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Colorimetric Determination of the Purity of 3′-Phospho Adenosine 5′-Phosphosulfate and Natural Abundance of 3′-Phospho Adenosine 5′-Phosphate at Picomole Quantities

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

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This work presents novel colorimetric methods not only to measure 3′-phospho adenosine 5′-phosphate (PAP) and 3′-phospho adenosine 5′-phosphosulfate (PAPS) in the range of picomoles, but also to determine the purity of PAPS or PAP contaminants in PAPS in the range of nanomoles. These methods exploit the availability of overexpressed phenol sulfotransferase (PST) and the fact that sulfuryl group transfer requires the use of PAP or PAPS as a cofactor or cosubstrate. Experimental results indicate that absorption at 400 nm due to the production of 4-nitrophenol (pNP) is catalyzed by PST when the sulfuryl group transfers from 4-nitrophenylsulfate (pNPS) to PAP or to 2-napthol. In the absence of an acceptor substrate, PAPS is hydrolyzed to PAP by PST and is determined by sulfation with pNPS before and after this reaction. The change of absorption of pNP at 400 nm corresponds to the amount of PAP that is hydrolyzed from PAPS. Moreover, a standard curve is constructed using authentic PAP and PAP-free PST. Furthermore, this curve is used to determine the amount of PAP in extracts of pig liver, rat liver, and *Escherichia coli*.

3′-Phospho adenosine 5′-phosphosulfate (PAPS)[2] is the donor of sulfuryl group for the enzymatic sulfation of biological materials and xenobiotics (1). Physiological sulfation by PAPS results in the production of 3′-phospho adenosine 5′-phosphate (PAP) which can act as cofactor for sulfuryl group transfer between other compounds (2). Phenol sulfotransferase (PST) is a dimeric protein, which contains two PAP binding sites (3). PAP is tightly bound to only one of these sites in a reaction involving a conformational change (3). Product inhibition of PST by PAP has been proposed previously (4, 5). Elucidating the catalytic mechanism of sulfotransferase requires developing a rapid spectrophotometric assay for PAP in the nanomolar to picomolar concentration range. A variety of methods have been developed for measuring PAPS and PAP (6–11). These methods require radioactive, ^{35}S (8) or ^{14}C (6), compounds or authentic PAPS as a standard and additional separation procedures (7–11). Commercially available PAPS usually contains significant amounts of PAP which may influence the results of many experiments when the concentrations of PAPS and PAP are crucial. We report here simple spectrophotometric methods which measure picomole amounts of PAP/PAPS, and determine the purity of PAPS, as well as PAP contamination in PAPS. In addition to a regular UV/VIS spectrophotometer, overexpressed, purified phenol sulfotransferase is the only enzyme required for these analyses.

PST catalyzes sulfuryl group transfer from PAPS to phenols, hydroxyl amines, or other substrates under physiological conditions (13) as shown by Eq. [1].

[1] To whom correspondence should be addressed at Institute of Biological Science and Technology, National Chiao Tung University, 75 Po-Ai Street, Hsinchu 30050, Taiwan. Fax: 886-3-5729288. E-mail: ysyang@cc.nctu.edu.tw.

[2] Abbreviations used: PAP, 3′-phospho adenosine 5′-phosphate; PAPS, 3′-phospho adenosine 5′-phosphosulfate; pNPS, 4-nitrophenyl sulfate; pNP, 4-nitrophenol; PST, phenol sulfotransferase; PMSF, phenylmethylsulfonyl fluoride; bis-tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane.

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PAPS + 2-naphthol ↔ PAP + 2-naphthyl sulfate [1]

This enzyme also catalyzes sulfuryl group transfer between two phenols as shown by Eq. [3], which is the combination of Eqs. [1] and [2]. Reaction [3] is PAP- or PAPS-dependent and the nucleotides are acting as coenzymes instead of as cosubstrates, as shown in Eqs. [1] and [2].

4-nitrophenyl sulfate + PAP ↔

4-nitrophenol + PAPS [2]

4-nitrophenyl sulfate + 2-naphthol ↔

4-nitrophenol + 2-naphthyl sulfate [3]

Binding of PAP and phenol sulfotransferase results in a stable enzyme–PAP binary complex (3). Release of PAP is obligatory and may be the rate-limiting step for the physiological reaction (4, 14). A tight binding of PAP and PST, K_d = 20 nM (3), indicates that a highly sensitive method can be developed to measure [PAP] using Reaction [3]. The fact that catalysis in Reaction [3] depends on the formation of an enzyme–PAP(S) complex accounts for why the enzymatic reaction depends on the amount of available PAP(S) when [E] $\gg$ [PAP(S)]. Thus, the minimum amount of PAP and PAPS that can be measured is near the amount of PST used in the standard assay. To measure the transfer activity among phenols (Reaction [3]), nmol to pmol of PST is required for a spectrophotometric assay (3). This is the sensitivity of PAP measurement that can be expected.

PAP can also be determined using Reaction [2], where the production of pNP from pNPS directly reflects the amount of sulfated PAP. Notably, this reaction only determines PAP and not PAPS. The sensitivity of this measurement is limited by the extinction coefficient of pNP, at the range of μM under neutral or alkaline conditions. This method can indirectly determine PAPS after hydrolysis, as indicated by Reaction [4], where it is also catalyzed by PST at alkaline pHs.

$$PAPS + H_2O \rightarrow PAP + H_2SO_4 \quad [4]$$

These two methods allow us to measure the amounts of PAP/PAPS from crude extracts of cells and determine the PAP and PAPS contents from commercial products.

MATERIALS AND METHODS

Chemicals. 4-Nitrophenyl sulfate (pNPS) was obtained from Merck (Germany). Some commercial pNPS includes higher amounts (up to around 1%) of 4-nitrophenol (pNP) contaminated than the others and, therefore, should be averted. PAP and PAPS were purchased from Sigma (U.S.A.). Dr. H. Nakajima of UNITICA, Japan, also kindly donated the PAPS. Hydroxyapatite (Bio-Gel HT) was purchased from Bio-Rad (U.S.A.). Alkaline phosphatase was purchased from Boehringer Mannheim (Germany). All other chemicals were obtained commercially at the highest purity possible.

Enzyme purification. Phenol sulfotransferase was purified as described elsewhere (3) with some modifications. A liquid chromatography system (Bio-Rad Bio-Logic) was used to provide pressure for a constant flow rate of column chromatography. PMSF was used as protease inhibitors to replace AMSF, antipain, and pepstatin A. Bio-Gel HT hydroxyapatite (Bio-Rad) was used instead of Spectra/gel HA (Spectrum, Los Angeles, CA), which is no longer commercially available. Purified enzyme with a purity of over 95%, as assessed by SDS–PAGE, after the first DEAE-sephacel chromatography, was used for the removal of PAP as described below.

Preparation of PAP-free sulfotransferase. The β-form of recombinant phenol sulfotransferase (3) was used as the PAP-free enzyme. Because the enzyme–PAP binary complex is more easily extracted than that of PAP-free enzyme during sonication, we collected the PAP-free enzyme by sonication as described elsewhere (3). Each fraction after 20 s sonication was collected and assayed for its PAP-dependent sulfotransferase activity. Fractions that required exogenous PAP for activity were collected and purified by DEAE sepharose chromatography. The isolation of PAP-free PST was facilitated by cell culturing, at 37°C with a low oxygen supply (speed of the incubator shaker was 100 rpm). Cells grown in this way typically yielded enzyme which was 70% in the PAP-free form.

Alternatively, enzyme bound PAP was removed by alkaline phosphatase. The enzyme solution after DEAE-sephacel chromatography was adjusted by methylamine buffer (200 mM, pH 11) to pH 9.5. Alkaline phosphatase and mercaptoethanol (to 5 mM final concentration) were then added. This mixture was stirred at room temperature for several hours until PAP-independent activity in the standard assay (Reaction [3]) was not detected. The PAP-free enzyme was further purified by hydroxyapatite chromatography.

Enzyme assay. Sulfotransferase activity was determined by changes of absorbency at 400 nm due to the production of pNP (ϵ = 10,500 M^{-1} cm^{-1} at pH 7.0) as described by Yang *et al.* (3), with minor modification. The PAP concentration used in the standard enzyme assay was 2 μM instead of 20 μM as described elsewhere (3). This modification did not appear to affect the enzymatic activity. A unit of enzyme activity represents the amount deemed necessary to catalyze the formation of 1 μmol of pNP per minute by the standard

assay. Specific activity is given in units per milligram of protein. Protein concentration was estimated on the basis of absorbency at 280 nm (3); 1 mg/ml PST produces 1.7 absorption unit for the homogeneous form of the enzyme based on the method of Gill and von Hippel (15). PAP-dependent enzyme activity (Reaction [3]) was determined by the standard assay condition in the presence and absence of PAP. The former assay determined the total enzyme activity and the latter determined that of the phenol sulfotransferase preparation already containing PAP.

Concentration of PAP and PAPS. Commercially available PAP or PAPS concentrations were determined by their absorption at 260 nm (ϵ = 15,100 M^{-1} cm^{-1} for adenosine).

Preparation of biological samples to determine PAP. The cell extract was prepared from pellets of 250 ml *Escherichia coli* cell culture which was mixed with 5 ml buffer (10 mM Tris-HCl at pH 7.4 plus 125 mM sucrose, 10% glycerol, 1 mM dithiothreitol, 1 mM PMSF, and 1 mM EDTA) and sonicated to break the cells as mentioned earlier for protein purification. Separate liver extracts were prepared from about 3.5 g each of frozen rat or pig liver, which was mixed with 10 ml buffer (10 mM Tris, pH 8) and 1.4 mM PMSF. This mixture was homogenized three times, each for 150 s, by Waring blender. Supernatants of cell and liver extracts were collected by centrifugation and used for PAP determination and for the assay of PST.

Measurement of PAP and PAPS at pmoles. The conditions to determine PAP/PAPS was the same as those of the standard assay except for that PAP content was varied and an excess amounts of PAP-free enzyme was predetermined. A linear standard curve was constructed using various amounts of authentic (commercial available) PAP or PAPS from 30 to 300 pmol.

Measurement of PAP at nmoles. PAP was determined by accepting sulfuryl group from an excess amount of pNPS catalyzed by PST to produce an equal amount of pNP. About 1 μmol PST (not necessary PAP-free) was mixed with 1 mM pNPS, 5 mM mercaptoethanol, and 100 mM bis-tris propane at pH 7. About 3 μM of PAP was initially added to saturate the enzyme–pNP–PAP ternary complex, and then the absorption at 400 nm was used as background. Changes of A_{400} following continuous addition of PAP were linear up to at least 15 μM of PAP. Calculated amounts of PAP added (determined by ϵ = 15,100 M^{-1} cm^{-1} at 260 nm) and the pNP produced (determined by ϵ = 10,500 m^{-1} cm^{-1} at 400 nm) were equal.

Hydrolysis of PAPS by phenol sulfotransferase. PST (about 25 μM) was incubated at 25°C with 210 μM PAPS and 13 mM Tris-HCl at pH 8. PAPS was completely hydrolyzed into PAP within 40 min, as determined by the PAP assay described earlier.

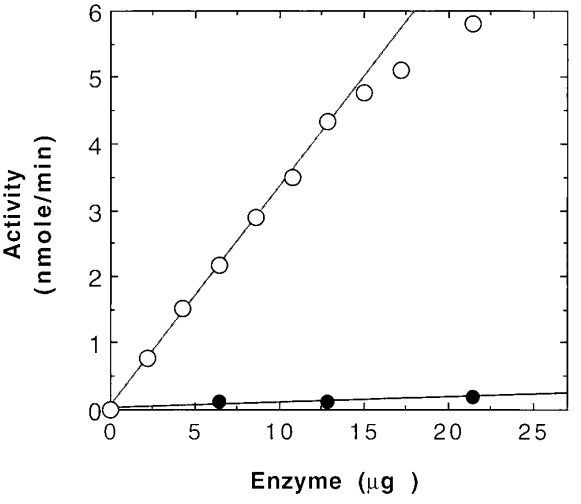

FIG. 1. Activity of phenol sulfotransferase with or without exogenous PAP. PAP-dependent (open circles) and PAP-independent (closed circles) activity of Reaction 3, catalyzed by PAP-free PST separated by sonication. The reaction condition is the same as that of standard assay for PAP-dependent activity, as described under Materials and Methods, except that 2 μM PAP was not added for PAP-independent assay. Each data point is the average of two measurements which differ by less than 10%.

RESULTS

Preparation of PAP-free sulfotransferase by sonication and treatment with alkaline phosphatase. Enzyme–PAP binary complex can be extracted more easily into a solution than that of PAP-free enzyme. PAP-free enzyme and PAP–enzyme binary complex were separated by sonication and were purified through DEAE-sephacel chromatography. Residual PAP content in PST was determined by the PAP-dependent standard assay, as described under Materials and Methods and shown in Fig. 1. According to this figure, the isolated PAP-dependent enzyme fraction still contained about 3% of PAP-independent enzyme as compared to the PAP-dependent enzyme activity. This amount of PAP-independent activity was close to the error of enzyme assay and was subtracted as background for PAP determination. To obtain PST 100% free of PAP-independent activity, we used alkaline phosphatase to remove residual PAP from the enzyme as described under Materials and Methods. Alkaline phosphatase-treated PST is active only in the presence of exogenous PAP and the PAP-independent activity is not observed ($\ll$1%). Both preparations of PST were used and found suitable for the determination of PAP/PAPS in the pmole range.

Linearity and sensitivity of PAP/PAPS-dependent activity of sulfuryl transfer. PAP is required for sulfuryl group transfer between two phenols (Reaction [3]), as shown in Fig. 1. Adding PAP to saturation increased sulfuryl transfer activity to a maximum; this condition

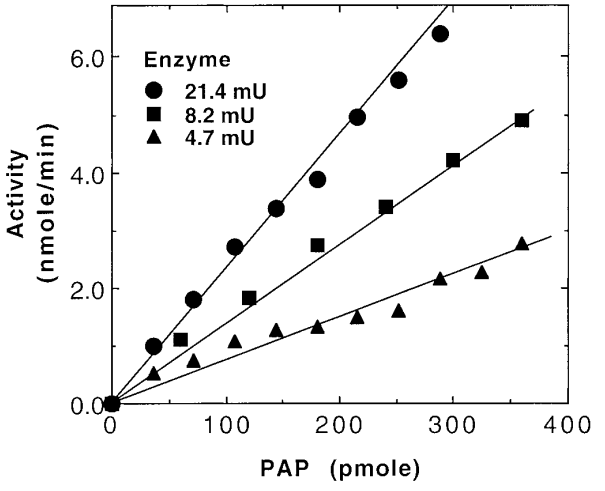

FIG. 2. Exogenous PAP-dependent enzymatic activity with a constant amount of PST. The reaction condition resembles that of the standard assay, except that the PAP concentration is varied and the amount of enzyme is predetermined. The amount of enzyme in mU used is calculated from the standard assay as described under Materials and Methods. Correlation (R^2) from the linear regression is 0.994, 0.991, and 0.947 for 21.4 (circles), 8.2 (squares), and 4.7 (triangles) mU of enzyme, respectively.

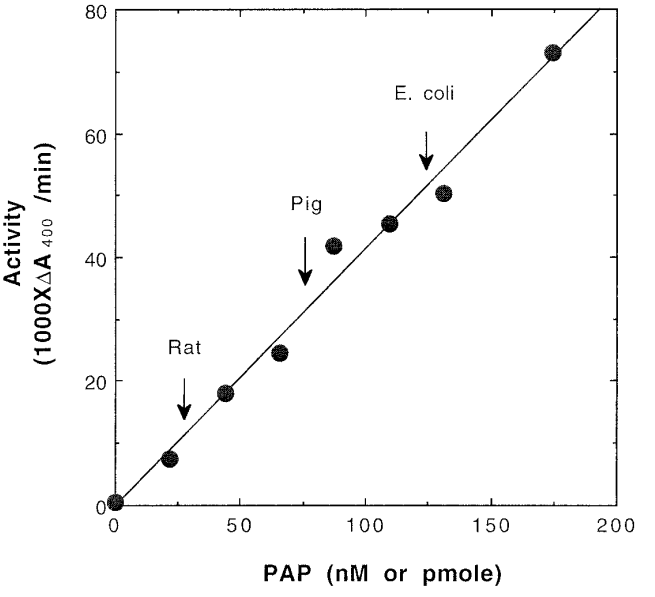

FIG. 3. Standard curve for PAP concentration and determination of PAP from cell extracts. Sixteen milliunits of PAP-free enzyme for each data point was used to construct the standard curve; 50 μl of cell extract obtained as described under Materials and Methods was used. PAP determination for each sample was duplicated with difference of less than 10%.

was used as standard assay for phenol sulfotransferase (3). Linear range for this assay was satisfactory, ranging from 0.1 to 5 mU/ml (about 4 to 200 pmol) of enzyme as shown in Fig. 1. Under the standard assay condition, the amount of PAP (2 μM) is at least 10 times more than that of the enzyme; additional PAP does not further extend the range of linearity. Also, the initial rate of more than 6 mU/ml of enzymatic activity is too fast for a routine spectrophotometer to measure for a standard assay.

This linear range for standard enzyme assay (shown in Fig. 1) also defines the maximum sensitivity to determine PAP/PAPS. Under the nonsaturating condition of PAP/PAPS with the enzyme, enzyme activity depends on both the nucleotides and the amount of enzyme used, as shown in Fig. 2. However, a linear relationship of enzyme activity and PAP concentration was still obtained with a constant amount of PST. A more linear response (as judged by a higher R^2 value) and increased sensitivity were obtained when more enzyme was used. According to Fig. 2, 4.7, 8.2, and 21.4 mU (or about 20, 40, and 100 nM) of PST were used with 30 to 350 pmol (or nM) of PAP. The sensitivity and the R^2 value can be further improved if a higher amount of enzyme is used. In this study, the PAP assay condition (as shown in Fig. 2) can adequately measure PAP from cell extracts.

Determination of PAP/PAPS from biological samples. A standard curve for PAP concentration was constructed using authentic PAP and 16 mU of PAP-free sulfotransferase as shown in Fig. 3. Similar results were obtained when PAP was replaced by PAPS (data not shown). Enzyme activities due to the addition of cell extracts are shown by the arrows in Fig. 3 and the corresponding amounts of PAP/PAPS found in the livers and bacteria were calculated and listed in Table 1. The PAP content of pET3c11 (plasmid contains PST cDNA)-transformed *E. coli* cells was calculated based on the amount of PAP attached to the α-form of recombinant PST (3). Overexpression PST in *E. coli* resulted in about 50 times more PAP than that found in untransformed cells (Table 1). This finding suggests that

TABLE 1

PAP Contents of Biological Samples and Their Effects on the Activity of Phenol Sulfotransferase

Source	PAP (nmol/g)	Phenol sulfotransferase (nmol dimer/g)[a]	
		− PAP	+ PAP
Pig liver	6.4	0.9	1.1
Rat liver	2.2	0.9	2.0
E. coli	8.1	0.5	0.7
E. coli (with pET3c11)	400[b]	400	800

[a] Denotes the determination by enzyme activity with or without exogenous PAP. The amount of active PST was calculated from its enzymatic activity.

[b] Denotes calculation from the amount of α enzyme (3).

PST may protect bound PAP, preventing its degradation. This method is capable of determining both PAP and PAPS in biological samples, although PAPS is unstable and may not exist in significant stable amounts.

Identification of the amount of free and enzyme-bound PAP in cell extracts. A majority, if not all, of the PAP is tightly bound to recombinant PST in pET3c11-transformed *E. coli* cells, as shown in Table 1. PAP in cell extracts may contain both free and enzyme-bound PAP, as determined by the PAP-independent assay (as shown in Table 1) for PST. Total native enzyme activity was determined by the standard assay for PST, which varies from 0.7 to 2.0 nmol enzyme dimer per gram of sample. The PAP amounts range from 2.2 to 8.1 nmol per gram, indicating that a large amount of free PAP can be found in the cell extracts. The PAP-independent activity of PST, i.e., less than that of the total enzyme activity, also indicates that even with the excess amount of available PAP in the extracts, not all the enzyme is in the PAP-bound state. This phenomenon is in contrast to pET3c11-transformed cells which contain large amount of PAP and is primarily, if not totally, enzyme bound. The calculated concentration of PAP from cell extracts under the assay condition is in the range of approximately 0.1 to 0.3 μM. This concentration happens to be close to the K_m value for the transfer reaction (Reaction [3]) under the standard assay condition.

Determination of the purity of PAPS. Commercial PAPS typically includes significant amounts of PAP and other impurities. The total amount of PAPS and PAP can be determined in the range described above. PAP alone can be determined spectrophotometrically (as shown by Reaction [2]), which is catalyzed by PST at a neutral pH. PAPS can also be determined by the same method after hydrolysis of the phosphosulfate (as shown by Reaction [4]), which is catalyzed by PST under alkaline conditions. Figure 4 summarizes the results of Reaction [2] using commercial PAPS as the source of PAP to determine its purity before and after enzymatic hydrolysis of PAPS. Upon hydrolysis of the PAPS by PST at pH 8, nearly 100% of PAP was obtained. By subtracting the absorption caused by impurities, 23%, as determined prior to hydrolysis, we determined that the commercial product contained 76% PAPS. This finding is consistent with HPLC and ^{31}P NMR analysis (data not shown) that 75 and 76% PAPS were found, respectively.

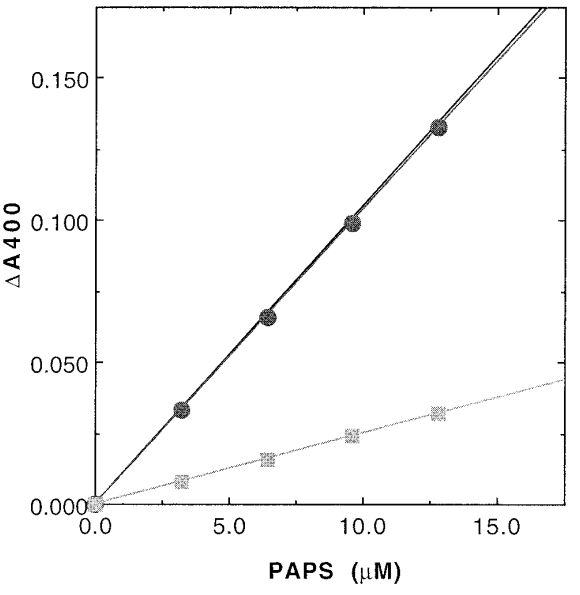

FIG. 4. Determination of PAPS content by direct sulfuryl group transfer from pNPS to PAP before and after the hydrolysis of PAPS. Commercial PAPS (3.2 , 6.4, 9.6 and 12.8 μM) was added into the reaction mixture after saturating the enzyme–pNP–PAP ternary complex with 3.2 μM PAP, as described under Materials and Methods. The top straight line denotes the theoretical amount of the change of absorption at 400 nm due to the sulfation of PAP (determined by ϵ = 15,100 M^{-1} cm^{-1} at 260 nm) and production of pNP (determined by ϵ = 10,500 M^{-1} cm^{-1} at 400 nm). Squares designate pNP production due to sulfation of commercial PAPS before hydrolysis. Circles designate pNP production due to sulfation of hydrolyzed PAPS (at pH 8 for 40 min by PST), as described under Materials and Methods. The absorption differences between circles and squares are equivalent to the amount of PAPS. Each data point was the average of two measurements with the difference of less than 10%.

DISCUSSION

PAP and PAPS are routinely used to assay sulfotransferase (3, 4). PAPS is a cosubstrate for all sulfotransferase and PAP is known to be a cofactor of sulfuryl transfer between phenols (2). The purity of PAPS or PAP in the biological samples can significantly influence measurement of the activity of sulfotransferase. In addition, knowing the contents of PAP and PAPS in the assay condition is of relevant concern. To elucidate the reactions and mechanism of sulfotransferase, this study presents a simple method to determine the purity of the PAPS/PAP and the trace amounts of nucleotides in biological samples. The two methods reported herein should facilitate the determination of PAP and PAPS in a biological sample, as well as in commercial products. In addition, catalytic amounts of PAP/PAPS are determined in picomoles, and a substrate amount of PAP is determined in nanomoles as discussed in the following.

PAP/PAPS is recycled in Reaction [3] which is the sum of Reactions [1] and [2]. In addition, only a catalytic amount of PAP or PAPS is necessary to reach maximum activity. Thus, in the presence of trace quantities of PAP, PAPS is formed from pNPS and, in turn, transfers to an excess of 2-naphthol. Under the reaction conditions presented herein, formation of pNP allows us to quantify the reaction as a function of the concentration of PAP. As Fig.

1 depicts, enzyme activity is determined at a relatively high concentration of PAP to enzyme (2 μM of PAP vs nM of enzyme). This defines the maximum sensitivity that can be expected. For the PAP assay, a linear relationship of enzymatic activity and concentration of PAP is obtained by using a relatively higher concentration of enzyme and PAP. According to Fig. 2, less than 100 pmol PST can be used to determine the pmol amount of PAP/PAPS. Since μmol or higher amounts of PST can be obtained easily, we were able to use excess enzyme for the assay of PAP.

This study also determines the amount of enzyme required to satisfy the linear relationship with [PAP] experimentally, as shown in Fig. 2. To determine [PAP] up to around 350 pmol, about 50 μg or 70 pmol (70 nM in 1 ml) of PST is sufficient. This figure also indicates that sensitivity of this PAP can be further increased by using greater amounts of PST. The maximum sensitivity of this assay should reach about 4 nM or 4 pmol when 1 ml of the total assay mixture is used. As expected, this sensitivity for E_2–PAP binary complex (3) is responsible for the transfer reaction (Reaction [3]). In addition, according to Fig. 1, enzymatic activity is observed in a linear range with less than 10 pmol to about 200 pmol of PST. The sensitivity can be enhanced to lower than single digits of pmol when the microplate reader is used to measure the production of pNP at 400 nm (i.e., about 200 μl of total volume is needed for the microplate reader). Further improvement of the sensitivity, to less than pmol, is also possible. Fluorogenic substrates, such as methylumbelliferyl sulfate, can be used to replace pNPS as sulfuryl group donor. We found that methods reported here are simple and sufficiently accurate for our purpose.

As mentioned earlier, PAP-free phenol sulfotransferase is a prerequisite to determine PAP or PAPS at pmol levels. Purification of PST by hydroxyapatite chromatography produces two enzyme fractions, one of which is PAP-free (3). This method requires at least two chromatographic separations of hydroxyapatite to obtain a relatively PAP-free enzyme. Alkaline phosphatase-treated sulfotransferase becomes PAP-free and is purified to homogeneity after hydroxyapatite chromatography with a single peak. Alkaline phosphatase removes PAP completely, as determined by PAP-dependent assay. However, change of pH and long incubation, followed by another chromatography, are cumbersome. According to our results, a simple preparation of relatively PAP-free (less than 5%) PST by sonication is sufficient for PAP determination according to our requirements.

Figure 3 and Table 1 indicate significant amounts of PAP in the extracts of liver and bacteria. However, a calculated amount of PAP from pET3c11 transformed cells is roughly 50 times more than that of untransformed cells (Table 1). Since all the observed PAP from the over-expressed system is enzyme bound, we believe that recombinant PST protects PAP to prevent it from metabolism. Cell extracts may contain both free and enzyme-bound PAP which can be determined by the standard assay in the absence of PAP, as shown in Table 1. Total amount of native PST in the extract is determined in the presence of excess exogenous PAP (the standard assay for PST), which varies from 0.7 to 2.0 nmol enzyme dimer per gram of sample. PAP-independent activity is also measured from native PST, which varies from 0.5 to 0.9 nmol enzyme dimer per gram of sample, while the total PAP amount ranges from 2.2 to 8.1 nmol per gram. Table 1 reveals that even though extracts from biological samples contain an excess amount of free PAP, exogenous PAP is still required for maximum activity. This result indicates that native enzymes may not contain tightly bound PAP. Calculated concentration of PAP from extracts under the enzyme assay condition ranged from 0.1 to 0.3 μM, which is near the K_m for transfer reaction (Reaction [3]), which may explain the amount of PAP-independent activity of native PST. In contrast to pET3c11 transformed cells, which contains a large amount of PAP, there is little free PAP available in the cell extracts. The interaction between the enzyme and PAP may be one reason that native enzyme differs from the recombinant enzyme (16, 17).

PAPS can replace PAP as a cofactor for sulfuryl transfer reaction (Reaction [3]) for the assays, as shown in Figs. 1, 2 and 3. This finding is expected because both PAP and PAPS are necessary for this reaction. The above method thus measures both PAP and PAPS. However, PAPS is unstable and can be hydrolyzed by phenol sulfotransferase. Thus, a significant amount of PAPS under physiological conditions is unlikely. Methods to determine PAP and PAPS independently are also developed and discussed in the following.

PAPS is routinely used to measure the physiological activity of sulfotransferase (Reaction [1]). However, commercial PAPS contains PAP, which is an inhibitor (4). The K_d of PAP and PST is in the nM range or lower for one of the isoforms (3). Therefore, even a small contaminate of PAP in PAPS may significantly influence the activity measured or the mechanism studied. The purity of PAPS can be determined, as shown in Fig. 4. PAP is determined at pH 7 (Reaction [2]) before and after hydrolysis of PAPS with PST at pH 8 (Reaction [4]). In addition, PAP and PAPS are used as substrates for Reactions [2] and [4] and, thus, the sensitivity of this assay is in the nmol range.

Importantly, the proper amount of enzyme must be used, as well as appropriate pHs for hydrolysis PAPS and sulfation of PAP. PST prefers to catalyze hydrolysis of PAPS under alkaline conditions and sulfuryl group transfer among phenols under a neutral or acidic condition. Little hydrolysis of PAPS can be observed at pH 7, which is the optimal condition for the sulfation of

PAP. Enzyme–PAP–pNP ternary complex produces extra absorbency near 400 nm (3). To avoid this interference, sufficient amounts of PAP were added to saturate the formation of the ternary complex as described under Materials and Methods.

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