3′-Phosphoadenosine 5′-Phosphosulfate Binding Site of Flavonol 3-Sulfotransferase Studied by Affinity Chromatography and ^{31}P NMR†

Frédéric Marsolais,‡ Mario Laviolette,§ Yoshimitsu Kakuta,|| Masahiko Negishi,|| Lars C. Pedersen,|| Michèle Auger,§ and Luc Varin*,‡

Department of Biology, Concordia University, 1455 de Maisonneuve Boulevard West, Montreal, Quebec, Canada H3G 1M8, Département de Chimie, Centre de recherche en sciences et ingénierie des macromolécules, Université Laval, Sainte-Foy, Québec, Canada G1K 7P4, and Pharmacogenetics Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709

Received September 17, 1998; Revised Manuscript Received December 17, 1998

ABSTRACT: The function of Lys-59, Arg-141, and Arg-277 in PAPS binding and catalysis of the flavonol 3-sulfotransferase was investigated. Affinity chromatography of conservative mutants with PAPS analogues allowed us to determine that Lys-59 interacts with the 5′ portion of the nucleotide, while Arg-141 interacts with the 3′ portion, confirming assignments deduced from the crystal structure of mouse estrogen sulfotransferase [Kakuta, Y., Pedersen, L. G., Carter, C. W., Negishi, M., and Pedersen, L. C. (1997) *Nat. Struct. Biol. 4*, 904−908]. The affinity chromatography method could be used to characterize site-directed mutants for other types of enzymes that bind nucleoside 3′,5′- or 2′,5′-diphosphates. ^{31}P NMR spectra of enzyme−PAP complexes were recorded for the wild-type enzyme and K59R and K59A mutants. The results of these experiments suggest that Lys-59 is involved in the determination of the proper orientation of the phosphosulfate group for catalysis.

Cytosolic sulfotransferases (ST)[1] catalyze the transfer of a sulfonate group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to hydroxyl or amino groups of acceptor substrates. In mammals, these enzymes play an important role in the detoxication of xenobiotics and endogenous metabolites, and generally possess a high affinity for acceptor substrates as compared with those of other types of enzymes involved in conjugation reactions (*1*). They also participate in the metabolic pathways of biologically active endogenous metabolites, such as steroid and thyroid hormones and catecholamine neurotransmitters (*2*−*6*). Sulfonation of these molecules modulates biological activity and, in the case of steroids, facilitates transport of hormonal precursors to target tissues, where they are further metabolized and bioactivated.

Cytosolic STs form a family of homologous enzymes with members present in mammals, plants, and bacteria. Amino acid sequence comparison of STs from different phyla allowed the identification of four well-conserved regions, with several strictly conserved amino acid residues (*7*, *8*). A few studies in which site-directed mutagenesis was used were successful in identifying amino acids that are involved in catalysis or in PAPS binding. Two residues located in conserved regions II and IV of the plant flavonol 3-ST (EC 2.8.2.25), Arg-141 and Arg-277, were shown to be required for cosubstrate binding (*9*, *10*). Replacement of His-98 with alanine or lysine in the rat hydroxysteroid ST-40 resulted in enzymes with undetectable catalytic activity (*11*). Similarly, results on mutants for the corresponding His-119 in the flavonol 3-ST suggested that this residue is involved in catalysis (*9*). Another residue, Lys-59 (located in region I), does not contribute to the binding affinity for PAPS but is required for catalysis (*10*). Replacement with alanine resulted in an essentially inactive enzyme with an affinity for PAPS similar to that of the wild-type enzyme, as demonstrated by the results of photoaffinity labeling experiments. Replacement with arginine resulted in a 15-fold decrease in specific activity, and a slight (2-fold) increase in affinity for PAPS. Finally, the results from 3′-phosphoadenosine 5′-phosphate (PAP)−agarose affinity chromatography experiments indicated that Lys-59 interacts with PAP.

The crystal structure of the mouse estrogen ST in complex with PAP and β-estradiol has recently been determined (*12*). The structural model confirms previous interpretations inferred from site-directed mutagenesis data. His-108 of estrogen ST (His-119 in the flavonol 3-ST) is positioned to interact with the hydroxyl group at position 3 of β-estradiol, and it has been proposed to act as a catalytic base abstracting a proton to activate the phenolic hydroxyl for nucleophilic attack on the sulfur of PAPS. The side chains of Arg-130 and Arg-257 (Arg-141 and Arg-277 in the flavonol 3-ST, respectively) bind the 3′-phosphate group of PAP and stabilize its negative charge. The structure of estrogen ST

† This work was supported by grants from the Natural Sciences and Engineering Research Council and le Fonds pour la Formation de Chercheurs et l'Aide à la Recherche. F.M. is the recipient of a postgraduate scholarship from the Natural Sciences and Engineering Research Council. Y.K. is a JSPS research fellow in biomedical and behavioral research at the NIH.

* Corresponding author. Phone: (514) 848-3396. Fax: (514) 848-2881. E-mail: varinl@clone.concordia.ca.

‡ Concordia University.

§ Université Laval.

|| National Institutes of Health.

[1] Abbreviations: PAP, 3′-phosphoadenosine 5′-phosphate; 2′,5′-PAP, 2′-phosphoadenosine 5′-phosphate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; ST, sulfotransferase.

10.1021/bi982239m CCC: $18.00
Published on Web 03/12/1999

reveals striking structural similarity with nucleotide kinases, including a P-loop fold corresponding to region I, where the conserved lysine is positioned to interact with the 5′-phosphate of PAP (*12*).

In this paper, we further investigate the function of Lys-59, Arg-141, and Arg-277 of the flavonol 3-ST in PAPS binding and catalysis. In the first part of this work, we used affinity chromatography with cosubstrate analogues to identify the site of interaction of amino acid side chains on the PAPS molecule. The results of these experiments confirmed that Lys-59 interacts with the 5′-phosphate group of PAP while Arg-141 interacts with the 3′ portion. We also studied the impact of Lys-59 mutations on PAP binding by comparing one-dimensional ^{31}P NMR spectra of enzyme−PAP complexes, and our results suggest that Lys-59 might determine the proper positioning of the cosubstrate at the active site for catalysis.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. The preparation of flavonol 3-ST mutants had been described previously (*9*, *10*). The mutant cDNAs were subcloned in the bacterial expression vector pQE30 (Qiagen), and the full-length coding sequence of the mutants was determined. Verification of the wild-type sequence by automated sequencing uncovered an error that introduced a gap of one amino acid residue in the alignment with other flavonol STs. The correction converts the sequence 68-WIC-70 to 68-LAFA-71, modifying the numbering of subsequent amino acid residues.

Expression of Recombinant ST. Cultures of the *Escherichia coli* strain XLI-blue expressing the wild-type or mutant cDNAs were started by inoculating with a 2.5% volume from an overnight culture. Cultures were grown in Luria-Bertani medium for 3 h at 30 °C, before the addition of the inducer, isopropyl thiogalactoside to a final concentration of 1 mM, and incubation was continued for an additional 3 h. Cells were pelleted by centrifugation and resuspended in 50 mM sodium phosphate (pH 8.0), 0.3 M NaCl, and 14 mM β-mercaptoethanol, and lysed by sonication. Cell debris was removed by centrifugation at 12000*g* for 15 min at 4 °C, and the supernatant was applied to nickel−nitrilotriacetic acid resin (Qiagen) equilibrated in the same buffer. The resin was washed with 50 mM sodium phosphate (pH 6.0), 0.3 M NaCl, and 14 mM β-mercaptoethanol, and the proteins were eluted with the same buffer containing 150 mM imidazole. Proteins were measured by the method of Bradford (*13*), and bovine serum albumin was used as the standard protein.

ST Assay and Determination of K_i. ST activity was determined immediately after nickel−agarose purification by monitoring the extent of incorporation of the label from [^{35}S]-PAPS (New England Nuclear) into the flavonol acceptor quercetin according to a previously described assay (*14*). Kinetic analysis was performed as described in ref *15*, with the following modification. Enzyme assays were performed in 50 mM sodium phosphate at pH 7.5 and 25 °C. K_i values for nucleotides were determined at a fixed quercetin concentration of 0.2 μM by varying the concentrations of both PAPS and inhibitor. The following concentrations of PAPS were used: 0.5, 0.25, 0.125, 0.083, and 0.0625 μM. The following concentrations of nucleotides were used: for PAP, 0.3, 0.2, and 0.1 μM; for ADP and ATP, 400, 200, and 100 μM; and for 2′,5′-PAP, 200, 100, and 50 μM.

Affinity Chromatography. Immediately after purification on nickel−agarose, the enzyme preparations were applied to a PD-10 column (Pharmacia) equilibrated with buffer A [25 mM Bis-Tris (pH 6.8) and 14 mM β-mercaptoethanol]. The eluted proteins were chromatographed on ADP−, ATP−, and 2′,5′-PAP−agarose columns (ca. 2 mL, Sigma Chemicals) equilibrated with buffer A, and washed with 3 column volumes of the same buffer. The bound proteins were eluted with linear gradients of 0 to 0.3 M NaCl in buffer A for ADP− and ATP−agarose, and 0 to 1 M NaCl in the same buffer for 2′,5′-PAP−agarose, at a flow rate of 0.5 mL/min. Protein absorbance was monitored at 280 nm with a Waters 486 tunable absorbance detector. To obtain reproducible results, affinity chromatography was performed with a Waters 625 LC HPLC system and a Waters AP minicolumn.

^{31}P NMR of Enzyme−PAP Complexes. After purification on nickel−agarose, enzyme preparations were applied to a PD-10 column equilibrated with buffer B [50 mM Tris (pH 7.5) and 14 mM β-mercaptoethanol]. The eluted proteins were chromatographed on a Protein Pak Q-8HR column (Waters) preequilibrated with buffer B and washed with 3 column volumes of the same buffer. The bound proteins were eluted with a linear gradient of 0 to 0.3 M NaCl in buffer B at a flow rate of 0.5 mL/min. The eluted proteins were concentrated twice on Centricon 30 membranes (Amicon) with deuterated buffer B (90% ^1H_2O/10% ^2H_2O). The ^{31}P NMR spectra were recorded on a Bruker AC-F 300 NMR spectrometer (Bruker Canada Ltd., Milton, ON) operating at a frequency of 121.50 MHz for ^{31}P and 300.13 MHz for ^{1}H. Samples were placed in 5 mm NMR tubes, and the spectra were recorded at room temperature (24 °C) under conditions of proton decoupling. A 45° pulse of 1.9 μs was used with a repetition time of 2.0 s and an acquisition time of 1.3 s. The spectral width was set to 12.2 kHz, and 32K data points were recorded for each free induction decay. Chemical shifts were referenced relative to external 85% H_3PO_4 at 0 ppm, and a 5 Hz line broadening was applied to all spectra. Nucleotide samples contained PAPS (Fluka) or PAP at 1 mM. Samples of enzyme complexes contained a slight molar excess of enzyme to ligand, ensuring almost complete binding of nucleotides.

Molecular Modeling of the Flavonol 3-ST. An amino acid sequence alignment of the flavonol 3-ST and the mouse estrogen ST was prepared using Pileup from the GCG package (*16*), and then refined visually with the help of a multiple sequence alignment of cytosolic STs (*17*). On the basis of the alignment, the side chains of the mouse estrogen ST crystal structure (*12*) were replaced with those of the flavonol 3-ST. The side chains were manually fit to best mimic the estrogen ST side chains with the least amount of steric contact with other residues.

RESULTS AND DISCUSSION

Affinity Chromatography. We have previously used affinity chromatography on PAP−agarose to characterize mutants of the flavonol 3-ST. The recombinant wild-type flavonol 3-ST binds strongly to PAP−agarose, and elutes at 0.78 M NaCl (*9*, *10*). Several lines of evidence suggested that binding of the enzyme to the chromatographic support is specific. The flavonol 3-ST purified from the plant is sensitive to product inhibition by PAP, a competitive inhibitor of PAPS

Table 1: Salt Concentration (M) Required for Elution of the Wild-Type and Mutant Flavonol 3-STs

enzyme	PAP−agarose[a]	ADP−agarose	ATP−agarose	2′,5′-PAP−agarose
wild-type	0.78	0.25	0.28	0.7
	0.05[b]	65[b]	265[b]	53[b]
K59R	0.78	0.22 (88%)	0.23 (82%)	0.66 (94%)
R141K	0.76 (97%)[c]	0.25	0.28	0.56 (80%)
R277K	0.78	0.25	0.28	0.68 (97%)

[a] From refs *9* and *10*. [b] K_i (μM) for nucleotide ligands. [c] Relative affinity compared to that of the wild-type enzyme.

for the active site of the enzyme, with a K_i (0.1 μM) slightly lower than the K_m for PAPS (0.18 μM) (*15*). This property explains the extensive use of PAP−agarose affinity chromatography in purifying cytosolic STs, and it has been shown that they can be specifically eluted from the chromatographic support with PAP or PAPS at concentrations not exceeding 1 mM (*18*−*20*). In a series of control experiments, mutant enzymes having a change in the net charge of the protein but kinetic parameters similar to those of the recombinant wild-type enzyme were chromatographed on PAP−agarose. No significant changes in chromatographic behavior were observed compared to the wild-type enzyme, ruling out the contribution of nonspecific ionic interactions (*10*).

When chromatographed on PAP−agarose, nonconservative mutants of Lys-59, Arg-141, and Arg-277 elute at significantly lower salt concentrations compared to the recombinant wild-type enzyme (*9*, *10*). In contrast, conservative mutants at these residues have an affinity for PAP−agarose similar to that of the recombinant wild-type enzyme (Table 1) (*9*, *10*). The apparent contradiction with the data for PAPS affinity can be explained by the fact that salt elution from PAP−agarose isolates the ionic component of the interaction with the ligand. This property allows quantification of the differences in specific ionic interactions between amino acids at the substrate binding site and the phosphate groups of the ligand.

Various nucleotides are known to act as competitive inhibitors of cytosolic STs (*21*). We reasoned that by correlating the chromatographic behavior of mutant and wild-type enzymes with the structural variation in nucleotide ligands, we might be able to identify the site of interaction on the PAPS molecule for a particular amino acid side chain. To validate this method, the mode of inhibition and K_i values for the various inhibitors used in this study were determined with the recombinant wild-type enzyme purified by nickel−agarose chromatography. Inhibition was studied at a fixed concentration of quercetin, by varying the concentrations of both PAPS and inhibitor. Reciprocal plots were drawn for each inhibitor, and K_i values were determined from slope replots. The mode of inhibition of ADP, ATP, and 2′,5′-PAP is competitive with respect to PAPS (*22*). There is at least a 1000-fold difference in K_i values between these analogues and PAP, suggesting that the high affinity is achieved mainly through the interaction with the 3′-phosphate group (Table 1).

The nickel−agarose-purified recombinant wild-type enzyme and conservative mutants of Lys-59, Arg-141, and Arg-277 were chromatographed on ADP−, ATP−, and 2′,5′-PAP−agarose and eluted with a linear salt gradient. The proteins eluted with good reproducibility between individual experiments. In all the chromatographic supports, including PAP−agarose, the ligand is cross-linked to the spacer arm via position N6 of the adenine ring, leaving the phosphate groups accessible. In the crystal structure of the mouse estrogen ST, the backbone oxygen of Thr-227 (Cys-246 in the flavonol 3-ST) is found in a proper position to form a hydrogen bond with position N6 of the adenine ring (*12*). Although the covalent bond with the spacer arm would remove this interaction, this structural constraint is kept constant and does not seem to alter the specificity of binding. When considering the affinity chromatography data, one should bear in mind that the position of the ligands in the active site will not necessarily coincide with that of PAP.

When chromatographed on ADP− and ATP−agarose, R141K and R277K elute at salt concentrations identical to that of the wild-type enzyme (Table 1). However, K59R displays a reduction in its affinity for ADP−agarose (88% of the wild-type value), which is even more pronounced for ATP−agarose (82%) (Table 1). Since there is a complementarity of charge between the side chain and the ligand, this effect cannot be explained by electrostatic repulsion. Instead, it must be attributed to steric factors, the side chain of arginine being bulkier than that of lysine. These results suggest that Lys-59 interacts with the 5′-phosphate, and probably the sulfate group of PAPS.

Another interesting change in chromatographic behavior is observed for R141K with 2′,5′-PAP−agarose. The affinity of R141K for 2′,5′-PAP−agarose falls to only 80% of the wild-type value, compared to 94% for K59R and 97% for R277K (Table 1). The most likely explanation for this effect is that the lysine side chain of the mutant is too short to interact with the phosphate in the 2′ position, compared with the arginine. Because of the weaker interaction of R141K with a 2′-phosphate, and the absence of an impact of structural variation in the 5′ portion of the ribose on its chromatographic behavior, Arg-141 appears to be involved in binding the 3′-phosphate of PAP.

The very weak fluctuations observed for R277K compared with those of the two other mutants suggest that there are limits in the sensitivity of the technique (Table 1). However, it has been shown that the contribution of Arg-277 to PAPS binding is weaker than that of Arg-141 (*9*), and this might explain at least in part the small variations in the affinity of R277K for the chromatographic supports. A more likely explanation for this phenomenon is that while Arg-277 interacts with the 3′-phosphate of PAP, it is better positioned than Arg-141 to interact with a 2′-phosphate group. In a molecular model of the flavonol 3-ST based on the coordinates of the crystal structure of the mouse estrogen ST (*12*), the main chain nitrogen of Arg-277 appears indeed to be in a proper position to interact with a 2′-phosphate group (Figure 1).

^{31}P NMR of Flavonol 3-ST−*PAP Complexes*. ^{31}P NMR is a powerful tool for studying the impact of mutations on nucleotide binding sites of enzymes, and has been used to characterize site-directed mutants of adenylate kinase (*23*). To further characterize the effect of mutations at Lys-59 on PAP binding, we studied the proton-decoupled one-dimensional ^{31}P NMR spectra of enzyme−PAP complexes. As a preliminary experiment for evaluating the impact of ligand binding to the wild-type enzyme, the chemical shifts of the ^{31}P resonances were determined for PAP, PAPS, and a

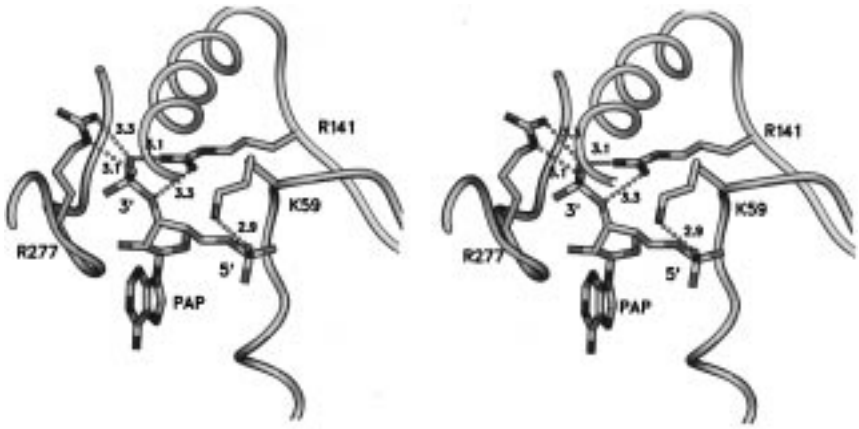

FIGURE 1: PAP binding site of the flavonol 3-ST, showing the site of coordination and bond lengths for Lys-59, Arg-141, and Arg-277. The flavonol 3-ST was modeled on the coordinates of the crystal structure of the mouse estrogen ST.

mixture of both nucleotides to allow the assignment of the individual phosphorus signals (Figure 2). The 3′-phosphate signal is shifted downfield by ca. 0.5 ppm from the 5′-phosphate position, consistent with previous ^{31}P NMR data for nucleoside 3′,5′-diphosphates (*24*, *25*). The addition of a 5′-sulfate group results in a large shielding effect on the 5′-phosphate signal (Figure 2).

R141K and R277K complexes could not be studied because of the low affinity of the mutant enzymes for PAP. In trial experiments, only ^{31}P resonances of unbound PAP could be observed. On the other hand, the relative intensities of signals representing bound and unbound PAP were similar for Lys-59 mutant and the wild-type enzyme complexes (data not shown), indicating that Lys-59 does not contribute to the affinity for PAP. In the ^{31}P NMR spectra of the wild-type enzyme in complex with PAP, the peaks of the two phosphorus atoms are superimposed (Figure 2). Therefore, binding has a shielding effect on the ^{31}P signals that is more pronounced for the 3′-phosphate than for the 5′-phosphate. In the K59R and K59A complexes, the values of both resonances are shifted downfield relative to that of the wild-type enzyme complex (Figure 2). The intermediate perturbations observed for the K59R complex compared to those observed for K59A are consistent with the partial complementation of catalytic activity in the arginine mutant (*10*).

The separation of the signals observed for the mutant enzyme complexes indicates that the two phosphates are affected to a different extent by the replacements of Lys-59. Although it is theoretically impossible to obtain a stable enzyme−substrate complex, attempts were made to assign the ^{31}P resonances in the mutants by studying enzyme−PAPS complexes. In the ^{31}P NMR spectra of the wild-type enzyme and K59R in complex with PAPS, only one signal could be observed unambiguously, making the data very difficult to interpret (data not shown). The PAPS complexes might be unstable due to substrate hydrolysis. On the basis of the crystal structure of the mouse estrogen ST (*12*) and the results of affinity chromatography with cosubstrate analogues, we propose that the most affected signal in the mutant complexes corresponds to the 5′-phosphate group. However, since both ^{31}P signals are affected, the chemical environment of the 3′-phosphate group appears to be modified as well. From these results, Lys-59 appears to determine the proper positioning of PAPS at the active site for catalysis.

CONCLUSION

Affinity chromatography with PAPS analogues, using nonspecific elution with salt, allowed us to demonstrate that Lys-59 interacts with the 5′-phosphate of PAP, and Arg-141 with the 3′-phosphate, confirming the assignments deduced from the crystal structure of the mouse estrogen ST (Figure 1) (*12*). The consistency between the two types of data validates the affinity chromatography method a posteriori. This method could be useful in characterizing site-directed mutants for other classes of enzymes that bind nucleoside 3′,5′- or 2′,5′-diphosphates, especially in the absence of a known three-dimensional structure.

The results presented in this paper also provide new insight into the function of the Lys-59 residue. Although this residue interacts with the 5′-phosphate group of PAP, it does not contribute to the affinity for PAPS (*10*) or PAP. This is not surprising since high-affinity binding depends on interactions with the 3′-phosphate group, as revealed by the analysis of K_i for nucleotide inhibitors. The 2-fold increase in the affinity for PAPS of K59R (*10*) is probably mediated by a slight change in position that strengthens the interaction between the 3′-phosphate group of PAP and the side chains of Arg-141 and Arg-277. The affinity chromatography data suggesting that the arginine side chain is too bulky for a proper interaction in the 5′ portion of the ribose, as well as the perturbations of both signals in the ^{31}P NMR spectra of the K59R−PAP complex, support this conclusion.

The most plausible mechanism of sulfonate transfer catalyzed by STs is a direct transfer involving nucleophilic attack at the sulfur of PAPS (*10*, *12*). The reduced affinity of K59R for ADP−agarose suggests that Lys-59 might interact with the sulfate group of PAPS, in addition to the 5′-phosphate. In a novel crystal structure of the mouse estrogen ST in complex with PAP and the transition state analogue vanadate, the side chain nitrogen of the corresponding lysine interacts with one equatorial oxygen of the vanadium ion as well as with the bridging oxygen of the phosphosulfate group (*26*). The main chain nitrogen of the same residue also makes contact with an equatorial oxygen of the transferred group. This suggests two possible catalytic roles for Lys-59. By interacting with the sulfate, Lys-59 might stabilize the transition state. In analogy with enzyme-catalyzed phosphoryl transfer reactions, the interaction might assist nucleophilic attack by reducing the negative charge

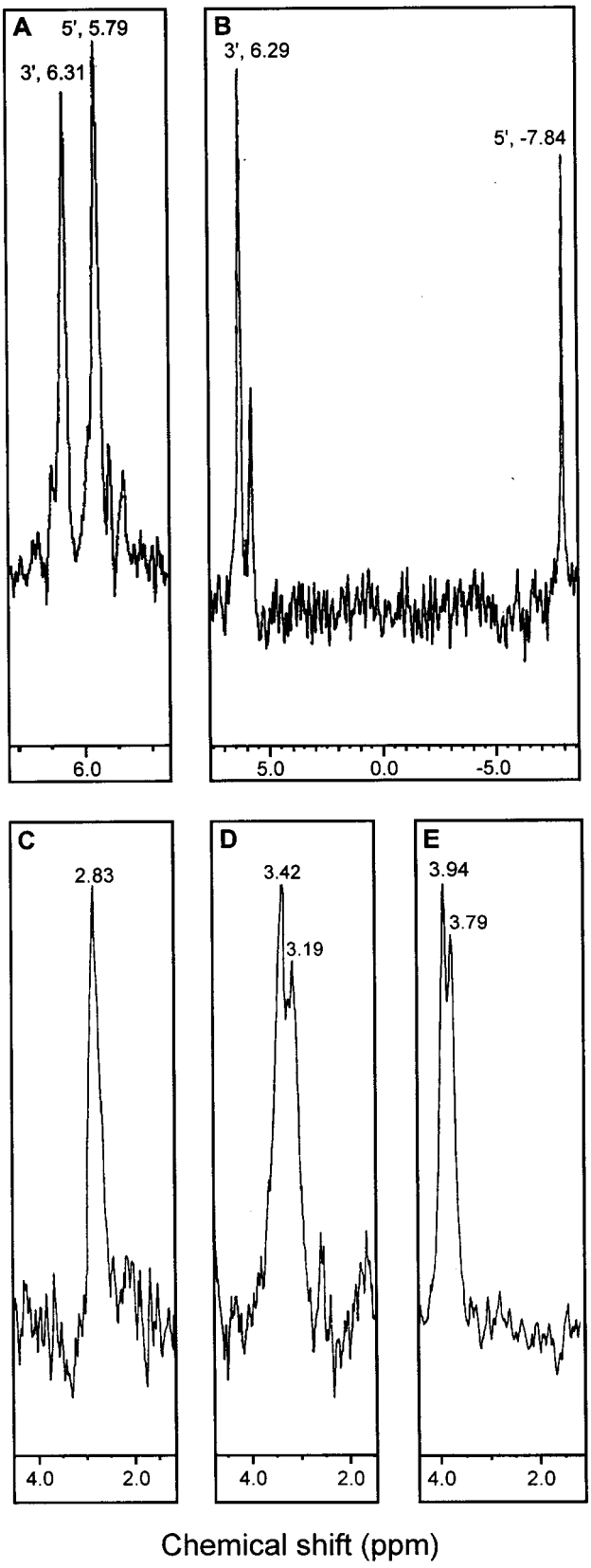

FIGURE 2: ^{31}P NMR spectra of (A) PAP, (B) a nonequimolar mixture of PAP and PAPS, (C) the wild-type enzyme−PAP complex, (D) the K59R−PAP complex, and (E) the K59A−PAP complex. The chemical shifts values (in parts per million) of the ^{31}P signals are indicated, with their assignments for spectra of the free nucleotides. The concentration of free nucleotides was 1 mM, 0.56 mM wild-type enzyme and 0.53 mM PAP, 0.58 mM K59R and 0.55 mM PAP, and 0.46 mM K59A and 0.44 mM PAP.

(*27*). An alternative function would be the stabilization of the developing negative charge on the bridging oxygen of the 5′-phosphate. From studies on the chemical hydrolysis and aminolysis of PAPS, it was proposed that in the transition state, the leaving group is highly polarized, possessing two almost fully formed negative charges (*28*). The catalytic function of Lys-59 might be similar to that of the invariant lysine present in the P-loop of adenylate kinases, which was proposed to accompany the transferred negative charge (*29*).

The ^{31}P NMR data for enzyme−PAP complexes suggest that altering interactions with the 5′-phosphate of PAP by mutating Lys-59 has an impact on 3′-phosphate binding. The direction of the shifts in ^{31}P signals observed for replacement with alanine or arginine is similar, indicating that the effects cannot be explained only by electrostatic interactions or steric factors. Lys-59 is probably required to determine a precise orientation of the phosphosulfate group for catalysis, and the 15-fold decrease in specific activity of the K59R mutant compared to that of the wild-type flavonol 3-ST (*10*) is probably due to the loss of optimal orientation.

ACKNOWLEDGMENT

We thank Maurice Boissinot for helpful discussions.

SUPPORTING INFORMATION AVAILABLE

Four figures showing Lineweaver−Burk plots and slope replots of the recombinant wild-type flavonol 3-ST inhibition by PAP, ADP, ATP, and 2′,5′-PAP and spectra of flavonol 3-ST−PAP, K59R−PAP, and K59A−PAP. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Mulder, G. J., and Jakoby, W. B. (1990) in *Conjugation reactions in drug metabolism* (Mulder, G. J., Ed.) pp 107−161, Taylor and Francis, New York.
2. Roy, A. K. (1992) *Proc. Soc. Exp. Biol. Med. 199*, 265−272.
3. Strott, C. A. (1996) *Endocr. Rev. 17*, 670−697.
4. Luu-The, V., Bernier, F., and Dufort, I. (1996) *J. Endocrinol. 150*, S87−S97.
5. Visser, T. J. (1994) *Chem.-Biol. Interact. 92*, 293−303.
6. Roth, J. A. (1986) *Trends Pharmacol. Sci. 7*, 404−407.
7. Varin, L., DeLuca, V., Ibrahim, R. K., and Brisson, N. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1286−1290.
8. Weinshilboum, R. M., and Otterness, D. M. (1994) *Handb. Exp. Pharmacol. 112*, 45−78.
9. Marsolais, F., and Varin, L. (1997) *Eur. J. Biochem. 247*, 1056−1062.
10. Marsolais, F., and Varin, L. (1995) *J. Biol. Chem. 270*, 30458−30463.
11. Homma, H., Ogawa, K., Hirono, K., Morioka, Y., Hirota, M., Tanahashi, I., and Matsui, M. (1996) *Biochim. Biophys. Acta 1296*, 159−166.
12. Kakuta, Y., Pedersen, L. G., Carter, C. W., Negishi, M., and Pedersen, L. C. (1997) *Nat. Struct. Biol. 4*, 904−908.
13. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
14. Varin, L., Barron, D., and Ibrahim, R. K. (1987) *Anal. Biochem. 161*, 176−180.
15. Varin, L., and Ibrahim, R. K. (1992) *J. Biol. Chem. 267*, 1858−1863.
16. Devereux, J., Haeberli, P., and Smithies, O. (1984) *Nucleic Acids Res. 12*, 387−395.
17. Rikke, B. A., and Roy, A. K. (1996) *Biochim. Biophys. Acta 1307*, 331−338.
18. Barnes, S., Buchina, E. S., King, R. J., McBurnett, T., and Taylor, K. B. (1989) *J. Lipid Res. 30*, 529−540.

19. Falany, C. N., Vazquez, M. E., Heroux, J. A., and Roth, J. A. (1990) *Arch. Biochem. Biophys. 278*, 312−318.
20. Driscoll, W. J., Martin, B. M., Chen, H. C., and Strott, C. A. (1993) *J. Biol. Chem. 268*, 23496−23503.
21. Rens-Damiano, S. S., and Roth, J. A. (1987) *J. Neurochem. 48*, 1411−1415.
22. Segel, I. H. (1975) *Enzyme kinetics*, pp 107−109, John Wiley and Sons, New York.
23. Byeon, I.-J. L., Shi, Z., and Tsai, M.-D. (1995) *Biochemistry 34*, 3172−3182.
24. Lee, C.-H., and Sarma, R. H. (1975) *J. Am. Chem. Soc. 97*, 1225−1236.
25. Barrio, J. R., Barrio, M. d. C. G., Leonard, N. J., England, T. E., and Uhlenbeck, O. C. (1978) *Biochemistry 17*, 2077−2081.
26. Kakuta, Y., Petrotchenko, E. V., Pedersen, L. C., and Negishi, M. (1998) *J. Biol. Chem. 273*, 27325−27330.
27. Cullis, P. M. (1987) in *Enzyme Mechanisms* (Page, M. I., and Williams, A., Ed.) pp 218−220, The Royal Society of Chemistry, London.
28. Bedford, C. T., Kirby, A. J., Logan, C. J., and Drummond, J. N. (1995) *Bioorg. Med. Chem. 3*, 167−172.
29. Abele, U., and Schulz, G. E. (1995) *Protein Sci. 4*, 1262−1271.

BI982239M