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酚亞硫酸基轉移酵素催化機制之研究與修改(1/2)

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中文摘要

關鍵詞：亞硫酸基轉移酵素；分子模擬；定點突變； 基因選殖與表達；酵素反應機制。

亞硫酸基轉移酵催化亞硫酸基轉移至生物系統中各種親和受體的反應。生物體中藉由硫酸化來進行調控的物質有蛋白質、醣蛋白、醣類、荷爾蒙、神經傳導物質及包括藥物及致癌物等化合物；因此，亞硫酸基轉移參與生物訊息之分辨與傳遞、荷爾蒙之調節、解毒作用及調節神經傳 導物質等重要生物反應。然而我們對於此酵素反應機制的了解卻非常有限。針對酚亞硫酸基轉移酵素催化反應管制與機制之研究，我們預計進行的相關實驗包括：核酸與酵素之親和性標記，酵素結構之分子模擬，氧化還 原對於此酵素生理與轉移反應之不同影響，決定此酵素之雙硫鍵位置，及定點突變以改變酵素特性。上述各種實驗方法，將可探討此酵素如何應用其結構上的變化來催化與調控亞硫酸基轉移反應，並進一步設計出使用不同核酸的新酵素。目前 我們的研究發現亞硫酸基轉移酵素中有一形態不穩定區(主要包括 63-Leu-Glu-Lys-Cys-Gly-Arg-68)，與核酸之催化反應無直接相關，但可被核酸與酵素之親和性標記。由分子模擬看出，此形態不穩定區位於核酸進出口，而定點突變此區的氨基酸後，嚴重影響到核酸與酵素的結合與釋放。其中 C66 形成酵素內之雙硫鍵後，生理反應活性 (Vmax)逐漸增加，亞硫酸基轉移反應(非生理反應)則僅 Km 增加。據此，我們可設計出抗氧化之酚亞硫酸基轉移酵素。酵素分子模擬顯示 C66，C82 及 C232 這三個胺基酸位置相當接近，可形成雙硫鍵；此外，K65 與 R68 經過定點突變後，也合乎根據酵素與反應物結合之三度空間立體結構所提出之反應機制。目前我們已建立催化亞硫酸基轉移機制及其管制的模式。

Keywords: Sulfotransferase, molecular modeling, site-directed mutagenesis, gene expression, enzyme mechanism.

Sulfotransferase catalyzes sulfuryl group transfer between nucleotide and a variety of nucleophiles that may be sugar, protein, xenobiotics and other small molecules. Nucleotides may serve as cosubstrate, cofactor, inhibitor or regulator in an enzyme catalyzed sulfuryl group transfer reaction. We are trying to understand how nucleotide regulates the activity of phenol sulfotransferase(PST) through the expression of two enzyme forms. The homogeneous rat recombinant PST was obtained from *E. coli* and the nucleotide copurified was examined. The nucleotide was completely removed from inactive PST in high salt and oxidative condition. Total enzyme activity was recovered following incubation in reductive environment. Many nucleotides are known to tightly bind to PST but only one nucleotide, 3'-phosphoadenosine 5'-phosphate (PAP), was identified to combine with PST by ion-pair RP-HPLC,

UV-visible spectra, ^{31}P NMR and ESI-MS and MS-MS spectrometry. In addition to the presence or absence of PAP, oxidation following reduction of PST was required to completely inter-convert the two forms of PST. According to the experimental results, a mechanism for the formation of the two enzyme forms was proposed.

成果報告內容

The following report funded by NSC 91-2311-B-009-002 is to be published in the June 24, 2003 issuse of *Biochemistry* (24).

Mechanism of Post-Translational Regulation of Phenol Sulfotransferase: Expression of Two Enzyme Forms through Redox Modification and Nucleotide Binding[†]

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Abbreviations used: Bis-tris propane, 1,3-bis[tris(hydroxymethyl) methylamino] propane; CD, circular dichroism; DTT, dithiothreitol; EDTA, (ethylenedinitrilo) tetraacetic acid; ESI-MS, electrospray ionization mass spectrometry; FAD, Flavin adenine dinucleotide; GSH, glutathione, reduced form; GSSG, glutathione, oxidized form; MS-MS, collision-induced dissociation tandem mass spectrometry; *m/z*, mass-to-charge ratio; β-NAD, β-nicotinamide adenine dinucleotide; β-NADH, β-nicotinamide adenine dinucleotide, reduced; NADP, nicotinamide adenine dinucleotide 3'-phosphate; NADPH, nicotinamide adenine dinucleotide 3'-phosphate, reduced; PAP, 3'-phosphoadenosine 5'-phosphate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; PMSF, phenylmethylsulfonyl fluoride; PNP, 4-nitrophenol; PNPS, 4-nitrophenyl sulfate; SDS, sodium dodecyl sulfate; TCEP, Tris(2-carboxyethyl) phosphine; Tris-HCl, tris(hydroxymethyl) aminomethane hydrochloride

It has been known for a long time that sulfation occurs in a biological system (*1*). Sulfotransferases (EC 2.8.2), which catalyze sulfuryl group transfer between nucleotide and a variety of nucleophiles, are responsible for all the known biological sulfation. The nucleotide, 3'-phosphoadenosine-5'-phosphosulfate (PAPS), is a biologically active form of inorganic sulfate that serves as the sulfate donor in various biological processes (*2*). Macromolecular substrates (such as proteins (*3*) and polysaccharides (*4*)) are metabolized by membrane-bound sulfotransferases. Small molecules, like xenobiotics (*5*) or endogenous compounds (such as hormones (*6*) and neurotransmitters (*7*) are metabolized by cytosolic enzymes. Sulfotransferases have been classified into several subfamilies according to the degree of the similarities of the deduced amino acid sequences (*8, 9*) or substrate specificities (*10*).

The regulation of the activity of sulfotransferase has not been well studied. Four phenol sulfotransferases (PST) have been separated and purified from rat liver (*11, 12*). PST has been cloned and expressed in *E. coli* (*13*). During the growth of the bacterial cells, the α- and β-forms of the sulfotransferase, are produced and both active. The ratio of two forms PST express is dependent on cell culture condition (*14*). The two forms are separable from each other by hydroxyapatite (*13*) and PAP-agarose chromatography (*15*). It has been shown that the interaction of a nucleotide with PST is important for the formation of the two different enzyme forms, but is not the only factor (*16*). Both forms of the recombinant enzyme are similar when compared by circular dichroism (CD) spectroscopy below 240 nm. Above 240 nm, the maximal difference is at about 260 nm with additional peaks at 280 and 290 nm. Addition of PAP with β-form PST gives similar CD spectrum to that of α-form PST (*16*).

The postulated nucleotide that pre-exist in α-form PST before purification has never been chemically and biochemically identified. The activity of β-form PST shows a hyperbolic dependence on PAP concentration in the nanomolar range, whereas the activity of α-form is not stimulated by the addition of nucleotide (*16*). The two forms of PST can also be distinguished by the PST assays and separated by chromatography (*13, 16*). PST activity can be measured by the physiological or the transfer reactions. The former reaction represents the transfer of sulfuryl group from PAPS to another co-substrate. The transfer reaction is the two-stage transfer of the sulfuryl group, for example, from PNPS to PAP and the subsequent transfers from PAPS to 2-naphthol (*16*). Only β-form PST is active toward both the physiological and the transfer reaction. The α-form PST is found unable to catalyze the physiological reaction, which requires the release of PAP to complete a turnover of a sulfuryl group transfer (*16*). Routine analysis of the activity of sulfotransferase generally measures only the physiological activity (*11, 12*) catalyzed by β-form PST and the α-form activity was mostly ignored (*15, 16*). The role of the two enzyme

forms in physiological condition is yet to be studied.

Complete conversion of the two enzyme forms has not been achieved previously *in vitro*. Part of the physiological activity (catalyzed only with β-form PST) can be generated following partial oxidation of α-form PST with glutathione, oxidized form (GSSG). However, prolong incubation with GSSG resulted in the loss of PST activity and incubation of PAP with β-form PST did not give complete α-form (*16, 17*). Factors involved in the transformation of the two forms of PST are the subject of this study. There are many nucleotides that bind tightly to PST. Some of the dissociation constants are in the nanomolar range (*15*). In addition to PAP, several other nucleotides are also found to be the cofactors or substrates of PST (*15*). PAP analogs, coenzyme A and its derivatives, are strong inhibitors for PST (*18, 19*). Cells contain numerous nucleotides. It is intriguing to learn what is the nucleotide irreversibly attached to PST and what is the mechanism responsible for the formation of the two PST forms from the expression of a single cDNA. Here we present a series of chemical and biochemical methods that identify the naturally produced nucleotide and study its effect on the formation of two PST forms.

EXPERIMENTAL PROCEDURES

Materials— 4-nitrophenyl sulfate (PNPS), 4-nitrophenol (PNP), 3'-phosphoadenosine 5'-phosphate agarose gel (PAP-agarose), 3'-phosphoadenosine 5'-phosphate (adenosine 3', 5'-diphosphate; PAP) and other nucleotide analogs were purchased from Sigma (USA). Tris(2-carboxyethyl) phosphine (TCEP) was obtained from Pierce (USA). DEAE sepharose fast flow and HiTrap desalting column were obtained from Amersham pharmacia biotech Asia pacific (Hong Kong). Hydroxyapatite ultrogel® was purchase from Biosepra (USA). All other chemicals were obtained commercially at the highest purity possible.

Enzyme assay— PST activity was determined by the change of absorbency at 400nm due to 4-nitrophenol (ε=10,500 $cm^{-1}M^{-1}$ at pH 7.0) as described previously (*16*). Standard assay is a transfer reaction that consists of 2-mercaptoethanol (5 mM), 4-nitrophenyl sulfate (5 mM), 2-naphthol (50 μM), PAP (2 μM) and bis-tris propane (100 mM at pH 7.0). The α-form activity was determined in the absent of PAP. The β-form activity was the difference between standard assay and α-form activities. Protein concentration of homogeneous form of PST was estimated on the basis of absorbency at 280 nm (ε=58,350 $cm^{-1}M^{-1}$ or 1.7 ml/mg cm^{-1}) (*20*).

Preparation of nucleotide from homogeneous PST— Recombinant rat phenol sulfotransferase was cloned into expression vector pET3c and transformed into *Escherichia coli* BL21 (DE3) PST was extracted by sonication, and purified by DEAE and hydroxyapatite liquid chromatography. Detailed methods were described previously (*13, 16*) except that a new hydroxyapatite chromatography (Hydroxyapatite ultrogel® from Biosepra) was used The purity of PST used for this study was examined by SDS polyacrylamide gel electrophoresis to be a single band and the purity was estimated over 95% or higher. Extra high purity PST was used for the extraction of nucleotide to prevent any possible contamination. Purified PST was further passed through a HiTrap column with desalting buffer (100 mM bis-tris propane at pH 7.0, 1 mM EDTA, 10% glycerol (v/v), and 125 mM sucrose) to remove

any unbound small molecules. Active fractions were pooled (a typical preparation contained 16 mg or 2.3×10^{-7} mole PST dimer; including 66% α-form) and mixed with 70% ethanol. The denatured and precipitated PST was removed by centrifugation. The supernatant was collected and concentrated with vacuum dryer (EZ550R, FTS system, USA). According to A_{260} (ε=15.1 $cm^{-1}mM^{-1}$ for adenine) (*21*), 1.4×10^{-7} mole of adenine nucleotide was recovered. The yield was 92% calculated base on one nucleotide per α-form PST dimer (*16*).

Separation of α- and β-form PST— The two forms of PST were separated by PAP-agarose chromatography as described previously (*15*) (with the following modification. The gel was incubated overnight with capture buffer which included DTT (1 mM), sucrose (125 mM), glycerol (10%), 2-mercaptoethanol (14 mM), EDTA (1 mM) and phosphate (25 mM at pH 6.8). Capture buffer (5-fold column volume) was used to equilibrate column. Unbound protein (α-form PST) was washed out with 10-fold column volume capture buffer, and bound PST (β-form PST) was eluted with capture buffer containing NaCl (0.3 M). This preparation of β-form PST was used as control to extract adenine nucleotide as described above and the existence of nucleotide was not found.

Ion-pair RP-HPLC analysis of nucleotides— Nucleotides were separated with a 5 μm (250mm) prepacked LiChrospher 100 RP-18 column (Merck, USA) and were monitored at 260nm with a UV-visible detector using a L-2000 HPLC system (Hitachi, Japan). The separation was achieved in an isocratic eluent at a flow-rate of 1 ml/min as previously described (*15, 22*) with some modification on the mobile phase buffer system. The separation buffer contained 10 mM TBHS (tetra-n-butyl-ammonium hydrogen sulfate) and 0.1 M phosphate at pH 5.8. HPLC mobile phase was 90% separation buffer and 10% acetonitrile, and they were mixed completely before running.

UV-visible spectral measurements— Nucleotides were dissolved in HPLC mobile phase solution as described above. The absorption spectra were recorded between 340 to 220 nm on a UV-visible spectrophotometer (U-3300, Hitachi, Japan) and the background absorption obtained by the HPLC mobile phase solution was subtracted. The scan parameters were 2.0 nm of slit, 120 nm/min of scan speed and 1 nm of sample interval.

^{31}P NMR spectroscopy— The ^{31}P NMR spectra were recorded on a VARIAN UNITYINOVA 500 NMR spectrometer (VARIAN, USA) operating at a frequency of 202.31 MHz. Samples were placed in 5 mm NMR tubes and the spectra were recorded at room temperature (25℃). A pulse of 4.7 μs was used with an acquisition time of 0.271 sec. The spectral width was set to 60.6 kHz, and 32,000 data points were recorded for each free induction decay. Chemical shifts were referenced relative to external 85% H_3PO_4 at 0 ppm.

Electrospray ionization mass spectrometry (ESI-MS)— ESI-MS investigations were carried out by means of quadrupole time-of-flight (Q-TOF) mass spectrometer (Micromass, UK) equipped with an electrospray ion source. Electrospray ionization mass spectrometry was performed in the positive-ion modes. Samples were dissolved in hydroxyapatite buffer (10 mM phosphate at pH 7.0, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 125 mM sucrose), and sample volumes of 20 μl/min were applied by loop injection. The liquid eluent was water/acetonitrile/1% formic acid (8/1/1, v/v). The collision energy of MS-MS spectra was 25 V. Nitrogen was used as nebulizing and drying gas. Mass spectra were acquired in scan range from *m/z* 200 to *m/z* 2500.

RESULTS & DISCUSSION

Identification of the nucleotide co-purified with PST— One of the main purposes of this study was to chemically identify the nucleotide that trapped and co-purified with PST. Alcohol (70%) was used to denature PST and to release the nucleotide from α-form PST. The adenine nucleotide recovered was equal to 0.92 PAP per PST dimer for α-form PST. None of the adenine nucleotide can be observed in a parallel experiment with β-form PST. This result was consistent with previous report (*16*), that fully active PST dimer require one PAP for the transfer reaction. The one PAP per PST dimer ratio is also previously determined by circular dichroism (*16*).

Under the selected HPLC condition, separation for a variety of nucleotides was obtained within 20 min, as shown in Figure 1. Other nucleotides that could be separated by this method were listed in Table 1. The ion-pair RP-HPLC profile gave only one peak from the extract of recombinant PST. The retention time of the extracted nucleotide was identical with that of commercial PAP at 6.15±0.04 min (Figure 1B). This result indicated that only one nucleotide was still tightly bound to purified PST after gel filtration to separate small molecules from purified PST. It has been reported previously (*15*) that many nucleotides bind tightly to PST at K_d in the nanomolar range. However, to remain bound to PST through a series of protein purification procedure, the nucleotide might need to be irreversibly trapped inside the enzyme. As reported later for the conversion of the two enzyme forms, we found that partial denaturation of PST by oxidation or high salt solution was required to remove the trapped nucleotide.

The UV-visible absorption spectrum of the isolated nucleotide was shown in Figure 2, which had the same spectrum as that of commercial PAP. In the previous study (*16*), the CD spectra of the two PST forms were compared. The major difference at 260 nm indicates that adenine may be involved in the binding of α-form PST. Biochemical test of this isolated nucleotide was examined as cofactor of β-form PST as described under "EXPERIMENTAL PROCEDURES" for standard assay. As compared to that of commercial PAP, identical PST activity was obtained. The concentration of the nucleotide was determined by A_{260} and the equivalent of 2 μM PAP (or A_{260} = 3.0×10^{-4}) was used as cofactor in standard assay condition. Specificities obtained were 2.01±0.05 and 1.90±0.11 μmole/min/mg for commercial PAP and nucleotide isolated from PST, respectively. This was the first time that the nucleotide in α-form PST was isolated and tested chemically and biochemically, and they were found to be identical.

The ^{31}P NMR spectrum of desalted PST was shown in Figure 3A. Figure 3B was the ^{31}P NMR spectrum of the solution containing the targeted nucleotide extracted from PST. The ^{31}P chemical shifts of PAP can be significantly different in distinct environments (*23*) and the variation between Figure 3A and 3B indicated that the

phosphorus signals were sheltered or interacted strongly with PST. Crystal structures of PAP and sulfotransferase complex (*24, 25*) show that both of the phosphate groups are strongly coordinated by neighboring amino acids in the enzyme binding site. Denaturation of PST released PAP into solution and gave the spectral differences (shown in Figures 3A and 3B). This observation confirmed our previous proposal that the PST bound nucleotide might be trapped inside the enzyme binding pocket. The proton-decoupled ^{31}P NMR spectrum gave three chemical shifts (Figure 3B), 3.86, 3.58 and 2.60 ppm from the extract of desalted PST. The two lower field chemical shifts were identical to those of 3'-phosphate and 5'-phosphate of PAP in Figure 3C (*15, 26*). Figure 3D was the mixture of the equal amount of enzyme extract and commercial PAP (determined by A_{260}). The third chemical shift shown in Figure 3B overlapped with that of sodium phosphate (data not shown). It is known that PAP is subjected to hydrolysis in aqueous solution (*15*); which may result in the presence of free phosphate in PST extract.

The positive-ion ESI-MS spectrum of purified PST dominated by different charged molecular ions was shown in Figure 4A. These signals were deconvoluted using the "MassLynx" program to yield molecular masses for the PST-PST (33,901 Da) and PST-PAP complex (34,327 Da) were appeared as shown in Figure 4B. Electrospray ionization mass spectrometry (ESI-MS) is a powerful tool to study biomolecular non-covalent interactions involving proteins with metals, ligands, peptides, proteins or oligonucleotides (*27-29*). This result further confirmed the strong interaction of PAP and PST. The other peaks of Figure 4B were salt clusters (*30-32*) with PST or PST-PAP complex. These salt cluster peaks disappeared when PST was passed through HiTrap desalting column with H_2O (data not shown). However, PST is unstable in pure water and precipitates easily. The MS-MS spectrum of *m/z* 428 produced two peaks, *m/z* 428 and *m/z* 136 that could be assigned to PAP and adenine, respectively (Figure 4C).

Conversion of the two PST forms— Table 2 detailed the procedures and results of complete transformation of α- to β-form PST. In a reverse process, Table 3 detailed the procedures and results of complete transformation of β- to α-form PST.

The ratio of α- to β-form PST expressed in *E. coli* is dependent on the cell growth condition. The percentage of α-PST can be as high as nearly 100% or as low as 30% and can be controlled by the growth temperature and oxygen supply (*14*). Incubation of PAP (20 μM) with 4 mg β-form PST (14 μM) produces only 60% α-form activity (*16*). Although PAP seems to help the refolding of denatured PST, only 4% of the total enzyme activity is recovered (*14*). In this study, 66% α-form PST was expressed in *E. coli* at 37°C. Accumulation of PAP is found in *E. coli* in the overexpressed α-PST (*21*). In animal liver, the concentration of PAP is in μM range

(*21*) and it is possible that the two enzyme forms are similarly regulated in mammalian cells.

As proposed above, PAP could be trapped inside PST so that the release of PAP could not be achieved without partially denaturing the enzyme. It has been shown that oxidation releases the tightly bound PAP (*17*) and high salt effects PAP-sulfotransferase interaction (*23, 33*). We were thus utilizing these strategies to exchange PAP and to interconvert the two enzyme forms (Tables 2 and 3). Oxidative modification at cysteines regulates PST activity and the K_d of PAP and PST is about 30-fold higher following incubation with 1 mM GSSG for 1 hour (*17*). However, oxidation of PST was found unable to completely transfer α-form to β-form PST and prolong oxidation inactivated the enzyme activity. In our experiments the α-form activity reduced to 20% (from 66% of total PST activity) with the incubation of 1 mM cystamine for 6 hours or 1 mM GSSG for 9 hours (data not shown). As shown in Table 2, a combination of oxidation and high salt treatment completely eliminated the activity of α-form PST (II to IV or II to V shown in Table 2). In such condition PAP could be removed from PST and then separated by ultrafiltration or gel filtration chromatography. PST was inactive in oxidative state (IV), but was completely reactivated (V) following reduction with TCEP as shown in Table 2.

Different degrees of transformation were obtained following the incubation of β-form PST (0.27 mg/ml or 4 μM dimer) with different PAP concentrations in reducing condition (Figure 5). The PAP to PST ratios were 1, 10, 100 and 1000 (i.e., 4 μM, 40 μM, 0.4 mM and 4 mM PAP, respectively). The two dissociation constants of PAP and PST are 31 nM and 152 μM (*15*). In such conditions, nearly all the first binding site of PST was occupied by a PAP as calculated and shown in Figure 5 as a solid circles. Accordingly, 3, 21, 72 and 96% of PST would contain the second PAP in such conditions (open circles in Figure 5). In our experiments, the transformations of β-form to α-form PST were in between the above calculated results. Repeated desalting (up to three times) did not change the β- to α-form ratio in these experiments. This result confirmed our proposal that PAP was irreversibly trapped inside α-form PST. It also indicated that the binding of PAP in both binding sites could facilitate the transformation of β- to α-form PST, i.e., to entrap one PAP inside PST.

Complete transformation (within experimental error) of β-form to α-form PST was achieved with the pretreatment of cystamine to inactivate PST following PAP incubation and reduction with TCEP (VII in Table 3). As has been shown previously (*16*), a PST_2-PAP (one PAP per PST dimer) complex is required for the catalysis of the transfer reaction. In a typical assay condition (about 20 nM PST was used) the calculated concentration of PST_2-PAP complex in the solution is only 6.2 nM for the

equal amount of PAP (20 nM) using K_d = 31 nM for PAP and PST determined previously (*15*). That is, only 31% enzyme activity would have been obtained if equal amount of PAP and PST were present due to the tight binding but did not irreversibly trapped inside PST as proposed for α-form. The treatment of PST with high salt concentration (0.3 M NaCl) did not increase the ratio of transformation significantly (VI in Table 3). It indicated that partial denaturation of PST by oxidation was required to facilitate the transformation.

Proposed model for the formation of the two forms of PST— In addition to this PST example (*13, 16*), expression of two protein forms from a single cDNA are found for other proteins. Rat liver malonyl-CoA decarboxylase (MCD) generates two isoforms from one cDNA (*34*), because it contains two potential translational start sites. However, two forms PST are indistinguishable in their sequence for they have identical first eight amino acids, molecular weight and western blot results (*13*). Another example is prion protein (PrP). The normal, cellular PrP (PrP^{c}) is converted into modified protein (PrP^{sc}) through a post-translational process whereby a portion of its α-helical and coil structure is refolded into β-sheet (*35, 36*). Their amino acid sequences and molecular weights are identical. Two forms structures can convert reversibly by oxidation-reduced method (*36, 37*). PST may be subjected to post-translational modification through redox regulation. In the absence of PAP, α-form PST is active in catalyzing the transfer reaction. However, partial oxidation is required for α-form PST to be able to catalyze the physiological reaction of sulfuryl group transfer (*17*), which requires the removal of PAP to complete a turnover of sulfuryl group transfer (*16*). In liver tissue, the concentrations of glutathione, reduced and oxidized form (GSH and GSSG), are in the mM ranges (*39*) and the concentration of PAP is in μM range (*21*). There are plenty of redox reagents and PAP available for the transformation of the two PST forms in physiological condition.

Scheme I illustrates how the two PST forms can be converted according to the results from this and previous studies. We propose that direct transformation between α- and β-form PST requires conformational change that can be facilitated by partial denaturation and renaturation through oxidation and reduction. The intermediate, the partially oxidized PST, is inactive for both the transfer and the physiological reactions. High salt seems to help the transformation but redox is crucial for the release and binding of PAP that induces conformational change of PST (*16*). Only the β-form PST is fully active for both the transfer and the physiological reactions. The binding of PAP does not transform the β-form to α- form PST directly. PAP needs to be irreversibly trapped inside PST to produce α-form. This makes α-form PST inactive toward the catalysis of the physiological reaction, which requires the release of PAP to complete a turnover.

A flexible loop, amino acid residue 64-68, which includes a cysteine at 66, may be accountable for the transformation of the two PST forms. The possible interaction of C66 with PST nucleotide binding site has been demonstrated by affinity labeling (*40*). Interestingly, crystal structures of sulfotransferases (*24, 25, and 41*) indicate that this highly conserved region is not part of the nucleotide-binding site. This region may be very flexible or disordered, so that cannot be resolved in the crystal structures (*24, 25*). As evidence in the crystal structure, this flexible region may be in the way of nucleotide entrance and releasing and thus affect two forms transformation of PST. It has been shown that in the presence of GSSG, C66 and C232 formed a disulfide bond (*17*). The K_d of PST and PAP is significantly increased in oxidation state (*17*). Mutation around C66, K65ER68G, also greatly affects the binding of PST and PAP (*16*). Detailed study concerning the effect of loop 64-68 on the formation of two PST forms is under investigation by site directed mutagenesis.

CONCLUSION

Although it has been demonstrated that many nucleotides can be tightly bound to PST and can be function as cofactor of PST for sulfuryl group transfer (*15*), only one nucleotide was found to trap inside PST. This nucleotide was identified as PAP according to ion-pair RP-HPLC, UV-visible spectra, ^{31}P NMR, ESI-MS and MS-MS spectrometry and biochemical test as cofactor of PST.

Partial denaturation of PST that results in its inactivation was required to remove the trapped PAP from α-form PST. This process can be induced by oxidation. Oxidation, reduction and the binding of PAP facilitate the transformation between α- and β-form PST.

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Table 1 HPLC retention times of nucleotides [a]

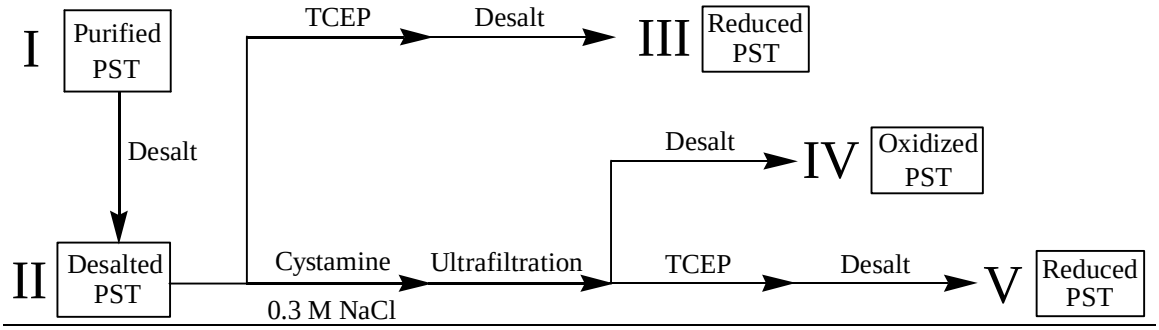
Compound	Retention time (min) [b]
β-NAD	3.00±0.02
Adenosine	3.27±0.04
NADP	4.00±0.03
Adenosine 5’-monophosphate	4.09±0.03
Adenosine 5’-diphosphate	5.19±0.05
Adenosine 3’-monophosphate	5.30±0.04
Adenosine 3’5’-cyclic monophosphate	5.68±0.06
Adenosine 2’5’-diphosphate	5.70±0.05
β-NADH	5.74±0.06
PAP	6.16±0.06
Adenosine 5’-triphosphate	6.82±0.07

Adenosine 5'-tetraphosphate	9.12±0.10
NADPH	9.63±0.12
FAD	20.37±0.18
Coenzyme A	25.93±0.25

[a] Separations were described under "EXPERIMENTAL PROCEDURES".

[b] The mean±standard deviation was obtained from triplicate HPLC separation.

Table 2 Complete transformation of α- to β-form PST [a]



PST step	Total specific activity (μmole/min/mg)	Specific activity of α-form PST (μmole/min/mg)	Composition of PST (%)	
			α	β
I. Purified PST	1.77±0.03 [f]	1.24±0.02	70	30
II. Desalted [b]	1.70±0.02	1.02±0.02	60	40
III. Reduced [c]	1.85±0.01	1.12±0.02	61	39
IV. Oxidized [d]	0.10±0.01	0.04±0.02	(40) [g]	(60) [g]
V. Reduced [e]	1.92±0.03	0.007±0.004	(0.4) [g]	99.6

[a] Assays and calculations of PST composition were performed as described under "EXPERIMENTAL PROCEDURES".

[b] Purified PST (I) following DEAE column was passed through a HiTrap desalting column to remove DTT and NaCl and to exchange buffer (100 mM bis-tris propane at pH 7.0, 1 mM EDTA, 10% glycerol, and 125 mM sucrose). The PST active fractions were pooled and concentrated by ultrafiltration.

[c] Desalted PST (II, 1 ml, 7.8 μM or 0.53 mg/ml) was incubated in TCEP (1 mM) for 6 hours at 25℃, then passed through HiTrap desalting column immediately.

[d] Another fraction of desalted PST (II, 5 ml, 7.8 μM or 0.53 mg/ml) was incubated with 1mM cystamine for 1 hour. This mixture was concentrated to by 1 ml ultrafiltration. PAP released from PST was removed by ultrafiltration from 10 ml to 1ml in the present of desalting buffer plus 1 mM cystamine and 0.3 M NaCl. The above ultrafiltration procedure was repeated 4 times. The inactive enzyme solution was run through a desalting column and was collected based on A_{280}.

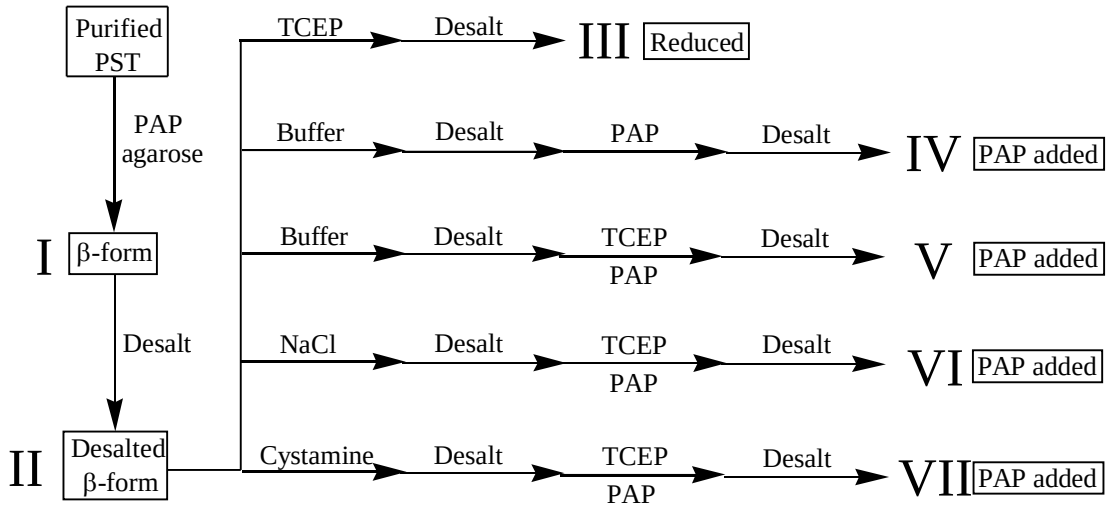
[e] Complete reactivation of PST was achieved by incubating the enzyme solution (1 ml, 4 μM or 0.27 mg/ml) with TCEP (1 mM) at 25℃ for 6 hours.

[f] The mean±standard deviation was obtained from triplicate activity assay.

[g] Due to the inactivation of PST by oxidation, the data was calculated from very low

specific activities.

Table 3 Complete transformation of β- to α-form PST [a]



PST step	Total specific activity (μmole/min/mg)	Specific activity of α-form PST (μmole/min/mg)	Composition of PST (%)	
			α	β
I. β-form PST	1.95±0.03 [g]	0.010±0.005	(0.5) [h]	99.5
II. Desalted [b]	1.90±0.04	0.009±0.005	(0.5) [h]	99.5
III. Reduced [c]	1.84±0.04	0.010±0.005	(0.5) [h]	99.5
IV. PAP added [d]	1.89±0.08	1.70±0.08	90	10
V. PAP added [d]	1.86±0.06	1.66±0.03	89	11
VI. PAP added [e]	1.86±0.07	1.69±0.03	91	9
VII. PAP added [f]	1.91±0.04	1.89±0.05	99	(1) [h]

[a] PST assay and separation of β-form PST (I) were performed as described under "EXPERIMENTAL PROCEDURES".

[b] The β-form PST was passed through a desalting column (HiTrap) to remove DTT that was added to stabilize PST. The resulting β-form PST solution contained 100 mM bis-tris propane at pH 7.0, 1 mM EDTA, 10% glycerol, and 125 mM sucrose.

[c] III was obtained by incubating the above enzyme (II, 1 ml, 4 μM or 0.27 mg/ml) with TCEP (1 mM) at 25℃ for 6 hours.

[d] The desalted β-form PST (1 ml, 10 μM or 0.68 mg/ml) was incubated with desalted buffer at 25℃ for 6 hours, and then passed through HiTrap desalting column. The active fractions were pooled (4 μM) and incubated with PAP (4 mM) and without (IV) or with (V) TCEP (1 mM) at 25℃ for 6 hours.

[e] Alternatively, the desalted β-form PST (1 ml, 10 μM or 0.68 mg/ml) was incubated with 0.3 M NaCl, first. Then the active fractions were pooled (4μM) and incubated with PAP (4 mM) and TCEP (1 mM) at 25℃ for 6 hours.

[f] The desalted β-form PST (1 ml, 10 μM or 0.68 mg/ml) incubating with cystamine (1 mM) at 25℃ for 6 hours and then with PAP (4 mM) and TCEP (1 mM) at 25℃ for 6 hours. The α-form PST was obtained following by a desalting column (Hitrap) to remove free PAP.

[g] The mean±standard deviation was obtained from triplicate activity assay.

[h] Calculated from the very small amount of the specific enzyme form.

Scheme 1

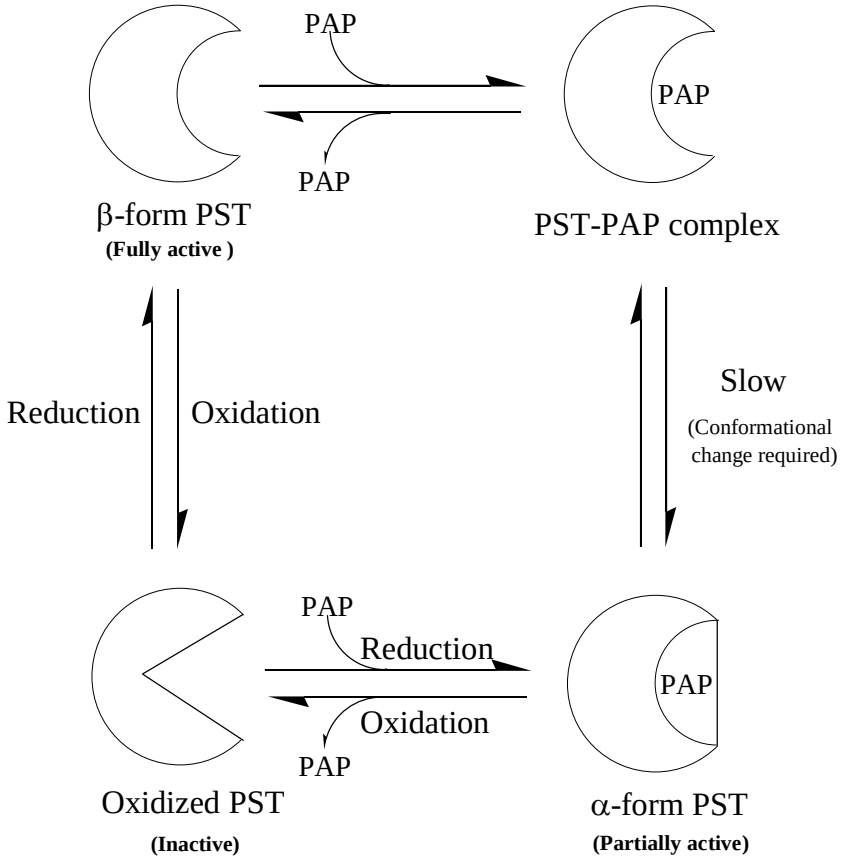


FIGURE LEGENDS

Figure 1. Ion-pair RP-HPLC separation of nucleotide. A 10 μl sample was injected for each run. (A) Commercial nucleotides included: 1 β-NAD; 2 adenosine; 3 adenosine 5'-monophosphate; 4 adenosine 5'-diphosphate; 5 adenosine 2', 5'-diphosphate; 6 PAP; 7 adenosine 5'-triphosphate; 8 adenosine 5'-tetraphosphate; 9 NADPH; (B) PST extract (A_{260}=0.44); (C) Commercial PAP (100 μM or A_{260}=1.5); (D) Mixture of enzyme extract and commercial PAP (A_{260}=0.22 for each)

Figure 2. UV-visible spectra of PAP and nucleotide extracted from PST. PAP (0.1 mM) was dissolved in HPLC mobile phase solution (90% separation buffer and 10% acetonitrile). The background of the solution in UV-visible spectra was subtracted. The PST extract following ion-pair RP-HPLC separation was collected at desired retention time (6.15 min) as shown in Figure 1B. The two spectra were normalized at 260 nm. The absorption of PST extract at 260 nm was 0.44.

Figure 3. ^{31}P NMR spectra. Purified PST was passed through a HiTrap column with sonication buffer (10 mM Tris-HCl at pH 7.0, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 125 mM sucrose). Nucleotides were obtained as described under "EXPERIMENTAL PROCEDURES" and dissolved in sonication buffer. (A) Desalted PST (20 mg/ml or A_{280}=34); (B) PST extract (A_{260}=0.5); (C) Commercial PAP (0.1 mM or A_{260}=1.5); (D) Mixture of enzyme extract and commercial PAP (A_{260}=0.38 for

each)

Figure 4. ESI-MS spectra. (A) ESI-MS spectrum of native PST; (B) Deconvoluted using the "MassLynx" program; (C) The MS-MS spectrum of *m/z* 428 peak from (A).

Figure 5. Addition of PAP partially transforms β--form to α-form PST. The β-form PST (4 μM or 0.27 mg/ml) was used to incubate with different concentrations of PAP (4 μM to 4 mM) in the presence of TCEP (1 mM). PST_2-PAP (●) and PST_2-PAP_2 (○) complexes were calculated using K_d1 =31 nM and K_d2 = 152 μM. The transformation ratio (▼ as % of α-form) was determined as describe under "EXPERIMENTAL PROCEDURES". The error bar was one standard deviation and calculated from triplicate activity assay.

Figure 1

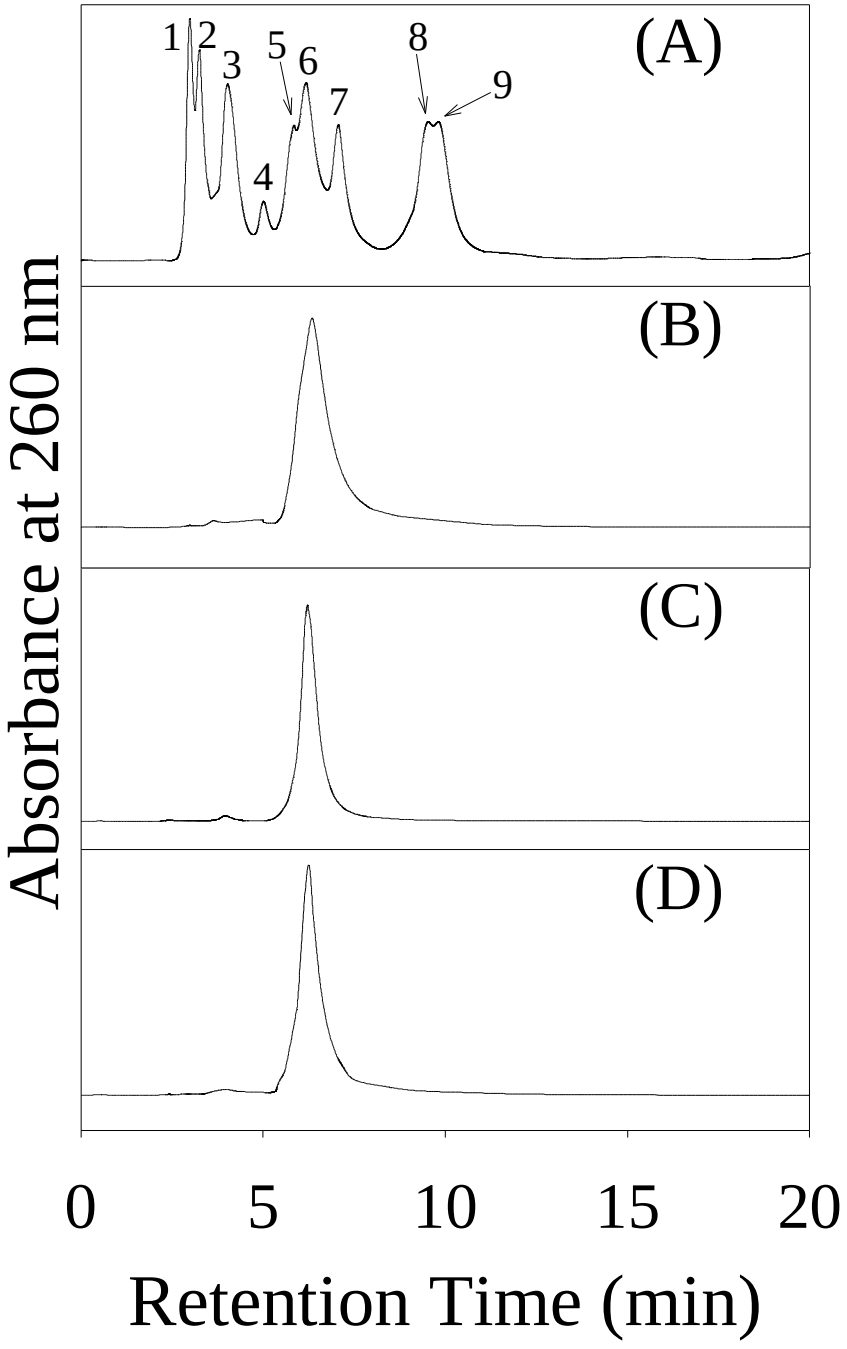


Figure 2

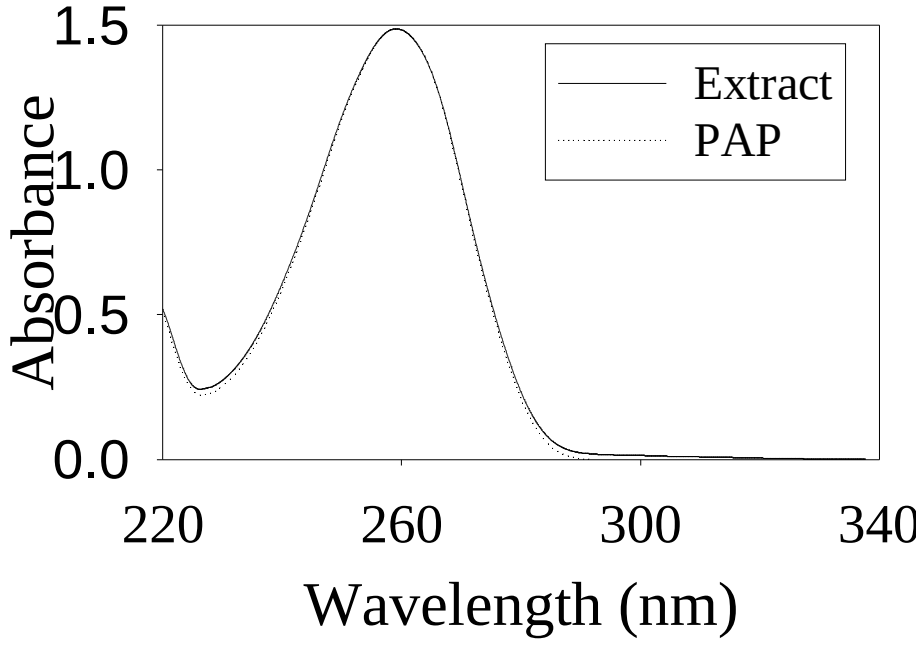


Figure 3

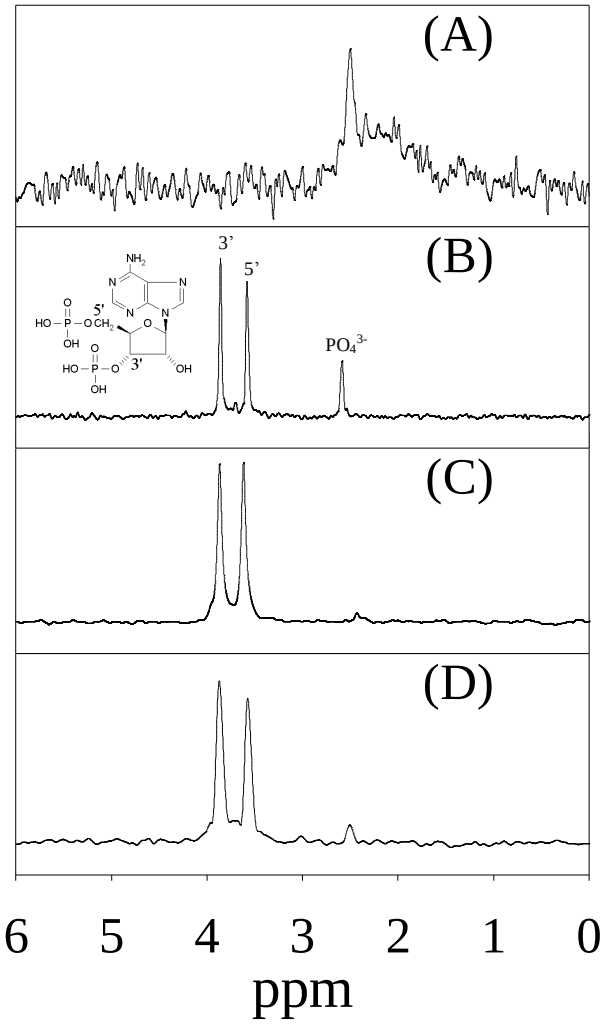

(A)
(B)
3'
5'
PO_4^{3-}
(C)
(D)
6
5
4
3
2
1
0
ppm

Figure 4

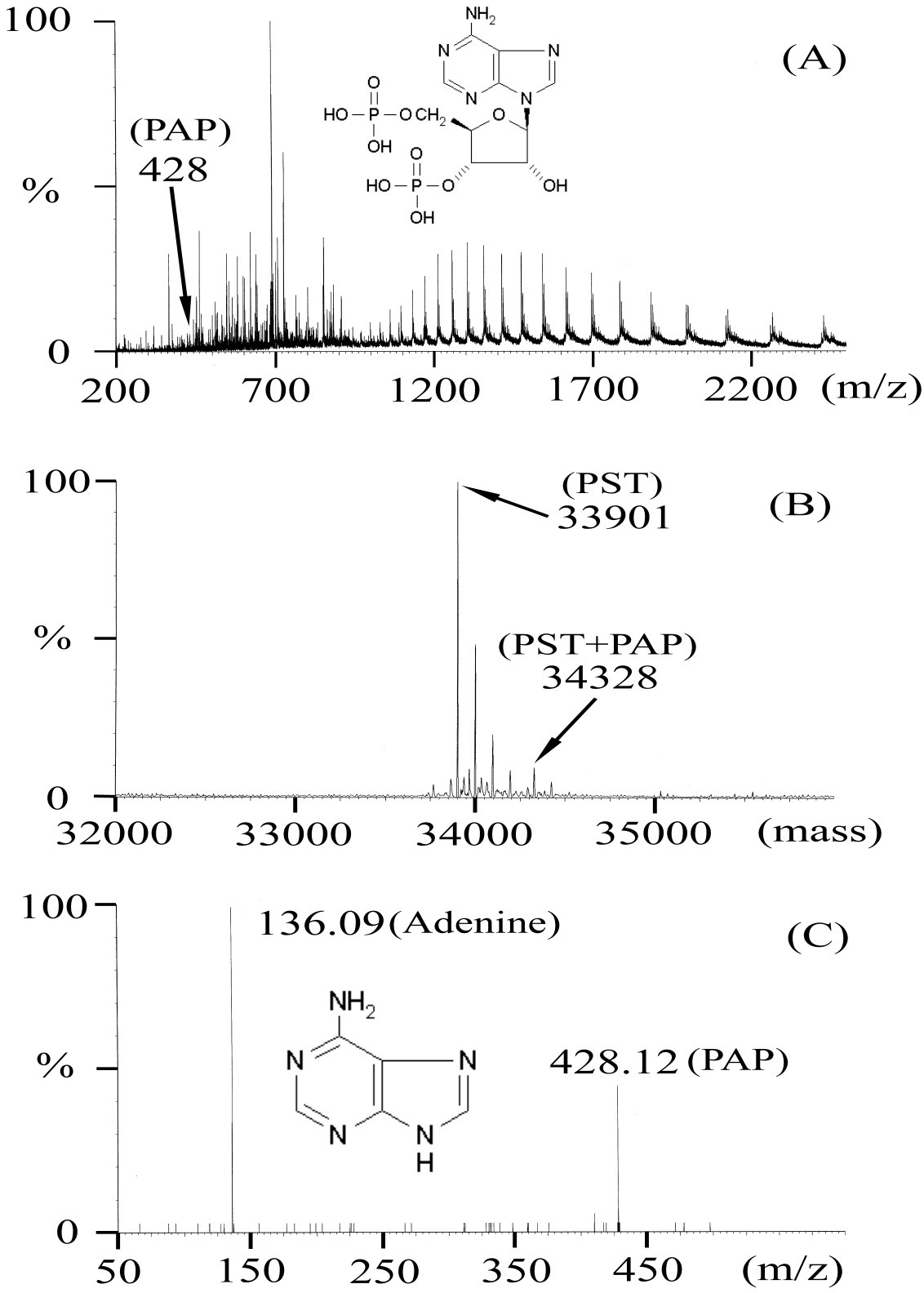
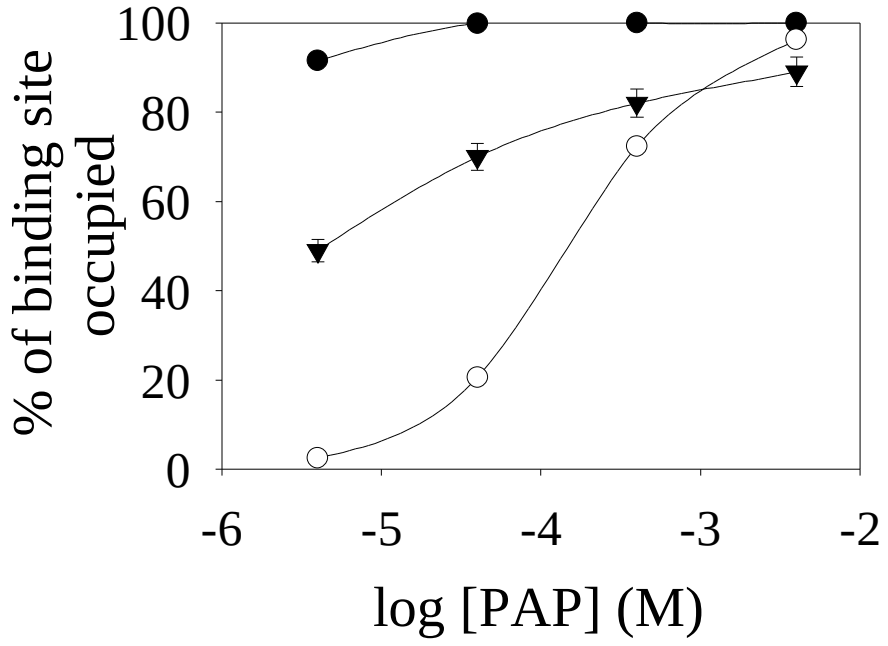

100
%
0
(PAP)
428
(A)
200
700
1200
1700
2200
(m/z)
100
%
0
(PST)
33901
(B)
(PST+PAP)
34328
32000
33000
34000
35000
(mass)
100
%
0
136.09(Adenine)
(C)
428.12 (PAP)
50
150
250
350
450
(m/z)

Figure 5



計畫成果自評

We feel that we have accomplished the proposed research goal. The second manuscript entitled “Site-directed mutagenesis reveals the regulation of the activity of phenol sulfotransferase by a flexible loop” which is funded by this grant is now in preparation and will be submitted to *Biochemistry* for publication before the end of this funded period.