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3′-Phosphoadenosine-5′-phosphosulfate: Photoaffinity Ligand for Sulfotransferase Enzymes

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SUMMARY

Sulfation is an important pathway in the biotransformation of many drugs, xenobiotic compounds, neurotransmitters, and hormones. The sulfate donor for these reactions is 3′-phosphoadenosine-5′-phosphosulfate (PAPS). We set out to determine whether PAPS might serve as a photoaffinity ligand for sulfotransferase enzymes. UV irradiation of [^{35}S]PAPS with partially purified human liver thermostable (TS) phenol sulfotransferase (PST) radioactively labeled a protein with a molecular mass of 35 kDa, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Photoaffinity labeling of TS PST with [^{35}S]PAPS did not require the presence of a phenolic substrate but rather was inhibited by *p*-nitrophenol, a sulfate acceptor substrate for TS PST. Inhibitors of TS PST enzymatic activity, including 3′-phosphoadenosine-5′-phosphate, ATP, ADP, and 2,6-dichloro-4-nitrophenol, also inhibited photoaffinity labeling of the 35-kDa protein with [^{35}S]PAPS, in a concentration-dependent fashion, with IC_{50} values of 14 μM, 2.1 mM, 7.7 mM, and 91 μM, respectively. The 35-kDa protein that was radioactively labeled by [^{35}S]PAPS in the presence of UV light coeluted with TS PST enzymatic activity during gel filtration high performance liquid chromatography. [^{35}S]PAPS was then used to photoaffinity label another sulfotransferase enzyme, the thermolabile (TL) form of PST partially purified from human liver. Therefore, [^{35}S]PAPS appears to be a photoaffinity ligand that could be used to study a variety of PAPS-dependent sulfotransferases. Photoaffinity labeling of TS and TL PST, as well as other PAPS-dependent sulfotransferases, should enhance our ability to purify this important group of enzymes and to determine amino acid sequences at or near their active sites.

Sulfate conjugation is an important pathway in the biotransformation of many drugs, xenobiotics, neurotransmitters, and hormones (1–3). Sulfation is also an important process in the posttranslational modification of certain biologically active peptides and proteins, such as the hormone cholecystokinin (4–6). PAPS is the sulfate donor for these reactions in vertebrates (7, 8). [^{35}S]PAPS has previously been used as a photoaffinity ligand for a Golgi membrane-bound protein thought to be a "PAPS transporter" (9). That observations raised the possibility that PAPS might also be a useful photoaffinity ligand for enzymes that catalyze sulfate conjugation.

PST (EC 2.8.2.1) catalyzes the sulfate conjugation of many phenolic drugs, xenobiotic compounds, and neurotransmitters (1–3). All human tissues that have been studied carefully contain at least two forms of PST, which differ in substrate specificity, sensitivity to inhibitors, physical properties, and regulation among individuals (10–16). One form of the enzyme is relatively thermostable (TS), is sensitive to inhibition by DCNP, and catalyzes the sulfate conjugation of micromolar concentrations of phenol, *p*-nitrophenol, and other "simple" phenols. The other form is relatively thermostable (TL), is resistant to DCNP inhibition, and catalyzes the sulfate conjugation of micromolar concentrations of dopamine and other phenolic monoamines. However, at higher concentrations phenol and *p*-nitrophenol are substrates for TL PST, and dopamine can serve as a substrate for TS PST at millimolar concentrations (11, 12, 17). The level of TS PST activity in human tissue is controlled by a common genetic polymorphism (18–20). In addition to the TS and TL forms of the enzyme, two isozymes of TS PST are found in human liver and brain, on the basis of the elution of two peaks of TS PST activity during ion exchange chromatography (12, 16). Individual subjects have one, the other, or both isozymes of TS PST in hepatic tissue (12). Those observations have led to speculation that the isozymes of TS PST might represent two different, genetically determined, structural forms or "allozymes" of the enzyme in human tissue (21, 22).

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ABBREVIATIONS: PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PST, phenol sulfotransferase; TS, thermostable; TL, thermolabile; DCNP, 2,6-dichloro-4-nitrophenol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IAP, 2-iodo-4-azidophenol; HPLC, high performance liquid chromatography; PAP, 3′-phosphoadenosine-5′-phosphate; BSA, bovine serum albumin; TEMED, *N,N,N′,N′*-tetramethylethylenediamine.

Photoaffinity labeling of human liver TS PST has been performed previously with a photoreactive phenolic substrate, [^{125}I]IAP (23). Because the biochemical properties and "pharmacogenetic" regulation of human liver TS PST have been well characterized and because photoaffinity labeling studies of this sulfotransferase enzyme have already been performed with the phenolic photoaffinity ligand [^{125}I]IAP, we chose TS PST to test in photoaffinity labeling experiments performed with [^{35}S]PAPS. We found that UV irradiation of [^{35}S]PAPS in the presence of partially purified human liver TS PST resulted in the labeling of a 35-kDa protein with properties identical to those of TS PST. These results indicate that PAPS can be used as a photoaffinity ligand for the study of PST and other PAPS-dependent sulfotransferases.

Materials and Methods

Tissue acquisition. Pathologically normal hepatic tissue was obtained, under guidelines approved by the Mayo Clinic Institutional Review Board, from patients who underwent clinically indicated partial hepatectomies for the removal of either primary or metastatic hepatic tumors. The tissue was stored at −80°. Hepatic tissue from four patients was thawed, pooled, and homogenized in 4 volumes of 5 mM potassium phosphate buffer, pH 7.5, with a Polytron homogenizer. The homogenate was centrifuged at 16,000 × *g* for 15 min at 4°, the supernatant from this step was centrifuged at 100,000 × *g* for 1 hr at 4°, and the supernatant from the final centrifugation step was used for the purification of human liver TS and TL PST.

PST purification. TS PST was partially purified as described by Otterness *et al.* (23). The purification procedure included, in sequence, DEAE-Sepharose CL-6B ion exchange, Affi-Gel Blue, and Heparin-Sepharose CL-6B chromatography. A similar procedure was used to separate TL from TS PST (12) and to purify TL PST from human hepatic preparations. This procedure resulted in the purification of TS PST by 700- to 800-fold and of TL PST by 300-fold over the activity present in a 100,000 × *g* supernatant preparation.

PST assay. Enzyme activity was assayed by the method of Foldes and Meek (24), as modified by Anderson and Weinshilboum (25) and adapted by Campbell *et al.* (12) for the measurement of human liver TS and TL PST activity. This assay is based on the sulfation of *p*-nitrophenol by TS PST or of dopamine by TL PST in the presence of [^{35}S]PAPS, the sulfate donor for the reaction. Samples that contained no sulfate acceptor substrate were used as blanks. After a 20-min incubation at 37°, the reaction was terminated by the precipitation of [^{35}S]PAPS and protein with barium hydroxide, barium acetate, and zinc sulfate. Each sample was centrifuged, the supernatant fluid was aspirated, Bio-Safe II liquid scintillation counting fluid was added, and radioactivity was measured in a Packard 1900CA liquid scintillation counter.

Protein assay. Protein concentrations were measured by the dye binding method of Bradford (26), with BSA as a standard.

Photoaffinity labeling with [^{35}S]PAPS. The reaction mixture for the photoaffinity labeling experiments consisted of 8.8 μg of partially purified enzyme, 17.6 mM potassium phosphate buffer, pH 6.5, and 50 nM [^{35}S]PAPS (specific activity, 90–340 Ci/mmol), in a volume of 42.5 μl in 12- × 75-mm siliconized culture tubes. In some experiments PST inhibitors such as PAP, ATP, ADP, DCNP, or NaCl were added, to study their effect on the covalent binding of [^{35}S]PAPS to proteins. Reaction mixtures were preincubated at 4° for 2 min and were then transferred to a Rayonet model RPR-100 photochemical chamber reactor. The samples were irradiated at 4° for 40 min, at a distance of 5.7 cm, with UV light that had a mean wavelength of 300 nm. The samples were then transferred to plastic microcentrifuge tubes, and 42.5 μl of SDS-PAGE sample buffer (0.25 M Tris·HCl buffer, pH 7.3, that contained 40% glycerol, 20% 2-mercaptoethanol, 9.2% SDS, and 0.005% bromophenol blue) were added to each sample. The samples were vortexed and were then heated at 95° for 10 min before SDS-PAGE.

Photoaffinity labeling with [^{125}I]IAP. Photoaffinity labeling with [^{125}I]IAP was performed with a slight modification of the method described by Otterness *et al.* (23). Specifically, the reaction mixture consisted of 3–5 μg of protein, 17.6 mM potassium phosphate buffer, pH 6.5, 4.0 μM nonradioactive PAPS, 0.1 μM IAP, and 4 nM [^{125}I]IAP (approximately 500,000 cpm), in a volume of 42.5 μl in 12- × 75-mm siliconized culture tubes. Reaction mixtures were preincubated at 4° for 2 min and were then transferred to a Rayonet RPR-100 photochemical chamber reactor. The samples were irradiated at 4° for 5 min, at a distance of 5.7 cm, with UV light that had a mean wavelength of 350 nm. The samples were then transferred to microcentrifuge tubes, and 42.5 μl of SDS-PAGE sample buffer were added to each sample. The samples were vortexed and were heated at 95° for 10 min before SDS-PAGE.

SDS-PAGE. SDS-PAGE was carried out in 1.5-mm-thick acrylamide slab gels with the discontinuous buffer system of Laemmli (27). The acrylamide concentration in the resolving gel was 10%, unless otherwise indicated. Electrophoresis was performed with a constant current of 30 mA/gel until the bromophenol blue marker reached the bottom of the gel (approximately 4 hr). Migration distances of protein standards and of proteins in the experimental samples were determined after staining with 0.05% Coomassie blue. Gels containing [^{35}S]PAPS-labeled proteins were immersed in RESOLUTION gel autoradiography enhancer and gently agitated for 40 min. After the RESOLUTION was decanted, the gels were immersed in cold water and gently agitated for 30 min to precipitate the fluor within the gels. The gels were then dried for 1 hr at 60° and autoradiographs were obtained with Kodak X-Omat AR X-ray film at −80°, with exposure times of 1 to 29 hr. Gels containing [^{125}I]IAP-labeled proteins were dried after destaining, and autoradiographs were obtained with Kodak X-Omat AR X-ray film with a Dupont Quanta III-T intensifying screen. The exposure time for these autoradiographs was 24 hr at −80°.

Computer analysis of autoradiographs. Densitometric analysis of autoradiographs was performed with the ANALYZE software (28). Use of this computer program made it possible to quantitate total pixel intensity within relevant bands of images that had been digitized with a Microtek MSF-300 scanner (Microtek Lab, Inc., Torrance, CA). Total pixel intensities of relevant bands were quantitated and normalized for background pixel intensity. It has been reported that fluorography methods used with β-particle-emitting isotopes may affect the quantitation of autoradiographic images (29). Therefore, we also analyzed some of the dried gels directly with an AMBIS radioanalytic imaging system (AMBIS Systems Inc., San Diego, CA). There were no systemic differences between results obtained with the two methods of quantitation; therefore, all data reported subsequently represent digitized results obtained from autoradiographs.

Data analysis. IC_{50} values for inhibition of labeling were calculated from least-squares best-fit polynomial equations for data obtained from the autoradiographs. The computer program NWA Statpak (Northwest Analytical, Inc., Portland, OR) was used to make these calculations. Molecular mass estimates for SDS-PAGE were determined by using this same program with data obtained from migration distances of protein standards.

Gel filtration HPLC. Gel filtration HPLC was performed at room temperature with a Waters 6000A pump, a 7.5-mm × 30-cm Waters Protein Pak 300 SW gel filtration column, and a Schoeffel model SF 770 Spectroflow UV monitor. The mobile phase consisted of 0.1 M potassium phosphate buffer, pH 6.1, at a flow rate of 0.9 ml/min. UV absorbance was monitored at 280 nm, and absorbance data were analyzed with a Hewlett Packard 3393A computing integrator. Samples were filtered through a 0.45-μm Gelman Acrodisc before HPLC analysis, a 50-μl aliquot of partially purified TS PST was injected onto the column, and 20-sec fractions were collected manually. Ten-microliter aliquots of each fraction were assayed for TS PST enzyme activity.

In a subsequent experiment, photolysis was conducted in the usual

fashion with [^{35}S]PAPS. These samples were then pooled and concentrated in an Amicon Centricon-10 microconcentrator before HPLC analysis. This procedure resulted in the removal of radioactive photolysis by-products and most of the unbound [^{35}S]PAPS. A blank sample that did not contain protein was also photolyzed and concentrated. This sample was included to determine the extent of removal of unbound [^{35}S]PAPS and its photolysis by-products by use of the Centricon-10 procedure. A 50-μl aliquot of each sample was then injected onto the HPLC column, and 20-sec fractions were collected manually. An aliquot of each of these fractions was mixed with Bio-Safe II liquid scintillation counting fluid, and radioactivity was measured in a Packard 1900CA liquid scintillation counter. Aliquots of selected fractions were also subjected to SDS-PAGE, followed by autoradiographic analysis. Blue dextran, thyroglobulin, ferritin, catalase, aldolase, albumin, ovalbumin, chymotrypsinogen A, and ribonuclease A were used to calibrate the HPLC column. The retention times of these standards were 5.06, 5.08, 6.19, 7.45, 7.47, 8.11, 8.72, 10.23, and 10.45 min, respectively.

Materials. [^{35}S]PAPS (1.6–2.4 Ci/mmol for PST enzyme assays and 90–340 Ci/mmol for photoaffinity labeling experiments) was purchased from New England Nuclear Corp. (Boston, MA). Dithiothreitol, *p*-nitrophenol, 2-mercaptoethanol, PAP, ATP, ADP, and BSA were obtained from Sigma Chemical Co. (St. Louis, MO). Disodium EDTA, barium acetate, barium hydroxide, zinc sulfate, and glycerol were purchased from Fisher Scientific Products (Fairlawn, NJ). DCNP was obtained from K and K Laboratories (Plainview, NY). Bio-Safe II liquid scintillation counting fluid was obtained from Research Products International Corporation (Mount Prospect, IL). Gel filtration molecular weight protein standards were purchased from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ). Acrylamide, bisacrylamide, TEMED, ammonium persulfate, bromophenol blue, Coomassie brilliant blue R-250, SDS, glycine, and SDS-PAGE molecular weight protein standards were obtained from Bio-Rad Laboratories (Richmond, CA). Tris base was obtained from Calbiochem (San Diego, CA). RESOLUTION gel autoradiography enhancer was purchased from EM Corp. (Chestnut Hill, MA).

Results

Introduction. This project consisted of four series of experiments. The first series of studies was designed to determine whether PAPS might be a photoaffinity ligand for TS PST and, if so, to determine optimal photolysis conditions. The second series of experiments was designed to study the relative specificity of [^{35}S]PAPS photoaffinity labeling by testing the ability of known inhibitors of TS PST enzymatic activity to inhibit photoaffinity labeling. The third set of studies was designed to determine whether the protein labeled with [^{35}S]PAPS was TS PST. This was accomplished by photoaffinity labeling partially purified TS PST and subjecting this preparation to gel filtration HPLC to determine whether TS PST enzymatic activity coeluted with the labeled protein. The fourth and final series of experiments was designed to determine whether [^{35}S]PAPS could also be used to photoaffinity label another sulfotransferase, TL PST.

[^{35}S]PAPS as a photoaffinity ligand. Exposure of partially purified human liver TS PST to UV light in the presence of [^{35}S]PAPS resulted in the radioactive labeling of a protein with an approximate molecular mass of 35 kDa by SDS-PAGE (Fig. 1, *lane B*). The molecular mass estimate of 35 kDa was the average value obtained from eight separate experiments. Although the absorption maximum for PAPS is 260 nm (30), photolysis was performed at 300 nm to minimize UV damage to proteins in the enzyme preparation (31). The apparent molecular mass of human liver TS PST estimated by gel

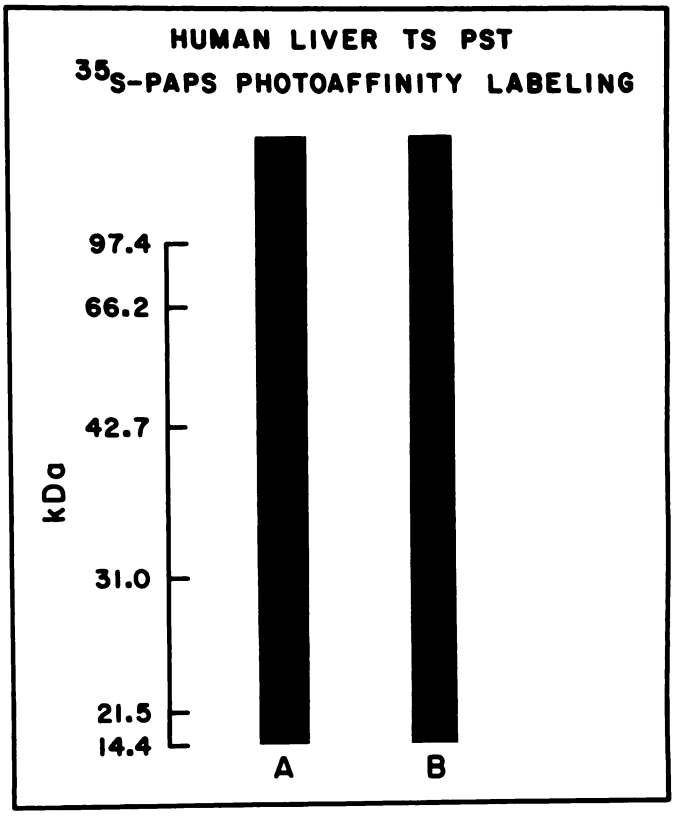

Fig. 1. SDS-PAGE of partially purified human liver TS PST. *Lane A*, Coomassie blue stain of the enzyme preparation used in a typical photolysis experiment. *Lane B*, autoradiograph obtained from the same preparation shown in *lane A*.

filtration chromatography performed in the course of previously published studies was reported to be 60 to 65 kDa (12). Therefore, the protein labeled with [^{35}S]PAPS might represent a subunit of human liver TS PST. Previous studies had shown that photoaffinity labeling of human liver TS PST with [^{125}I]IAP resulted in the radioactive labeling of a protein doublet with molecular masses estimated at 32 and 34 kDa (23). As we will demonstrate subsequently, the 35-kDa protein that was radioactively labeled with [^{35}S]PAPS migrated with [^{125}I]IAP-labeled TS PST during SDS-PAGE.

Effect of time of photolysis. The quantity of radioactivity incorporated into the 35-kDa protein in the TS PST preparation increased with increasing time of photolysis with [^{35}S]PAPS for up to 50 min (Fig. 2). Photolysis of TS PST without [^{35}S]PAPS being present resulted in a time-dependent decrease in enzymatic activity (Fig. 2A). A photolysis time of 40 min was chosen to optimize the quantity of radioactivity incorporated into the protein, while at the same time minimizing the loss of enzymatic activity during irradiation.

Effect of *p*-nitrophenol. Photoaffinity labeling of TS PST performed with the phenolic ligand [^{125}I]IAP was greatly enhanced in the presence of PAPS (23). However, *p*-nitrophenol, a model phenolic sulfate acceptor substrate for TS PST (11, 12), was not required for photoaffinity labeling of the 35-kDa protein with [^{35}S]PAPS. In fact, when concentrations of *p*-nitrophenol from 0.0001 to 100 mM were tested, this compound inhibited the photoaffinity labeling of TS PST in a concentration-dependent fashion, with an IC_{50} value of approximately 145 μM (Fig. 3).

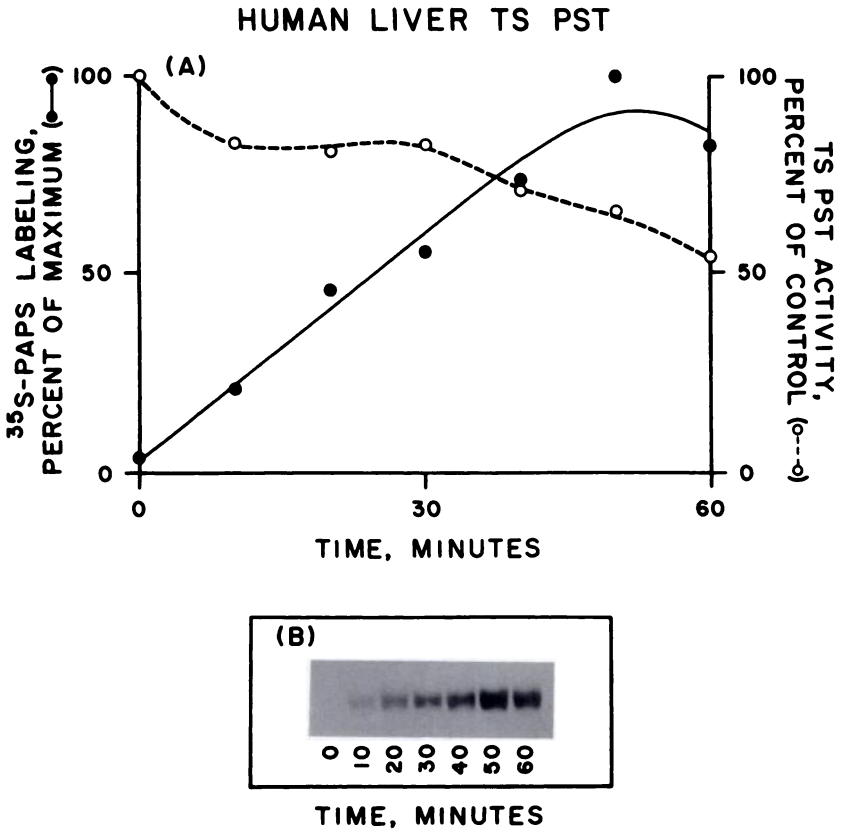

Fig. 2. Effect of photolysis time on radioactive labeling of proteins by [^{35}S]PAPS. A, Computer-analyzed data; B, autoradiographs. Each band shown in B is a separate lane in the 30- to 40-kDa region from SDS-PAGE. The data points (●) shown in A were obtained by computer analysis of the autoradiographs depicted in B. TS PST enzymatic activity (○) is also shown in A. See text for details.

Effect of PAP. PAP is a product of the reaction catalyzed by PST, and PAP competitively inhibits the enzyme reaction (3, 16). The IC_{50} value for inhibition of partially purified human liver TS PST by PAP in the presence of 4 μM *p*-nitrophenol and 0.4 μM PAPS was 0.31 μM (23). Concentrations of PAP from 0.0001 to 10 mM were tested to determine the effect of this compound on the photoaffinity labeling of TS PST by [^{35}S]PAPS (Fig. 4). PAP inhibited labeling of the 35-kDa protein in a concentration-dependent fashion, with an IC_{50} value of approximately 14 μM.

Effect of ATP and ADP. Compounds such as ATP and ADP that are structurally related to PAPS also inhibit PST enzymatic activity (32). K_i values for the inhibition of partially purified human brain TS PST by ATP and ADP were reported to be 23.2 and 30.0 μM, respectively (32), and IC_{50} values determined for the inhibition of our partially purified human liver TS PST by ATP and ADP were 52.7 and 61.7 μM, respectively. Concentrations of these two compounds from 0.001 to 50 mM were tested to determine their effect on the photoaffinity labeling of TS PST. Both ATP and ADP inhibited photoaffinity labeling of the 35-kDa protein in a concentration-dependent fashion. Approximate IC_{50} values for inhibition of labeling were 2.1 and 7.7 mM for ATP and ADP, respectively.

Effect of DCNP. DCNP also inhibits TS PST enzymatic activity (10, 12). The IC_{50} value for inhibition of partially purified human liver TS PST by DCNP was 0.17 μM (23). Concentrations of DCNP from 0.0001 to 10 mM were tested to determine the effect of DCNP on the photoaffinity labeling of TS PST. DCNP inhibited photoaffinity labeling of the 35-kDa protein in a concentration-dependent fashion, with an approximate IC_{50} value of 91 μM (Fig. 5). However, when DCNP was present during photolysis, two additional proteins with molecular mass values lower than 35 kDa were labeled with [^{35}S]PAPS (Fig. 5B). This phenomenon was observed only when photoaffinity labeling was performed in the presence of DCNP. The molecular basis of this behavior remains unclear.

Effect of NaCl. NaCl inhibits TS PST enzymatic activity (16, 33). The IC_{50} value for the inhibition of partially purified human liver TS PST by NaCl was 78 mM (23). Concentrations of NaCl from 0.016 to 1.0 M were tested to determine the effect of this compound on the photoaffinity labeling of TS PST. NaCl inhibited labeling of the 35-kDa protein in a concentration-dependent fashion, with an approximate IC_{50} value of 580 mM. Therefore, all of the TS PST inhibitors tested, including PAP, ATP, ADP, DCNP, and NaCl, were also capable of inhibiting the photoaffinity labeling of the 35-kDa protein in a concentration-dependent fashion.

Gel filtration HPLC. The purpose of this series of experiments was to determine whether TS PST enzymatic activity coeluted during gel filtration HPLC with the protein that was photoaffinity labeled with [^{35}S]PAPS. In the first experiment, a sample of partially purified TS PST was subjected to gel filtration HPLC, and enzymatic activity was measured. TS PST enzymatic activity eluted at 8.16 min, the same time as BSA, with a molecular mass of 66.2 kDa (Fig. 6A). A sample of TS PST that had been photoaffinity labeled with [^{35}S]PAPS was then subjected to gel filtration HPLC. This sample contained two peaks of radioactivity (Fig. 6B). Radioactive peak I eluted at the same time as did TS PST enzymatic activity. When peak I was precipitated with trichloroacetic acid and the precipitate was analyzed by SDS-PAGE, it contained the 35-kDa protein, as detected both with Coomassie blue stain and by autoradiography (Fig. 7). Radioactive peak II was also sub-

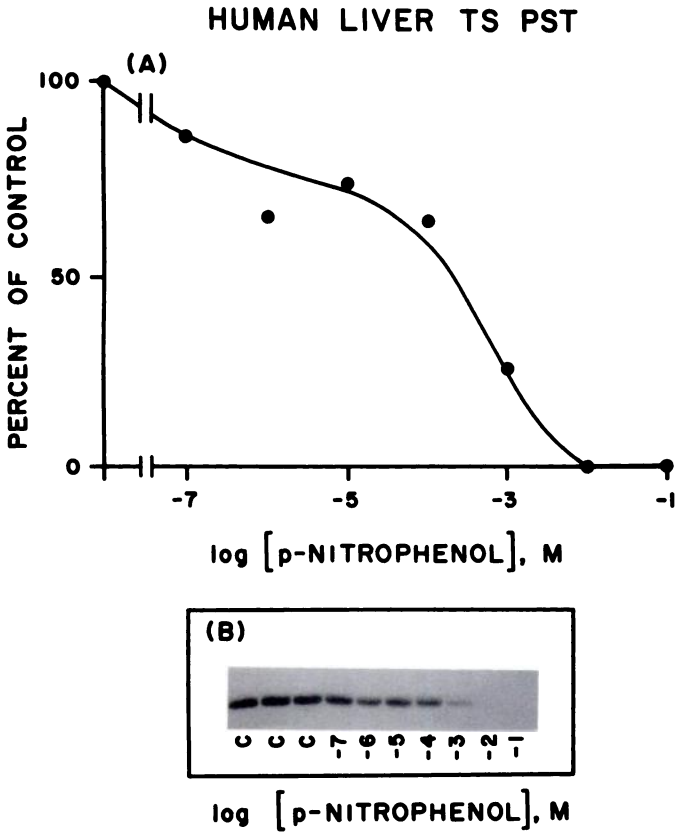

Fig. 3. Effect of *p*-nitrophenol concentration on radioactive labeling of proteins by [^{35}S]PAPS. A, Computer-analyzed data; B, autoradiographs. Each protein band in B is a separate lane in the 30–40-kDa region from SDS-PAGE. *Numbers* in B, concentrations; *C*, control samples with no *p*-nitrophenol present. Each data point shown in A was obtained by computer analysis of the autoradiograph depicted in B.

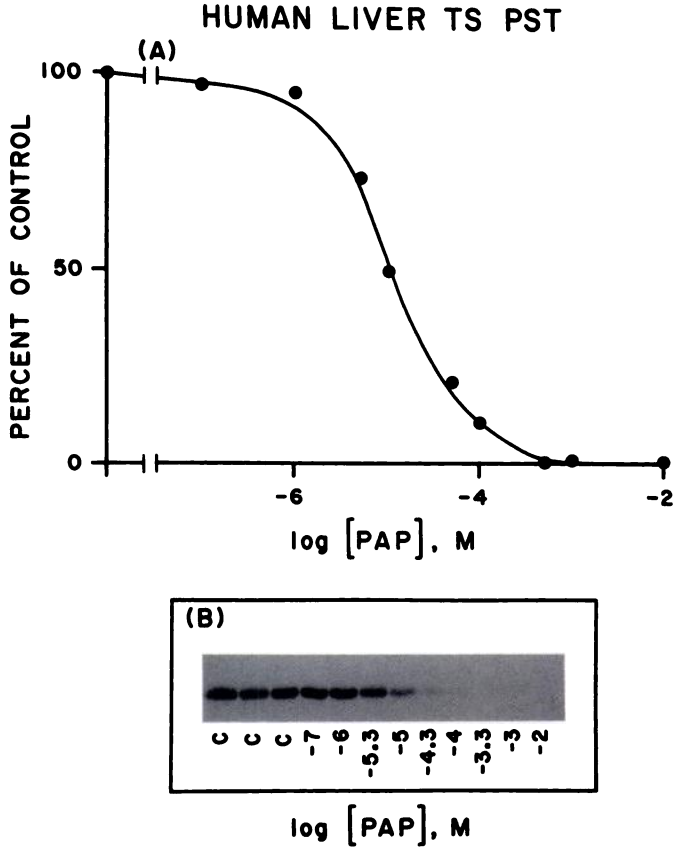

Fig. 4. Effect of PAP concentration on radioactive labeling of proteins by [^{35}S]PAPS. A, Computer-analyzed data; B, autoradiographs. Each protein band in B is a separate lane in the 30–40-kDa region from SDS-PAGE. *Numbers* in B, concentrations; *C*, control samples with no PAP present. Each data point shown in A was derived by computer analysis of the autoradiograph depicted in B.

jected to trichloroacetic acid precipitation, followed by SDS-PAGE analysis. No protein was detected in this lane by Coomassie blue stain, and no radioactivity was present (Fig. 7). A blank sample that was subjected to photolysis but that did not contain protein also contained radioactivity that eluted during HPLC at the same time as radioactive peak II in the photolyzed sample that contained enzyme (Fig. 6C). Therefore, the radioactivity present in peak II appeared to represent a by-product of the photolysis of [^{35}S]PAPS. In the control sample, a protein with a lower molecular mass was also slightly labeled with [^{35}S]PAPS (Fig. 7), probably as a consequence of prolonged exposure to [^{35}S]PAPS during the lengthy Centricon separation procedure (23). The results of these experiments demonstrated that TS PST enzymatic activity coeluted during gel filtration HPLC with the 35-kDa protein that was photoaffinity labeled by [^{35}S]PAPS.

Photoaffinity labeling of TL PST with [^{35}S]PAPS. To test the possibility that [^{35}S]PAPS might also serve as a photoaffinity ligand for other sulfotransferases, partially purified human liver TL PST was used as a second potential target for this photoreactive ligand. We reported previously that purified human small intestinal TL PST has a molecular mass of 69 kDa, as estimated by gel filtration chromatography, and that it is composed of a 35.5-kDa monomer (13), with a slightly larger molecular mass than that of the monomer for human hepatic TS PST. Previous studies have demonstrated that ion exchange chromatography can separate TS from TL PST in human hepatic preparation (12). It should be emphasized that the TL PST used in the present experiments had also been separated from TS PST during enzyme purification by the use of ion exchange chromatography. The TS PST preparations used in these experiments were devoid of TL PST enzymatic activity and vice versa. We found that the partially purified human hepatic TL PST preparation, like human small intestinal TL PST (13, 34), contained a protein with a molecular mass slightly greater by SDS-PAGE than that of the TS PST monomer (Fig. 8). When the same photolysis conditions were used that resulted in the radioactive labeling of TS PST with [^{35}S]PAPS, this slightly larger protein species in the TL PST preparation was also radioactively labeled (Fig. 8).

Photoaffinity labeling of TS and TL PST with [^{125}I]IAP. [^{125}I]IAP was designed as a phenolic photoaffinity ligand for human TS PST (23, 35). We also compared the photoaffinity labeling of both partially purified human hepatic TS and TL PST with [^{125}I]IAP. As expected, photolysis of [^{125}I]IAP in the presence of TS PST resulted in the radioactive labeling of a protein doublet that co-migrated with the 35-kDa protein labeled by [^{35}S]PAPS (Fig. 8). However, under identical photolysis conditions [^{125}I]IAP did not label any protein in the TL PST preparation (Fig. 8).

Discussion

Sulfate conjugation is an important pathway in the biotransformation of many drugs, xenobiotic compounds, neurotrans-

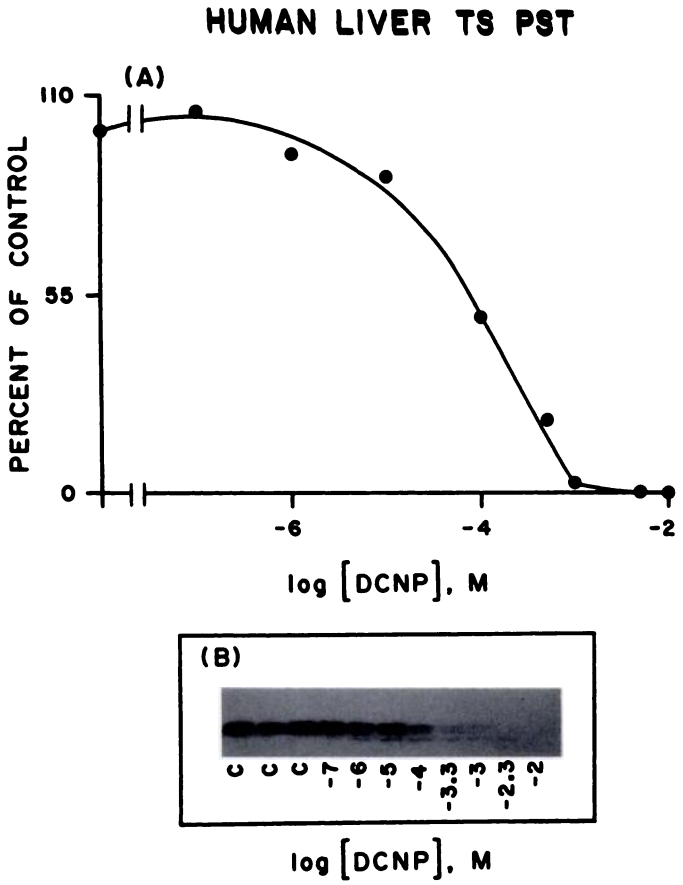

Fig. 5. Effect of DCNP concentration on radioactive labeling of proteins by [^{35}S]PAPS. A, Computer-analyzed data; B, autoradiographs. Each protein band in B is a separate lane in the 30–40-kDa region from SDS-PAGE. *Numbers* in B, concentrations; *C*, control samples with no DCNP present. Each data point in A was obtained by computer analysis of the autoradiograph shown in B.

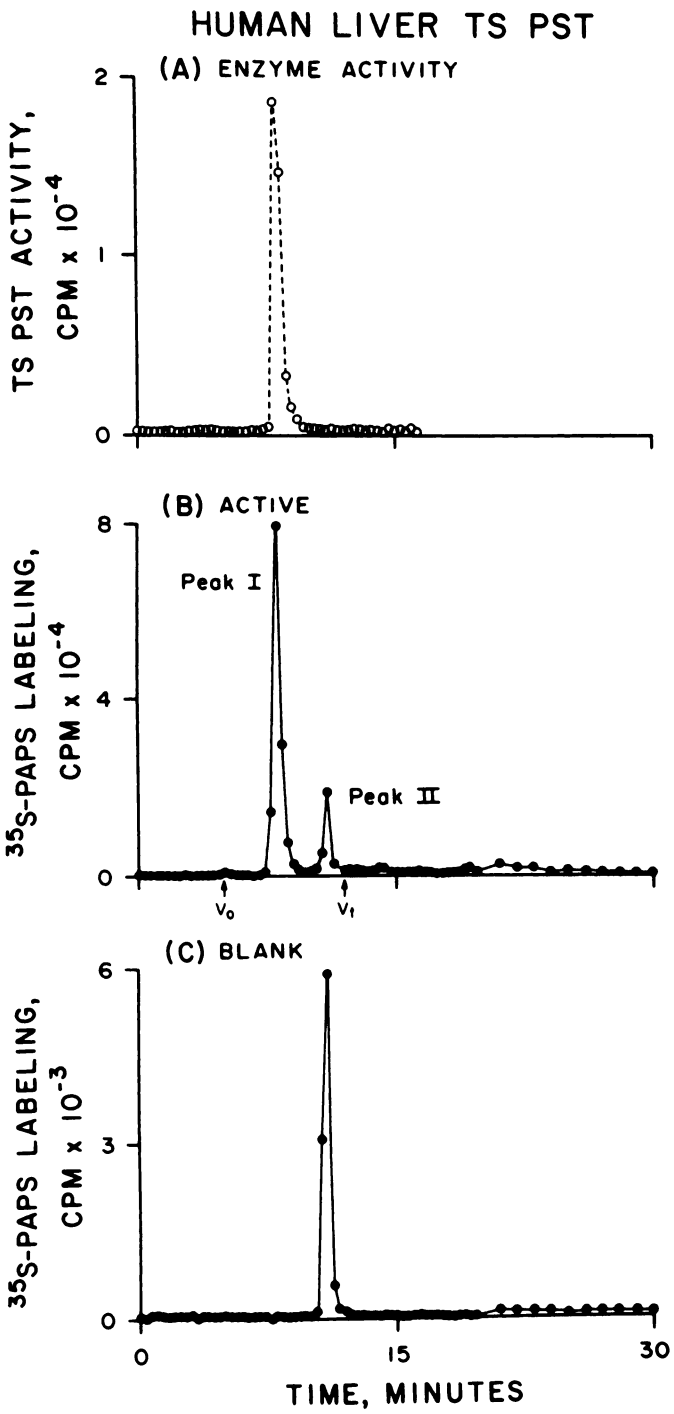

Fig. 6. Gel filtration HPLC of TS PST. A, TS PST enzyme activity in fractions obtained during gel filtration HPLC of an unphotolyzed enzyme preparation. B, HPLC of a photoaffinity-labeled preparation of TS PST (active). C, HPLC of a buffer sample with [^{35}S]PAPS photolyzed in the absence of TS PST (blank). Radioactivity shown in B and C was measured in fractions obtained during gel filtration HPLC. Note the difference in *y*-axes for B and C. See text for details.

mitters, and hormones (1–3). PST is the most important of the sulfotransferases involved in drug metabolism (2, 36). All human tissues that have been studied carefully contain at least two forms of PST, which have been designated TS and TL PST. These two forms differ in their substrate specificities, physical properties, and regulation among individuals (10–16). Human liver TS PST appears to be a dimer composed of subunits of between 32 and 35 kDa in molecular mass, whereas human TL PST appears to be a slightly larger homodimer made up of monomers of between 34 and 35.5 kDa (13, 23, 37–40).

We demonstrated previously that the photoreactive phenolic compound [^{125}I]IAP could be used as a photoaffinity ligand for human liver TS PST (23). Photoactivation of that ligand with human liver TS PST resulted in the radioactive labeling of two proteins with apparent molecular masses of approximately 32 and 34 kDa (23). Because TS PST is an important and well characterized sulfotransferase and because a phenolic photoaffinity ligand for the enzyme had already been developed, we chose TS PST for use in photoaffinity labeling experiments performed with [^{35}S]PAPS. The direct photoaffinity labeling of other proteins with adenosine derivatives such as ATP, ADP, *S*-adenosyl-L-methionine, and PAPS has been described previously (9, 41, 42). The mechanism of the photoactivation of purines and of purine nucleosides is thought to involve the C-8 position of the purine ring system (43). We found that exposure of partially purified human liver TS PST to UV light in the presence of [^{35}S]PAPS resulted in the radioactive labeling of a single 35-kDa protein. The binding of [^{35}S]PAPS was inhibited in a concentration-dependent fashion by inhibitors of TS PST enzymatic activity, including PAP, ATP, ADP, DCNP, and NaCl. IC_{50} values for inhibition of photoaffinity labeling by all of these compounds were uniformly 1 to 2 orders

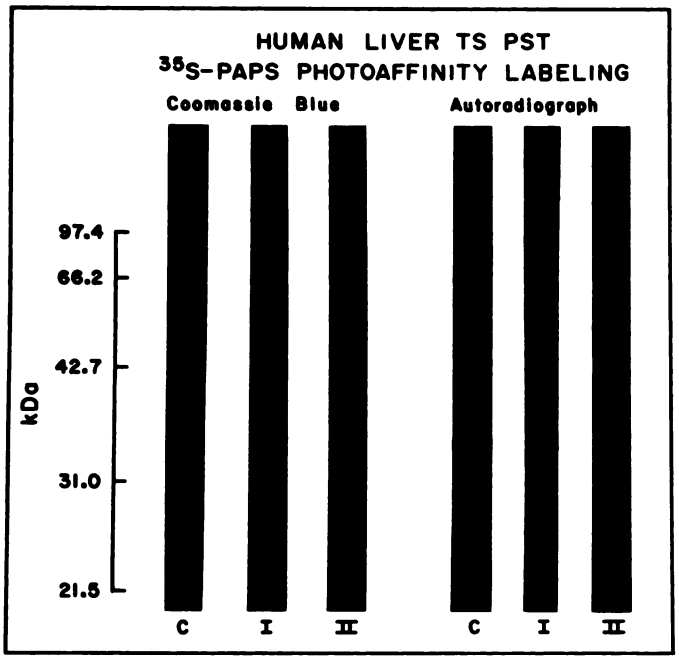

Fig. 7. SDS-PAGE of fractions collected during gel filtration HPLC of photoaffinity-labeled TS PST. *C*, "control" preparation that was applied to HPLC. *Roman numerals*, radioactive HPLC peaks shown in Fig. 6B.

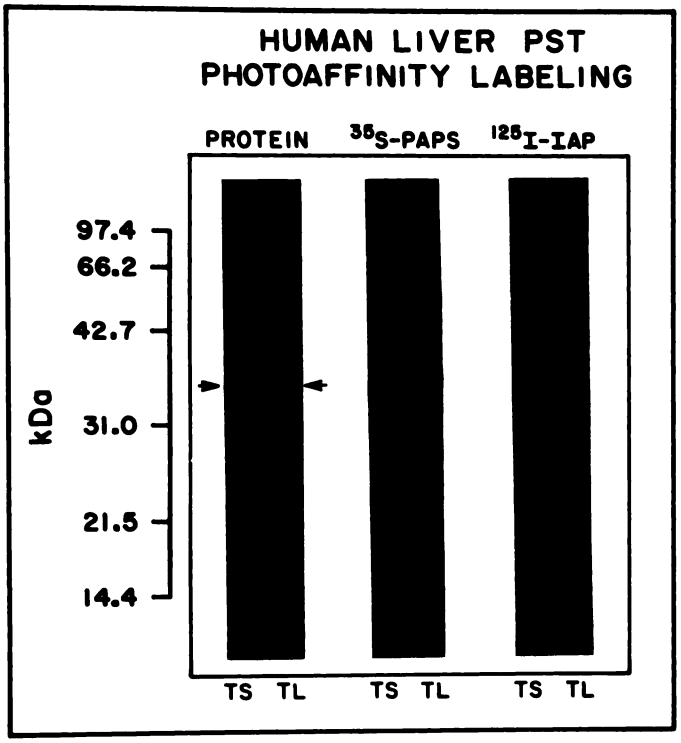

Fig. 8. Photoaffinity labeling of TS and TL PST with [^{35}S]PAPS and [^{125}I]IAP. Partially purified human liver TS and TL PST were photolyzed in the presence of either [^{35}S]PAPS or [^{125}I]IAP, and photolyzed preparations were analyzed on 12.5% acrylamide gels. *Arrows*, protein species stained with Coomassie blue that also were radioactively labeled by photolysis with [^{35}S]PAPS. See text for details.

of magnitude higher than were IC_{50} values for the inhibition of enzymatic activity. Similar results have been reported for inhibition of the photoaffinity labeling of the methyl-conjugating enzyme phenylethanolamine-*N*-methyltransferase by its methyl donor, *S*-adenosyl-L-methionine (42). Many other photoaffinity labeling studies have tested the effects on labeling of only a single high concentration of an inhibitor. We went beyond the use of a single concentration and attempted to determine the concentration dependence of the inhibition of [^{35}S]PAPS photoaffinity labeling of TS PST. However, it is probably inappropriate to compare directly IC_{50} values for compounds that reversibly inhibit an enzymatic reaction with values for inhibition of the photoaffinity labeling of the enzyme. The important observation is that compounds that interfere with catalysis of the PST reaction also uniformly interfere with the covalent binding of [^{35}S]PAPS, an observation in support of the relative specificity of PAPS as a photoaffinity ligand.

Photoaffinity labeling of TS PST with [^{35}S]PAPS did not require the presence of a phenolic co-substrate. In fact, labeling was inhibited by *p*-nitrophenol, a model sulfate acceptor-substrate for the enzyme (11, 12). Whether this inhibition was due to competition between the two co-substrates at the active site or to the possible photoactivation of *p*-nitrophenol remains unclear. Although substrate kinetic studies of guinea pig liver PST were reported to demonstrate that the reaction proceeded via a rapid equilibrium, random, Bi-Bi mechanism (44), similar studies of PST from rat and human brain supported a sequential, ordered, Bi-Bi reaction mechanism, with PAPS as the first substrate to bind to the enzyme (16, 45). Photoaffinity labeling of TS PST by [^{125}I]IAP was greatly enhanced in the presence of PAPS (23). That observation, as well as the results of our present experiments performed with [^{35}S]PAPS, appear to support the initial binding of PAPS. [^{35}S]PAPS has been used on at least one previous occasion as a photoaffinity ligand, with labeling of a protein in the Golgi membrane (9). Even though the authors of that report speculated that the radioactively labeled protein might represent a PAPS "transporter," Golgi membrane is also the location of a tyrosylprotein sulfotransferase (46). Because the protein that was radioactively labeled in those studies had a molecular mass of approximately 34 kDa (9), the labeled protein might represent the Golgi membrane tyrosylprotein sulfotransferase rather than a PAPS transport protein.

The reason that photoaffinity labeling of human liver TS PST with [^{125}I]IAP resulted in the labeling of a protein doublet (23), whereas photoaffinity labeling of an identical enzyme preparation with [^{35}S]PAPS resulted in the labeling of only a single species with molecular mass of 35 kDa, remains unclear, but there are several possible explanations for this behavior. For example, the molecular mass of human hepatic TS PST is approximately 65 kDa (12). Therefore, the enzyme might be a heterodimer and [^{125}I]IAP might label both subunits, whereas [^{35}S]PAPS might be capable of labeling only one. Obviously, the molecular basis for this behavior must ultimately be determined by the isolation and characterization of the protein species labeled by both [^{125}I]IAP and [^{35}S]PAPS.

The development of photoaffinity labeling techniques for human TS and TL PST will enhance our ability to purify these enzymes to homogeneity and to obtain amino acid sequence information, especially with regard to their active sites. The fact that PAPS can be utilized as a photoaffinity ligand for sulfotransferases should also make it possible to use a similar approach to study any PAPS-dependent sulfotransferase. Information obtained from such experiments should both increase our understanding of the mechanism of sulfate conjugation and make it possible to apply the techniques of molecular biology

to the study of TS PST, TL PST, and other sulfotransferase enzymes.

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