Site-selected Mutagenesis of a Conserved Nucleotide Binding HXGH Motif Located in the ATP Sulfurylase Domain of Human Bifunctional 3′-Phosphoadenosine 5′-Phosphosulfate Synthase*

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3′-Phosphoadenosine-5′-phosphosulfate (PAPS) synthase is a bifunctional protein consisting of an NH₂-terminal APS kinase and a COOH-terminal ATP sulfurylase. Both catalytic activities require ATP; the APS kinase domain involves cleavage of the β-g phosphodiester bond of ATP, whereas the ATP sulfurylase domain involves cleavage of the α-β phosphodiester bond of ATP. Previous mutational studies have suggested that β-g phosphodiesterase activity involves a highly conserved NTP-binding P-loop motif located in the ATP sulfurylase domain of PAPS synthases. Sequence alignment analysis of PAPS synthases and the superfamily of TagD-related nucleotidylyltransferases revealed the presence of a highly conserved HXGH motif in the ATP sulfurylase domain of PAPS synthases, a motif implicated in the α-β phosphodiesterase activity of cytididylyltransferases. Thus, site-selected mutagenesis of the HXGH motif in the ATP sulfurylase domain of human PAPS synthase (amino acids 425–428) was performed to examine this possibility. Either H425A or H428A mutation produced an inactive enzyme. In contrast, a N426K mutation resulted in increased enzymatic activity. A G427A single mutant resulted in only a modest 30% reduction in catalytic activity, whereas a G427A/H428A double mutant produced an inactive enzyme. These results suggest an important role for the HXGH histidines in the ATP sulfurylase activity of bifunctional PAPS synthase and support the hypothesis that the highly conserved HXGH motif found in the ATP sulfurylase domain of PAPS synthases is involved in ATP binding and α-β phosphodiesterase activity.

In the course of sulfonation, inorganic sulfate must be activated prior to being transferred to an acceptor molecule (1), and the activated sulfate molecule is 3′-phosphoadenosine 5′-phosphosulfate (PAPS).1 The activation of inorganic sulfate to form PAPS results from the concerted action of two enzymes (1). The first step is catalyzed by ATP sulfurylase (ATP:sulfate adenyllyltransferase, EC 2.7.7.4) and involves the reaction of inorganic sulfate with ATP to form adenosine 3′-phosphosulfate (APS) and inorganic pyrophosphate. This reaction results in the formation of a high energy phosphoric-sulfuric acid anhydride bond that is the chemical basis for sulfate activation (2). The second step involves APS reacting with another molecule of ATP to form PAPS and ADP and is catalyzed by APS kinase (ATP:adenyllysulfate 3′-phosphotransferase, EC 2.7.1.25). ATP sulfurylase and APS kinase cloned from bacteria, Fungi, yeast, and plants are found on separate polypeptide chains; however, in mammalian species, the marine worm, and the fruit fly, gene fusion has occurred, and the two enzymes are integral to bifunctional PAPS synthase. It has been clearly established that the NH₂-terminal region of human (h) PAPS synthase constitutes the APS kinase domain, whereas the ATP sulfurylase domain is located in the COOH-terminal portion of this bifunctional protein (3). That is, the NH₂-terminal 268 amino acids of hPAPS synthase (623 amino acids) when overexpressed and purified functions independently as an APS kinase (3), whereas the overexpressed and purified COOH-terminal 405 amino acids of hPAPS synthase functions independently as an ATP sulfurylase.2

A highly conserved P-loop NTP-binding motif (GXXGXGK(S/T)) is present within the NH₂-terminal APS kinase domain of human (3), guinea pig,2 mouse (4), marine worm (5), and Drosophila (6) PAPS synthase. The P-loop motif is highly conserved among NTP-binding proteins where it is involved in binding the phosphate moiety and in the cleavage of the β-g phosphodiester bond of NTP (7, 8). It was recently reported that site-selected mutagenesis of the P-loop motif in mouse PAPS synthase markedly impaired APS kinase activity (9). The latter finding would be in keeping with the hypothesis that the P-loop motif located near the NH₂ terminus of PAPS synthase is involved in cleavage of the β-g phosphodiester bond of ATP and transfer of the terminal phosphoryl group of ATP to the 3′-hydroxyl position of adenosine 5′-phosphosulfate.

In contrast to the NH₂-terminal APS kinase domain of PAPS synthase, the COOH-terminal ATP sulfurylase domain does not and would not necessarily be expected to contain a classical P-loop motif for ATP binding. The reason being that although the COOH-terminal ATP sulfurylase domain of PAPS synthase binds ATP as does the NH₂-terminal domain, the ultimate fate of the bound ATP is not the same. That is, APS kinase catalyzes the removal of the terminal γ-phosphate for transfer to the acceptor molecule, APS, whereas ATP sulfurylase catalyzes the removal of the β-g diphosphate (inorganic pyrophosphate) of ATP and condensation of the formed AMP with inorganic sulfate to form APS. It might, therefore, be expected that the latter function would require a different type of nucleotide-binding site, one that would function as an α-β phosphodiesterase rather than a β-g phosphodiesterase. Inspection of the

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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; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PEI, polyethyleneimine cellulose.

2 K. V. Venkatachalam and C. A. Strott, unpublished data.
data base search (10) output for the COOH-terminal domain of hPAPS synthase revealed a HXGH motif that is conserved throughout the PAPS synthase family. This motif has been previously characterized as the signature of a large family of (predicted) nucleotidyltransferases, prototyped by the Bacillus subtilis cytidylyltransferase TagD; all functionally characterized proteins of this family cleave the $\alpha$-$\beta$ phosphate bond of an NTP (11).

In this paper, we report that site-selected mutagenesis of the HXGH motif implicates this motif in the ATP sulfurylase reaction of bifunctional hPAPS synthase, a finding consistent with the concept that the HXGH sequence is involved in ATP binding and $\alpha$-$\beta$ phosphodiesterase activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Radionucleotides [$a$-$^{32}$P]ATP for DNA sequencing, inorganic $[^{35}S]$S[S0$_4$]I ($300$ Ci/mmol), and $[^{35}S]$PAPS for enzyme assays were purchased from NEN Life Science Products. Oligonucleotides were obtained from Life Technologies, Inc. and Gene Probe Technology (Gaithersburg, MD). Site-directed mutagenesis kit was obtained from Stratagene (La Jolla, CA). Version-2 sequencing kit was obtained from U. S. Biochemical Corp. Agarose was purchased from FMC BioProducts (Rockland, ME) containing 50 mM KCl, 1 mM dithiothreitol, 10% glycerol, and 1.2 mg/ml lysozyme), and the cell suspension was transferred to a microcentrifuge tube (1:1000). Cells were collected by centrifugation and lysed using lysis buffer as before, and the clear supernatant was added to induce expression as noted above. Cells were collected by centrifugation (10,000 g at 25 °C for 7 min, and lysates were centrifuged for 15 min at 10,000 g). The contents were then poured into small columns and washed with buffer A (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 10% glycerol, 1 mM dithiothreitol) until the $A_{260}$ nm was less than 0.01. The columns were subsequently washed with six column volumes of buffer A containing 10 mM imidazole and eluted with 1 ml of buffer A containing 100 mM imidazole.

**PAPS Synthase Assay**—Enzyme activity was determined in a total volume of 10 ml consisting of 3 ml of sample, 3 ml of reaction buffer (150 mM Tris-HCl, pH 8.0, 50 mM KCl, 15 mM MgCl$_2$, 3 mM EDTA, and 45 mM dithiothreitol), 1 ml of 50 mM ATP, and 3 ml of inorganic $[^{35}S]$S[S0$_4$]I (-3.4 $\mu$Ci). Reactions were carried out for 30 min at 37 °C and stopped by placing aliquot reaction tubes in boiling water for 5 min. Aliquots (1 ml) were transferred to PEI-TLC plates and developed using 0.9 M LiCl as the solvent system. Following chromatography, the PEI-TLC plates were dried and exposed overnight to x-ray film (Eastman Kodak Co.). The respective spots for PAPS, APS, and $S_4$ were excised, and the radioactivity was determined by liquid scintillation.

Western Blot Analysis—Expressed purified proteins (30 ml from 1 ml peak fractions) were resolved by SDS-polyacrylamide gel electrophoresis using 12% polyacrylamide gels obtained from Novex, Inc. (San Diego, CA) and electroblotted onto Immobilon-P membranes from Millipore (Bedford, MA). Membranes were soaked in a solution of 0.1% bovine serum albumin in phosphate-buffered saline containing 0.1% Tween 20 (blocking buffer) for 45 min by gentle shaking, after which they were exposed to primary antibody (1:1000), followed by secondary antibody (3:10000) and then developed with E. coli extract (0.5 mg/ml), for 1 h. The primary antibody was generated against a COOH-terminal peptide fragment of human PAPS synthase as previously reported (3). Blots were washed three times with wash buffer (1X phosphate-buffered saline with 0.01% Tween 20) and incubated with the secondary antibody in blocking buffer (1:5000) for an additional 1 h. Finally, the membranes were subjected to three washes in wash buffer and two additional washes in reaction buffer (0.1 M Tris-HCl, pH 8.0). The blots were developed by color reaction according to the manufacturer’s instructions (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD).

**Amino Acid Sequence Alignment Analyses**—Searches of the protein sequence data base (the nonredundant data base at the NCBI) were performed using the gapped BLAST program, the position-specific iterative BLAST (PSI-BLAST) program, the position-specific iterative BLAST (PSI-BLAST) program (10, 12). Multiple alignments were constructed using either the Clustal W program (13) or the MACAW program (14, 15).

**RESULTS**

**Amino Acid Sequence Analyses**

Initially, the presence of the HX/G/A/H motif that is strictly conserved in the COOH-terminal, ATP sulfurylase domain of the PAPS synthases as well as ATP sulfurylases was noticed by visual inspection of the data base search outputs and multiple alignments of this domain (data not shown). Data base searches started with the PAPS synthase sequences because the queries failed to detect significant similarity to any members of the TagD superfamily, although limited similarity to some of them was detectable when the search was performed using the PHI-BLAST program that combined BLAST analysis with the detection of the HX/G/A/H pattern (12). By contrast, in a search initiated with the sequence of the nucleotidyltransferase domain of the NadR protein from Mycobacterium tuberculosis using the PSI-BLAST program (10), members of the PAPS synthase superfamily were retrieved at a statistically significant level (p < 0.01) within 8–10 search iterations (10, 16). Although quite subtle (11% identity on a 189-amino acid overlap), the alignment between NadR and PAPS synthases encompassed almost the whole nucleotidyltransferase domain (data not shown). Multiple alignment analysis using the MACAW program (14, 15) showed that not only the HX/G/A/H but also the surrounding hydrophobic positions that form the basis of a predicted $\beta$-loop-$\alpha$ structural unit were conserved in the PAPS synthases and the TagD superfamily (Fig. 1).

**Expression of Wild Type and Mutant hPAPS Synthase**

Western Blotting—Expression of partially purified wild type and mutant protein preparations was clearly demonstrated.
NTP-binding Motif in ATP Sulfurylase Domain of PAPS Synthase

FIG. 1. Multiple alignment of the predicted NTP binding site. Analysis using the MACAW program involved selected bifunctional PAPS synthases and ATP sulfurylases (upper block) and TagD superfamily nucleotidylyltransferases (lower block). The conserved HXGH motif is shown by reverse type, and additional conserved residues are shown by bold type. In the consensus line, h indicates hydrophobic residues and s indicates small residues. PAPS SYN, PAPS synthase; ATP SUL, ATP sulfurylase; CITC, acetate:SH-citrate lyase ligase; RIBF, riboflavin kinase/FMN adenylyltransferase; TAGD, glyceraldehyde-3-phosphate cytidylyltransferase; PANC, pantothenate synthase; CTPT, CTP-phosphocholine cytidylyltransferase; NADR, NAD operon regulator; KDTB, lipopolysaccharide core biosynthesis protein; DROME, Dro sophila melanogaster; YEAST, Saccharomyces cerevisiae; CHLRE, Chlamydomonas reinhardtii; ARATH, Arabidopsis thaliana; SYNSP, Synechocystis sp.; ECOLI, E. coli; MYCPN, Mycoplasma pneumoniae; BACSU, B. subtilis; MYCTU, M. tuberculosis. The first column of numbers represents the gene identification; the second column of numbers represents the amino acid residue location.

FIG. 2. Immunoblot of bacterially expressed wild type and mutant hPAPS synthase proteins purified by column chromatography. Overexpression of plasmid constructs, preparation of cell extracts, purification of fusion proteins, and Western analysis were carried out as described under “Experimental Procedures.” The primary antibody was developed against a COOH-terminal-specific peptide of hPAPS synthase. Samples applied were: lane 1, wild type; lanes 2–6, mutants H425A, N426K, G427A, H428A, and G427A/H428A, respectively; lane 7, mock control. Protein applications for lanes 1–7 were: 0.27, 0.3, 0.1, 0.1, 0.15, 0.2, and 0.5 μg/50 μl of sample volume, respectively.

FIG. 3. Relative overall PAPS synthase specific activities in cellular extracts of wild type (WT), mutant constructs, and mock control. Overexpression of plasmid constructs, preparation of cell extracts, and PAPS synthase assays were carried out as described under “Experimental Procedures.”

that the HXGH motif is located.

A unifying functional feature of the TagD nucleotidylyltransferase superfamily and the aminoacyl-tRNA synthetases is their α-β phosphodiestérase activity. It has been proposed that the conserved HXGH motif in this superfamily is involved in NTP binding (23), an hypothesis strengthened by recent mutational analyses (24). Our finding that mutating either H425A or H428A of the HXGH motif in hPAPS synthase produced an inactive enzyme is comparable with the results of similar mutations carried out with CTP:phosphocholine cytidylyltransferase (24). The G427A mutation of hPAPS synthase resulted in approximately a 30% reduction in overall enzymatic activity, a finding also comparable with a similar mutation carried out with CTP:phosphocholine cytidylyltransferase (24). Thus, as with the TagD superfamily, mutational studies with hPAPS synthase support the proposition that the highly conserved HXGH motif in the ATP sulfurylase domain of PAPS synthases is involved in NTP binding.

Crystal structures of aminoacyl-tRNA synthetases indicate that the HIGH sequence is located in the first loop of a βαβ unit, which allows the imidazole nitrogens to form hydrogen-binding contacts with the phosphate oxygens of ATP (11). Mutational studies of the HXGH motif in CTP:phosphocholine cytidylyltransferase also implicate the involvement of similar contacts with CTP (24). It follows that the results of the mutational studies involving the HXGH motif in hPAPS synthase should be analogously interpreted. Whereas the H425A and H428A mutants of hPAPS synthase produced inactive en-
zymes, only a modest reduction (~30%) in PAPS synthase activity was noted with the G427A mutant compared with the wild type enzyme. On the other hand, the N426K mutant yielded an increase in PAPS synthase activity, suggesting that the ε-NH₂ group of lysine creates a more efficient enzyme than the amide NH₂ of asparagine, perhaps by creating enhanced hydrogen contact with ATP.

During PAPS formation by bifunctional PAPS synthase (ATP sulfurylase/APS kinase) two molecules of ATP are required, one for each reaction. Site-selected mutagenesis of a P-loop motif (GXXGXGK(T/S)) located in the NH₂-terminal, APS kinase domain of mouse PAPS synthase markedly impaired APS kinase activity (9), a finding in keeping with the purported role of the NTP-binding P-loop motif in the cleavage of the β-γ phosphodiester bond of ATP and transfer of the terminal phosphoryl group to the 3'-hydroxyl position of APS. In contrast to the NH₂-terminal, APS kinase domain, the COOH-terminal, ATP sulfurylase domain of PAPS synthase, acting as an α-β phosphodiesterase during the formation of APS from ATP and inorganic sulfate, requires a different type of NTP-binding site. Based on the results of site-selected mutagenesis of hPAPS synthase, we propose that the domain homologous to the TagD superfamily nucleotidylyltransferases and in particular, the completely conserved HXGH motif located in the ATP sulfurylase domain of all bifunctional PAPS synthases fulfills such a function.

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