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Nucleotide Binding and Sulfation Catalyzed by Phenol Sulfotransferase

En-Shyh Lin and Yuh-Shyong Yang[1]
Department of Biological Science and Technology, College of Science, National Chiao Tung University, Hsinchu, Taiwan, Republic of China

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The sulfation of a nucleotide is an indispensable step for the sulfuryl group transfer in a biological system. The product and cosubstrate of sulfotransferase in physiological condition are adenosine 3′,5′-bisphosphate (PAP) and 3′-phospho adenosine 5′-phosphosulfate (PAPS), respectively. We find that ribose and adenine, two major parts of the adenosine nucleotide, bind tightly to phenol sulfotransferase (PST) separately, and various nucleotides also bind tightly to PST. We determine the dissociation constants of a variety of nucleotides and examine their potential as cofactors or cosubstrates of PST. Using 4-nitrophenyl sulfate as the sulfuryl group donor, three nucleotides, adenosine 5′-monophosphate (AMP), adenosine 2′,5′-bisphosphate (2′,5′-PAP), and adenosine 2′:3′-cyclic phosphate 5′-phosphate (2′:3′-cyclic PAP), are shown here for the first time to be sulfated at 5′-phopho position by a PST catalyzed reaction. Spectrophotometry, HPLC, and ^{31}P NMR are used to determine the activity of PST and identify the sulfated nucleotides. The V_{max} of PST and K_m of these nucleotides are determined when they are used as cofactors or cosubstrates for the sulfuryl group transfer. The existence and possible physiological significance of these newly reported binding and sulfation of nucleotides by PST in biology is yet to be discovered.

It has been known for a long time that sulfation occurs in a biological system (1, 2). Compounds that involve sulfation and the hydrolysis of sulfate esters include drugs, carcinogens, other xenobiotics (3, 4), hormones (5), bile acids (6), neurotransmitters (7), glycoproteins, glycosaminoglycans, and saccharides (8). Unlike a phosphorylation reaction (9), the mechanism of an enzymatic sulfuryl group transfer has yet to be understood (10, 11). However, the resolution of crystal structures of three sulfotransferases (12, 13, 14) produces important advances for our understanding in the reaction of these enzymes.

A sulfated nucleotide, 3′-phospho adenosine 5′-phosphosulfate (PAPS), is used for physiological sulfation which is catalyzed by sulfotransferases (EC 2.8.2). As shown in Eq. 1, sulfotransferases are responsible for all the known sulfuryl group transfer reactions (3). As shown in Reaction 2, sulfotransferase with the catalytic amount of PAP can carry out a two-step sulfuryl group transfer (10, 15, 16). Phenol sulfotransferase catalyzes both reactions (15), with the activity in the physiological direction (Reaction 1) almost 10-fold slower than that in the transfer reaction (Reaction 2). The binding of PAP (17) and oxidation/reduction of cysteine (18) (which in turn affect the PAP binding) may be the reason for the rate difference of these two reactions.

$$\text{PAPS} + \text{HO-R} \rightarrow \text{PAP} + \text{R-OSO}_3^- \qquad [1]$$

$$\text{R-OSO}_3^- + \text{R′-OH} \rightarrow \text{R-OH} + \text{R′-OSO}_3^- \qquad [2]$$

The sulfation of nucleotides other than PAP catalyzed by sulfotransferase has not been reported before. A recently published procedure (16) for the measurement of the trace amount of PAP was found to be very useful for the initial screening of other potential cofactors for a sulfuryl group transfer as in Reaction 2. Following the initial screening, we are able to confirm, by HPLC and NMR, that three other adenine nucleotides can also be cofactors or substrates of PST.

Abbreviations used: PAPS, 3′-phospho adenosine 5′-phosphosulfate; PAP, 3′-phospho adenosine 5′-phosphate or adenosine 3′, 5′-bisphosphate; 2′, 5′-PAP, adenosine 2′, 5′-bisphosphate; 2′:3′-cyclic PAP, adenosine 2′:3′-cyclic phosphate 5′-phosphate; AMP, adenosine 5′-monophosphate; ADP, adenosine 5′-diphosphate; pNPS, 4-nitrophenyl sulfate; pNP, 4-nitrophenol; PST, phenol sulfotransferase.

[1] To whom correspondence should be addressed at Department of Biological Science and Technology, National Chiao Tung University, 75 Po-Ai Street, Hsinchu, Taiwan, ROC. Fax: 886-3-5729288. E-mail: ysyang@cc.nctu.edu.tw.

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MATERIALS AND METHODS

Chemicals. PAP[1], PAPS, 2′,5′-PAP, 2′:3′-cyclic PAP, adenosine, adenine, D-ribose 5′-phosphate, AMP, ADP, guanosine 5′-monophosphate, and other nucleotide analogs were purchased from Sigma; 4-nitrophenyl sulfate (pNPS) was obtained from Merck. Some of the commercial pNPS contained a higher amount of 4-nitrophenol (pNP) contamination (to about 1%) and was avoided. DEAE-sephacel was obtained from Pharmacia. All other chemicals were obtained commercially at the highest purity possible.

Preparation and purification of PAP free phenol sulfotransferase. Recombinant phenol sulfotransferase was purified as described earlier (19) and modified latter (16). The β-form of PST, which is PAP free, was used for this study. The purity of the recombinant PST was more than 95% based on SDS–PAGE and contained less than 1% of PAP (based on the standard enzyme assay with or without PAP as described below).

Enzyme assay. Enzyme activity was determined by the change of absorbency at 400 nm due to pNP (ϵ = 10,500 $cm^{-1}M^{-1}$ at pH 7.0) as described previously (15). By the standard assay, a unit of enzyme activity represents the amount required to catalyze the formation of 1 μmol of 4-nitrophenol per min. Specific activity is given in units per mg of protein. Protein concentration of homogeneous forms of PST was estimated on the basis of absorbency at 280 nm (ϵ = 58,350 $M^{-1}cm^{-1}$ or 1.7 ml/mg cm^{-1}) (20). The amount of enzyme bound PAP was determined by the standard assay in the presence and absence of PAP (16).

Nucleotides as cofactors of PST. The reaction condition for the screening of nucleotides (as a cofactor of sulfuryl group transfer catalyzed by PST) was the same as that of the standard assay except that PAP was replaced by other nucleotides.

Nucleotides as substrates of phenol sulfotransferase. PAP or other nucleotides were incubated with pNPS (1 mM), mercaptoethanol (5 mM), and Bis-tris propane (100 mM) at pH 7, then PST was added to start the reaction. Enzyme activity was determined by the change of absorbency at 400 nm due to pNP (ϵ = 10,500 $cm^{-1}M^{-1}$ at pH 7.0). A unit of enzyme activity of reverse physiological reaction represents the amount required to catalyze the formation of 1 μmol of 4-nitrophenol per min by the this assay. Specific activity is given in units per mg of protein.

Concentration of PAP and other nucleotides. The concentrations of nucleotides, adenosine, and for guanosine were determined by their absorption at 260 nm, ϵ = 15,100 $M^{-1}cm^{-1}$ and at 276 nm, ϵ = 9000 $M^{-1}cm^{-1}$, respectively.

Dissociation constants of nucleotides and PST determined by fluorescence spectrophotometer. An aliquot amount of nucleotides was added into the solution containing PST (0.16 to 6.5 μM), 50 mM potassium phosphate at pH 7.0, and 2 mM 2-mercaptoethanol with a final volume of 2 ml in a quartz cuvettes of 1 cm square cross-section. The decrease in intrinsic fluorescence of protein was measured at 340 nm upon excitation at 280 nm and 25°C with a Hitachi Spectrofluorimeter (F-4500). Each data point was duplicated, and the difference was within 10%. An aliquot amount of nucleotides was added to a predetermined concentration of PST to obtain at least 10 data points (ΔF) for each K_d. The dissociation constant was obtained by the equation: $\Delta F = \Delta F_{max} - K_d(\Delta F/[\text{nucleotide}])$.

NMR (^{31}P) spectra of nucleotides. A Bruker MSL-300 NMR Spectrometer was used to determine a ^{31}P NMR spectra of nucleotides. Deuterium from a D_2O solvent was used for field/frequency stabilization. Phosphoric acid (85%) in a concentric capillary (2 mm O.D.) was used as a primary ^{31}P reference. The ^{31}P NMR spectra of commercially available nucleotides were obtained in the solution which contains 1 mM K_3PO_4, 10 mM bis-tris propane at pH 7.0, and 1 mM nucleotides, in a final volume of 0.6 ml. After sulfation catalyzed by PST, the spectra of nucleotides were obtained in the solution which initially include 2 mM PAP or other nucleotides, 1.3 μM PST, 50 mM mercaptoethanol, 10 mM pNPS, 0.1 mM K_3PO_4, and 50 mM Bis-tris propane at pH 7.

HPLC analysis of nucleotides. Nucleotides were separated with a 5 μm (250 mm) prepacked LiChrospher 100 RP-18 column (Merck) and were detected at 260 nm with a UV-Vis detector using a D-7000 HPLC system (Hitachi). Separation was achieved in an isocratic condition which contains 100 mM tetra-n-butyl-ammonium hydrogen sulfate (TBHS) and 0.1 M phosphate at pH 5.8 (21) at a flow rate of 0.9 ml/min.

TABLE 1

Dissociation Constants of Nucleotides and Sulfotransferase[a]

	Kd1 (mM)[b]	Kd2 (μM)[c]
PAP	31 ± 4[d]	152 ± 1
2′,5′-PAP	119 ± 7	134 ± 1
2′,3′-cyclic PAP	15.9 ± 0.1	136 ± 1
Adenosine	50 ± 0.3	225.6 ± 0.5
AMP	9.2 ± 0.3	164 ± 4
Adenosine 5′-monosulfate	30 ± 1	220 ± 1
Adenosine 5′-phosphoramide	77 ± 4	188 ± 1
ADP	38 ± 1	75.0 ± 0.3
ATP	79 ± 1	122 ± 1
Adenosine 5′-tetraphosphate	51 ± 1	116 ± 2
GMP	65 ± 1	50 ± 1
D-Ribose	284 ± 16	2460 ± 30
D-Ribose 5′-phosphate	13 ± 2	4020 ± 40
Adenine	75.4 ± 0.1	417 ± 0.5

[a] Dissociation constants were determined by fluorescence as described under Materials and Methods.
[b] The β form of PST (60 nM enzyme dimer) was used.
[c] The β form of PST (6.5 μM enzyme dimer) was used.
[d] Equation $\Delta F = \Delta F_{max} - K_d(\Delta F/[\text{nucleotide}])$ was used to calculate the dissociation constants. The range of error is Kd*(1 − R^2), while R^2 is the correlation constant.

RESULTS

Two distinct dissociation constants for nucleotides and phenol sulfotransferase were determined and are shown in Table 1. Phosphates at the 2′, 3′, or 5′ position (compare PAP, 2′, 5′-PAP, AMP, ADP, ATP, and adenosine) of the nucleotides do not affect their binding with enzyme (both Kd_1 and Kd_2 do not alter significantly). As indicated by the first dissociation constants (listed in the lower section of Table 1), only part of the nucleotide (adenine, D-ribose or D-ribose 5′-phosphat alone) is enough to bind to PST tightly. Different nucleotides, such as GMP, also bind to PST tightly. As shown in the lower section of Table 1 (D-ribose and D-ribose 5′-phosphate), the base is important for binding with PST and thus removal of adenine significantly affects the second dissociation constant.

All the compounds listed in Table 1 were tested as cofactors for the sulfuryl group transfer between pNPS and β-naphthol. The positive ones are listed in Table 2, and their K_m and V_{max} were determined (Table 3). Un-

TABLE 2

Nucleotides as Cofactors of Phenol Sulfotransferase[a]

pNPS + β-naphthol → pNP + β-naphthyl sulfate (1) + (2)
pNPS + nucleotide → pNP + sulfated nucleotide (1)
Sulfated nucleotide + β-naphthol → nucleotide + β-naphthyl sulfate (2)

Cofactors	K_m (μM)[b]	V_{max} (nmole/min mg)[b]
PAP	0.4 ± 0.1	270 ± 30
2′,3′-cyclic PAP	2.3 ± 0.1	182 ± 4
2′,5′-PAP	67 ± 6	116 ± 5
AMP	4300 ± 400	144 ± 4

[a] The activity measured is the combined reactions of (1) and (2) as shown above.

[b] The data was obtained by non-linear regression from the initial rates determined as described under Materials and Methods.

like the K_ds of various nucleotides listed in Table 1, which are all in the same order of magnitude, K_ms of the four nucleotides listed in Table 2 varied up to 10,000-fold. V_{max}s of PST remain relatively constant with different nucleotides as the cofactor.

Positive result of the transfer reaction implies that nucleotides listed in Table 2 must be sulfated. The sulfated nucleotides were identified by HPLC and ^{31}P NMR as shown in Figs. 1 and 2. As shown in Fig. 1A, an extra peak which was identical to PAPS (obtained commercially, data not shown) was observed using pNPS as the sulfuryl group donor and PAP as the sulfuryl group acceptor (in the absence of β-naphthol). Three new peaks were also detected when PAP was replaced by 2′,5′-PAP (Fig. 1B), AMP (Fig. 1C), or 2′:3′cyclic,5′-PAP (Fig. 1D); and, were assigned as 2′,5′-PAPS, APS and 2′:3′cyclic,5′-PAPS, respectively. Under the conditions described under Materials and Methods, less charged compounds move faster (less retention time). The elution times shown in Fig. 1 turned out as expected, i.e., after being incubated with pNPS and PST (as shown in Fig. 1), the sulfated nucleotides appeared after the nucleotides. Commercially available compounds, PAP, PAPS, 2′,5′-PAP, AMP, 2′:3′cyclic,5′-PAP were used as markers to identify the peaks appeared in Fig. 1 (data not shown). Peaks eluted around 5 min (labeled as E in Fig. 1) are due to the buffer and compounds used for enzymatic reaction.

As shown in Fig. 2, sulfation of adenine nucleotides at 5′-phospho position was confirmed by ^{31}P NMR spectra of the sulfated nucleotides. Figure 2A showed the ^{31}P chemical shifts of PAPS with a small contamination of PAP. Chemical shifts at 2′-, 3′-, 5′-, and 3′:5′cyclic phosphates were determined by commercially available compounds (PAP, 2′,5′-PAP, AMP, and 2′:3′cyclic,5′-PAP). In addition to the increase of a unique chemical shift at −10 ppm for 5′-phosphosulfate (Figs. 2B, 2C, 2D, and 2E), the progress of nucleotide sulfation also showed the decrease of chemical shift at 4.2 ppm for 5′-phosphate (data not shown). Phosphate peaks at 3 ppm in Figs. 2B, 2C, and 2D were added exogenous after sulfation and used as internal standard.

The K_m and V_{max} of the sulfation of nucleotides catalyzed by PST were determined and listed in Table 2. Similar to that of the transfer reaction (nucleotides were used as cofactor), V_{max}s of PST remained to be relatively stable while K_m of different nucleotides varied up to more than 1000-fold. The lack of a phospho group at 2′- or 3′-position in adenosine nucleotide resulted in a significant increase of K_m for both the transfer and reverse physiological reactions.

DISCUSSION

The interaction of nucleotides with sulfotransferase is an important phenomenon for the study of this family of enzymes. AMP-, ATP-, and PAP-agarose have been used successfully as affinity chromatographies for the purification of sulfotransferase (22, 23). Recently, ADP-, ATP-, and 2′,5′-PAP-agarose were used for the analysis of flavonao 3-sulfotransferase and its mutants (24). However, binding of different nucleotides with sulfotransferase has not been well characterized. Dissociation constants listed in Table 1 indicate that even D-ribose or adenosine, part of the nucleotide, tightly binds to phenol sulfotransferase at the first binding

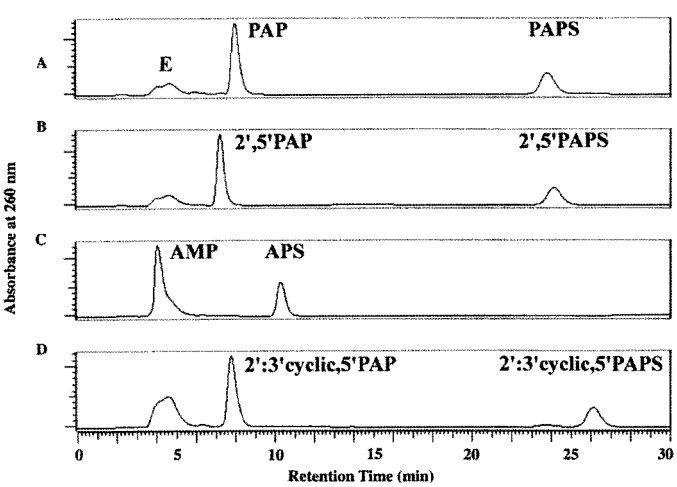

FIG. 1. HPLC analysis of nucleotide sulfation catalyzed by phenol sulfotransferase. (A) Sulfation of 3′-phospho adenosine 5′-phosphate. (B) Sulfation of 2′-phospho adenosine 5′-phosphate. (C) Sulfation of adenosine 5′-monophosphate. (D) Sulfation of adenosine 2′,3′-cyclic phosphate 5′-phosphate. The reaction mixture included nucleotide (200 μM), pNPS (1 mM), PST (0.25 mg for reactions A–C and 1 mg for reaction D, β form), and bistris propane (100 mM) at pH 7 was incubated at 25°C for 2 h. The procedure for HPLC analysis of the nucleotides and sulfated nucleotides is described under Materials and Methods. The controls of the above reactions, i.e., in the absence of PST, pNPS, and nucleotides, respectively, were conducted (data not shown) to ensure that the observed sulfated nucleotides were the products of the expected reactions.

sites. Several other nucleotides, such as uridine 5′-monophosphate (data not shown) and guanosine 5′-monophosphate, also tightly bind to phenol sulfotransferase at similar dissociation constants. Variations of phosphate at 2′, 3′, or 5′ positions of a sugar ring do not affect the dissociation constants of adenine nucleotide with PST. These observations explain why different nucleotides can be used as functional parts of affinity chromatographies for the purification of sulfotransferase; they also suggest that other type of affinity chromatography can be developed for the purification and analysis of sulfotransferases. We concluded that both the ribose and the base (adenine or another base) might bind to the enzyme independently. Adenine is especially important for the binding of the nucleotide to the second binding site of phenol sulfotransferase. Phosphate that is at the 2′, 3′, or 5′ position of the adenine nucleotide is not important in binding with PST; however, the efficiency of nucleotide sulfation is dependent on the presence of adenine and 3′-phosphate as will be discussed later.

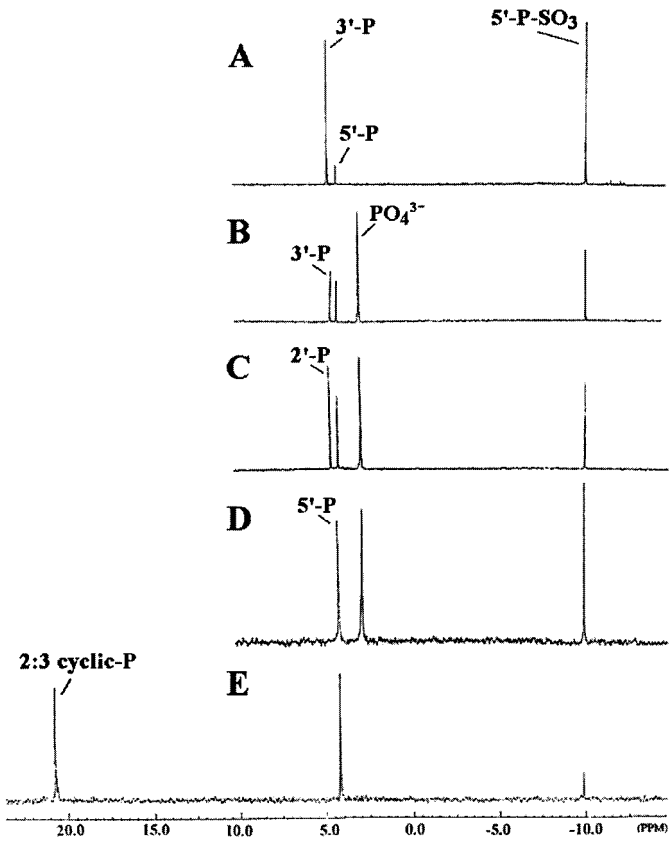

FIG. 2. ^{31}P NMR analysis of the sulfation of nucleotides catalyzed by phenol sulfotransferase. (A) 3′-phospho adenosine 5′-phosphosulfate. (B) Sulfation of 3′-phospho adenosine 5′-phosphate. (C) Sulfation of 2′-phospho adenosine 5′-phosphate. (D) Sulfation of adenosine 5′-monophosphate. (E) Sulfation of adenosine 2′,3′-cyclic phosphate 5′-phosphate. The reaction mixture included nucleotide (2 mM), pNPS (10 mM), phenol sulfotransferase (0.08 mg for reactions B–D and 0.4 mg for reaction E, β form), and bistris propane (50 mM) at pH 7 was incubated at 25 C for 2 h. Exogenous K_3PO_4 (0.1 mM) was added into reactions B–D. The procedure for obtaining ^{31}P NMR spectra of the nucleotides and sulfated nucleotides is described under Materials and Methods. Controls of the above reactions, i.e., in the absence of PST, pNPS, and nucleotides, respectively, were conducted (data not shown) to ensure that the observed sulfated nucleotides were the products of the expected reactions.

TABLE 3

Nucleotides as Substrates of Phenol Sulfotransferase[a]

pNPS + nucleotide → pNP + sulfated nucleotide

Substrates	K_m (μM)	V_{max} (nmole/min mg)
PAP	0.9 ± 0.5	30 ± 3
2′,3′-cyclic PAP	58 ± 20	42 ± 4
2′,5′-PAP	77 ± 12	56 ± 3
AMP	1280 ± 200	51 ± 2

[a] The data were obtained by nonlinear regression from the initial rates determined as described under Materials and Methods.

Two types of activated sulfate, APS and PAPS, are used for PAPS synthase and sulfotransferase, respectively; but it has not been reported that PST utilizes nucleotides other than PAP and PAPS. For all the known sulfotransferases, while the substrate spectrum of PST is very wide on the phenol part (25), the specificity for the nucleotide is generally thought to be limited to only PAP or PAPS. Herein, the sulfation of nucleotides which is catalyzed by PST is clearly demonstrated by the results of a HPLC and ^{31}P NMR analysis (Figs. 1 and 2). PST appears to be very useful for the screening of nucleotides for the activation of a sulfate.

It is interesting to compare the Km, Kd, and the concentration of PAP and AMP in the cell. The concentration of PAP/PAPS in the cell is in μM range (16). However, in most cells, the total concentration of adenosine phosphates (AMP, ADP, or ATP) is usually in the range of 2 to 10 mM (26). This indicates that AMP can be just as effective as a substrate or cofactor as PAP in a physiological condition for PST usage. Kds listed in Table 1 indicate that phenol sulfotransferase may be tightly bound to many nucleotides *in vivo*. It is known that the binding of PAP induces a conformational change of phenol sulfotransferase (15), and a ternary complex of E-PAP-phenols has been proposed as the inhibitory pathway for a physiological reaction of PST. The effect of nucleotides (other than PAP) on the enzyme conformation and inhibition is now under investigation.

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