0026-895X/89/060856-10$02.00/0

MOLECULAR PHARMACOLOGY, 36: 856–865

Human Liver Thermostable Phenol Sulfotransferase: Photoaffinity Labeling with 2-Iodo-4-azidophenol

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Received February 27, 1989; Accepted September 25, 1989

SUMMARY

Phenol sulfotransferase (PST) catalyzes the sulfate conjugation of phenolic drugs, neurotransmitters, and xenobiotic compounds. Human tissues contain at least two forms of PST, which differ in their substrate specificities, inhibitor sensitivities, physical properties, and regulation. One form of the enzyme is thermostable (TS) and catalyzes the sulfate conjugation of micromolar concentrations of "simple" phenols. The other form of PST is thermolabile and catalyzes the sulfate conjugation of micromolar concentrations of dopamine and other phenolic monoamines. Quantitative structure-activity relationship (QSAR) analyses of substrate kinetic data obtained with purified human liver TS PST made it possible to design a photoreactive substrate for this form of the enzyme. Because of the very high affinity of TS PST for 2-halogenated phenols, 2-iodo-4-azidophenol (IAP) was synthesized and tested for this purpose. The K_m predicted for IAP on the basis of QSAR analysis was 95 nM. The apparent K_m determined experimentally was 52 nM. UV irradiation of partially purified human liver TS PST in the presence of [^{125}I]IAP and 3′-phosphoadenosine-5′-phosphosulfate, the sulfate donor for the reaction, resulted in the radioactive labeling of two proteins, with molecular weights of 32,000 and 34,000, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Inhibitors of the reaction catalyzed by TS PST, including 2,6-dichloro-4-nitrophenol-3′-phosphoadenosine-5′-phosphate and NaCl, as well as 2-iodophenol, a competing substrate, inhibited the photolabeling of both of these proteins by [^{125}I]IAP in a concentration-dependent fashion. Partially purified TS PST was then radioactively labeled with [^{125}I]IAP and was subjected to gel filtration high performance liquid chromatography to verify that the photoaffinity-labeled proteins detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis coeluted with TS PST enzyme activity. Photoaffinity labeling of TS PST will be useful in studies of the molecular characteristics of the protein and its active site, as well as in the purification of this important drug-metabolizing enzyme.

PST (EC 2.8.2.1) catalyzes the sulfate conjugation of many phenolic and catechol drugs, xenobiotic compounds, and neurotransmitters (1–3). All human tissues that have been studied in detail contain at least two forms of PST (4, 5). These forms differ in their substrate specificities, sensitivities to inhibitors, physical properties, and regulation among individuals (6–9). One form of the enzyme is thermostable and catalyzes the sulfate conjugation of micromolar concentrations of phenol, *p*-nitrophenol, and other "simple" phenols. The other form is relatively thermolabile and catalyzes the sulfate conjugation of micromolar concentrations of dopamine and other catechol and phenolic monoamines. However, at higher concentrations phenol and *p*-nitrophenol are substrates for TL PST (8, 10), and at high concentrations dopamine can serve as a substrate for the TS form of the enzyme (4). It has been suggested that the two forms of PST be referred to as the P (phenol-metabolizing) and M (monoamine-metabolizing) forms of the enzyme (7). However, because of significant overlap in their substrate specificities, they will be referred to subsequently as TS and TL PST, based on the unequivocal differences in their thermal stabilities. In addition to the TS and TL forms of the enzyme, two isozymes of TS PST are present in human liver and brain, on the basis of the elution of two peaks of TS PST activity during ion exchange chromatography (4, 11). These isozymes differ in their thermal stabilities, and it has been shown that individual subjects have one, the other, or both isozymes of TS PST in hepatic tissue (4). This observation raises the possibility of two different, genetically determined, structural forms or "allozymes" of TS PST in human tissue.

Partially purified human liver TS PST has been used in

This work was supported in part by National Institutes of Health Grants GM 28157 (R.M.W.), GM 35720 (R.M.W.), DK 32878 (L.J.M.), and DK 34988 (L.J.M.), and by National Institutes of Health contract ES 55110 (R.M.W.).

ABBREVIATIONS: PST, phenol sulfotransferase; TS, thermostable; TL, thermolabile; PAPS; 3′-phosphoadenosine-5′-phosphosulfate; HPLC, high performance liquid chromatography; IAP, 2-iodo-4-azidophenol; QSAR, quantitative structure-activity relationship; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TLC, thin layer chromatography; BSA, bovine serum albumin; TEMED, *N,N,N′N′*-tetramethylethylenediamine; DCNB, 2,6-dichloro-4-nitrophenol; PAP, 3′-phosphoadenosine-5′-phosphate; TCA, trichloroacetic acid.

QSAR analyses to study the characteristics of the active site of the enzyme and to make it possible to predict K_m values of phenolic substrates for this form of the enzyme (12). Based on the results of those QSAR analyses, we designed a photoaffinity substrate for human liver TS PST. The goals of the experiments to be described subsequently included the design, synthesis, and testing of this photoaffinity ligand, IAP, as a specific photoreactive substrate for partially purified human liver TS PST. The availability of a probe for the active site of TS PST should enhance our ability to study the molecular characteristics of this important drug-metabolizing enzyme.

Materials and Methods

Tissue acquisition. Human hepatic tissue was obtained from patients who underwent clinically indicated partial hepatectomies for the removal of either primary or metastatic tumors. This tissue was obtained under guidelines approved by the Mayo Clinic Institutional Review Board. Pathologically normal hepatic tissue was stored at −80°. Tissue from seven 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 16,200 × *g* for 15 min at 4°. The supernatant from this step was then centrifuged at 100,000 × *g* for 1 hr at 4°, and the supernatant from the final centrifugation step was used to purify human TS PST.

TS PST purification. TS PST was partially purified using modifications of the methods described by Campbell *et al.* (4) and by Sundaram *et al.* (5). The procedure included, in sequence, ion exchange, Affi-Gel Blue, and heparin-Sepharose chromatography.

Ion exchange chromatography. Supernatant fractions after centrifugation of human liver homogenates at 100,000 × *g* for 1 hr were applied to a 5.0 × 7.0 cm column of DEAE-Sepharose CL-6B that had been equilibrated with 5 mM potassium phosphate buffer, pH 7.5, which contained 3 mM DTT and 0.2 mM EDTA (buffer A). The column was eluted with 250 ml of buffer A that contained 100 mM NaCl, followed by a 1000-ml gradient from 100 to 300 mM NaCl in buffer A. Fractions of 8.3 ml were collected, and 10-μl aliquots of 100-fold-diluted fractions were used to assay TS PST activity. Fractions that contained peak TS PST activities were pooled and applied to an Affi-Gel blue column.

Affi-Gel Blue chromatography. Pooled fractions from the ion exchange step that contained the peak of TS PST activity were applied to a 2.6 × 27 cm column of Affi-Gel Blue (100–200 mesh) that had been equilibrated with buffer A. The column was eluted with a 400-ml gradient from 0 to 1.0 M KCl in buffer A, followed by a 250-ml "step" to 1.5 M KI in buffer A. Because the iodide ion is more chaotropic than is the chloride ion (13), KI was more effective than KCl in eluting a sharp peak of PST from the column. Fractions of 8.3 ml were collected, and 10-μl aliquots of 100-fold-diluted fractions were used to assay TS PST activity. Fractions from the Affi-Gel blue column that contained peak TS PST activity were pooled and desalted by application to a 2.6 × 68 cm column of Sephadex G-25 that was eluted with buffer A. Fractions from the G-25 column that contained protein were pooled and concentrated in an Amicon pressure concentrator with a YM10 membrane.

Heparin-Sepharose CL-6B chromatography. The desalted concentrated pool of Affi-Gel Blue fractions that contained the peak of TS PST activity was applied to a 0.9 × 13 cm Heparin-Sepharose CL-6B column that had been equilibrated with buffer A. The column was eluted with 50 ml of buffer A, followed by a 200-ml gradient from 0 to 1.0 M NaCl in buffer A. Fractions of 8.3 ml were collected, and 10-μl aliquots of 100-fold-diluted fractions were used to assay TS PST activity. Fractions that contained peak TS PST activities were pooled and stored at 4° for subsequent use in the photoaffinity labeling experiments.

PST assay. PST activity was assayed by the method of Foldes and Meek (14) as modified by Anderson and Weinshilboum (15) and by Campbell *et al.* (4) for the measurement of human liver TS PST activity. The assay is based on the sulfation of *p*-nitrophenol by 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 incubation for 20 min, the reaction was terminated by the precipitation of PAPS and protein with barium hydroxide, barium acetate, and zinc sulfate. Each sample was then centrifuged, the supernatant fluid was aspirated, 3a70 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 (16) with bovine serum albumin as a standard.

Conductivity measurements. Conductivity was measured with a YSI model 31 conductivity bridge.

Synthesis of 4-azidophenol. All reactions described in this and in subsequent synthetic steps were performed under long wavelength yellow light produced by Sylvania Gold F40/GO fluorescent bulbs. As a first step in the synthesis of 4-azidophenol, 2 mmol of 4-aminophenol were dissolved in 10 ml of 1.0 N HCl. This solution was cooled to 0–5° in an ice bath, and 2.2 mmol of sodium nitrite were added. After stirring for 10 min at 0–5°, 4 mmol of sodium azide were added and the temperature of the solution was allowed to rise to room temperature. A white insoluble product separated from the solution after approximately 30 min. The product was filtered, washed with 1.0 N HCl, and dried *in vacuo*. The final yield was 25 mg (9%). The product invariably darkened when dried. Caution must be exercised in dealing with this product because attempts to "scale up" the reaction resulted in violent decomposition of the product during the drying step.

The product of this reaction gave a single spot during TLC on silica gel plates with a solvent system composed of chloroform/methanol/acetic acid (9:1:0.1, v/v). The product was detected by iodine vapor, and its R_F was 0.64. The product also gave a single symmetrical peak with a retention time of 19.98 min during reverse phase HPLC. To perform HPLC, the sample was injected onto a 5-μm 0.46 × 15 cm Altex C18 reverse phase column. The mobile phase consisted of 0.1 M triethylammonium acetate buffer, pH 5.0, with a flow rate of 1 ml/min. A 50-min linear gradient from 10 to 60% acetonitrile was used. UV absorbance at 254 nm was monitored, to detect the elution of phenolic compounds.

Synthesis of [^{125}I]IAP. The 4-azidophenol synthesized with the procedure described in the preceding section was the starting material for the synthesis of IAP. In a typical reaction, 10 μg of 4-azidophenol was iodinated with Na^{125}I using the solid phase oxidant *N*-chlorobenzenesulfonamide (IODO-BEADS). The 4-azidophenol was dissolved in 20 μl of methanol. Subsequently, 60 μl of 0.2 M sodium borate buffer, pH 9.0, 1 mCi of Na^{125}I, and one IODO-BEAD were added to the solution. After 30 sec, the reaction mixture was diluted with 0.9 ml of 0.1 M triethylammonium acetate, pH 5.0. The mixture was aspirated from the reaction tube and was placed on an octadecylsilane precolumn (Pierce Chemical Co.). HPLC purification was then accomplished with a Vydac 218TP54 C18 reverse phase column with the same elution conditions used to perform HPLC of 4-azidophenol. The elution of radioactive products was monitored with a Beckman model 170 Radioisotope Detector. Under these conditions, the retention time of [^{125}I]IAP was 36.37 min.

Synthesis of [^{127}I]IAP. 4-Azidophenol was also the starting material for the synthesis of [^{127}I]IAP, i.e., nonradioactive IAP. Specifically, 4.6 mg of 4-azidophenol was dissolved in 3.4 ml of a solution that consisted of 1 part of 0.2 M sodium borate buffer, pH 9.0, 1 part of methanol, and 1.4 parts of acetonitrile. A saturated solution of iodine in acetonitrile (15–20 μl) was then added until color persisted for 5 sec. The reaction product was purified with a semipreparative C18 reverse phase Vydac 218TP1010 column. The solvent system and elution conditions were the same as those described for 4-azidophenol except for the fact that the flow rate was 4 ml/min. Under these conditions, the reaction product eluted at 33.49 min. The product of this reaction co-migrated with [^{125}I]IAP during TLC on silica gel plates with chlo-

roform as the solvent. Finally, the synthetic [^{127}I]IAP and [^{125}I]IAP coeluted during reverse phase HPLC (Fig. 1). Specifically, 20 μmol of [^{127}I]IAP and 48,000 cpm of [^{125}I]IAP were dissolved in acetonitrile/triethylammonium acetate buffer (1:1, v/v). This mixture was applied to a Beckman ultrasphere C18 reverse phase HPLC column. Absorbance at 254 nm was monitored, and 1-ml fractions of eluent were collected. Radioactivity was measured in these fractions, and it coeluted with the peak in UV absorbance at 254 nm (Fig. 1).

The structure of IAP was verified by comparing the NMR spectrum of its *O*-sulfate derivative with that of the *O*-sulfate derivative of 4-azidophenol. The sulfate derivatives were prepared by treatment of the parent compounds with sulfur trioxide-pyridine complex in pyridine, followed by HPLC purification. Protons adjacent to either the phenolic group or the azide group are easily differentiated with the sulfate derivative, whereas they have very similar chemical shifts in the parent compound. NMR was performed with a General Electric Omega 500 MHz instrument. Data for the 4-azidophenol sulfate included the following: ^{1}H NMR (D_2O), δ 7.10 (2, d, J = 9.2) and 7.30 (2, d, J = 9.2). Data for the IAP sulfate included: ^{1}H NMR (D_2O), δ 7.05 (1, dd, J = 9.0, 2.7), 7.39 (1, d, J = 9.0), and 7.61 (1, d, J = 2.7). These NMR spectra showed the loss of one proton *ortho* to the phenolic group in the iodinated compound, thus confirming that the compound was IAP.

Photoaffinity labeling. The reaction mixture for the photoaffinity labeling experiments consisted of 10.7 μg of partially purified P PST, 11.3 mM potassium phosphate buffer, pH 6.5, 4.0 μM PAPS, 0.1 μM IAP, and 1.2 nM [^{125}I]IAP (approximately 500,000 cpm) in a volume of 155 μl in 12 × 75 mm siliconized culture tubes. In some experiments, PST inhibitors such as PAP, DCNP, and NaCl or a competing substrate, 2-iodophenol, were added to study their effect on the binding of [^{125}I]IAP 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 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 plastic microcentrifuge tubes. BSA, 10 μg in 10 μl, was added to each sample as a carrier protein, and proteins were precipitated by the addition of 165 μl of 20% TCA. Samples were placed on ice for 15 min and were then centrifuged at 4° for 5 min, the supernatants were discarded, and the protein pellets were resuspended in 0.125 M Tris·HCl buffer, pH 6.8, that contained

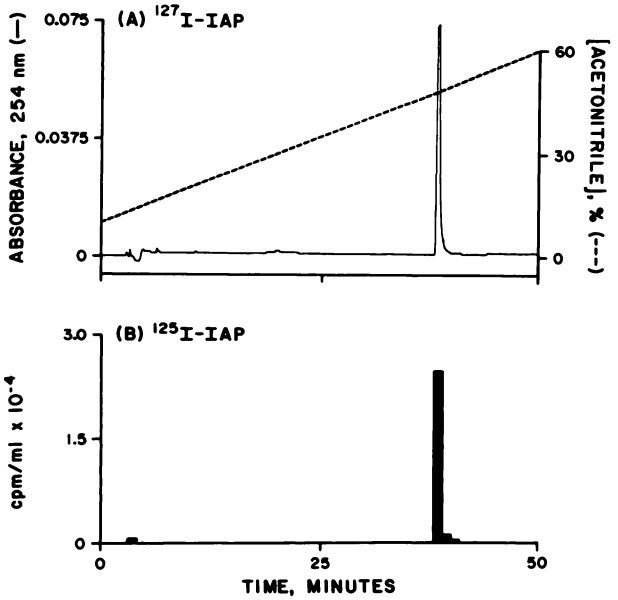

Fig. 1. Reverse phase HPLC of [^{127}I]IAP and [^{125}I]IAP. A mixture of radioactive [^{125}I]IAP and nonradioactive [^{127}I]IAP was subjected to HPLC through a reverse phase C18 column. A, [^{127}I]IAP was detected by the measurement of UV absorbance at 254 nm. B, [^{125}I]IAP was detected by the measurement of radioactivity in 1-ml fractions of the HPLC eluent.

4% SDS, 10% glycerol, 0.01% bromophenol blue, and 0.1 M dithiothreitol.

Gel electrophoresis. SDS-PAGE was carried out in 1.5-mm thick acrylamide slab gels with the discontinuous buffer system of Laemmli (17). The acrylamide concentration in the resolving gel was 10%. 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 standard proteins and of proteins in the sample were determined after staining with 0.05% Coomassie blue. Autoradiographs of gels were obtained with Kodak X-Omat AR X-ray film with a Dupont Quanta III-T intensifying screen. Exposure times were 2–3 days at −80°.

Computer analysis of autoradiographs. Densitometric analysis of the autoradiographs was performed using the ANALYZE software (18). Use of this computer program made it possible to quantitate total pixel density within relevant bands of images that had been digitized with a Microtek MSF-300G scanner (Gardena, California). Total pixel density of relevant bands was quantitated and "normalized" for the pixel density contributed by gel background.

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 6.0, 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 45-μl aliquot of TS PST purified through the heparin-Sepharose step was injected onto the column, and 20-sec fractions were collected manually. Twenty-five-microliter aliquots of each fraction were assayed for TS PST activity. A 50-μl aliquot of a sample of TS PST that had undergone photoaffinity labeling with [^{125}I]IAP was also subjected to HPLC analysis under the same conditions. Before HPLC analysis, an Amicon Centricon-10 Microconcentrator was used to concentrate the photoaffinity-labeled protein and to separate unbound [^{125}I]IAP from protein. The radioactivity of each HPLC fraction was measured in a Beckman Gamma 4000 γ-counter. Blue dextran, catalase, aldolase, albumin, ovalbumin, chymotrypsinogen A, and ribonuclease A were used to calibrate the HPLC column. The retention times of these standards were 4.90, 7.38, 7.39, 7.97, 8.60, 10.07, and 10.28 min, respectively.

Kinetic analysis. Michaelis (K_m) constants were calculated by the method of Wilkinson (19) with a computer program written by Cleland (20). An IBM PC computer was used to perform these calculations. IC_{50} values for enzyme inhibitors were estimated from semilogarithmic plots of inhibition data.

Materials. [^{35}S]PAPS (2.1–2.4 Ci/mmol) was purchased from the New England Nuclear Corp. (Boston, MA). Dithiothreitol (Cleland's reagent), *p*-nitrophenol, and BSA were obtained from the Sigma Chemical Co. (St. Louis, MO). Disodium EDTA, barium acetate, barium hydroxide, zinc sulfate, and glycerol were purchased from Fisher Scientific Products (Fairlawn, NJ). 3a70 liquid scintillation counting fluid was obtained from Research Products International Corporation (Mount Prospect, IL). DEAE-Sepharose CL-6B, Sephadex G-25 coarse, heparin-Sepharose CL-6B, and gel filtration molecular weight protein standards were purchased from Pharmacia Fine Chemicals Inc. (Piscataway, NJ). Affi-Gel Blue, acrylamide, bis-acrylamide, 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 buffer was purchased from Calbiochem (San Diego, CA). Sodium azide and 4-aminophenol were obtained from Aldrich Chemical Co. (Milwaukee, WI). Sodium nitrite was purchased from Mallinckrodt (Paris, KY) and Na^{125}I (2000 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL).

Results

Introduction. This project consisted of four series of experiments. The first studies involved the design and synthesis of a photoaffinity ligand for TS PST. The second set of experiments involved the partial purification of human hepatic TS PST to test with the photoaffinity ligand. The third series of studies was performed to test IAP as a substrate and as a photoaffinity ligand for partially purified human liver TS PST. Finally, [^{125}I]IAP was used to radioactively label partially purified TS PST, which was subjected to gel filtration HPLC to verify that the protein bands labeled with the photoaffinity reagent were TS PST.

Design of the photoaffinity ligand. Previously published QSAR analyses performed with a series of substituted phenolic substrates for human liver TS PST made it possible to develop an equation that accurately predicted K_m values of this form of the enzyme for phenols (12). The following equation was derived by the authors of those previous studies (12) from data obtained with 35 phenolic substrates for partially purified human liver TS PST:

$$\log 1/K_m = 0.92\ (\pm 0.18)\ \log P - 1.48\ (\pm 0.38)$$
$$MR'_4\ -0.64\ (\pm 0.41)\ MR_3 + 1.04\ (\pm 0.63)$$
$$MR_2 + 0.67\ (\pm 0.44)\ \sigma^- + 4.03\ (\pm 0.42)$$

In this equation K_m is the Michaelis constant, P is the octanol-water partition coefficient, MR is the molar refractivity of the substituent at the position indicated, and σ is the Hammett constant. K_m values predicted by this equation were correlated with those observed experimentally for the 35 phenolic substrates tested with a correlation coefficient of 0.95 (12).

Among the 35 phenols studied in previous experiments, the compounds with the lowest K_m values for TS PST were those with a halogen in the 2-position (12). 2-Halogen analogs possessed the desired electron-attracting, hydrophobic, and bulk properties required for optimal activity, as predicted by the equation derived on the basis of data from all 35 compounds. Because a radioactively labeled photoaffinity ligand was desired 2-iodophenol was a candidate compound because of the high specific activity of radioactive iodine. The azido moiety required for photoactivation could be synthetically placed most easily at the 4-position, so IAP was an attractive candidate compound to be tested as a photoaffinity ligand. The structure-activity relationship equation predicted that the affinity of TS PST for IAP would be very high, with a K_m value of 95 nM. Therefore, IAP was synthesized and tested as a possible photoaffinity ligand for human liver TS PST.

Partial purification of TS PST. Human liver TS PST was partially purified by ion exchange, Affi-Gel Blue, and heparin-Sepharose chromatography (Fig. 2) before photolysis, to decrease possible nonspecific labeling of proteins that might be present in a crude tissue preparation. SDS-PAGE of this preparation is shown in Fig. 3, *lane B*. The gel contained three major protein bands that stained with Coomassie blue, with apparent molecular weights of approximately 30,000, 33,000, and 60,000. The same preparation after addition of the BSA that was used as a protein carrier in the photoaffinity experiments is shown in Fig. 3, *lane C*. The only difference between the bands stained with Coomassie blue in Fig. 3, *lanes B* and *C*, is the presence of BSA in *lane C* at an approximate molecular weight of 67,000. This partially purified preparation of human liver TS PST was utilized in the photoaffinity labeling experiments to be described subsequently.

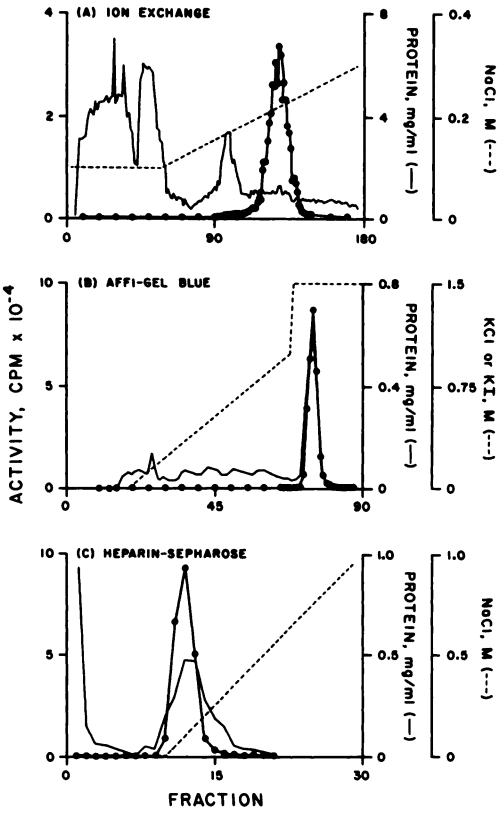

Fig. 2. Purification of human liver TS PST. Enzyme activities, protein concentrations, and salt gradients are shown in fractions obtained during DEAE-Sepharose CL-6B ion exchange chromatography (A), Affi-Gel Blue dye ligand chromatography (B), and heparin-Sepharose chromatography (C). ●, TS PST activity. See text for details.

IAP as a TS PST substrate. The K_m value of TS PST for IAP predicted by the QSAR studies was 95 nM. IAP was synthesized and tested as a substrate for TS PST. Eight concentrations of IAP from 1.95 to 250 nM were studied in the presence of 0.4 μM [^{35}S]PAPS. A double-inverse plot was constructed with data obtained from this experiment (Fig. 4), and the apparent K_m calculated from the data was 52 nM, a value very similar to the predicted figure of 95 nM. Because of substrate inhibition (Fig. 4), only data obtained with the five substrate concentrations from 1.95 to 31.25 nM were used to calculate the apparent K_m value.

Initial photolysis experiments. Exposure of partially purified human liver TS PST to UV light for 5 min at 4° in the presence of [^{125}I]IAP and PAPS resulted in the radioactive labeling of two proteins, with approximate molecular weights of 32,000 and 34,000 by SDS-PAGE (Fig. 3, *lane D*). The apparent molecular weight of human liver TS PST estimated by gel filtration chromatography performed in the course of previously published studies was 60,000 to 65,000 (4). Therefore, the two radioactively labeled proteins might represent subunits of human liver TS PST. TL PST purified from human small intestine is a dimer composed of apparently identical molecular weight 35,500 subunits (5). It has been reported that TL PST purified from human brain and platelet is also a dimer made up of identical molecular weight 34,000 monomers (21, 22), and it was recently reported that human liver TS PST is a dimer of molecular weight 32,000 monomers (23). The subunit

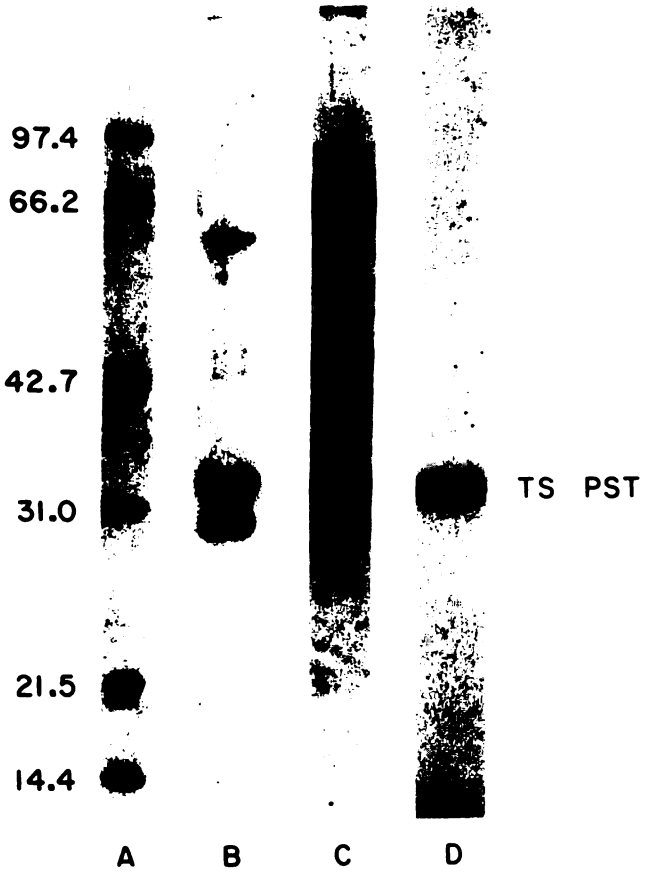

Fig. 3. SDS-PAGE of purified human liver TS PST. *Lanes A–C* were stained with Coomassie blue protein stain. *Lane A* contains molecular weight standards, *lane B* contains partially purified human liver TS PST, *lane C* shows the protein profile of a typical photolysis experiment in which BSA has been added as a carrier protein, and *lane D* shows the autoradiographs of a typical photolysis experiment. See text for details.

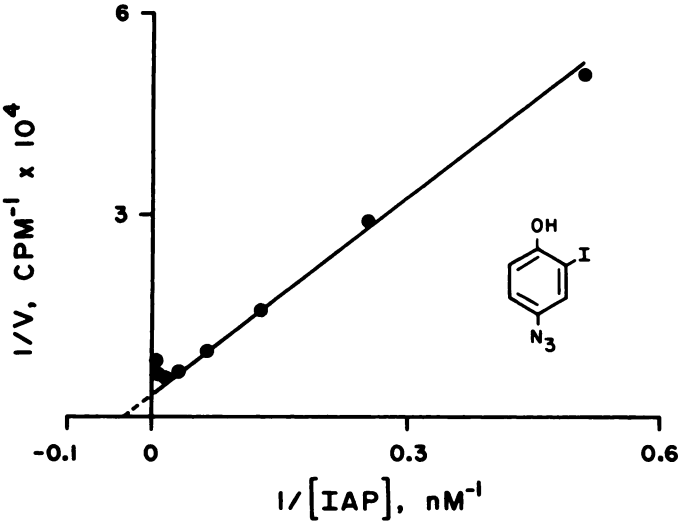

Fig. 4. Double inverse plot of IAP as a substrate for purified human liver TS PST. Each point is the mean of three determinations. The structure of IAP is shown in the *inset*.

of purified human jejunal TL PST can be clearly distinguished by SDS-PAGE from the radioactively labeled proteins found in this experiment performed with purified human hepatic TS PST. In addition, our purified TS PST preparation contained no TL PST enzymatic activity. Both the molecular weight 32,000 and 34,000 proteins were radioactively labeled in all photoaffinity experiments. Therefore, these two bands will be referred to subsequently as a "doublet." Computer analyses of data obtained from autoradiographs in these experiments were performed using the entire protein doublet because resolution of the molecular weight 32,000 and 34,000 proteins was inadequate to allow separate analysis of each band. In all experiments, the two bands appeared to have approximately equal density on the X-ray film.

Prephotolysis of the [^{125}I]IAP for 5 min at 4°, followed by photolysis in the presence of enzyme, resulted in a 74% reduction of incorporation of radioactivity into the protein doublet. When the ligand was prephotolyzed in the presence of 1 mM diethylamine, a scavenger for electrophiles (24), radioactivity incorporated into the protein doublet was reduced by 91%. Photoactivation of [^{125}I]IAP with partially purified TS PST in the absence of PAPS resulted in a 74% reduction in labeling of the protein doublet. Finally, when the enzyme preparation was irradiated for 5 min in buffer, but in the absence of IAP, it lost approximately 27% of its enzymatic activity.

Effect of time of photolysis. The quantity of radioactivity incorporated into the protein doublet increased with increasing time of photolysis up to at least 5 min (Fig. 5). Approximately 94% of the IAP had been photolyzed after 5 min of UV light exposure. This value was determined by photolyzing IAP in the absence of protein, removing aliquots at increasing time intervals, analyzing these aliquots by HPLC, and measuring the peak height of the parent compound during HPLC.

Effect of DCNP. DCNP is an inhibitor of TS PST (4, 7).

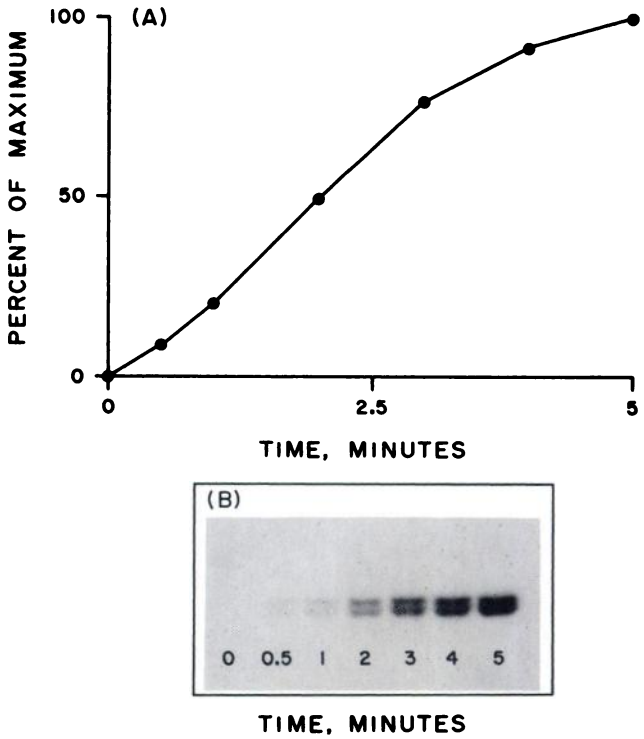

Fig. 5. Effect of time on the incorporation of [^{125}I]IAP into the protein doublet. Computer-analyzed data are shown in A, and autoradiographs are shown in B. Each doublet shown in B is a separate lane in the molecular weight 30,000-35,000 region from SDS-PAGE. The data points shown in A were obtained by computer analysis of the autoradiographs depicted in B.

Partially purified human liver TS PST activity was measured in the presence of eight concentrations of DCNP, from 0.08 to 10 μM, and an average IC_{50} value of 0.17 μM was determined on the basis of the results of two experiments. Concentrations of DCNP of 0.001–100 μM were then tested to determine the effect of the enzyme inhibitor on the photoaffinity labeling of TS PST (Fig. 6). DCNP inhibited labeling of the protein doublet with [^{125}I]IAP in a concentration-dependent fashion, with an IC_{50} value of approximately 0.17 μM.

Effect of PAP. PAPS is the sulfate donor for the reaction catalyzed by PST, and PAP is a product of the sulfation reaction (3). PAP competitively inhibits the PST reaction (11). Partially purified TS PST activity was measured in the presence of nine concentrations of PAP, from 0.02 to 5.0 μM, and an average IC_{50} value of 0.31 μM was determined on the basis of the results of two experiments. Concentrations of PAP of 0.0001–10 mM were then tested to determine the effect of PAP on the photoaffinity labeling of TS PST (Fig. 7). PAP inhibited the labeling of the protein doublet in a concentration-dependent fashion, with an IC_{50} value of approximately 15 μM.

Effect of 2-Iodophenol. 2-Iodophenol is a substrate for TS PST (12). The apparent K_m of the enzyme for 2-iodophenol is 17 nM (12). Concentrations of 2-iodophenol from 0.0001 to 1 mM were tested to determine the effect of this competing substrate on the photoaffinity labeling of TS PST (Fig. 8). 2-Iodophenol inhibited the labeling of the protein doublet with [^{125}I]IAP in a concentration-dependent fashion, with an IC_{50} value of approximately 3 μM.

Effect of NaCl. NaCl inhibits TS PST (11, 25). Partially purified TS PST activity was measured in the presence of seven concentrations of NaCl, from 0.007 to 0.5 M, and an average

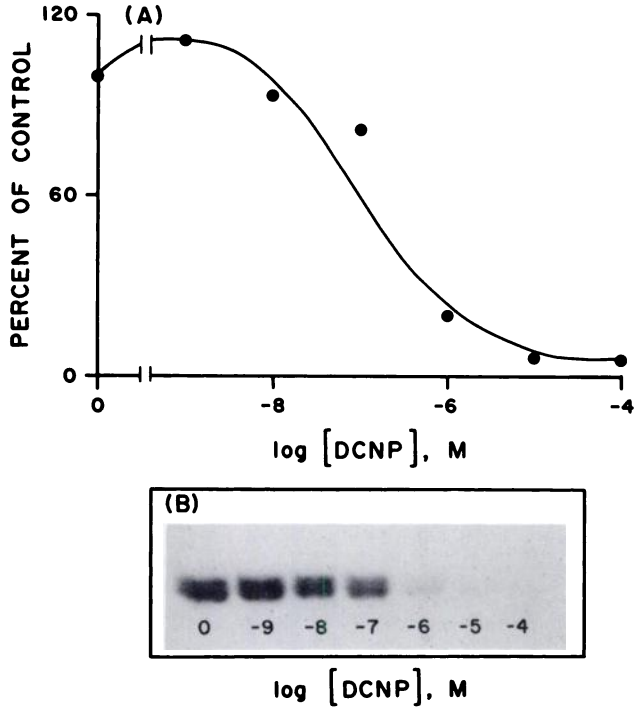

Fig. 6. Effect of DCNP concentration on the incorporation of [^{125}I]IAP into the protein doublet. Computer-analyzed data are shown in A, and autoradiographs are shown in B. Each doublet shown in B is a separate lane in the 30,000–35,000 region from SDS-PAGE. The data points shown in A were obtained by computer analysis of the autoradiographs depicted in B.

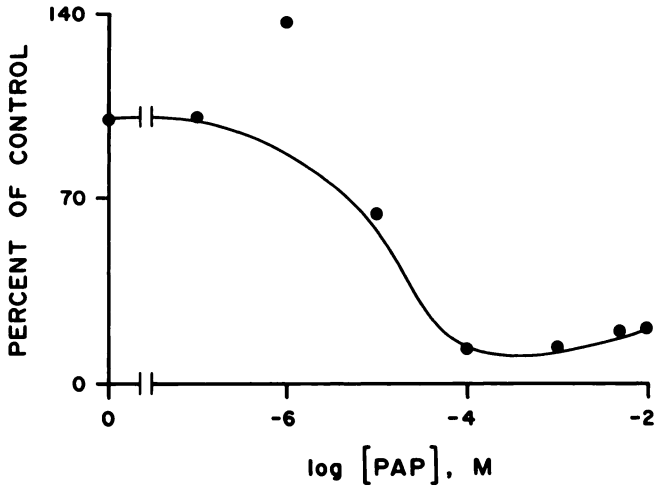

Fig. 7. Effect of PAP concentration on the incorporation of [^{125}I]IAP into the protein doublet. Each data point was derived by computer analysis of an autoradiograph of the protein doublet in a separate lane from SDS-PAGE.

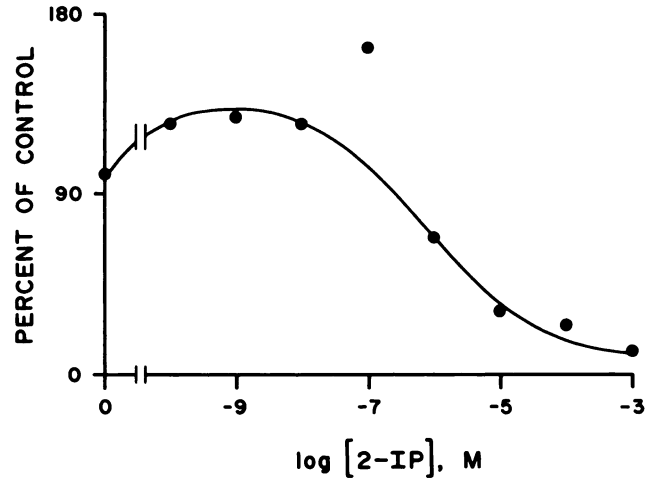

Fig. 8. Effect of 2-iodophenol (*2-IP*) concentration on the incorporation of [^{125}I]IAP into the protein doublet. Each data point was derived by computer analysis of an autoradiograph of the protein doublet in a separate lane from SDS-PAGE.

IC_{50} value of 78 mM was determined on the basis of the results of two experiments. NaCl concentrations from 0.02 to 2.0 M were then tested to determine the effect of NaCl on the photoaffinity labeling of TS PST (Fig. 9). NaCl inhibited labeling of the protein doublet in a concentration-dependent fashion, with an IC_{50} value of approximately 570 mM.

Effect of TCA. We consistently observed labeling of a protein doublet in all of our experiments. To be certain that this observation was not a result of the treatment of the samples after photoaffinity labeling, a control experiment was designed to test the effect of extracting the TCA from the photoaffinity-labeled samples and/or of boiling the samples before SDS-PAGE. The protein doublet was still present when TCA was extracted from the sample with diethyl ether before application of the sample to the gel or when TCA was extracted and the sample was boiled for 5 min before electrophoresis. These results indicated that the appearance of the photoaffinity-labeled protein doublet was not a result of the treatment of the samples after photoaffinity labeling.

Gel filtration HPLC of TS PST. It was important to

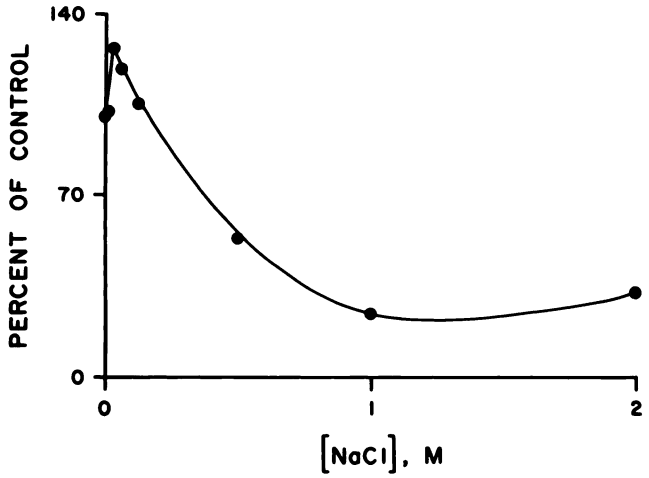

Fig. 9. Effect of NaCl concentration on the incorporation of [^{125}I]IAP into the protein doublet. Each data point was derived by computer analysis of an autoradiograph of the protein doublet in a separate lane from SDS-PAGE.

determine whether the photoaffinity-labeled protein doublet actually represented TS PST. Therefore, a sample of partially purified TS PST was analyzed by gel filtration HPLC to determine the elution of enzymatic activity from this column, so that it could be compared with the elution of [^{125}I]IAP-labeled protein obtained after photolysis. TS PST activity eluted at 8.33 min (Fig. 10A). A sample of partially purified TS PST was then subjected to photoaffinity labeling, but, instead of adding BSA and precipitating the protein with TCA in the usual fashion, protein was separated from [^{125}I]IAP and noncovalently bound ^{125}I-reaction products by centrifugation in an Amicon Centricon-10 Microconcentrator. This procedure required 4 hr to accomplish, unlike the usual procedure that separated protein from noncovalently bound ligand and ^{125}I-reaction products within 1–2 min. An aliquot of photoaffinity-labeled supernatant from the Centricon was then subjected to gel filtration HPLC. Six peaks of radioactivity eluted from the HPLC column (Fig. 10B). Each of these radioactive peaks isolated by HPLC was subjected to TCA precipitation and was then analyzed by SDS-PAGE. Peak II, the peak which eluted at 8.33 min, was visualized on the gel both by Coomassie blue staining and by autoradiography (Fig. 11). This peak contained radioactively labeled proteins with apparent molecular weights of approximately 32,000-34,000. None of the other peaks of radioactivity that eluted from the HPLC column could be detected on the gel either by Coomassie blue staining or by autoradiography. Peaks IV–VI eluted from the gel filtration HPLC column beyond the total column volume (V_t). The radioactivity in Peaks III–VI did not precipitate with TCA, an observation that suggested that these radioactive peaks might represent small molecular weight species that were generated from the radioactive ligand during photolysis but that they were not covalently bound to proteins. Finally, a control photolysis sample in which all substituents of the photoaffinity labeling reaction mixture were present except for partially purified TS PST was also analyzed by HPLC. This sample contained radioactive Peak III and other late-eluting radioactive peaks (Fig. 10C). These observations once again suggested that the late-eluting radioactive peaks found during HPLC analysis of photoaffinity-labeled partially purified TS PST were due to the formation of small molecular weight radioactive

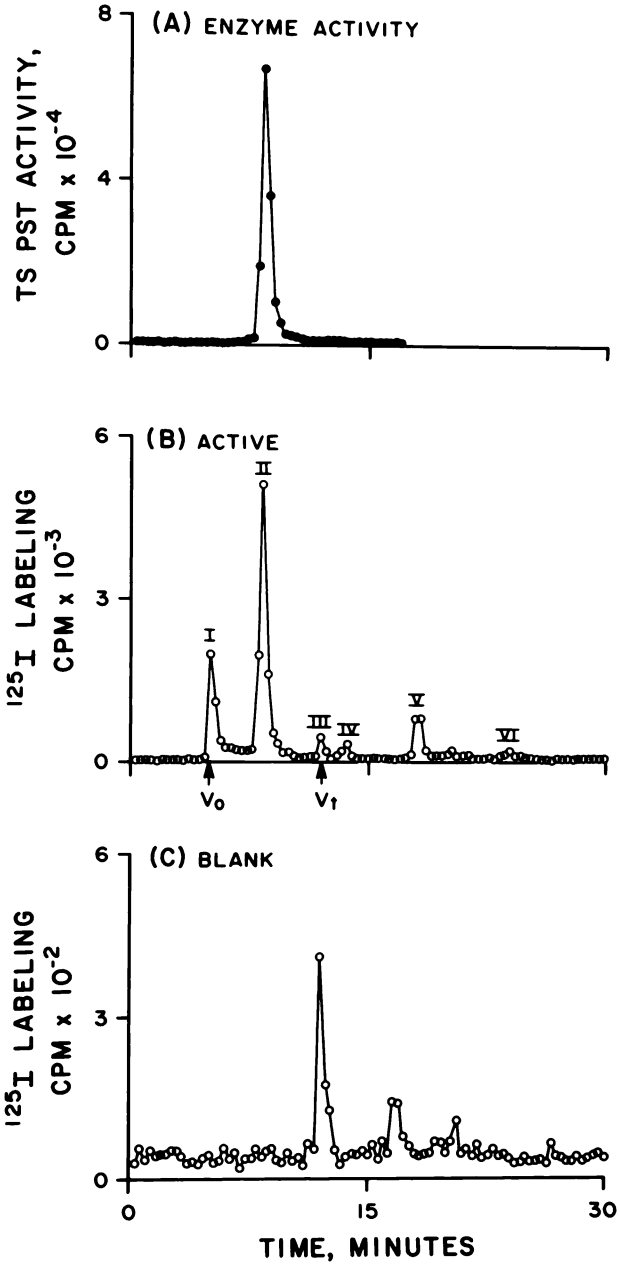

Fig. 10. Gel filtration HPLC of TS PST. A, TS PST enzyme activity in fractions obtained during gel filtration HPLC of an unphotolyzed preparation of TS PST. B, HPLC of a photoaffinity-labeled preparation of TS PST. C, HPLC of a sample photolyzed with [^{125}I]IAP in the absence of TS PST (blank). Radioactivity was measured in the fractions obtained during gel filtration HPLC. Note the difference in *y*-axes in B and C. See text for details.

compounds and not to nonspecific labeling of other proteins with [^{125}I]IAP. Radioactive Peak I eluted with the void volume (V_o); so this radioactive peak seemed to represent a high molecular weight species. Of the radioactivity associated with Peak I, 40% did not precipitate with TCA, suggesting that a large part of the radioactivity was noncovalently associated with this protein. No radioactivity could be detected in the Peak I preparation by SDS-PAGE (Fig. 11) nor could any protein be detected, even when the gel was examined with silver stain. Therefore, we concluded that Peak I did not contain sufficient protein to detect, within the limits of sensitivity of the silver staining technique, and that the radioactivity asso-

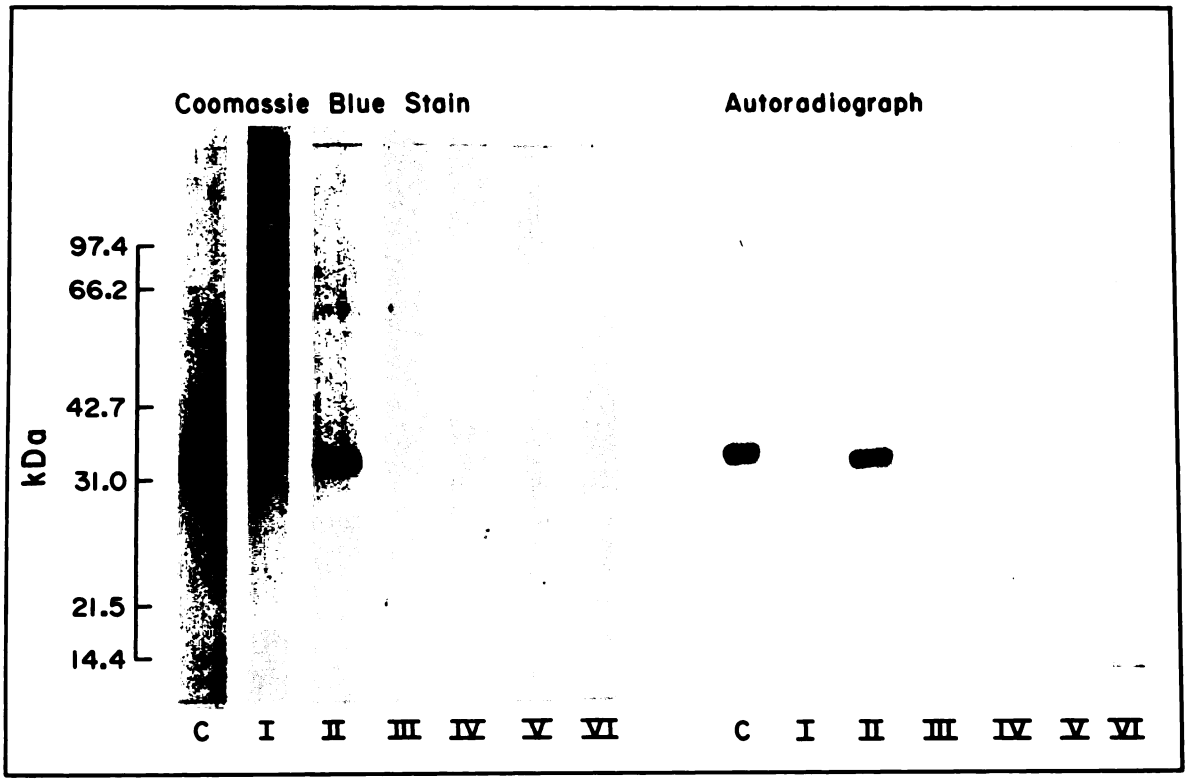

Fig. 11. SDS-polyacrylamide gel electrophoresis of photoaffinity-labeled TS PST radioactive fractions collected during gel filtration HPLC. C represents the control preparation applied to HPLC; *roman numerals* correspond to radioactive HPLC peaks shown in Fig. 10B. Coomassie blue stain for protein is shown on the *left* and autoradiographs of the same fractions are shown on the *right*. See text for details.

ciated with this peak did not appear to be covalently bound. In summary, this experiment demonstrated that TS PST enzymatic activity coeluted during gel filtration HPLC with photoaffinity-labeled proteins that gave rise to the molecular weight 32,000 and 34,000 doublet that was seen on SDS-PAGE.

Discussion

Sulfate conjugation is an important pathway in the metabolism of phenolic and catechol drugs, xenobiotics, and endogenous compounds (1–3). PST catalyzes the sulfation of many phenols, and multiple forms of PST are present in both human and animal tissues (4–8, 14, 26). Individual variations in the level of activity of both TS and TL PST in the human platelet are controlled by inheritance (27–29), and individual differences in levels of platelet TS PST activity are correlated with individual variations in the activity of this form of the enzyme in human cerebral cortex, liver, and small intestinal mucosa (30–32). In addition, TS PST in the platelet demonstrates individual genetic variations in its thermal stability (29, 33), a type of variation that usually reflects differences in protein structure (34, 35). These inherited differences in platelet TS PST thermal stability also reflect individual variations in the thermal stability of TS PST in other tissues, including cerebral cortex, liver, and small intestinal mucosa (30–32). Two isozymes of TS PST are present in human liver and brain (4, 11). Hepatic tissue from individual subjects contain one, the other, or both isozymes, and the two isozymes differ in their thermal stability (4). These observations have led to the suggestion that a structural gene polymorphism, a polymorphism that is responsible for at least two different structural forms of TS PST in human tissue, might be one mechanism by which inheritance controls TS PST in human tissue (29, 36, 37).

QSAR studies were performed previously with partially purified human liver TS PST to study the characteristics of the active site of the enzyme and to make it possible to predict K_m values for phenolic substrates (12). With the information obtained from those studies, we designed a photoreactive substrate, IAP, for TS PST. The apparent K_m value for IAP was 52 nM, similar to the predicted value of 95 nM. In the presence of UV light and PAPS, the cosubstrate for TS PST, [^{125}I]IAP was bound to two proteins with apparent molecular weights of 32,000 and 34,000. The binding of [^{125}I]IAP was specific for the protein doublet and was inhibited by DCNP, PAP, and NaCl, compounds that inhibit TS PST enzymatic activity. 2-Iodophenol, a competing substrate for TS PST, was also able to inhibit the photoaffinity labeling of the doublet by [^{125}I]IAP. In addition, binding was greatly enhanced in the presence of PAPS, the sulfate donor for the reaction. Kinetic studies of guinea pig liver PST showed that the reaction proceeded via a rapid equilibrium random Bi-Bi mechanism (38). However, kinetic 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 (11, 39). The results of our photoaffinity labeling experiments seem to support the initial binding of PAPS, because the extent of photoaffinity labeling with phenolic substrate was greatly enhanced in the presence of PAPS.

The biochemical significance of the protein doublet labeled by [^{125}I]IAP is presently unclear. PST from rat tissues is composed of subunits with apparent molecular weights of

33,500-35,000, and human TL PST appears to be a homodimer made up of molecular weight 34,000 to 35,500 monomers (5, 21, 22, 40). Therefore, the two radioactively labeled proteins in the doublet found in our experiments might represent subunits of TS PST. Because our purified enzyme was obtained from a pooled human liver preparation, the presence of the doublet could be due to structural variants of TS PST among individuals. That is an especially interesting possibility because of the existence of isozymes of TS PST in human hepatic tissue (4). Another possibility is that TS PST might be a heterodimer. This explanation implies either that both subunits contain active sites or that the active site lies between the two subunits and that the photoaffinity ligand is able to bind to either subunit. A third possible explanation is that the smaller subunit is a proteolytic product of the larger protein or that TS PST might undergo posttranslational modification, which alters its size, and, thus, its migration during SDS-PAGE. Finally, we cannot exclude the possibility of copurification of another soluble sulfotransferase that is also capable of being labeled with IAP. Obviously, each of these possibilities will have to be explored in the course of future studies.

Development of photoaffinity labeling techniques for human TS PST should enhance our ability to purify the enzyme to homogeneity and to obtain amino acid sequence information, especially with regard to the active site. For example, Yamato *et al.* (41) have determined the amino acid sequence of the active site of human pseudocholinesterase by labeling the active site serine with [^{3}H]diisopropylfluorophosphate, followed by isolation of radioactively labeled peptide fragments and analysis of their amino acid sequences. Similar information with regard to PST would both enhance our understanding of the biochemistry of the sulfate conjugation of phenols and would help make it possible to apply the techniques of molecular biology to the study of this important drug-metabolizing enzyme.

Acknowledgments

We thank Robert T. Abraham, Elizabeth Hadac, and Janis W. Schlager for their advice and Luanne Wussow for her assistance with the preparation of this manuscript. We also thank Corwin Hansch, Department of Chemistry, Pomona College (Claremont, CA) for calculation of the K_m value predicted for IAP on the basis of QSAR analysis. Finally, we thank Gerald T. Bratt, Department of Biochemistry, University of Minnesota, for access to the NMR instrument.

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