Chemical Modification and Site-directed Mutagenesis of Conserved HXXH and PP-loop Motif Arginines and Histidines in the Murine Bifunctional ATP Sulfurylase/Adenosine 5'-Phosphosulfate Kinase*

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The sulfurylase domain of the mouse bifunctional enzyme ATP sulfurylase/adenosine 5'-phosphosulfate (APS) kinase contains HXXH and PP-loop motifs. To elucidate the functional importance of these motifs and of conserved arginines and histidines, chemical modification and site-directed mutagenesis studies were performed. Chemical modification of arginines and histidines with phenylglyoxal and diethyl pyrocarbonate, respectively, renders the enzyme inactive in sulfurylase, kinase, and overall assays. Data base searches and sequence comparison of bifunctional ATP sulfurylase/APS kinase and monofunctional ATP sulfurylases shows a limited number of highly conserved arginines and histidines within the sulfurylase domain. Of these conserved residues, His-425, His-428, and Arg-421 are present within or near the HXXH motif whereas His-506, Arg-510, and Arg-522 residues are present in and around the PP-loop. The functional role of these conserved residues was further studied by site-directed mutagenesis. In the HXXH motif, none of the alanine mutants (H425A, H428A, and R421A) had sulfurylase or overall activity, whereas they all exhibited normal kinase activity. A slight improvement in reverse sulfurylase activity (<10% residual activity) and complete restoration of forward sulfurylase was observed with R421K. Mutants designed to probe the PP-loop requirements included H506A, R510A, R522A, R522K, and D523A. Of these, R510A exhibited normal sulfurylase and kinase activity, whereas R522A and R522K showed no sulfurylase activity, and H506A had normal sulfurylase activity but produced an effect on kinase activity (<10% residual activity). The single aspartate, D523A, which is part of the highly conserved GRD sequence of the PP-loop, affected both sulfurylase and kinase activity. This mutational analysis indicates that the HXXH motif plays a role only in the sulfurylase activity, whereas the PP-loop is involved in both sulfurylase and kinase activities. Residues specific for sulfurylase activity have also been distinguished from those involved in kinase activity.

Recently there has been increased interest in the sulfate-activating bifunctional enzyme, ATP sulfurylase/APS kinase (PAPS synthetase). With the recent cloning of two isoforms of sulfurylase/kinase (SK) from both mouse, MSK1 (1) and MSK2 (2), and human, HSK1 (3, 4) and HSK2 (3), increased efforts are directed toward understanding the structure-function relationship of the bifunctional enzyme. This enzyme was initially identified by Sugahara and Schwartz (5–7) as the site of the brachymorphic defect in mice. The enzyme was subsequently purified to homogeneity and complete kinetic, functional, and structural analyses of the two-step pathway revealed that both activities reside on a single bifunctional protein that uses a channeling mechanism to efficiently produce PAPS (8–12). We have also reported the cloning of two mouse bifunctional enzyme isoforms, MSK1 (1) and MSK2 (2), and have shown that a point mutation in the kinase portion of MSK2 renders the enzyme inactive, causing murine brachymorphism (2).

Sequence alignment of various monofunctional sulfurylase and kinase enzymes from lower organisms and plants, and bifunctional enzymes from animals reveal the presence of several highly conserved regions (1). Many of these conserved residues are present in motifs that have been implicated in ATP binding (P-loop) (13), phosphoryl transfer (FISP) (14), phosphodiester-cleavage (15), and pyrophosphate binding (PP-loop) (16). Recently, we reported on the generation of several site-directed mutants in the ATP-binding motif of the kinase domain (17), as well as the expression of truncated, monofunctional and rearranged domains of the bifunctional enzyme (18). Continuing our elucidation of the important binding motifs and functional residues in the bifunctional enzyme, we have extended our analysis to the sulfurylase portion of the enzyme, where enzyme inactivation via chemical modification, in conjunction with sequence alignment, aided in identifying potentially important arginine and histidine residues (19). Specific critical arginine and histidine residues were then determined through site-directed mutagenesis studies (3).

Two conserved motifs present in the sulfurylase portion of the bifunctional enzyme are the PP-loop and an unnamed motif suggested to be involved in phosphodiester cleavage (20–22). The PP-loop, a new P-loop-like motif, was first recognized by Bork and Koonin (16) through comparison of a series of ATP pyrophosphatase domains. A modified version of the PP-loop is found in the mouse SIK ATP sulfurylase region from residues 514 to 532 in the form GANFYIVGRDPAGMPHPET (hhXhhXhhXhhXhhhhXhhXhX), where h is a hydrophobic residue and t is a.

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1 The abbreviations used are: APS, adenosine 5'-phosphosulfate; PAPS, 3'-phosphate 5'-phosphosulfate; PCR, polymerase chain reaction; IMAC, tris-imidazole buffer; DEP, diethylpyrocarbonate; SK, sulfurylase/kinase.
turn residue. The PP-loop was found to be present in monofunctional ATP sulfurylases, as well as Escherichia coli NtrL protein and Bacillus subtilis OutB protein (23). The PP-loop is often found in association with other functional motifs/domains, like the P-loop motif (present in the kinase portion) in PAPS synthetases (17) or an amidotransferase domain, a citrulline-aspartate ligase domain, and a nitrilase/amidase domain (16). The other motif, HXXH, is highly conserved among the monofunctional ATP sulfurylases and bifunctional PAPS synthetases, and through a sequence comparison with TagD-related nucleotidyltransferases, it is hypothesized that this motif is participating in the α-β phosphodiesterase activity (21). The PP-loop and HXXH motives contain several highly conserved arginines and histidines, both of which have functional side chains.

Histidine is particularly interesting since its pKᵢ is near physiological pH, allowing the residue to function as either a proton donor or a proton acceptor (24–26). Histidines have been implicated in metal ion binding (27) and electrostatic stabilization of intermediates (28). The catalytic importance of arginine has been shown in nucleotide substrate binding (29), and it may form a catalytic triad with serine and asparagine (30). Arginine can also have structural importance, as demonstrated by its role in conformational changes in murine leukemia virus reverse transcriptase (31). Evidence supporting a putative role for arginines and histidines in the ATP sulfurylase/APS kinase reaction mechanism comes from studies of both yeast and fungal ATP sulfurylases, which are inactivated by phenylglyoxal, which modifies arginines, and by diethyldithiocarbamate, which targets histidines (32). Finally, in order to assess the putative roles of these motifs in MSK1, overall assays alone, as were recently reported for human SK1 (15), are necessary but not sufficient. Since PAPS synthetase is a bifunctional enzyme with at least two reactive centers and multiple binding sites, several factors could lead to loss of overall activity, including loss of kinase activity, loss of sulfurylase activity, or inefficient transfer of APS from sulfurylase to kinase. Furthermore, the possibility that a mutation in one functional portion of the protein can affect the other reaction, as we previously found for a P-loop mutation present in the kinase portion, which resulted in loss of sulfurylase activity (17), complicates interpretation. Thus, in order to more definitively assess the functionality of these motifs in the sulfurylase reaction, individual sulfurylase and kinase assays, as well as overall assays, were performed.

**EXPERIMENTAL PROCEDURES**

### Reagents

Diethylpyrocarbonate, ATP, APS, phenylglyoxal, and ATP-agarose were purchased from Sigma, Ultrapure PAPS was from Dr. S. Singer, University of Dayton; BCA protein assay reagent was from Pierce. EasyTides® (ρ-pDCCTP (3000 Ci/mmole) used for radiolabeling, [α-32P]ATP used for DNA sequencing, and PAP3S (3'-phosphoadenosine 5'-phosphosulfate) were purchased from NEN Life Science Products. [14C]APS was generated from [32P]APS as described (33). Restriction enzymes were obtained from New England Biolabs, unless otherwise indicated. T4 polynucleotide kinase was from Promega, AmpliTag® was supplied by Perkin Elmer, and Pfu DNA polymerase was obtained from Stratagene. All enzymes were used in the buffers recommended by the suppliers. Thrombin protease for removal of the histidine tag was purchased from Novagen, pET 15b bacterial expression vector was from Novagen, and the general cloning vector pBluescript was obtained from Stratagene. The PCR 2.1 vector for direct ligation of PCR products was a component of the Invitrogen TA Cloning® kit. The Sequenase Quick Denature kit used for dideoxynucleotide termination DNA sequencing was supplied by U. S. Biochemical Corp. DNA used for sequencing was prepared using the Promega Wizard (Plus) Miniprep kit. Plasmid DNA used for transformation was generated with the Qiagen Maxiprep kit. Radiolabeled probes were made with the Stratagene Prime-It II random hexamer kit. Invitrogen Original TA Cloning® kit was used for construction of some of the clones. Purification of DNA from agarose gels was performed with the Qiagen QIAquick kit.

Metal chelate resin (His-bind®) for gravity purification of the expressed protein was purchased from Novagen. A similar resin (PolyHis) for high pressure liquid chromatography (HPLC) was obtained from Amersham Molecular Biochemicals. HPLC was performed on a Rainin Dynamax SD-200 system networked to a Macintosh IICi computer.

### Methods

#### Preparation of Rat Chondrosarcoma Enzyme—

The rat chondrosarcoma enzyme preparation was used in all chemical and kinetic assays and modification studies was prepared from rat chondrosarcoma as described through ammonium sulfate precipitation, and chromatography on S-300, hydroxylapatite, and ATP-affinity columns as described earlier (11, 19, 33).

#### Modification of Histidyl Residues by Diethyl Pyrocarbonate—

Stock DEP (diethylpyrocarbonate) solution (6.9 M) was diluted in absolute ethanol under nitrogen to working concentrations and kept on ice. ATP-purified enzyme was diluted into 25 mM NaH₂PO₄-KH₂PO₄ buffer, pH 6.5, 10% glycerol, 1 mM dithiothreitol, 1 mM EDTA prior to modification. The amount of DEP/ethanol added to the enzyme preparation was kept to a minimum (usually at a ratio of 1:30 of final volume), in order to minimize degradation of enzyme activity or side reactions with DEP. The carbethoxymethylation reaction was carried out with loss of sulfurylase activity and by increasing absorbance at 242 nm. The UV absorbance profiles were measured by scanning from 320 nm to 210 nm in a Perkin-Elmer spectrophotometer.

#### Modification of Arginyl Residues by Phenylglyoxal—

Phenylglyoxal was dissolved in deionized water to a stock concentration of 100 mM. The reaction with ATP-purified enzyme was performed at 25 °C, and inactivation was monitored by loss of sulfurylase or kinase activity compared with control. The enzyme was also covalently labeled with [14C]phenylglyoxal and examined by amino acid analysis. [14C]Phenylglyoxal (50 μCi) was dissolved in 0.5 ml of water, and 18 μCi was added to approximately 500 μg of ATP-purified enzyme preparation. The mixture was incubated for 90 min, dialyzed, and assayed after modification to determine the extent of inactivation compared with unmodified controls. The sample was electrophoresed in two lanes on a 10% SDS-gel with a third lane of unmodified enzyme; the gel was then transferred to polyvinylidine difluoride for 70 min. The 56-kDa bands were excised and analyzed for amino acid composition. L-Arginine in water was modified with 2.0 μmol of unlabeled phenylglyoxal for 30 min at 25 °C, dried in a SpeedVac, and examined by amino acid analysis as a control.

#### Construction of Site-directed Mutants—

In order to create the appropriate site-directed mutants, a four-primer, two-reaction PCR protocol was used, as described (17). Briefly, two overlapping PCR fragments were generated incorporating the codon for the new amino acid. A second PCR reaction generated the final 1.9-kilobase band which was ligated into the Pet15b expression vector. Following transformation into the host strain Top10F*, positive clones were identified and the entire insert as well as junctions with the vector were sequenced to verify the mutagenesis and to ensure that no additional mutations had spontaneously arisen. This methodology was employed in the construction of all site-directed mutants (R421A, R421K, H425A, H428A, R468A, H506A, R510A, R522A, R522K, and D523A).

#### PCR—

All PCR reactions were performed in a Perkin-Elmer GeneAmp 2400 thermal cycler using either Tag polymerase from Perkin-Elmer or Pfu polymerase from Stratagene. Standard cycling parameters included a 1–5 min preincubation at 94 °C followed by 20 cycles of 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C. Following the cycling step, a 10-min extension step at 72 °C allowed completion of all unfin- ished transcripts.

#### Sequence Analysis—BLAST 2.0 and PSI-BLAST protein sequence similarity searches were performed via the National Center for Biotechnology Information web server (34). Nucleotide sequence from each clone was assembled, and deduced amino acid sequence was generated and analyzed using the computer programs SEQED, LINEUP, TRANS- LATE, GAP, COMPARE, and DOTPLOT of the Wisconsin (GGC) program vector for multiple alignment.

#### Protein Purification—

The Novagen pET15b system was used for bacterial expression of the cloned murine bifunctional enzyme. All DNA fragments to be expressed were ligated into the NdeI and XhoI sites of the plasmid, and all clones were sequenced in their entirety before transformation into JM109 DE3 cells by the CaCl₂ method. Expressed protein was purified as described (17). Briefly, overnight bacterial cultures were centrifuged and the resultant pellet sonicated in Tris buffer.
containing 5 mM imidazole (50 mM Tris, pH 7.9). Following removal of bacterial debris by ultracentrifugation, the supernatant was loaded onto a 1-ml affinity column of nitrotriacetic acid resin charged with nickel, which binds to the imidazole moiety of histidine residues to capture the expressed protein. Columns were washed with 15 volumes of IMAC 20, 4 volumes of IMAC 30, and 4 volumes of IMAC 50. At this point, the protein was eluted with 4 column volumes of IMAC 200.

Enzyme Assays—The Pierce BCA protein assay reagent was used to determine the protein concentration for enzyme assays. The sulfurylase reaction was assayed in the physiologically reverse direction (10). Standard assays contained 50 mM NaH₂PO₄-K₂HPO₄ (pH 7.8), 12 mM MgCl₂, 0.5 mM dithiothreitol, 5 mM NaF, 0.2 mM P₂O₇, (containing 6.7 μCi of [³²P]P), 0.1 mM APS, and 50 μl of enzyme preparation. The reaction was initiated by addition of 50 μl of enzyme in IMAC 5 or buffer A (25 mM NaH₂PO₄-K₂HPO₄ (pH 7.8), 1 mM EDTA, and 1 mM dithiothreitol), incubated at 37 °C and terminated by addition of ice-cold charcoal solution. The samples were pelleted, followed by three washes (0.05 M sodium imidazole, pH 7.0, with 2.23 g of Na₄P₂O₇/500 ml), extracted with 50% ethanol, 0.4% NH₄OH, 0.1% Tween 80 and centrifuged. ATP- incorporated radioactivity in an aliquot of the supernatant was determined by scintillation counting and the specific activity calculated.

The standard kinase assay contained 10 μM [³⁵S]APS, 250 mM ATP (pH 7.0), 5 mM MgCl₂, 10 mM ammonium sulfate, and 12 μl of enzyme and was brought up to 25 μl with buffer A (9). The reaction was stopped with ice-cold ethanol, and PAPS (product) formation was measured by paper electrophoresis and scintillation counting. The coupled (overall) assay (10) contained 0.4 μM [³⁵S]H₂SO₄, 10 mM ATP, 20 mM MgCl₂, 22 mM Tris-HCl (pH 8.0), and 10 μl of enzyme preparation in a total volume of 25 μl. The reaction was started by addition of the enzyme and terminated by addition of ice-cold ethanol and freezing. Product formation was measured as described for the kinase assay.

RESULTS

Modification of Histidyl and Arginyl Residues—The first reaction in the synthesis of PAPS is a substitution reaction catalyzed by ATP sulfurylase and involves conversion of ATP to APS by addition of SO₄⁻², followed by the release of the leaving group, pyrophosphate. Motifs previously implicated in the α-β phosphodiester cleavage reaction and binding/stabilization of ATP/PP, are present in the sulfurylase portion of the bifunctional enzyme, and sequence comparisons suggested that functionally important residues are located in these motifs. In order to determine whether arginines or histidines are catalytically important residues are located in these motifs. In order to determine whether arginines or histidines are catalytically important residues are located in these motifs. In order to determine whether arginines or histidines are catalytically important residues are located in these motifs. In order to determine whether arginines or histidines are catalytically important residues are located in these motifs.

DEP reacts specifically and stoichiometrically with histidine and the extent of modification can be determined spectrophotometrically by monitoring the increase in absorbance at 242 nm (24). The difference spectra (Fig. 1A) recorded at various time intervals during the modification of the enzyme preparation confirms that histidy1 residues are being modified. Since there are no other changes in the spectral scans before and after treatment, DEP most likely reacts specifically with histidines. Concomitant with spectral changes, modification of histidines by DEP inactivated both the ATP sulfurylase and APS kinase functions (Fig. 1B). In addition, the decrease in activity was dependent on DEP concentration and incubation time (data not shown). These results suggest that the histidine(s) being modified by DEP are vital for both sulfurylase and kinase activity.

Modification of proteins by phenylglyoxal is highly specific for arginyl residues (35). Phenylglyoxal inactivated both the ATP sulfurylase and APS kinase activities of the bifunctional enzyme in a time-dependent manner, which was similar in extent and rate (Fig. 1C). In order to verify that arginyl residues were the target of modification, the enzyme was incubated with [¹⁴C]phenylglyoxal and subsequently examined by amino acid analysis. After a 60-min incubation, there was an 80% decrease in sulfurylase activity. The [¹⁴C]-labeled residue cochromatographed with the phenylglyoxal-modified L-arginine control upon amino acid analysis. A combination of MgATP and SO₄⁻² provided some protection to the sulfurylase activity from inactivation (data not shown), indicating that binding of ATP involves one or more arginyl residues.
Since the chemical modification studies suggested arginine and histidine are important for sulfurylase activity, a strategy was devised for identifying which residues to target for mutagenesis analysis. Advanced BLAST and PSI-BLAST programs were used to search data bases for protein sequences having high similarity to MSK1; the 86 proteins identified using BLAST were mainly bifunctional ATP sulfurylase/APS kinase from higher organisms and plants. The first iteration step of PSI-BLAST identified the same set of 86, but in the second iteration step 381 protein sequences were retrieved. The second group (295) were mainly P-loop containing proteins. Relatively few sequences were reported with similarity to the sulfurylase portion of the bifunctional enzyme. Conservation of histidine and arginine positions was evaluated by comparing the number of sequences with conserved residues to the total number of sequences retrieved for that portion of enzyme.

**Fig. 2.** Percentage of conservation of histidines (A) and arginines (B) in the ATP sulfurylase portion of the bifunctional enzyme ATP sulfurylase/APS kinase. Percentage of conservation was calculated by comparing the number of sequences with conserved position to the total number of sequences retrieved for that portion of enzyme.

**Table 1**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Sulfurylase assay</th>
<th>Kinase assay</th>
<th>Overall assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol ATP min⁻¹ mg⁻¹</td>
<td>pmol PAPS min⁻¹ mg⁻¹</td>
<td>nmol APS min⁻¹ mg⁻¹</td>
</tr>
<tr>
<td>1MSK</td>
<td>24.4</td>
<td>160.8</td>
<td>2.4</td>
</tr>
<tr>
<td>R421A</td>
<td>0.02</td>
<td>209.8</td>
<td>0.9</td>
</tr>
<tr>
<td>R421K</td>
<td>2.1</td>
<td>251.7</td>
<td>3.3</td>
</tr>
<tr>
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<td>202.8</td>
<td>1.1</td>
</tr>
<tr>
<td>H428A</td>
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<td>254.6</td>
<td>ND</td>
</tr>
<tr>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>D523A</td>
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<td>55.9</td>
<td>0.2</td>
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</table>
conservation for histidines and arginines in the sulfurylase portion of MSK1 is shown in Fig. 2 (A and B, respectively). Three histidines which show >90% conservation are His-425, His-428, and His-506, the first two of which lie in the HXXH motif, while His-506 lies in the PP-loop. Percentage of conservation results for arginine shows five highly conserved arginines in the sulfurylase domain. Three of these highly conserved arginines are present in or are near previously identified motifs. Arg-421 precedes the HXXH motif, while Arg-510 preceeds, and Arg-522 is present in, the PP-loop. A sample of retrieved sequences were chosen to show the conservation of histidines and arginines in these motifs (Fig. 3).

The targets for the first set of mutagenesis studies were the phosphodiester cleavage motif, having a consensus sequence HXXH, and Arg-421, which is present at position 4 to this site. Site-directed mutations generated were R421A, R421K, H425A, and H428A. Following expression and purification, these mutant enzymes were assayed for reverse ATP sulfurylase, APS kinase, and overall activities. Extracts of cells bearing these mutant enzymes were assayed for reverse ATP sulfurylase activity (Table I). Both of the HXXH motif mutants, H425A and H428A, showed complete loss of reverse sulfurylase activity (Table I). Both of the HXXH motif mutants, H425A and H428A, show complete loss of reverse sulfurylase activity, kinase activity (Table I). The sulfurylase and kinase portions have different rates of inactivation by DEP. In either case, the sulfurylase and kinase reactions appear to involve distinct histidines. Sequence alignment and percent-conservation results suggested that kinase histidines are less conserved compared with sulfurylase histidines (3, 17). Specifically, three histidines (His-425, His-428, and His-506) which show an exceptionally high degree of conservation (90%) are present in the sulfurylase region and found to be either proximal to, or part of, previously identified sequence motifs. Two of the highly conserved histidines occurred in the HXXH motif and the third, His-506, preceded the PP-loop. The roles of these histidines were further analyzed by site-directed mutagenesis.

Histidine has been shown to be involved in the binding of substrates as well as general acid/base catalysis (25, 36). DEP, a histidine-specific compound, inactivates enzymes by generating a non-reactive N-carbethoxyhistidyl derivative (24). Although we were unable to determine the total number of modified histidyl residues in the native sulfurylase/kinase enzyme, the inactivation experiments suggested the presence of histidines at the active site(s) of PAPS synthetase. As depicted in Fig. 1, the sulfurylase and kinase portions have different rates of inactivation by DEP. Since DEP only modifies the unprotonated form of histidine (24), it is possible that the pKa for the essential sulfurylase histidine(s) is higher than the pKa for the essential kinase histidine(s), thus explaining why DEP inactivates the sulfurylase activity at a slower rate than the kinase. However, it is also possible that the kinase histidine is simply more solvent-accessible, allowing easier chemical modification by DEP. In either case, the sulfurylase and kinase reactions appear to involve distinct histidines. Sequence alignment and percent-conservation results suggested that kinase histidines are less conserved compared with sulfurylase histidines (3, 17). Specifically, three histidines (His-425, His-428, and His-506) which show an exceptionally high degree of conservation (>90%) are present in the sulfurylase region and found to be either proximal to, or part of, previously identified sequence motifs. Two of the highly conserved histidines occurred in the HXXH motif and the third, His-506, preceded the PP-loop. The roles of these histidines were further analyzed by site-directed mutagenesis.

Arginine has also been implicated as an essential residue for the function of a number of enzymes, playing a general role as a positively charged component in enzymatic recognition sites.
for anionic substrates (37). Frequently, essential arginines interact with the negatively charged phosphoryl moiety of phosphate-containing substrates, as confirmed by x-ray crystallographic studies of staphylococcal nuclease (38) and lactate dehydrogenase (39). Modification by the arginine-specific phenylglyoxal affects both sulfurylase and kinase activities at a similar rate and to a similar degree. Interestingly, essential arginyl residues have been shown to react significantly faster than other arginines in aspartate transcarbamylase (40). Presumably, this selectivity represents an increase in the reactivity of the essential arginine, as well as a decrease in reactivity of other polar arginyl residues, at positions removed from the active site, which would be interacting with other residues in the protein or with the solvent (41). Therefore, the modification of "active site" arginines involved in binding or catalysis would be favored over "structural" arginines. Substrate protection experiments suggest that this is the case for the bifuncional sulfurylase/kinase enzyme, with essential arginines present in the active site(s) of both ATP sulfurylase and APS kinase (19).

Considering that all SK substrates (and products) contain phosphoryl moieties, the putative participation of multiple arginines in the bifunctional enzyme is not surprising. When percentage of conservation of various arginines in the MSK1 sulfurylase domain was determined, five highly conserved (>90%) arginines were found. Selective targeting of four of these arginines, Arg-421, Arg-510, Arg-522, and Arg-468, for mutational analysis was based on their presence in sequences with respect to the two conserved motifs. Arg-421 preceded the HXXH motif in a region spanning residues 421–428, Arg-510 preceded the PP-loop, Arg-522 was contained within the PP-loop, and Arg-468 was approximately midway between both motifs.

The results of our individual assays showed that the R421A mutant enzyme is inactive in the reverse sulfurylase assay, completely active in the kinase assay, and exhibits no significant overall activity. R421K, which retains the positive charge, showed an improvement in the reverse sulfurylase activity compared with R421A, but still exhibits only 10% activity compared with MSK1. Kinase activity for R421K was comparable to R421A, while synthesis of PAPS in the overall reaction was not improved and was similar to R421A. Interestingly, synthesis of APS in the forward sulfurylase reaction was fully re-
stored, indicating that R421K can synthesize APS and PP, but presumably cannot either transfer APS or release PP, because synthesis of PAPS is still reduced. This is corroborated by the partial restoration of the reverse sulfurylase activity when a positive charge is introduced at this site. These results suggest Arg-421 participates in binding of APS or PP. Histidine mutants H425A and H428A showed no reverse or forward sulfurylase activity, normal kinase activity and no PAPS synthesis, indicating that the sulfurylase portion is rendered inactive in both reaction directions. These experiments indicate that His-425, His-428, and Arg-421 are all playing critical roles in the sulfurylase reaction. Since the HXXH motif, like the P-loop (13), is organized in a potential loop structure, it is quite possible that these residues are involved in a catalytic or substrate binding step.

A second set of mutations was performed in the region spanning residues 514–532, which constitutes a modified version of the PP-loop (16). Substituting an alanine for the arginine midpoint in the PP-loop sequence (R522A) ablated ATP sulfurylase activity while leaving the kinase activity intact. The arginine to lysine mutation R522K, which preserves the positive charge, did not cause any improvement in ATP sulfurylase activity in either direction compared with R522A, indicating that this position has a strict requirement for arginine. The alanine either direction compared with R522A, indicating that this did not cause any improvement in ATP sulfurylase activity in the PP-loop (16). Substituting an alanine for the arginine mid-ranging residues 514–532, which constitutes a modified version of the substrate binding step.

Possible that these residues are involved in a catalytic or substrate binding step. For efficient functioning of the sulfurylase domain, pyrophosphate (a highly negatively charged leaving group) would need stabilizing electrostatic interactions provided by positively charged residues. Considering that stabilizing interactions are required during the transient process of the reaction, it is likely that a residue with transient/delocalized positive charge would be preferred. Thus, the strict requirement of arginine at residue 522 may be due to its four positively charged neighbors. Thus, the differential affects observed between R421K and R522K suggest that arginine is critical at the 522-position. Asp-506 and Asp-523, which flank the PP-loop, are highly conserved in monofunctional ATP sulfurylases from lower organisms and plants, suggesting that these residues may be important either for sulfurylase activity or structure. Thus, the result obtained, i.e. a decrease in kinase activity for the H506A and D523A mutant enzymes is rather surprising. H506A showed normal reverse sulfurylase, decreased kinase (<10%), and higher than normal forward sulfurylase (~3-fold) activities, and no overall PAPS synthesis. The results for the D523A mutant were also interesting, as it decreased sulfurylase (in both directions), kinase (~35%), and overall PAPS synthesis activities. These results are even more intriguing considering the fact that both pyrophosphate and aspartate are negatively charged, suggesting that Asp-523 is not directly involved in binding to phosphate groups. Most likely, the binding of phosphate moieties is mediated either through a water molecule or through other positively charged side chains. Since D523A exhibited a significant decrease in kinase activity, aspartate may be binding to some portion of the substrates which is essential for both the sulfurylase and kinase activities. The interacting parts of the substrates critical for both activities, ATP/APS, are the adenine nucleotide base and the ribose sugar. It is plausible that Asp-523 might be involved in hydrogen-bonding either to the nucleotide group or to a hydroxyl group on the ribose ring. The recently solved crystal structure of xanthine-guanine phosphoribosyltransferase shows that in the substrate-bound enzyme crystal structure, an aspartate group, with the help of a lysine, is hydrogen-bonded to the N7-protonated tautomeric form of the adenine base (42). A similar situation may pertain to the sulfurylase/kinase mechanism and is under investigation.

Secondary structure predictions using segment-oriented and nearest neighbor based programs revealed that the PP-loop is unstructured (43, 44). Lack of a defined structure in the PP-loop motif may bring these two residues His-506 and Asp-523 into proximity and through interdomain interactions allow them to participate in the kinase reaction. However, our previous studies on the individual domains of the bifunctional enzyme, MSK1, showed that recombinant monofunctional domains can function independently, suggesting lack of any cross-participation of sulfurylase domain residues in kinase activity and vice versa (18). Alternatively, His-506 and Asp-523 may be involved in channeling the intermediate APS to the kinase portion in the fused bifunctional SK. These anomalous interdomain effects need to be resolved; availability of a crystal structure (in progress) would greatly aid in understanding these apparent anomalies.

Finally, mutation of the highly conserved intermotif residue Arg-468 to alanine also had a significant effect on sulfurylase activity in both reaction directions and on overall PAPS synthesis, while kinase activity was normal. Residue 468 is not part of a functionally defined motif; however, it and the other arginine, histidine, and aspartate residues studied here are not only highly conserved themselves but are embedded in largely hydrophobic sequences which are very strongly conserved (nearly identical) over the entire range of known SK sequences. Mutation of individual residues may well perturb the larger assemblage in ways that affect the action of other functional residues. Obtaining the three-dimensional structure of the enzyme will be vital to understanding the interplay of these motifs. In sum, both the sulfurylase and kinase portion of the bifunctional enzyme require ATP but cleave different bonds in ATP, i.e. sulfurylase carries out an α-β phosphodiesterase reaction and the kinase performs a β-γ phosphodiesterase reaction. The present study, and our previous detailed examination of the kinase specific P-loop (17), provides evidence on the identity of residues participating in each type of cleavage reaction. We have also begun to dissect which residues participate exclusively in each half-reaction, aided by our recent studies on the monofunctional and rearranged domains of ATP sulfurylase/APS kinase (18). Finally, determining the way in which those residues identified in one enzyme domain, which influence the activity of other enzyme domain, produce their effects will be critical to understanding the unique channeling mechanism of this bifunctional enzyme.

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