Characterization of the Phosphorylated Enzyme Intermediate Formed in the Adenosine 5′-Phosphosulfate Kinase Reaction†

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ABSTRACT: Adenosine 5′-phosphosulfate (APS) kinase (ATP:APS 3′-phosphotransferase) catalyzes the ultimate step in the biosynthesis of 3′-phosphoadenosine 5′-phosphosulfate (PAPS), the primary biological sulfuryl donor. APS kinase from *Escherichia coli* is phosphorylated upon incubation with ATP, yielding a protein that can complete the overall reaction through phosphorylation of APS. Rapid-quench kinetic experiments show that, in the absence of APS, ATP phosphorylates the enzyme with a rate constant of 46 s^{-1}, which is equivalent to the V_{max} for the overall APS kinase reaction. Similar pre-steady-state kinetic measurements show that the rate constant for transfer of the phosphoryl group from E-P to APS is 91 s^{-1}. Thus, the phosphorylated enzyme is kinetically competent to be on the reaction path. In order to elucidate which amino acid residue is phosphorylated, and thus to define the active site region of APS kinase, we have determined the complete sequence of *cys*C, the structural gene for this enzyme in *E. coli*. The coding region contains 603 nucleotides and encodes a protein of 22 321 Da. Near the amino terminus is the sequence ^{35}GLSGSGKS, which exemplifies a motif known to interact with the β-phosphoryl group of purine nucleotides. The residue that is phosphorylated upon incubation with ATP has been identified as serine-109 on the basis of the amino acid composition of a radiolabeled peptide purified from a proteolytic digest of ^{32}P-labeled enzyme. We have identified a sequence beginning at residue 147 which may reflect a PAPS binding site. This sequence was identified in the carboxy terminal region of 10 reported sequences of proteins of PAPS metabolism.

3′-Phosphoadenosine 5′-phosphosulfate (PAPS)[1] is the major donor of sulfuryl (SO_3) groups in biological systems (Huxtable, 1986; Falany, 1991). The biosynthesis of PAPS from ATP and inorganic sulfate occurs in two steps: first, ATP sulfurylase (ATP:sulfate adenylyltransferase) catalyzes the reaction of ATP and sulfate to yield adenosine 5′-phosphosulfate and pyrophosphate, and second, APS kinase (ATP:APS 3′-phosphotransferase) catalyzes the ATP-dependent phosphorylation of the 3′-hydroxyl of APS (Robbins & Lipman, 1958). Cloning of the structural genes for APS kinase and ATP sulfurylase from *Escherichia coli* has been reported (Leyh et al., 1988). We recently described purification of APS kinase from an overproducing strain of *E. coli* and characterization of the physical and steady-state kinetic properties of the enzyme (Satishchandran & Markham, 1989). Purified APS kinase was found to be phosphorylated upon incubation with ATP at a stoichiometry of one phosphoryl group per subunit (E-P). The phosphoryl group of E-P could be transferred either to APS to yield PAPS or to ADP to form ATP, suggesting that E-P may be an intermediate in the APS kinase reaction. Involvement of a phosphorylated enzyme in the reaction would rationalize the complex steady-state kinetic behavior of the enzyme, which reflects a combination of ping-pong and sequential pathways (Satishchandran & Markham, 1989). Phosphorylated enzyme intermediates are unusual in phosphoryl-transfer reactions where the acceptor is a small molecule (Knowles, 1980). The only simple kinase reaction which has been shown to proceed through a phosphorylated enzyme intermediate is catalyzed by nucleoside diphosphate kinase, which shows classical ping-pong kinetics with a single substrate binding site for donor and acceptor (Garces & Cleland, 1969). Identification of the amino acid which becomes phosphorylated in *E. coli* APS kinase is of particular interest since the APS kinase from *Penicillium chrysogenum* is not detectably phosphorylated by incubation with ATP (Renosto et al., 1991). Here we report the complete sequence of the *cys*C gene that encodes APS kinase and identification of the phosphorylated residue.[2]

MATERIALS AND METHODS

APS was synthesized and purified as described previously (Baddiley et al., 1957; Yount et al., 1966; Satishchandran & Markham, 1989). APS kinase was purified to electrophoretic homogeneity from the overproducing *E. coli* strain JM83/pTL3/pGP1-2 following the published method; the enzyme concentration was determined using the extinction coefficient of 1.047 $(mg/mL)^{-1}$ cm^{-1} at 280 nm (Satishchandran & Markham, 1989). Other compounds were purchased from commercial sources.

DNA Sequencing. The synthetic oligonucleotides used as primers for DNA sequencing were prepared by Applied Biosystems Instruments at the core facility of the Institute for Cancer Research. Double-stranded plasmid DNA was sequenced using the Sequenase II kit from US Biochemicals following the instructions of the manufacturer. DNA fragments were separated electrophoretically on denaturing polyacrylamide gels prepared with the GIBCO-BRL Sequencing Gel kit. The entire sequence was obtained using both strands of previously reported plasmids as templates.

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[1] Abbreviations: APS, adenosine 5′-phosphosulfate; E-P, phosphorylated APS kinase; GCG, Genetics Computer Group; HPLC, high-performance liquid chromatography; PAP, 3′-phosphoadenosine 5′-phosphate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PEI, polyethyleneimine; TFA, trifluoroacetic acid; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

[2] A preliminary report of these studies was presented at the Fourth International Congress of Chemistry in New York, 1991.

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DNA Sequence Analysis. Individual sequences were assembled and the final data analyzed using the GCG suite of computer programs (Devereux et al., 1984). The final sequence has been deposited in GenBank as Accession Number M86936. Searches for similar sequences in the computer databases used the FASTA program (Pearson, 1990) with GenBank Release 69.0. In a search for a potential PAPS binding motif, the three available APS kinase sequences [*met*14 of *Saccharomyces cerevisiae* (Korch et al., 1991), *nod*Q of *Rhizobium meliloti* (Schwedock & Long, 1990) and *cys*C], the six published sulfotransferase sequences (Nash et al., 1988; Ozawa et al., 1990; Roche et al., 1991; Ogura et al., 1989; Varin et al., 1991), and a homologous aging-related protein (Chaterjee et al., 1987) were aligned in pairwise fashion using BESTFIT, and then as group using the PILEUP program. The final alignment was displayed using the program MALIGNED (Clark, 1992). The potential motif was tested against the PIR-Protein database Release 30.0 and Swissprot Release 19.0 using the FINDPATTERNS program; all possible residues were allowed at the X positions. In order to identify potential known motifs in APS kinase, the deduced APS kinase sequence was inspected using the program MOTIFS with the ProSite motif database (Release 8.0).

Amino Terminal Protein Sequence. Purified APS kinase (1.5 nmol) was sequenced by Dr. E. Burke on an Applied Biosystems 4778 instrument at the Peptide Sequencing Facility of the Albert Einstein College of Medicine, Bronx, NY.

Identification of the Type of Phosphorylated Residue. ^{31}P NMR spectra were recorded on a Bruker AM–300 spectrometer operating at 121.45 MHz. Samples contained 50 mM Hepes/KOH, pH 8.0 with 10% D_2O for field-frequency locking. Chemical shifts are referenced to 85% phosphoric acid; positive chemical shifts are downfield with respect to the reference.

Identification of the Phosphorylated Peptide. A 10-nmol sample of enzyme was phosphorylated by incubation with 1 mM [γ-^{32}P]ATP (20 mCi/mmol) for 0.5 h at 25 °C in 0.1 mL containing 50 mM Hepes/KOH, 50 mM KCl, and 2 mM $MgCl_2$. After removal of nucleotides by gel filtration on Sephadex G-50 using 50 mM sodium phosphate and 2 mM EDTA, pH 7.8, as the eluant, the phosphorylated enzyme was concentrated using a Amicon Centricon 10 device. The enzyme was treated with 5 μg of staphylococcal protease V8 in 0.1 mL of 50 mM sodium phosphate, 2 mM EDTA, and 0.1 mg/mL TPCK, pH 7.8, for 4 h at 37 °C. Peptides were then separated by reverse-phase HPLC on a Vydac C-18 column (218TP54) attached to a Waters Model 600 instrument. The column was eluted at a flow rate of 1 mL/min with a gradient between water-containing 395 μL/L TFA (solvent A) and acetonitrile-containing 360 μL/L TFA (solvent B). The overall gradient was composed of several linear gradients as described by Jaffe et al. (1992). Elution was monitored at 214 nm. Fractions were collected and radioactivity was determined. The sole radioactive peak eluted 98 mL after injection and corresponded to a peak absorbing at 214 nm which was well resolved from other peaks. The composition of the peptide was determined by Dr. W. Abrams at the Protein Analytical Laboratory of the University of Pennsylvania School of Dental Medicine using a Maxima (c) Dynamic Solutions instrument (Millipore).

Rapid Chemical Quench Kinetic Studies. Rapid-quench experiments used a KinTek RQF-3 quench flow instrument (KinTek Instruments, University Park, PA). The dead time of the instrument was determined to be 2 ms by calibration using the base-catalyzed hydrolysis of benzylidenemalononitrile as described by the instrument manufacturer. To measure the rate of E-P formation, 62.5 μM enzyme was mixed with 0.5 mM [γ-^{32}P]ATP (20 Ci/mmol) (final concentrations) in 50 mM Hepes, 50 mM KCl, 1 mM $MgCl_2$, and 10% glycerol, pH 8.0, at 25 °C. After incubation times varying between 2 and 500 ms, the reactions were terminated by mixing with 10% TCA (final concentration) in 10% glycerol. The quenched reactions were collected in a tube containing 0.1 mg of bovine serum albumin. After incubation on ice for 30 min, the protein precipitate was collected on glass fiber filters (Millipore), the filters were washed extensively with 10% TCA, and the extent of protein phosphorylation was quantified by scintillation counting. In experiments measuring the rate of transfer of the phosphoryl group of E-P to APS, 12.5 μM E-P (prepared as described above) was mixed with 100 μM [^{35}S]APS (10 Ci/mmol) (final concentrations). Reactions were stopped by mixing to a final concentration of 50 mM EDTA, 200 mM KOH, and 10 μM PAP in 10% glycerol. The reaction products were seperated on PEI TLC plates using 1 M LiCl as the solvent. The radioactivity associated with the spots on the TLC plate was quantified using a two-dimensional AMBIS radioactivity detector. The rate constants were obtained by fitting the data to a first-order rate equation using the program EnzFitter (Elsevier Biosoft).

RESULTS

Rapid Chemical Quench Kinetic Studies. To verify whether the phosphoenzyme formed in the APS kinase reaction was kinetically viable as an intermediate on the normal reaction pathway, we carried out rapid-quench experiments to determine the rate of phosphorylation of APS kinase by saturating concentrations of ATP. When ATP at a concentration of ~100 times the K_m was mixed with APS kinase, E-P was formed with a rate constant of 46 s^{-1} ((Figure 1A). Since V_{max} under these conditions is ~45 s^{-1}, phosphorylation of the protein is kinetically competent to be on the overall reaction pathway at all substrate concentrations. In other rapid-quench experiments when E-P was mixed with APS the rate constant for PAPS formation was 91 s^{-1}, (Figure 1B) well in excess of V_{max}. The results suggest that phosphorylation of the enzyme is the rate-limiting step in the overall reaction.

Sequence of cysC. Since the phosphorylated enzyme is a kinetically viable reaction intermediate, we sought to locate the site of phosphorylation. This laboratory previously reported the cloning of a 9.4-kb DNA fragment which contains the *cys*DNC operon from *E. coli* (Leyh et al., 1988). The first two genes of this operon, *cys*DN, encode the two subunits of ATP sulfurylase and the third gene is the structural gene for APS kinase. The location of *cys*C on the cloned DNA was deduced by deletions of the 9.4-kb chromosomal fragment (Leyh et al., 1988). To allow the identification of a proper reading frame for *cys*C, the amino terminal sequence of the purified protein was determined, yielding the sequence ALHDENVV. Previous insertion mutagenesis studies indicated that the promoter for the *cysDNC* operon precedes *cys*D and thus is approximately 2.5-kB upstream of *cys*C. Therefore, the identification of a promoter for *cys*C was not attempted, and only the coding region of *cys*C was sequenced. The complete DNA sequence of *cys*C and the deduced protein sequence are shown in Figure 2. The N-terminal methionine is not present in the purified enzyme. The open reading frame codes for protein of 22 321 Da, in good agreement with the value of 21 kDa estimated by SDS–polyacrylamide gel electrophoresis (Satishchandran & Markham, 1989). The native protein undergoes dimer–tetramer interconversions depending on experimental conditions and the phosphorylation state (Satishchandran & Markham, 1989).

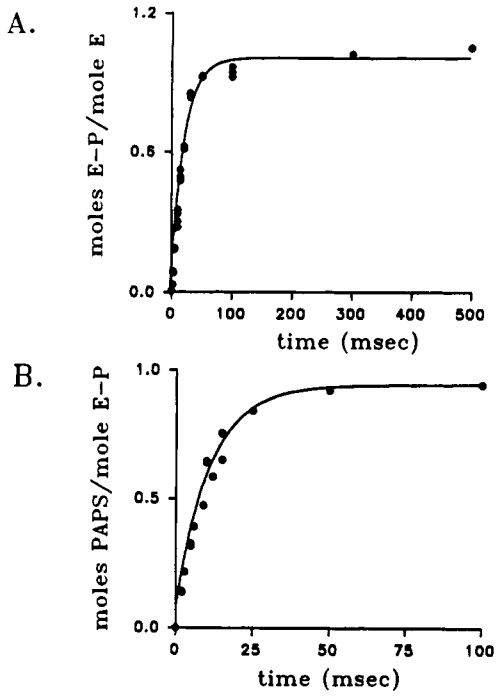

FIGURE 1: Rapid quench kinetics of phosphoryl group transfer. (A) Rate of phosphorylation of APS kinase by ATP. The solutions contained final concentrations 62.5 μM APS kinase and 0.5 mM [γ-^{32}P]ATP in 50 mM Hepes/KOH, 50 mM KCl, 1 mM $MgCl_2$, and 10% glycerol, pH 8.0. After incubation for the times shown, the reactions were quenched by mixing with 10% TCA (final concentration) in 10% glycerol. The ^{32}P present in the precipitated protein was quantified as described in Materials and Methods. (B) Rate of phosphorylation of APS by E-P. Solutions contained 100 μM [^{35}S]-APS, 12.5 μM APS kinase in 50 mM Hepes/KOH, 50 mM KCl, 1 mM $MgCl_2$, and 10% glycerol, pH 8.0. The reactions were quenched with 50 mM EDTA, 200 mM KOH, and 10 μM PAP (final concentrations) in 10% glycerol. The lines drawn are the best fits to a first-order rate equation, with rate constants of 46 (A) and 91 s^{-1} (B). The standard deviations in the rate constants are 4% (A) and 9% (B).

Examination of the codon utilization (Table I) indicates that the fourth and fifth codons, encoding histidine and aspartate, are among those least (<1%) used in *E. coli*, while most other codons are among the more commonly employed. The use of low-frequency codons at the beginning of the coding region and the absence of a consensus ribosome binding sequence (Shine & Dalgarno, 1974) within the 15 nucleotides preceding the start codon might account for the ~70-fold lower production of APS kinase compared to the *cys*DN gene products, even when RNA production is driven using a bacteriophage T7 promoter and T7 RNA polymerase. The presence of sequence motifs suggesting locations of substrate binding sites is discussed below.

Identification of the Phosphorylated Residue Type. We had previously reported that the phosphoryl linkage in E-P is quite stable, surviving gel filtration and SDS–gel electrophoresis. Before attempting to determine which residue was phosphorylated, we sought to elucidate the type of residue to which the phosphoryl group was attached, as this knowledge would aid in the choice of an appropriate protease and of chromatographic conditions for peptide isolation. The ^{31}P NMR spectrum of a 0.1 mM solution of the phosphorylated enzyme showed a single peak at 4.3 ppm, demonstrating that the phosphoryl group was present in a phosphomonoester linkage (Vogel, 1989); however, the line was too broad (~10 Hz) for ^{1}H coupling to be resolved in the absence of ^{1}H decoupling. The ^{31}P NMR spectrum of E-P denatured by incubation for several hours at 25 °C showed that the 4.3 ppm NMR resonance was replaced by a sharper new peak at 4.8 ppm, a chemical shift also characteristic of a phosphomonoester. In the absence of ^{1}H decoupling, the 4.8 ppm resonance was a triplet ($^3J_{P-H}$ = 6.5 Hz) indicating a CH_2–O–P linkage. The chemical shift of 4.3 ppm, the P–H coupling constant, and multiplicity coincide with those of a phosphoserine standard under these conditions. Thus the NMR data conclusively identify the phosphorylated residue as a serine, one of 11 serine residues in the sequence. Due to local environmental effects in the native protein, the ^{31}P resonance is shifted downfield by 0.5 pppm from free phosphoserine; unfortunately, the interpretation of small changes in ^{31}P chemical shifts is not yet unambiguous (Gorenstein, 1989).

Identification of the Phosphorylated Serine. To identify the serine which is phosphorylated, ^{32}P-labeled E-P was proteolytically cleaved with *Staphylococcal* V8 protease. Peptides were separated by reverse-phase HPLC. A single peak containing ^{32}P was obtained. The composition of the peptide was determined and is shown in Table II where it is compared to the predicted amino acid composition for the peptide encompassing residues 98–113. An excellent correlation is observed, while no other predicted peptides gave compositions that were remotely similar to the observed composition. Since there is only one serine in this peptide, the phosphorylation site is identified as serine-109.

Sequence Motifs in cysC. When the deduced protein sequence of APS kinase was compared with the collection of consensus sequences present in the ProSite database (Release 8.0), a perfect match with the purine nucleotide binding site consensus sequence G-x-x-x-x-G-K-S (Walker et al., 1982) was seen starting at residue 35, as GLSGSGKS (Figure 2, in box). The motif is also present in the APS kinase sequences from *S. cerevisiae* and *R. meliloti* (Korch et al., 1991; Schwedock & Long, 1990). The oncogene product H-*ras*p21 displays a motif similar to the purine nucleotide binding motif in APS kinase. The 1.35-Å X-ray crystal structure of H-*ras*p21 complexed with β-γ-imido-GTP reveals the primary interaction of the amino acids in the purine nucleotide binding motif to be with the β-phosphoryl group of the nucleotide (Pai et al., 1990). Our observation that the serine that is phosphorylated in the APS kinase reaction is not the serine present in this motif is consistent with the interactions predicted from the studies on *ras*p21.

We compared the deduced *E. coli* APS kinase sequence with those of *S. cerevisiae* and the C-terminal region of the *nod*Q protein [a subunit of a bifunctional ATP sulfurylase/APS kinase (Schwedock, 1991)]. The *E. coli* enzyme has 47.9% identity over a 200 amino acid region to the yeast enzyme and 54.9% identity over a 195 amino acid region to the C-terminal portion of the *R. meliloti* enzyme. The ATP consensus sequence and the sequence FISP containing serine-109 are identical in all three enzymes. The phosphorylation of the *S. cerevisiae* or the *R. meliloti* proteins has not been reported, nor have the mechanisms of these enzymes been extensively studied.

In an effort to discern a motif which might be related to PAPS binding, we compared the APS kinase sequences with sequences available for enzymes which are involved in PAPS utilization: bovine estrogen sulfotransferase (Nash et al., 1988), rat hydroxysteroid sulfotransferase (Ogura et al., 1989), rat alcohol sulfotransferase (Ogura et al., 1990), rat aryl sulfotransferase (Ozawa et al., 1990), *R. meliloti* nodulation factor sulfotransferase (Roche et al., 1991), two plant (*Flaveria chloraefolia*) flavol sulfotransferases (Varin et al., 1992), *E. coli* PAPS reductase (*cysH*) (Ostrowski et al., 1989), and a rat senescence-related protein with high sequence homology to the mammalian sulfotransferases (Chaterjee et al., 1987).

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     -10            1
CTG GGG GGA TAA ATA ATG GCG CTG CAT GAC GAA AAC GTC GTC TGG CAT AGC
                    Met Ala Leu His Asp Glu Asn Val Val Trp His Ser      12

CAT CCG GTC ACT GTG CAA CAA CGC GAG CTA CAC CAC GGT CAT CGT GGT GTA
His Pro Val Thr Val Gln Gln Arg Glu Leu His His Gly His Arg Gly Val      29

               100            ATP MOTIF
GTG CTG TGG TTT ACC GGC CTC TCC GGG TCC GGT AAA TCA ACG GTC GCC GGG
Val Leu Trp Phe Thr GLY Leu Ser Gly Ser GLY LYS SER Thr Val Ala Gly      46

GCG CTG GAG GAG GCG TTA CAT AAA CTC GGC GTC AGT ACG TAT CTG CTG GAT
Ala Leu Glu Glu Ala Leu His Lys Leu Gly Val Ser Thr Tyr Leu Leu Asp      63

           200
GGC GAC AAT GTT CGC CAC GGA TTA TGC AGC GAT CTC GGT TTT AGC GAT GCC
Gly Asp Asn Val Arg His Gly Leu Cys Ser Asp Leu Gly Phe Ser Asp Ala      80

GAT CGT AAA GAG AAT ATC CGT CGC GTC GGT GAA GTG GCG AAT TTG ATG GTT
Asp Arg Lys Glu Asn Ile Arg Arg Val Gly Glu Val Ala Asn Leu Met Val      97

         300                              P-Ser
GAA GCC GGA CTG GTG GTG CTG ACC GCA TTT ATC TCG CCA CAC CGC GCC GAA
Glu Ala Gly Leu Val Val Leu Thr Ala Phe Ile Ser Pro His Arg Ala Glu     114

CGC CAG ATG GTT CGC GAA CGC GTA GGA GAA GGG CGC TTT ATC GAA GTG TTT
Arg Gln Met Val Arg Glu Arg Val Gly Glu Gly Arg Phe Ile Glu Val Phe     131

       400
GTC GAT ACG CCG CTG GCG ATT TGC GAA GCC CGC GAT CCC AAA GGC TTA TAT
Val Asp Thr Pro Leu Ala Ile Cys Glu Ala Arg Asp Pro Lys Gly Leu Tyr     148

                    PAPS MOTIF
AAG AAA GCG CGT GCC GGT GAA CTG CGC AAC TTT ACG GGA ATA GAT TCC GTT
Lys LYS ALA Arg Ala GLY Glu Leu Arg ASN PHE THR Gly Ile Asp Ser Val     166

    500
TAC GAA GCG CCT GAA TCG GCA GAA ATT CAT CTC AAT GGT GAA CAA TTA GTA
Tyr Glu Ala Pro Glu Ser Ala Glu Ile His Leu Asn Gly Glu Gln Leu Val     183

ACA AAT TTG GTA CAG CAA TTA TTA GAT CTG TTG AGA CAG AAC GAT ATT ATC
Thr Asn Leu Val Gln Gln Leu Leu Asp Leu Leu Arg Gln Asn Asp Ile Ile     200

 600
AGA TCC TGA
Arg Ser End
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FIGURE 2: Sequence of *cysC* and deduced amino acid sequence of APS kinase. The serine phosphorylated upon incubation with ATP is enclosed in double lines. A sequence consistent with an ATP binding motif and the region containing a sequence present in sequences of 10 reported PAPS-utilizing proteins are boxed. For both motifs, the residues agreeing with the consensus are capitalized in bold type. Numbers above the sequence refer to nucleotides, while numbers at the right refer to amino acids.

A common sequence, K(A/G)xxGxxx(N/E)x(0 or 1)FT, is present in all these sequences (Figure 3), with the exception of the PAPS reductase, which has no sequences resembling this motif. Comparison of groups consisting solely of either APS kinases or sulfotransferases was not useful in these analyses since within these groups the sequences are too similar to one another. This motif begins between 35 and 69 residues from the carboxy terminus of each protein. This motif did not appear either in the sequence of yeast ATP sulfurylase (Cherest et al., 1987) or in the *nod*P component of the bifunctional *R. meliloti* ATP sulfurylase/APS kinase (Schwedock & Long, 1990; Schwedock, 1991). In order to test whether this motif might be useful in identifying PAPS-utilizing proteins, the consensus was tested against the databases PIR-protein and Swissprot with all possible combinations of residues in the X positions; only the proteins used in constructing the sequence were found, suggesting that this may be a PAPS-related motif. Since no PAPS binding sites have yet been identified by affinity labeling, the functional significance of this motif is unclear.

Table I: Summary of Codon Usage for *cysC*[a]

Phe	TTT	6	Ser	TCT	0	Tyr	TAT	2	Cys	TGT	0
Phe	TTC	0	Ser	TCC	4	Tyr	TAC	1	Cys	TGC	2
Leu	TTA	6	Ser	TCA	1		TAA	0[b]		TGA	1[b]
Leu	TTG	3	Ser	TCG	2		TAG	0[b]	Trp	TGG	2
Leu	CTT	0	Pro	CCT	1	His	CAT	6	Arg	*CGT*	4
Leu	CTC	4	Pro	CCC	1	His	CAC	4	Arg	*CGC*	10
Leu	CTA	1	Pro	CCA	1	Gln	CAA	4	Arg	CGA	0
Leu	*CTG*	10	Pro	*CCG*	2	Gln	CAG	3	Arg	CGG	0
Ile	*ATT*	3	Thr	ACT	1	Asn	AAT	5	Ser	AGT	1
Ile	*ATC*	4	Thr	*ACC*	2	Asn	AAC	3	Ser	*AGC*	3
Ile	ATA	1	Thr	*ACA*	1	Lys	AAA	5	Arg	AGA	2
Met	ATG	3	Thr	ACG	4	Lys	AAG	1	Arg	AGG	0
Val	*GTT*	4	Ala	GCT	0	Asp	GAT	9	Gly	*GGT*	7
Val	GTC	7	Ala	*GCC*	6	Asp	GAC	2	Gly	*GGG*	4
Val	GTA	4	Ala	GCA	2	Glu	GAA	13	Gly	GGA	4
Val	*GTG*	6	Ala	*GCG*	7	Glu	GAG	4	Gly	GGG	3

[a] The high usage codons utilized in *E. coli* are shown in italic, while the low usage codons are underlined (Zhang et al., 1991). [b] Termination codons.

Table II: Predicted and Observed Amino Acid Compositions of the Phosphorylated Peptide[a]

	residues 98–113			residues 98–113	
	predicted	obsd		predicted	obsd
Ala	3	3.3	Ser + P-Ser	1	1.3
Gly	1	1.3	Pro	1	1.3
Leu	2	2.1	His	1	0.9
Val	2	1.7	Arg	1	1.0
Thr	1	1.2	Glu	1	0.8
Phe	1	0.8	all others	0	<0.2

[a] Predictions are based on the deduced amino acid sequence of the protein. No other peptides have similar compositions. Compositions are the average of three determinations and are normalized to arginine. P-Ser is unstable when subjected to acid hydrolysis, and the P-ser/serine ratio was variable in different determinations [P-ser/(P-ser + Ser) = 0.4–0.6]. Serine is the product of P-Ser hydrolysis, and therefore, P-Ser and Ser are presented together.

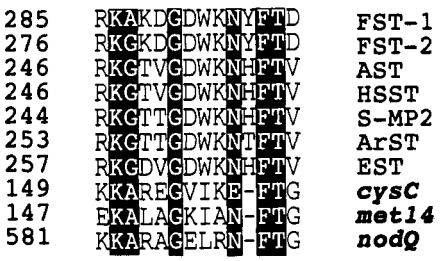

FIGURE 3: Alignment of sequences of PAPS-utilizing proteins in region of proposed PAPS binding site. The set of residues in the consensus is shown in white upon black. From top to bottom the sequences are as follows: flavol sulfotransferase-1, flavol sulfotransferase-2, alcohol sulfotransferase, hydroxysteroid sulfotransferase, senescence-related marker protein 2, aryl sulfotransferase, estrogen sulfotransferase, *E. coli* APS kinase, *S. cerevisiae* APS kinase, and *R. meliloti nod*Q protein (APS kinase domain).

DISCUSSION

These results demonstrate that both phosphorylation of APS kinase on serine-109 by ATP and transfer of the phosphoryl group of E-P to APS are rapid enough to represent steps in the mechanism, even at V_{max} conditions. APS kinase contains an ATP binding motif near the amino terminus and an apparent PAPS binding motif near the carboxy terminus. The serine that is phosphorylated is located between these motifs. Previous studies have indicated that, at very low (physiological) APS concentrations, the APS kinase reaction can occur by a ping-pong kinetic mechanism where no ternary E·ATP·APS complex forms, while at higher APS concentrations a E·ATP·APS complex occurs on the reaction pathway (Satishchandran & Markham, 1989). The phosphorylated enzyme can participate in either steady-state route. Although the steady-state kinetic behavior of the APS kinase from *P. chrysogenum* is quite similar to that of the *E. coli* enzyme, the fungal enzyme apparently does not form a stable phosphoenzyme (Renosto et al., 1991). However, the formation of an APS-dependent transient phosphorylated enzyme intermediate has not been excluded (Renosto et al., 1991). The sequence of the fungal enzyme has not been reported; thus the structural similarity of the two enzymes is as yet unclear. Whether the phosphorylated *E. coli* enzyme is involved in the overall reaction under all conditions remains to be determined by site-directed mutagenesis of serine-109 and from elucidation of the stereochemical course of phosphoryl transfer. If the phosphorylated protein is an obligatory intermediate in the APS kinase reaction, the distant sequence locations of the ATP and PAPS binding motifs might suggest that the two substrate binding sites are not close enough in space to allow direct phosphoryl transfer and suggest a role for the phosphoserine analogous to the biotin "swinging arm" mechanism (Northrup & Wood, 1969; Wood, 1972).

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