PURIFICATION AND CHARACTERIZATION OF A CANINE LIVER PHENOL SULFOTRANSFERASE

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Abstract:

Species differences in the metabolism of xenobiotics can present significant problems for safety and efficacy assessment during the development of new pharmaceutical agents. Identification of animal models for human metabolism and/or toxicology of any particular compound would significantly reduce the extent and cost of animal testing of novel candidate pharmaceuticals. Sulfation is an important pathway for metabolism of xenobiotics and potent endogenous compounds and is catalyzed by members of the sulfotransferase enzyme family. We have purified a phenol sulfotransferase from male dog liver cytosol which sulfates simple phenolic compounds such as 1-naphthol and 4-nitrophenol. On SDS-polyacrylamide gel electrophoresis, the protein had a subunit molecular weight of approximately 32,000 Da and was 34,200 Da by electrospray mass spectrometry. Immunoblot analysis with an anti-peptide antibody specific for the human phenol-sulfating form of phenol sulfotransferase (P-PST, 1A1) suggested the protein was highly homologous to the human P-PST enzyme. This was supported by amino acid sequence analysis of four peptides derived from the purified enzyme and by comparison with sequences of other phenol sulfotransferases, which showed the highest identity with human and monkey orthologs. Our data illustrate the high degree of conservation of phenol sulfotransferases across mammalian species and suggest that this dog liver enzyme is more closely related to the human P-PST than equivalent proteins in rats and mice.

Interspecies differences in the pharmacokinetics, metabolism, and toxicity of xenobiotics are a significant problem in the development of new pharmaceuticals (2–4), and only through a thorough understanding of the molecular events underpinning these differences will we eventually be able to accurately and confidently apply information obtained in animal species to the human situation. Of the many interrelated factors that determine the toxicity and metabolic fate of a xenobiotic in a given species, the enzymes that make up the body’s chemical defense mechanism are among the most important and also among the least well understood.

To protect against a toxic environment, animals have evolved a number of enzyme systems whose function can be viewed as the elimination of xenobiotics through biotransformation to less reactive, more water-soluble compounds (5, 6). Conjugation with sulfate is one of these protective mechanisms and is an important pathway in the metabolism of many drugs and other xenobiotics, and also of endogenous compounds including steroids and bile acids, neurotransmitters, and thyroid hormones (7–9). To date, much of our knowledge of the sulfotransferase (SULT) enzymes responsible for these reactions, in particular those relating to xenobiotic metabolism, has come from studies in rat and man. In these species two subfamilies of sulfotransferase have been described, phenol sulfotransferases and hydroxysteroid sulfotransferases (8, 9). In man the phenol SULTs can be further subdivided into a monoamine sulfating form (often called M-PST) which sulfates dopamine and other catechol neurotransmitters and two closely-related forms (often referred to as P-PST and 1A2) which have a preference for many simple phenols, hydroxyarylamines and iodothyronines (8, 9). In rat this subdivision is not so clear, with the main phenol sulfotransferase (known variously as ASTIV or 1A1) able to sulfate both simple phenols and catecholamines such as dopamine. Additionally, a SULT that preferentially sulfates L-dopa and tyrosine has recently been identified (10). These differences between the major rat and human xenobiotic-metabolizing SULTs indicated that the rat may not be a particularly suitable model for studying human sulfation; therefore, we have begun investigation of SULTs in other species to try and identify the species that most closely resembles man.

The beagle dog is commonly used in drug metabolism and toxicity studies within the pharmaceutical industry. Previous in vitro studies using dog liver cytosol suggested that dog phenol sulfotransferases may closely resemble the human liver enzymes (11, 12). We have undertaken a study of the SULT enzyme complement found in dog liver to assess whether the dog provides a better representation of the enzymes found in man than the rat does and hence whether it is a suitable model species for human drug metabolism.

Here we report the purification and partial sequencing of a 34kDa phenol-metabolizing SULT enzyme from male dog liver cytosol which shows distinct similarity to the human P-PST enzyme in terms of immunological cross-reactivity, amino acid sequence, and substrate specificity.
Materials and Methods

Chemicals. 1-[1-14C]Naphthol (53mCi/mmol) was purchased from Amer sham U.K. Ltd. (Little Chalfont, UK), and 3'-phosphodeoxenosine 5'-phosphos phate (PAP)5S was from Du Pont/NEN (Stevenage, UK). Nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate (p-toluidine salt), ultra labeled substrates, PAPS, and all chromatography media were purchased from Sigma (Poole, UK). Scintillation fluid (Emulsifier Safe) was from Canberra Packard (Pangbourne, UK). All other reagents were of analytical grade and were purchased from commonly used local suppliers.

Tissue Preparation. Liver tissue from adult male beagle dogs was obtained from animals forming part of safety assessment studies of pharmaceuticals carried out at Glaxo Wellcome, as required by national and international regulatory authorities. No animal was sacrificed specifically for this work. Liver was immediately frozen in liquid nitrogen and stored at −70°C. Tissue cytosols were prepared by differential centrifugation and all operations were performed at 1−4°C. Thirty percent (w/v) homogenates were prepared in 10mM Tris/HCl, pH 7.4, 250mM sucrose, 3mM 2-mercaptoethanol and centrifuged at 10,000g for 15 min. The resulting supernatants were subject to further centrifugation at 105,000g for 60 min to yield the cytosolic fraction.

The lipid layer was removed by straining through glass wool and the supernatants stored at −70°C until used (within 2 months).

Enzyme assays. 1-naphthol SULT activity (13) was assayed in a final volume of 250 µl containing 1-[1-14C]naphthol (10 µM, 0.05 µCi), 200 µM PAPS and column fractions (or liver cytosol) in a buffer comprising 30 mM potassium phosphate, pH 5.5, containing 5 mM dithiothreitol and 2 mg/ml BSA. After incubation for 15 min at 37°C the reaction was terminated by the addition of 200 µl 0.6 M glycine/0.4 M trichloroacetic acid, followed by 3 ml chloroform. After shaking for 2 min the tubes were centrifuged at 3,000g for 2 min to separate the phases. A 200 µl aliquot of the aqueous phase was subjected to liquid scintillation counting in 3 ml Emulsifier Safe.

SULT activity towards all other substrates was assayed using the PAPS assay as described previously (14), with the following modifications. SULT activity was measured in a final volume of 150 µl containing 0.02 µCi PAPS, 50 µM PAPS, 20 µg liver cytosol (or 10 µl column fractions), and substrate (6.7 µM for dopamine) in a buffer of 10 mM potassium phosphate pH 7.4. After incubation for 20 min at 37°C the reaction was stopped by adding 200 µl each of 100 mM barium acetate, 100 mM barium hydroxide, and 100 mM zinc sulfate. The tubes were mixed and centrifuged at 10,000g for 2 min. An aliquot (0.5 ml) of the aqueous layer was subjected to liquid scintillation counting in 4 ml Emulsifier Safe.

Purification of phenol sulfotransferase. The purification procedure was adapted from methods previously applied to the purification of rat SULT enzymes (13, 15, 16). Approximately 25 g of male dog liver were used to prepare a cytosolic fraction as described above. The cytosol obtained was applied directly to a column of DEAE-Sephadex Fast Flow (40 cm × 2.6 cm) at a flow rate of 1 ml/min in 10 mM triethanolamine, 250 mM sucrose, 3 mM 2-mercaptoethanol, pH 7.4. After incubation for 20 min at 37°C the reaction was stopped by adding 200 µl each of 100 mM barium acetate, 100 mM barium hydroxide, and 100 mM zine sulfate. The tubes were mixed and centrifuged at 10,000g for 2 min. An aliquot (0.5 ml) of the aqueous layer was subjected to liquid scintillation counting in 3 ml Emulsifier Safe.

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Determination of molecular weight by electroscopy mass spectroscopy. An aliquot of the SULT protein solution (20 µl) was injected onto a Purific R/HR 320 µm i.d. 5 cm column equilibrated in aqueous 0.1% TFA. The salts and buffers were removed by flowing aqueous 0.1% TFA through the column at a flow rate of 40 µl/min. The protein was eluted directly onto the mass spectrometer by passing acetonitrile containing 0.1% TFA through the column.

Protein sequence analysis. Sample concentration. Five hundred µl of protein sample were concentrated to dryness on an Amicon 3 kDa molecular weight cut-off spin-filter (12,000 rpm, for approximately 30 min), resuspended in 100 µl 120 mM Tris-HCl, 8% SDS, pH 8.0 and collected by spinning the inverted filter.

Gel analysis. One-hundred µl of gel buffer (1 M Tris-HCl pH 8.45, 24% Glyceral, 0.015% Coomassie blue G, 0.005% Phenol Red) were added before running on a 10–20% Tris/tricine gel (Novex, R & D Systems, Abingdon, UK). To 150 µl 100mM Tris, 100mM tricine, 0.1% SDS pH 8.0 a microcatalytic spattula tip of mercuric acetate acid (a free radical scavenger) was added to form the cathode buffer. The sample was warmed at 37°C for 30 min and applied in 50 µl aliquots to the gel, along with 15 µl aliquots of protein molecular weight standards (Novex). The gel was run at constant voltage (125 mV) for approximately 90 min.

Spotting. Gels were washed for 5 min in blotting buffer (containing 10mM CAPS pH 11.0, 10% methanol in Milli-Q water) before being transferred to ProBlot PVDF (PE-Applied Biosystems, Warrington, UK) by blotting for 60 min at 150 mA constant current.

Staining. PVDF blots were washed in water and air dried prior to vacuum drying for 30 min in a desiccator and staining with sulforhodamine B (0.05% sulforhodamine B in 30% methanol, 0.2% acetic acid) for 30 sec. After destaining with several changes of water, proteins appeared as bright red bands, which were excised and sequenced by Edman degradation on an ABI476A protein sequencer (PE- Applied Biosystems).

In situ trypsin digestion of proteins. Two hundred to four hundred µl aliquots of the sample were loaded onto pre-conditioned PCS hydrophobic columns (Protein Chemistry Systems, Hewlett-Packard, Stockport, UK). All salts and buffers were removed by washing with 2% trifluoroacetic acid (TFA-2 mL). The columns were then equilibrated in digestion buffer (50 mM ammonium bicarbonate/acetonitrile 1:4 at pH 8.3). Trypsin (2 mg) was added to the digestion buffer (1 µl), and this solution allowed to flow through the column, which was then left at 37°C overnight. After this time individual PCS columns were placed in line to a 1 mm Aquapore RP-300 C18 reversed phase column via an adaptor. Peptide fragments were separated by gradient elution at a flow rate of 15 µl/min beginning at 0% B and rising to 60% B in 60 min, where solvent A = 0.1% TFA and solvent B = 0.1% TFA/acetonitrile.

Analysis of Purified Protein

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DEAE-Sepharose column fractions from the gradient separation of dog liver cytosolic proteins were assayed for sulfotransferase activity towards dopamine (■) and 1-naphthol (○) as described in Materials and Methods. The NaCl concentration in selected fractions (▲) was estimated using a conductivity meter (Hanna Instruments, Leighton Buzzard, UK).

Detection was at 215 nm. Peptide fragments were collected and sequenced by MS/MS techniques using a SCIEX API III fitted with a modified source to allow nano electrospray ionization (nano-ESI).

**Results**

**Purification of a Phenol Sulfotransferase from Male Dog Liver.** Anion exchange chromatography of male dog liver cytosol on DEAE-Sepharose allowed the separation of two peaks of sulfotransferase enzyme activity, the first containing primarily dopamine sulfating activity and a second peak which preferentially sulfated 1-naphthol (fig. 1). Occasionally a shoulder appeared on the 1-naphthol SULT activity peak, as illustrated in fig. 1. However, we have no evidence to suggest that the whole peak contained anything other than a single SULT enzyme. As this second peak eluted immediately after the first peak, care was taken to ensure that fractions pooled contained only the SULT activity towards 1-naphthol. This pooled protein fraction was further purified by gel filtration, FPLC/anion exchange, and affinity columns, and the results of a representative purification experiment for this enzyme activity are shown in table 1. In this experiment, a total of 280 μg of the SULT exhibiting 1-naphthol sulfating activity were purified 371-fold from liver cytosol with a 3.2% yield and a final specific activity of 482 nmol/min/mg.

The purified enzyme appeared to exist as a single polypeptide species with a subunit molecular weight of approximately 32,000 Da, as shown by Coomassie blue staining following SDS-PAGE (fig. 2). Immunoblot analysis of the purified enzyme (fig. 3) revealed that it reacted with an antibody prepared against a peptide derived from the P-PST which has been shown to cross-react only with human P-PST and not the human M-PST enzyme (20). As this antibody has a high specificity for the human P-PST enzyme, it would suggest that the dog liver SULT we have purified shares a high degree of similarity to this isoform of human SULT.

**Substrate Specificity and Kinetic Properties of Purified Dog Liver PST.** The sulfation of a range of substrates by the purified dog liver SULT enzyme towards was also assessed and compared with data obtained using liver cytosol (table 2). These compounds were chosen principally because they are considered to be able to distinguish the human P-PST and M-PST enzymes (23), although there is some overlap. For example, 4-nitrophenol (in the μM range) is specific for human P-PST, whereas dopamine, tyramine, and 5-HT are specific for M-PST. 1-Naphthol is sulfated by both P-PST and M-PST, as is vanillin (depending on the substrate concentration). 1-Naphthol appeared to be the most specific substrate (of those tested) for the dog liver PST purified here since the enzyme activity was purified 370-fold relative to cytosol, and dopamine was the least specific since the sulfation capacity of the purified enzyme for this compound was only enriched 33-fold. The purified enzyme had no discernible activity towards steroids such as estrone and dehydroepiandrosterone (not shown). Vanillin, 4-nitrophenol, tyramine, and 5-hydroxytryptamine were also sulfated by the purified SULT. The data indicate that additional phenol SULTs exist in dog liver cytosol, and we already have evidence for an enzyme that has increased selectivity towards specific substrates.

**TABLE 1**

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (nmol/min/mg)</th>
<th>Total Activity (nmol/min)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>3270</td>
<td>1.3</td>
<td>4251</td>
<td>100</td>
<td>1</td>
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<tr>
<td>DEAE Sepharose</td>
<td>182</td>
<td>2.3</td>
<td>418.6</td>
<td>10</td>
<td>1.8</td>
</tr>
<tr>
<td>Sephacryl S200</td>
<td>11</td>
<td>22.8</td>
<td>250.8</td>
<td>6</td>
<td>17.6</td>
</tr>
<tr>
<td>Mono Q</td>
<td>3</td>
<td>54.8</td>
<td>164.4</td>
<td>3.9</td>
<td>42.2</td>
</tr>
<tr>
<td>ADP Agarose</td>
<td>0.28</td>
<td>482</td>
<td>135</td>
<td>3.2</td>
<td>371</td>
</tr>
</tbody>
</table>

Sulfotransferase enzyme activity was measured in cytosol and pooled column fractions as described in Materials and Methods.
After electrophoretic separation of proteins and transfer to nitrocellulose, blots were probed with anti-human P-PST peptide antibody and interactions visualized using alkaline phosphatase immunostaining. Lane 1, human liver cytosol (20 μg); lanes 2 and 3, dog liver cytosol (20 μg - from different animals); lane 4, purified dog liver PST (2.4 μg). The sizes of the immunoreactive polypeptides (in kDa) are indicated.

for dopamine and that can be separated from the PST purified here by anion exchange chromatography (fig. 1 and E. A. Oddy et al., unpublished). As with other sulfotransferases, there appeared to be some substrate inhibition at high concentrations of substrate (not shown).

**Protein Sequence Analysis.** Analysis of the purified enzyme by electrospray mass spectrometry indicated mass species of 34,217.22 ± 10.64 Da and 34,289.75 ± 12.20 Da. This differs from the approximately 32,000 Da polypeptide observed following gel electrophoresis; however, the human P-PST enzyme also migrates aberrantly (2,000 Da smaller than its calculated mass) on SDS-PAGE (24). The subunit mass determined by mass spectrometry agrees well with the calculated subunit molecular weight of the protein derived from a dog liver PST cDNA clone (34,114 Da) whose sequence has recently been deposited in the EMBL nucleotide sequence database (accession number D29807) and which probably codes for the enzyme purified here. The higher masses measured by mass spectrometry with the purified protein could result from N-terminal modifications (see below), or possibly from 2-mercaptoethanol adducts that we have recently identified with the human M-PST protein (unpublished results). Chemical sequencing of this polypeptide following transfer to PVDF membrane was unsuccessful, suggesting that the protein was N-terminally blocked. Leaving the blotted protein overnight on the sequencer and treating with TFA vapor caused fragmentation of the protein and the appearance of a number of sequenced fragments. The partial sequence string of one of these, PAPRL(I/L), was used to search protein sequence databases, and four sulfotransferase sequences were matched. It was possible to identify two credible sequence strings in the amino acid data by comparison with conserved regions of the four aligned sequences. These were PAPRL(I/L)KTH and HPVLYLFYEDM. One of the peptides derived from the tryptic digestion of the protein was analyzed by MS/MS. Every protein has a characteristic peptide mass fingerprint following digestion, and this can be used to search a database of theoretically digested proteins obtained from sequence databases. Fragment ions of a peptide obtained during MS/MS allowed amino acid sequence information to be inferred about the peptide. MS/MS analysis of the peptide gave a [MH+H] ion of m/z ratio 710.2 that corresponded to a peptide of mass 1418.4 Da. A series of fragment ions were produced that allowed the complete sequence of the peptide to be determined as DVAVSYYHFYR. Chemical sequencing of another peptide obtained by tryptic digestion gave GIP(LV)IXFAEAL. Amino acid sequence alignment of the four peptide sequences obtained with other mammalian phenol sulfotransferases confirm this dog liver SULT protein to be a member of the phenol sulfotransferase enzyme family (fig. 4). All four peptides demonstrated very high identity with corresponding regions of these other phenol sulfotransferases. Comparison with the derived amino acid sequence from a recently-reported dog liver phenol sulfotransferase cDNA clone (EMBL Accession number D29807) showed 100% identity within those residues that were unambiguously determined by sequencing.

**Discussion**

Study of the substrate specificity, biochemical properties, and cDNA-derived amino acid sequences of an ever-increasing number of mammalian sulfotransferases has revealed that these cytosolic enzymes fall into two distinct classes. The first class, commonly known as phenol sulfotransferases, sulfates a wide range of phenols, amines, catechols, and estrogens, while the second class, commonly known as hydroxysteroid sulfotransferases accepts a diverse range of steroids, bile acids, cholesterol, and xenobiotic alcohols. While this distinction is generally applicable across the mammalian species studied so far, there are distinct species differences in the substrate specificities and biochemical properties of the different isoenzymes within each subfamily. These differences at the molecular and biochemical level are likely to contribute significantly to the interspecies differences observed in the conjugation of xenobiotics in vivo, which impact significantly on the acceptance and applicability of animal models of human drug metabolism. It is central to the usefulness of animal models that they accurately reflect the human situation since toxicology and metabolism studies and the safety assessment of pharmaceutical agents are dependent on them.

**TABLE 2**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cytosol</th>
<th></th>
<th>Purified PST</th>
<th></th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Naphthol (1.3–133)</td>
<td>7</td>
<td>1.3</td>
<td>2</td>
<td>482</td>
<td>370</td>
</tr>
<tr>
<td>Vanillin (0.13–6.7)</td>
<td>7</td>
<td>0.17</td>
<td>1</td>
<td>32.6</td>
<td>191</td>
</tr>
<tr>
<td>4-Nitrophenol (0.13–6.7)</td>
<td>0.4</td>
<td>0.38</td>
<td>1</td>
<td>49.8</td>
<td>131</td>
</tr>
<tr>
<td>Tyramine (133–1333)</td>
<td>500</td>
<td>0.15</td>
<td>225</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>5-HT (13.3–133)</td>
<td>740</td>
<td>0.13</td>
<td>118</td>
<td>8.2</td>
<td>63</td>
</tr>
<tr>
<td>Dopamine (1.33–67)</td>
<td>2</td>
<td>0.13</td>
<td>4</td>
<td>4.3</td>
<td>33</td>
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</tbody>
</table>

Enzyme activities were determined in duplicate, over the range of substrate concentrations in parentheses, on a single sample of dog liver cytosol or purified phenol sulfotransferase using either 1-[1-14C]naphthol or PAP35S (all other substrates) as described in Materials and Methods. Kₘ and V_max values were calculated using the Regression software package (Blackwell Scientific).
The beagle dog is a routinely used species for toxicology and metabolism studies within the pharmaceutical industry. However, the sulfotransferase enzyme complement of the dog has not been studied in any great detail, and it is not known how similar the sulfotransferase enzymes found in dog are to those in man. Acetaminophen and 17α-ethynylestradiol sulfotransferase activities were found to be higher in dog than in human liver cytosol (25), and chromatographically-separable forms of phenol sulfating activity have been shown in dog liver cytosol (11, 12). Christ and Walle (12) suggested that two of the fractions they separated may represent orthologs of the P- and M-forms of human PST based on the use of pentachlorophenol as a selective inhibitor.

Enzyme assays revealed that dog liver cytosol sulfates both simple phenols like 1-naphthol and catecholamines such as dopamine, and we were able to separate the enzyme activities responsible by anion exchange chromatography. These data are compatible with the existence of canine orthologs of human P- and M-PST although the purified dog liver PST characterized here did also retain the ability to sulfate dopamine, which is a specific substrate for human M-PST, in addition to other (less specific) M-PST substrates such as 5-HT and tyramine (23). We were able to purify the enzyme preferentially sulfating 1-naphthol to apparent homogeneity, and on the basis of substrate preference and more particularly the peptide sequence analysis and reaction with an antibody specific for human P-PST, we believe the protein we have isolated to be orthologous to the human P-PST enzyme known as 1A2 (26, 27), since the two proteins are identical in the region from which the peptides were derived (20).

In conclusion, we have purified and characterized a new member of the phenol sulfotransferase enzyme family from dog liver cytosol. The high degree of biochemical and molecular similarity between this protein and the human sulfotransferase enzyme known as P-PST indicates a necessary evolutionary conservation of function and suggests that the dog may be a pertinent animal model for human sulfotransferases.

Acknowledgments. We are grateful to Dr Rob Cooke (Biomolecular Structure Department, Glaxo Wellcome Medicines Research Center) for helpful discussions.

Using representative cDNA-derived amino acid sequences from the EMBL sequence database, the four peptide sequences obtained from the dog liver PST purified here were aligned using Gene Jockey II software on an Apple Macintosh computer. *Accession number of cDNA sequence in EMBL nucleotide sequence database from which amino acid sequences were derived. † Numbers before and after peptide sequence represent position in relevant PST protein relative to the N-terminal methionine residue. Residues underlined indicate variations from the peptides derived from purified dog liver PST.

### References

### Table

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Dog Liver SULT Peptide 1</th>
<th>Dog Liver SULT Peptide 2</th>
<th>Dog Liver SULT Peptide 3</th>
<th>Dog Liver SULT Peptide 4</th>
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<tr>
<td>-</td>
<td>L02235 House PST†</td>
<td>14 GIPLXLYFAEL</td>
<td>103 PAPRLLUTH</td>
<td>137 DTVVSYHTY</td>
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<td>-</td>
<td>X52883 Rat PST‡</td>
<td>14 GIPLXLYFAEL</td>
<td>96 PAPRLLUTH</td>
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<td>-</td>
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**Fig. 4.** Alignment of dog liver phenol sulfotransferase peptide sequences with other mammalian phenol sulfotransferases.


