Deletion and Site-directed Mutagenesis of the ATP-binding Motif (P-loop) in the Bifunctional Murine Atp-Sulfurylase/Adenosine 5’-Phosphosulfate Kinase Enzyme*

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The P-loop is a common motif found in ATP- and GTP-binding proteins. The recently cloned murine ATP-sulfurylase/adenosine 5’-phosphosulfate (APS) kinase contains a P-loop (residues 59–66) in the APS kinase portion of the bifunctional protein. A series of enzymatic assays covering the multiplicity of functions of this unique protein (reverse ATP-sulfurylase, APS kinase, and an overall assay) were used to determine the effect of deleting or altering specific residues constituting this motif. In addition to the full-length cDNA construct (1MSK), two deletion mutants that progressively shortened the N terminus by 34 amino acids (2MSK) and 70 amino acids (3MSK) were designed to examine the effects of translation initiation before (2MSK) and after (3MSK) the P-loop. The 2MSK protein possessed sulfurylase and kinase activity equivalent to the full-length construct, but 3MSK exhibited no kinase activity and reduced sulfurylase activity. In light of the evident importance of this motif, a number of site-directed mutants were designed to investigate the contribution of key residues. Mutation of a highly conserved lysine in the P-loop to alanine (K65A) or arginine (K65R) or the following threonine (T66A) to alanine ablated APS kinase activity while leaving ATP-sulfurylase activity intact. Three mutations (G59A, G62A, and G64A) addressed the role of the conserved glycines as follows: G64A showed diminished ATP-sulfurylase activity without effect on APS kinase activity only, whereas G62A had no effect on either activity. G59A caused a significant decrease in ATP-sulfurylase activity without effect on APS kinase activity. A series of highly conserved flanking cysteines (Cys-53, Cys-77, and Cys-83) were mutated to alanine, but none of these mutations showed any effect on either enzyme activity.

The P-loop motif is a highly conserved feature of ATP- and GTP-binding proteins where it has been implicated in cleavage of the β-γ-phosphate bond of the NTP (1). Also known as the Walker type A motif, the consensus sequence has been variously described as (G/A)XXGK(T/S) (2), GXGXXK (3), GXXXXGKS (4), and GXXGXGKS (5). This flexible motif generally joins a β-strand and an α-helix to form a pocket into which the phosphate groups can insert. Whereas the N-terminal glycine and C-terminal lysine are absolutely conserved, there is some variability permitted in the intervening amino acids. The lysine side chain has been implicated in hydrogen bonding to the γ-phosphate as demonstrated by x-ray analysis of skeletal myosin bound to an ATP analog (MgADP and beryllium fluoride) (6–8). There is often a serine or threonine following the lysine which provides a hydroxyl group to bind the divalent cation associated with the bound nucleotide (1).

Despite the importance of the terminal lysine, a conservative change to arginine has been shown in some instances to allow attenuated function. Following arginine substitution for the conserved P-loop lysine, the PrtD integral membrane ATP-binding cassette component of the Erwinia chrysanthemi metalloprotease secretion system demonstrated a 10-fold decrease in ATPase activity (9). Similarly, the VirB11 protein of Agrobacterium tumefaciens which also possesses ATPase activity and a Walker consensus sequence is able to mediate DNA transfer with only slight perturbation following a lysine to arginine mutation (10). A nonconservative switch to alanine, however, prevents DNA transfer from the host cell to other plant cells. A conflicting report by Driscoll et al. (3) demonstrated that the nonconserved lysine to alanine substitution in guinea pig estrogen sulfotransferase had no effect on the $K_m$ of the enzyme, suggesting that there is some flexibility in residue requirements.

Another enzyme system in which both ATP and PAPS binding are critical is the sulfate activation pathway, consisting of an ATP-sulfurylase (ATP:sulfate adenylyltransferase, EC 2.7.7.4) reaction that catalyzes the addition of inorganic sulfate to ATP to form APS, followed by an APS kinase (ATP:adenyllysulfate 3’-phosphotransferase, EC 2.7.1.25) reaction which transfers phosphate from ATP to APS to form PAPS (see Reaction 1).

$$\text{ATP} + \text{SO}_4^{2-} \rightarrow \text{APS} + \text{PP}_i$$

$$\text{APS} + \text{ATP} \rightarrow \text{ADP} + \text{PAPS}$$

**REACTION 1**

Since PAPS is the sole source of donor sulfate for all sulfoconjugation reactions in higher organisms, the importance of the sulfate activating enzymes cannot be overstated. We recently cloned the murine ATP-sulfurylase/APS kinase (PAPS synthetase) from brain and showed it to be a fused bifunctional protein through its sequence homology to separate plant and fungal sulfurylases and kinases and by assays of the protein expressed in COS-1 cells (11). The organization of the fused

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The abbreviations used are: PAPS, 3’-phosphate 5’-phosphosulfate; APS, adenosine 5’-phosphosulfate; PCR, polymerase chain reaction; PAGE, polyacrylamide electrophoresis.
enzyme includes an APS kinase-homologous domain toward the N terminus (Fig. 1) and an ATP-sulfurylase-homologous domain toward the C terminus. A P-loop motif, GLSGAGKT, is found in the APS kinase portion of the protein from residues 59 to 66. Furthermore, there are three invariant cysteines that flank this motif, cysteine 53 which is upstream from the P-loop and cysteines 78 and 83 which are downstream. Although disulfide bonds have not been previously associated with the P-loop motif, it is possible that in this enzyme they confer structural stability.

Our recent cloning and expression of the murine sulfurylase/APS kinase (11) provides an opportunity to address specific questions regarding the molecular details of substrate binding to the bifunctional enzyme as well as the relationship of the two active centers in the fused protein. Our initial studies have focused on the P-loop domain, shown to be critical to many enzymatic activities that bind ATP. Here we report the expression of the bifunctional murine brain ATP-sulfurylase/APS kinase in a bacterial host and the effects of deletion or site-specific mutation of essential P-loop residues on the multiple catalytic functions of this enzyme, as assessed by three independent assays as follows: ATP-sulfurylase activity, APS kinase activity, and the overall PAPS synthetase activity. This constitutes the first study that systematically mutated every conserved P-loop residue; the data indicate which of the eight positions mutations can be tolerated and in some cases the chemical nature of amino acid side chain allowed.

**EXPERIMENTAL PROCEDURES**

**Construction of pET-15b/1MSK**—The murine brain ATP-sulfurylase/APS kinase coding sequence was obtained from reverse transcriptase-PCR of RNA isolated from 2-day-old C57B mouse brain using the sense primer NdeI N1 = nucleotides 1–20 (5’–GGAATTCCATATG-GAGATTCCTGGGAGTCTG-3’) which included the initiation methionine, and the complementary strand primer XhoI N3 = nucleotides 1906 to 1886 (5’–GCTCTAGACTCGAGTTAGGCTTTCTCTAAGG-3’). Each primer included a 5’ restriction site to facilitate insertion into the expression vector. Following digestion with NdeI and XhoI, the 1.9-kilobase coding sequence, designated 1MSK, was ligated into an NdeI/XhoI-digested Novagen pET-15b expression vector plasmid and transformed into Invitrogen Top10F bacterial cells. Positive clones were identified by both restriction mapping and DNA sequencing of the entire insert.

**Site-directed Mutagenesis**—The pET-15b/1MSK construct was used as the template DNA in all mutagenic PCR reactions. Mutations were produced by 2-step, four primer PCRs in which the N-terminal and C-terminal oligonucleotide primers were identical to those used to generate the pET-15b/1MSK insert (12). Amino acid substitutions are listed in Table I. Briefly, two separate amplification reactions (one using NdeI N1 and the mutant antisense primer and the other using the mutant sense primer and XhoI N3) were done simultaneously for each mutation construct. The two reaction products were electrophoresed on a 1% agarose TAE gel, and the product DNA bands were excised, pooled, and purified using the Qiaquick gel extraction kit. This purified DNA was used as the template DNA for a second PCR amplification using the NdeI N1 and XhoI N3 primers. The resulting 1.9-kilobase band was gel-purified and digested with NdeI and XhoI prior to ligation into the NdeI and XhoI sites of pET-15b. The desired ligation products were cloned first into Top10F bacterial cells and subsequently into BL21 (DE3).
for protein expression.

DNA Sequencing—All oligonucleotide primers were purchased from the University of Chicago oligonucleotide core facility. Manual dideoxynucleotide DNA sequencing was performed according to the protocol of the Life Technologies, Inc. T7 polymerase Quick Denature kit using α-35S-DATP from NEN Life Science Products. Approximately 1 μg of closed circular plasmid DNA was denatured in the presence of 25 pmol of sequencing primer. Extension times varied between 10 and 15 min at which point the reaction was terminated. All samples were heated to 70 °C prior to loading onto a 6% acrylamide TBE gel. Dried gels were exposed to Kodak Biomax MR single emulsion x-ray film overnight, and the film was developed the following day. Some automated DNA sequencing was done using the Dye Terminator Cycle Sequencing Ready Reaction Kit from Perkin-Elmer and an ABI PRISM 377 DNA sequencer (Perkin-Elmer).

Polymerase Chain Reaction (PCR)—All PCR amplifications were performed in a Perkin-Elmer GeneAMP 2400 thermal cycler using Taq polymerase from Perkin-Elmer. Standard cycling parameters included a 1–5-min preincubation at 94 °C followed by 20–30 cycles of 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C. Following the cycling phase, there was a final extension for 10 min at 72 °C.

Protein Expression—The Novagen pET-15b system was chosen for bacterial expression of the cloned murine brain bifunctional enzyme. All DNA fragments to be expressed were inserted into the NdeI/XhoI doubly-digested plasmid, and all clones were sequenced in their entirety before transformation into BL21 DE3 cells by the CaCl2 method. A bly-digested plasmid, and all clones were sequenced in their entirety. Cell culture precipitation was maintained overnight, and the film was developed the following day. Some automated DNA sequencing was done using the Dye Terminator Cycle Sequencing Ready Reaction Kit from Perkin-Elmer and an ABI PRISM 377 DNA sequencer (Perkin-Elmer).

Enzyme Assays—The optimal protein concentration for assessing enzymatic activity was found to be between 0.3 and 1.0 μg/ml, depending on the assay. Therefore, following gravity column purification, the protein concentration was determined using the Pierce BCA protein assay and diluted with phosphate buffer to a stock concentration of 20 μg/ml for use in all three assays. All assays were performed at least three times; presented data are representatives of individual results. Negative controls were assayed including vector without insert and no vector (DE3 cells alone); for both cases, counts were routinely less than 10% of those obtained with expressed IMSK crude extract.

The ATP-sulfurylase assay results in formation of ATP, the reverse of the physiological direction (14), due to the unfavorable K<sub>cat</sub> of the forward reaction. Standard sulfurylase assays contained 50 μM NaH<sub>2</sub>PO<sub>4</sub>-K<sub>2</sub>PO<sub>4</sub>, pH 7.8, 12 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 5 mM NaF, 0.2 mM Na<sub>2</sub>P<sub>O</sub> <sub>7</sub> (containing 6.7 μCi of [35P]P, 0.1 mM APS, and 50 μM ATP in a total volume of 1 ml. The generation of ATP from APS and PP<sub>i</sub> is quantified.

The standard kinase assay contained 80 μM [α-32P]APS, 0.5 mM ATP, pH 7.0, 5 mM MgCl<sub>2</sub>, 10 mM ammonium sulfate, and 12 μl of enzyme and was brought to a total volume of 25 μl with buffer A (25 mM NaH<sub>2</sub>PO<sub>4</sub>-K<sub>2</sub>PO<sub>4</sub>, pH 7.8, 1 mM dithiothreitol, 1 mM EDTA, and 10% glycerol) (15). Conversion of the labeled substrate to PAPS is monitored by paper electrophoresis which separates the two charged species.

The standard coupled assay assesses the overall reaction with ATP and sulfate as substrates, measuring the production of APS and PAPS. The 25-μl standard overall reaction volume contained 0.4 μM [35S]SO<sub>4</sub> <sub>2-</sub>, 10 mM ATP, 20 mM MgCl<sub>2</sub>, 22 mM Tris-HCl, pH 8.0, and 15 μM nonradioactive Na<sub>2</sub>HPO<sub>4</sub>-K<sub>2</sub>PO<sub>4</sub>. This preparation (15) is valuable not only as a measure of the ability of the enzyme to synthesize PAPS from ATP and SO<sub>4</sub><sup>-2</sup> but also as an assay of the forward sulfurylase reaction.

Assays were performed in duplicate, and each experiment was repeated at least three times. Kinetic parameters were obtained as described (14).

RESULTS

Expression of the Murine ATP-Sulfurylase/APS Kinase in Bacteria—Since previous expression studies of the recombinant sulfurylase/kinase enzyme were performed in a eukaryotic expression system (11) where proper folding and modification would be expected to occur normally, the correct expression of the recombinant enzyme, physically and functionally, had to be demonstrated in the newly developed bacterial expression system. Following purification of the soluble fraction on the histidine affinity resin, an expressed protein corresponding to the expected molecular weight was visible on SDS-PAGE (Fig. 2). To ascertain that the affinity purified protein was in fact that protein which was expressed from our construct and not a bacterial contaminant, a Western blot using a nickel-conjugated alkaline phosphatase “antibody” was performed; a single band was visualized corresponding to the expressed product at approximately 70 kDa (Fig. 2). Further confirmation that this protein was the ATP-sulfurylase/APS kinase enzyme was obtained by 32P-radiolabeled substrate analog binding using dialdyde-ATP (16), which labeled a single band at the same molecular weight (Fig. 2). Finally, the 70-kDa band was absent in extracts from induced cells lacking a plasmid construct. Thus, the following data for the bacterial expression system synthesized a recombinant plasmid-specific protein which could be purified and labeled with ATP as expected (16).
reaction assay. To verify that overexpression of the recombinant protein was accompanied by an increase in enzyme activity, homogenates were first assayed. Soluble extracts obtained from DE3 cells, DE3 cells with empty vector, and DE3 cells transfected with 1MSK exhibited the following activities: sulfurylase, 0.03, 0.05, and 5.13 nmol/min-mg, respectively; and kinase, 0.01, 0.02, and 0.68 pmol/min-mg, respectively. For all subsequent assays the expressed recombinant proteins were purified as described.

Analysis of pET-15b/1MSK and Deletional Mutants—To assess the contribution of the P-loop motif to enzyme function, three cDNA constructs were designed. The first, 1MSK, included the initiator codon ATG at position one. Two further constructs, 2MSK and 3MSK, began at the second and third methionine codons in the cDNA, respectively. Translation at the 2MSK transcript is initiated upstream of the P-loop motif, whereas the 3MSK mutant protein entirely lacks the P-loop. Enzyme activities of the 1MSK and 2MSK constructs were found to be nearly identical in all three assays (Table II); however, the 3MSK construct exhibited decreased reverse sulfurylase activity (66% of wild type) and complete absence of kinase and overall activities.

Analysis of Site-directed Mutants—Although a great deal of experimental work has focused on the P-loop motif in various systems, a comprehensive site-specific analysis of essential residues has not yet been accomplished in any single study reported in the literature. Accordingly, in this study, all of the conserved residues were individually altered, and the effect of each change on sulfurylase/kinase enzyme activities was assessed (Table III and Fig. 3). The lysine residue at the C-terminal end of the P-loop motif has been implicated in NTP binding (1). Substitution of Lys-65 with alanine had some effect on sulfurylase activity (80% residual activity; 105% activity; 2.58 mmol of ATP/min-mg). Interestingly, the N-terminal glycine (Gly-59), which is highly conserved as the

![Fig. 2. Visualization of 1MSK expressed protein.](image)

**Table II**

| Construct | Sulfurylase assay | Kinase assay | Overall assay |
|-----------|------------------|--------------|--------------|
|           | µmol ATP min⁻¹ mg⁻¹ | pmol PAPS min⁻¹ mg⁻¹ | nmol APS min⁻¹ mg⁻¹ | nmol PAPS min⁻¹ mg⁻¹ |
| 1MSK      | 24.4             | 160.8        | 2.4          | 11.7          |
| 2MSK      | 23.1             | 157.5        | 2.5          | 12.5          |
| 3MSK      | 16.2             | 1.8          | 4.7          | 0.09          |

**Table III**

| Construct | Sulfurylase assay | Kinase assay | Overall assay |
|-----------|------------------|--------------|--------------|
|           | µmol ATP min⁻¹ mg⁻¹ | pmol PAPS min⁻¹ mg⁻¹ | nmol APS min⁻¹ mg⁻¹ | nmol PAPS min⁻¹ mg⁻¹ |
| 1MSK      | 24.4             | 160.8        | 2.4          | 11.7          |
| C53A      | 22.6             | 175.3        | 4.2          | 22.7          |
| G59A      | 1.9              | 242.2        | 0.7          | 0.6           |
| G62A      | 23.9             | 180.1        | 6.0          | 7.8           |
| G64A      | 25.8             | 191.2        | 9.9          | 1.0           |
| K65A      | 20.5             | 6.2          | 6.8          | 0.1           |
| K65R      | 23.3             | 5.3          | 8.6          | 0.02          |
| T66A      | 24.1             | 25.6         | 7.9          | 0.2           |
| C78A      | 23.5             | 178.0        | 2.9          | 25.6          |
| C83A      | 22.6             | 195.8        | 1.2          | 10.1          |
by substituting alanine for each cysteine individually. None of these mutations appeared to have any detrimental effects on the kinase, sulfurylase, or overall activities (Table III). When the marine worm (Urechis caupo) (17) and Drosophila sulfurylase/kinase DNA sequences recently became available, it was noted that neither Cys-78 nor Cys-83 were conserved in these species; Cys-78 was replaced by a serine in both cases, and Cys-83 was replaced by a threonine in the worm and by alanine in the fruit fly. The variance among the fused enzymes of non-mammalian species is of particular interest considering their conservation in the more widely divergent subsets described above. This lack of conservation indicates that flexibility exists for these residues.

### DISCUSSION

The bifunctional sulfurylase/kinase that we originally purified from rat chondrosarcoma (16, 18, 19) and recently cloned from mouse brain (11) would be expected to require binding sites for at least two molecules of ATP and one molecule of APS/PAPS. The single P-loop motif in the APS kinase domain which has been shown in other proteins to bind ATP, and perhaps PAPS, was therefore targeted for mutagenesis. However, based on the reaction stoichiometry a second nucleotide-binding site may be expected to occur. The P-loop extending from residues 59 to 66 in the murine sulfurylase/kinase fits the Walker type A consensus motif GXXGXXK/T/S. The sequence and location of this motif is highly conserved across the known fused sulfurylase/kinases where it is found from residues 59 to 65 in mouse (11) and human, from residues 44 to 51 in worm (17), and from residues 54 to 61 in fruit fly.⁹

Two deletion mutants were designed to assess the contribution of the P-loop to enzyme activity. The small (34-amino acid) N-terminal deletion of the 2MSK construct did not affect enzyme activity; however, complete deletion of the P-loop in the 3MSK construct not only eliminated kinase activity but also decreased sulfurylase activity. Although the structural consequences of deleting 70 amino acids from the N termini have not been established for this protein, the ability of the 3MSK sulfurylase domain to exhibit significant activity would suggest that the remainder of the fused protein is structurally stable.

To more accurately dissect the contribution of the P-loop motif, a series of site-specific mutants was created and assayed. The observed detrimental effect of the K65A and K65R substitutions (Table II) is well supported in the literature. The conserved lysine has been implicated in hydrogen bonding to the γ-phosphate as discussed previously (6–8). In such a structure, alanine would clearly be unable to compensate for the charged lysine residue. Arginine, although possessing a different structure, is a candidate for providing the necessary charge stabilization. The inability of the arginine substitution to restore activity to the fused sulfurylase/kinase cannot be directly attributed to a defect in hydrogen bonding. The bulky arginine side chain may be sufficient to disrupt the NTP binding pocket or even the entire N-terminal end of the protein, thereby abating enzyme activity. Logan and Knight (21) also found that arginine could not substitute for the conserved lysine in Escherichia coli RecA.

In myosin, EF-Tu, RAS, and the G-proteins, the coordination sphere of the magnesium ion includes two oxygen atoms from the β- and γ-phosphates of the bound trinucleotide, the hydroxyl side chain from the threonine or serine at position 8, and two water molecules (1). In the APS kinase reaction the Mg²⁺ associated with the ATP molecule is potentially stabilized by

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⁹ A. Deyrup, S. Krishnan, B. Singh, and N. B. Swartz unpublished observations.

³ D. Jullien and E. Kas, EMBL accession number Y12861.
the Thr-66 hydroxyl group. Substitution with alanine substantially reduced kinase activity in the fused sulfurylase/kinase which suggests that the position of the magnesium ion in this enzyme may be critical. In addition to lacking non-bonding electrons, however, alanine differs from threonine vis à vis molecular volume and polarity. Further dissection of the roles of steric effects could be accomplished through alternative substitutions with valine and serine.

The structural flexibility conferred by the cluster of glycines in the P-loop has been implicated in binding of the nucleotide triphosphate as allowing adjacent amino acids to form backbone hydrogen bonds with the β- and γ-phosphate groups (1). However, changing either the initial (Gly-59) or second glycine (Gly-62) to an alanine did not decrease kinase activity. Substitution of a bulkier residue or proline may prove necessary to disrupt enzyme function. In the case of familial persistent hyperinsulinemic hypoglycemia of infancy, a spontaneous mutation of the second glycine to a valine in the P-loop motif of the sulfonyleurea receptor causes the pathological state (22), and therefore, valine would also be a logical choice for further glycine mutagenesis. However, in many ATP-binding proteins, the second glycine of the motif (in our case Gly-62) is not considered a feature of the motif (4, 23). Therefore, the role of the second glycine appears to be highly variable and, furthermore, appears dispensable in the fused sulfurylase/kinase.

In contrast to the G64A mutation in the kinase domain which affects kinase activity, the G59A substitution in the kinase domain completely ablates sulfurylase activity in both directions and thus overall PAPS activity but does not affect the kinase reaction when exogenous APS is supplied. The loss of activity for the G59A mutation is especially surprising considering that, in an early report, Saraste et al. (23) found that the first residue of the P-loop could be either an alanine or a glycine (23). An initial alanine is found predominantly among the P-loops of elongation factors and phosphoglycerate kinases as well as in a thymidine kinase (23). However, the inability of an alanine to satisfactorily substitute for this initial glycine has been demonstrated in the E. coli RecA protein (21).

The loss of function of the G59A mutant suggests that there may be some interaction between the N- and C-terminal domains of the protein or that the P-loop is folded into close contact with a critical portion of the sulfurylase domain. Models have been proposed for several enzymes in which nucleotide triphosphate binding and hydrolysis control specific conformational changes critical to protein function. Thus the role of the P-loop most likely involves not only the provision of a hydrophobic binding pocket for NTP but also a flexible structure that can undergo a conformational change in response either to substrate binding or an interaction with another protein or domain (24). In turn, the conformational change may control access of subsequent substrates to the active site, modification of binding affinities, or relocation of reactive groups toward the reaction center of the bound substrate. These actions are not mutually exclusive and could be operating to varying degrees or combinations to facilitate catalysis not only at the kinase active site but at the sulfurylase site as well.

Moreover, the mechanistic interdependence, if any, of the two activities has not yet been established for the fused enzyme. Modification of the sole nucleotide-binding site therefore also offered the opportunity to explore the relationship of the two functions. Since Gly-59 appears to play an important role in the interaction between the sulfurylase and kinase domains, the effect of this mutation on sulfurylase activity measured in both the forward and reverse directions indicates that this glycine is involved in stabilizing a structure necessary for nucleotide triphosphate binding, a function common to reactions in both directions, rather than in \( \text{SO}_4^{2-} \) binding. This possibility is highlighted by a specific mutation we have made in the sulfurylase domain which affects the forward sulfurylase reaction only with no effect on the reverse reaction, suggesting that this residue participates predominately in \( \text{SO}_4^{2-} \) binding. A full understanding of the relationship between the two portions must await a more detailed structural analysis of our mutants, which is beyond the scope of the present study.

The P-loop in the sulfurylase/kinase is particularly interesting considering that in addition to a role in ATP binding, a variation of this motif (GXXGXXK) has also been implicated in PAPS binding (3, 25). The bifunctional ATP-sulfurylase/APS kinase would be expected to bind two molecules of ATP for each complete enzymatic reaction, as well as one molecule of PAPS; however, only a single P-loop, with all the consensus features, has been identified in the bifunctional enzyme. It is possible, therefore, that there is an additional nucleotide-binding site which is not a P-loop motif or is a more modified version. Additional searches using FINDPATTERNS has confirmed no additional motifs of the form GXXXXK; however, another ATP/GTP binding motif, DXXG (26–28), is found starting at residues 169 and 523. The potential contribution of these motifs is under investigation. Alternatively, the single P-loop may be used to bind the ATP required for each reaction. Since rat ATP-sulfurylase/APS kinase employs intermediate channeling to pass the label APS intermediate from the ATP-sulfurylase active site to the APS kinase active site (29), a possible mechanism to facilitate channeling might involve substrate binding to a single site resulting in a conformational change which serves to bring the subsequent active site into proximity. Because the P-loop is a potential binding site for both ATP (substrate) and PAPS (product), it is a likely candidate for binding one or more of the sulfurylase/kinase reactants and/or products, and further investigation of the roles of the various residues that constitute this important motif is necessary.

In summary, this study constituted a molecular dissection of a putative motif previously considered important in ATP- and PAPS-binding proteins. Our results demonstrate that while the consensus residues Gly-59, Gly-64, Lys-65, and Thr-66 are strictly required, the Gly-62 position can tolerate substitution. Furthermore, we were able to group the sulfurylase/kinase mutants into three general phenotypic categories as follows: those deficient in sulfurylase activity only, kinase activity only, or those that exhibited minimal loss of either activity. In this set of mutants the other potential phenotypic category, i.e. normal sulfurylase and kinase individual activities, but reduced overall activity was not observed; however, we have obtained such a mutant from a set of substitutions in the sulfurylase region.

A final point of interest is that in all proteins bearing functional P-loop motifs, this sequence is located near the N terminus of the protein where it forms a loop between a β-strand and an α-helix. This arrangement is maintained in the murine, human, and worm fused sulfurylase/kinase that have a domain organization in which the kinase domain is positioned at the N terminus of the bifunctional enzyme, and the sulfurylase domain is toward the C terminus. This order is the reverse of the gene order in the E. coli operon (30) and the Penicillium chrysogenum sulfurylase with a fused C-terminal portion similar to kinase sequence (20), which are all structurally ordered as in the reaction sequence. Therefore, placing the kinase at the N terminus in the fused sulfurylase/kinase of higher organisms may allow the same flexible orientation of the P-loop motif seen in other ATP- and GTP-binding proteins. Had the sulfurylase and kinase been fused in the order found in the arrangement of the genomes of lower organisms, the P-loop would be buried in
the sequence and most likely more conformationally constrained.

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