; cf. Fig. 7), full-length PAPS synthase demonstrated a sigmoidal response indicating cooperative substrate binding (endogenous COS-1 cell PAPS synthase as well as COS-1 cell-expressed hPAPS synthase; cf. Fig. 9A). The physiological significance of this finding remains to be ascertained. Interestingly, however, the bacterial overexpressed and purified full-length hPAPS synthase protein exhibited a hyperbolic response curve (cf. Fig. 9B). This finding suggests that endogenous COS-1 cell PAPS synthase as well as expressed hPAPS synthase were either covalently modified in some fashion (maybe phosphorylation) or were acted upon by some endogenous factor to produce the cooperative binding curve seen in Fig. 9A. In contrast, the bacterial overexpressed and purified hPAPS synthase would not have been subject to a similar cellular modification.

PAPS synthase has been cloned for the marine worm, Urechis caupo (GenBank™ accession no. L39001) (21), mouse (GenBank™ accession no. U34483) (22), and guinea pig (GenBank™ accession no. AF004875). An amino acid alignment of these PAPS synthase sequences with hPAPS synthase reveals that there are 421 identities (69%) and 96 similarities for 85% homology indicating a high degree of conservation for this crucial protein. Although hPAPS synthase is 68% identical to worm PAPS synthase, it is 95% and 98% identical to guinea pig and mouse PAPS synthase, respectively.

Is there an adaptive advantage in the fusion of ATP sulfurylase and APS kinase into a single polypeptide chain in higher eukaryotes? Although a number of advantages have been listed for the existence of multifunctional proteins in general (36) as well as ATP sulfurylase and APS kinase in particular (37), the answer to the foregoing question remains obscure. The fusion protein could expedite channeling (direct transfer) of APS from ATP sulfurylase to APS kinase (7), an idea which takes into consideration the substantial energy barrier that exists in APS formation (9), as well as the purported instability of APS in cells (7). Channeling, however, can also be achieved by ATP sulfurylase and APS kinase as distinct polypeptides associating noncovalently in a multifunctional complex as has been suggested for E. coli (11). On the other hand, filamentous fungi appear not to demonstrate channeling of APS between the two enzyme systems (37). Nevertheless, the development of a fused protein system could represent an evolutionary step creating a more efficient pathway that is able to overcome inherent obstacles to the synthesis of activated sulfate and production of the crucial universal sulfonate donor molecule, PAPS. In our analysis, the catalytic efficiency of the active NH2-terminal domain of hPAPS synthase was many-fold higher than that of the complete protein, which is contrary to what would be expected if the advantage of protein fusion is to improve catalytic efficiency. Thus, at least based on the APS kinase kinetic data, it appears that the fusion of ATP sulfurylase and APS kinase did not produce a catalytic advantage. We could not, however.

\[ \frac{V_{\text{max}}}{K_{\text{M}}}, \text{ APS} \]

**TABLE II**

Kinetic values for ATP and APS substrates

| Construct | $K_a$ ATP | $K_a$ APS | $V_{\text{max}}$ ATP | $V_{\text{max}}$ APS | $V_{\text{max}}/K_a$ ATP | $V_{\text{max}}/K_a$ APS |
|-----------|-----------|-----------|----------------------|---------------------|-------------------------|-------------------------|
| Full-length | 0.2       | 0.6       | 222                  | 33                  | 1120                    | 555                     |
| NH2-terminal | 1.5       | 0.4       | 50                   |                     |                         |                         |

* Determined as difference between COS-1 cell endogenous control and expressed values (cf. Fig. 9A).
perform kinetic analyses for ATP sulfurylase, since the forward reaction toward the formation of APS is quite slow. Also, the backward reaction toward the formation of ATP and SO$_4$ from PP$_i$ and APS is extremely complicated at low PP$_i$ concentrations because of the presence of a ubiquitous inorganic pyrophosphatase that competes for the PP$_i$, in converting it to inorganic phosphate. Thus, purified preparations of the full-length and the active COOH-terminal domain of hPAPS synthase will be necessary to perform kinetic analyses and further examine the question of catalytic advantage of the fused genes for ATP sulfurylase and APS kinase. Fusion of the ATP sulfurylase and APS kinase genes into a single gene might produce an adaptive advantage. Therefore, at first glance, fusion of ATP sulfurylase and APS kinase would not appear to have necessarily created a regulatory advantage. However, in multicellular organisms, the creation of unique cis-elements and trans-factors allows for differential tissue-specific expression of PAPS synthase during growth and development, as well as in response to acute and/or chronic metabolic needs.

Although the biochemistry of ATP sulfurylase and APS kinase is fascinating and complex, their evolution presents intriguing variations on a theme (Fig. 10). For instance, the number of genes involved varies; in E. coli, there are three genes; in the nitrogen-fixing nodulating bacteria, filamentous fungi, yeast, and plants there are two genes; and in animal species there is but one gene. ATP sulfurylase is encoded by two genes in E. coli and the nodulating bacteria. In filamentous fungi, yeast, and plants, ATP sulfurylase is encoded by a single gene. Interestingly, APS kinase, which is encoded by a single gene in all species, can be either a distinct catalytic entity (E. coli, fungi, yeast, and plants) or melded with ATP sulfurylase as a fusion protein (nitrogen-fixing bacteria and animals). It is further intriguing that the COOH-terminal region of ATP sulfurylase in the filamentous fungi is highly homologous to APS kinase (although it is devoid of APS kinase activity) and, in this sense, is analogous to the nod Q fusion proteins of the nodulating bacteria. It is interesting to note that the ATP sulfurylase of filamentous fungi is allosterically inhibited by PAPS, whereas the yeast and bacterial enzymes are not so affected by PAPS. This is thought to be because of the presence of a PAPS binding motif in the COOH terminus of fungal ATP sulfurylase (the same motif that is present in APS kinase noted above), a motif not present in the bacterial and yeast enzymes (14).

The apparent orientation of intrinsic ATP sulfurylase and APS kinase in hPAPS synthase is in contrast to what is found in bacteria, fungi, yeast, and plants. In bacteria, ATP sulfurylase and APS kinase are encoded by contiguous genes, and, as indicated in Fig. 10, ATP sulfurylase precedes APS kinase. Additionally, the structural organization of the fused nod Q protein of symbiotic nitrogen-fixing bacteria is similarly oriented, i.e. ATP sulfurylase is located in the NH$_2$-terminal domain and APS kinase activity in the COOH-terminal domain of the bifunctional protein (cf. Fig. 10). Analogously, although lacking APS kinase catalytic activity, the COOH-terminal region of ATP sulfurylase in filamentous fungi is highly homologous to APS kinase of other species as well as APS kinase of filamentous fungi (contains the triple nucleotide binding motifs discussed previously for APS kinase); it has been suggested that this region of fungal ATP sulfurylase evolved from APS kinase (14). Thus, gene fusion appears to have occurred in the symbiotic nitrogen-fixing bacteria and filamentous fungi (similar to the case with animal species), however, with a difference. In the symbiotic nitrogen-fixing bacteria, straight forward gene fusion occurred, which created a bifunctional protein; in filamentous fungi, there appears to have been gene duplication as well as gene fusion, and the latter failed to result in a bifunctional protein. A priori, it might be expected that for the fused hPAPS synthase protein (and by analogy worm and mouse PAPS synthase), ATP sulfurylase activity would be located within the NH$_2$-terminal region of the protein and APS kinase in the COOH-terminal region. Instead, the opposite is found. APS kinase activity is located in the NH$_2$-terminal domain and ATP sulfurylase activity in the COOH-terminal domain.

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