Enzymic sulphation of dopa and tyrosine isomers by HepG2 human hepatoma cells: stereoselectivity and stimulation by Mn²⁺

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HepG2 human hepatoma cells, labelled with [³⁵S]sulphate in media containing L-3,4-dihydroxyphenylalanine (L-dopa), (D-dopa), DL-*m*-tyrosine or D-*p*-tyrosine, were found to produce the [³⁵S]sulphated forms of these compounds. Addition to the labelling media of *m*-hydroxybenzylhydrazine, an aromatic amino acid decarboxylase inhibitor, greatly enhanced the production of L-dopa O-[³⁵S]sulphate and DL-*m*-tyrosine O-[³⁵S]sulphate, with a concomitant decrease in the formation of dopamine O-[³⁵S]sulphate and *m*-tyramine O-[³⁵S]sulphate. With 3'-phosphoadenosine 5'-phospho[³⁵S]sulphate as the sulphate donor, HepG2-cell cytosol was shown to contain enzymic activity catalysing the sulphation of L-dopa, D-dopa, L-*m*-tyrosine, D-*m*-tyrosine, L-*p*-tyrosine and D-*p*-tyrosine. The pH optimum of the enzyme, designated dopa/tyrosine sulphotransferase, was determined to be 8.75 with D-*m*-tyrosine as the substrate. The enzyme exhibited stereoselectivity for the D-form of dopa or

tyrosine isomers. Addition of 10 mM MnCl₂ to the reaction mixture resulted in a remarkable stimulation of dopa/tyrosine sulphotransferase activity, being as high as 267.8 times with D-*p*-tyrosine as the substrate. Quantitative assays revealed L-dopa, D-dopa and D-*m*-tyrosine to be better substrates than L-*p*-tyrosine. When the HepG2-cell cytosol was subjected to DEAE Bio-Gel and hydroxyapatite column chromatography, dopa/tyrosine sulphotransferase was co-eluted with the thermolabile 'M-form' phenol sulphotransferase. Furthermore dopa/tyrosine sulphotransferase displayed properties similar to that of the M-form phenol sulphotransferase with respect to thermostability and sensitivity to 2,6-dichloro-4-nitrophenol. Whether the M-form phenol sulphotransferase is truly (solely) responsible for the dopa/tyrosine sulphotransferase activity present in HepG2 cells remains to be clarified.

INTRODUCTION

In addition to xenobiotics, some endogenous low-molecular-mass compounds have also been shown to be substrates for sulphation in mammals [1–3]. For these compounds, sulphation is believed to increase their water solubility by endowing them with (additional) charged properties and thereby facilitating their excretion from the body. One such compound is the free tyrosine O-sulphate (TyrS), which was first detected in human urine over 40 years ago [4]. Following the finding on the excretion of free TyrS in human and other mammalian urine [4–6], a great number of studies performed to demonstrate the sulphation of the amino acid form of L-*p*-tyrosine, however, have all turned out to be unsuccessful [1,7–10]. With the discovery of the widespread occurrence of the post-translational protein modification by tyrosine sulphation [11], it has become increasingly accepted that the free TyrS excreted in mammalian urine is probably derived from the turnover (degradation) of tyrosine-sulphated proteins *in vivo* [6,12,13].

By employing radioactive 3'-phosphoadenosine 5'-phospho[³⁵S]sulphate (PAP[³⁵S]) as the sulphate donor, we have recently obtained unequivocal evidence indicating enzymic sulphation of L-*p*-tyrosine in several mammalian cell lines [14,15]. A controversial issue regarding this finding, however, is why should mammalian cells carry out the sulphation of an amino acid needed for protein synthesis? To convert L-*p*-tyrosine into L-*p*-TyrS, a compound destined for excretion [4–6], would seem to be counterproductive in terms of cellular economy. The question that then ensues is does L-*p*-tyrosine truly represent the physio-

logical substrate for the enzyme, initially designated the 'tyrosine sulphotransferase' [14]. In this regard, it should be pointed out that, although none of the previously identified aryl (phenol) sulphotransferases could utilize unmodified tyrosine (with free α-amino and α-carboxyl groups), some of them have been shown to be capable of catalysing the sulphation of esterified tyrosine (see Figure 1 for the chemical structure) or tyrosine residues located at the N-termini of small peptides [1,16]. In searching for the physiological substrates of the 'tyrosine sulphotransferase,' we focused on tyrosine analogues or derivatives in which both

Figure 1 Chemical structures of the 3,4-dihydroxyphenylalanine (dopa) and tyrosine isomers shown to be substrates for dopa/tyrosine sulphotransferase in HepG2 cells

The structures of the L-isomers of dopa and tyrosine are shown. As described in the Results section, D-isomers can also be used as substrates by dopa/tyrosine sulphotransferase.

Abbreviations used: TyrS, tyrosine O-sulphate; MEM, minimum essential medium; dopa, 3,4-dihydroxyphenylalanine; PAPS, 3'-phosphoadenosine 5'-phosphosulphate; DCNP, 2,6-dichloro-4-nitrophenol; DTT, dithiothreitol; Ampso, 3-[dimethyl(hydroxymethyl)methylamino]-2-hydroxypropanesulphonic acid; Caps, 3-(cyclohexylamino)-1-propanesulphonic acid.

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the α -amino and α -carboxyl groups remained unblocked. The candidate substrates (see Figure 1) thus fall into three categories: (i) the positional (*ortho*-, *meta*- or *para*-) isomers of tyrosine, (ii) the hydroxylated derivative of tyrosine, i.e. 3,4-dihydroxyphenylalanine (dopa), and (iii) the stereoisomers (L- and D-forms) of compounds in the first two categories.

Using HepG2 human hepatoma cells as a model, we have demonstrated in the current study the production of [35 S]sulphated forms of L-dopa, D-dopa, DL-*m*-tyrosine and D-*p*-tyrosine when the cells were metabolically labelled with [35 S]sulphate in the presence of these compounds. The activities of the enzyme, present in the HepG2-cell cytosol and now designated 'dopa/tyrosine sulphotransferase,' with different dopa and tyrosine isomers as substrates, were characterized. The relationship of dopa/tyrosine sulphotransferase to previously identified human sulphotransferases, e.g. the thermostable 'simple phenol (P)-form' and the thermolabile 'monoamine (M)-form' phenol sulphotransferases [17,18], was investigated by examining their elution patterns on DEAE and hydroxyapatite column chromatography, their thermostability and sensitivity to inhibition by 2,6-dichloro-4-nitrophenol (DCNP), as well as their presence in human blood platelets.

MATERIALS AND METHODS

Materials

L-*p*-Tyrosine, D-*p*-tyrosine, DL-*m*-tyrosine, DL-*o*-tyrosine, tyramine, dopamine, L-dopa, D-dopa, *m*-hydroxybenzylhydrazine, *p*-nitrophenol, ninhydrin, aprotinin, antipain, benzamidine, soyabean trypsin inhibitor, PMSF, ATP, AMP, Mes, Hepes, Taps, 3-[dimethyl(hydroxymethyl)methylamino]-2-hydroxypropanesulphonic acid (Ampso), 3-(cyclohexylamino)-1-propanesulphonic acid (Caps), EDTA (disodium salt), dithiothreitol (DTT), DCNP, minimum essential medium (MEM), fetal bovine serum, penicillin G and streptomycin sulphate were products of Sigma Chemical Company (St. Louis, MO, U.S.A.). Bio-Gel HTP hydroxyapatite and DEAE Bio-Gel A were from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Carrier-free Na₂³⁵SO₄ was from ICN Biomedicals (Irvine, CA, U.S.A.). Chromatogram cellulose TLC plates were from Eastman Kodak Company (New Haven, CT, U.S.A.). HepG2 human hepatoma cells (ATCC HB 8065) were from American Type Culture Collection (Rockville, MD, U.S.A.). All other chemicals were of the highest grade commercially available.

Cell culture

HepG2 human hepatoma cells were routinely maintained in MEM supplemented with 10% fetal bovine serum, penicillin G (30 μ g/ml) and streptomycin sulphate (50 μ g/ml), in a 5% CO₂ atmosphere at 37 °C. Confluent cells, grown in 100 mm tissue culture dishes or in individual wells of 24-well culture plates, were used in the following experiments.

Metabolic labelling of HepG2 cells with [35 S]sulphate in the presence of dopa or tyrosine isomers

Confluent HepG2 cells grown in individual wells of a 24-well culture plate, preincubated in sulphate-free (prepared by omitting streptomycin sulphate and replacing MgSO₄ with MgCl₂) phenylalanine- and tyrosine-deficient MEM for 12 h, were labelled with 0.25 ml aliquots of the same medium containing [35 S]sulphate (0.3 mCi/ml) and 1 mM L-dopa, D-dopa, DL-*m*-tyrosine, D-*p*-tyrosine or DL-*o*-tyrosine, with or without 0.1 mM *m*-hydroxybenzylhydrazine, an aromatic amino acid decarboxylase inhibitor [19]. At the end of 20 h labelling, the

media were collected for analysis of [35 S]sulphated forms of dopa and tyrosine isomers based on the procedure described below.

Preparation of cell homogenate and cytosolic fraction

Confluent HepG2 cells, grown in 80 100 mm tissue culture dishes, were scraped off, suspended in 60 ml of 20 mM Hepes/NaOH, pH 7.0, containing 250 mM sucrose, 30 μ g/ml aprotinin and 1 mM PMSF, and homogenized using a Dounce tissue grinder placed on ice. The crude homogenate was centrifuged at 140 000 *g* for 2 h at 4 °C. The supernatant containing the cytosolic proteins was dialysed at 4 °C overnight against 10 mM Hepes/NaOH (pH 7.0) containing 250 mM sucrose. The dialysed fraction was either subjected to fractionation using DEAE and hydroxyapatite column chromatography or directly used in the enzyme assays for various analyses as described below.

DEAE Bio-Gel and Bio-Gel HTP hydroxyapatite chromatography

Unless otherwise indicated, all buffer solutions used in the column chromatography described below were at pH 7.4 and supplemented with 1 mM DTT. All operations were carried out at 4 °C. The dialysed HepG2-cell cytosolic fraction (approx. 50 ml) was applied to a DEAE Bio-Gel A column (1.5 cm $\times$ 10 cm) pre-equilibrated with 10 mM Tris/HCl. The bound proteins were eluted with an NaCl gradient composed of 75 ml each of 0 and 175 mM NaCl solution containing 10 mM Tris/HCl. The eluted fractions were assayed for dopa/tyrosine sulphotransferase and P-form and M-form phenol sulphotransferase activities using the procedures described below. The fractions containing dopa/tyrosine sulphotransferase activity were combined and directly loaded on to a Bio-Gel HTP hydroxyapatite column (1.5 cm $\times$ 10 cm) pre-equilibrated with 10 mM Tris/HCl. The bound proteins were eluted with a linear gradient composed of 75 ml each of 1 and 125 mM potassium phosphate buffer. The eluted fractions were again assayed for the three sulphotransferase activities.

Preparation of platelets

The method of Anderson and Weinshilboum [20] for the isolation of platelets was used. Briefly, 30 ml of heparinized human blood was mixed with 30 ml of PBS and centrifuged at 4 °C for 10 min at 200 *g*. The supernatant collected was centrifuged at 4 °C for 10 min at 16 300 *g*. The pellet was rinsed twice, each time followed by centrifugation, with 0.9% NaCl. The rinsed platelet-containing pellet was resuspended in 0.5 ml of 20 mM Hepes/NaOH (pH 7.0) containing 250 mM sucrose, 30 μ g/ml aprotinin and 1 mM PMSF, and homogenized in a Dounce homogenizer placed on ice. The crude homogenate prepared was centrifuged at 4 °C for 10 min at 1000 *g*. The supernatant collected was used for the enzyme assays described below.

Enzyme assays for dopa/tyrosine sulphotransferase and P-form and M-form phenol sulphotransferases

Dopa/tyrosine sulphotransferase activities were assayed using PAP[35 S] as the sulphate donor. The standard assay mixture, with a final volume of 50 μ l, contained 50 mM Ampso, pH 8.75, 250 mM sucrose, 5 mM DTT, phosphatase inhibitors (25 mM NaF and 1 mM AMP), protease inhibitors (30 μ g/ml aprotinin, 30 μ g/ml antipain, 300 μ g/ml benzamidine and 30 μ g/ml soyabean trypsin inhibitor), 2 μ M PAP[35 S] (15 Ci/mmol) and 1 mM dopa or tyrosine isomers tested. In some assays, 0.01 mM

m-hydroxybenzylhydrazine was also included. The reaction was started by the addition of 10 μ l of the HepG2-cell cytosol, allowed to proceed at 37 °C for 60 min, and terminated by heating at 100 °C for 3 min. The precipitates formed were cleared by centrifugation, and the supernatant was subjected to analysis of dopa *O*-[³⁵S]sulphate (dopa[³⁵S]) or Tyr *O*-[³⁵S]sulphate (Tyr[³⁵S]) as described below. P-form and M-form phenol sulphotransferase activities were assayed under similar conditions, except that 10 μ M *p*-nitrophenol (for the P-form enzyme) or 50 μ M dopamine (for the M-form enzyme) was used as the substrate and that 50 mM Hepes, pH 7.0, was used as the buffer system.

To examine the pH-dependence of dopa/tyrosine sulphotransferase activity, different buffers (50 mM Mes at pH 5.5, 6.0 and 6.5; 50 mM Hepes at pH 7.0, 7.25, 7.5, 7.75 and 8.0; 50 mM Taps at pH 8.0, 8.25, 8.5, 8.75 and 9.0; 50 mM Ampso at pH 8.5, 8.75, 9.0, 9.25 and 9.5; 50 mM Caps at pH 10 and 11) were used to replace 50 mM Ampso (pH 8.75) normally included in the reaction mixture. To study the effect of Mn²⁺, different concentrations of MnCl₂ were included in the reaction mixtures. Controls with 10 mM EDTA or 10 mM MnCl₂ plus 10 mM EDTA were concomitantly prepared.

To examine the thermostability of the three sulphotransferase activities, aliquots of the HepG2-cell cytosol were subjected to heat-treatment at 43 °C for different lengths of time, and used in the assays described above. To determine the inhibitory effects of DCNP on the three sulphotransferase activities, aliquots of the HepG2-cell cytosol were preincubated with different concentrations of DCNP at 37 °C for 15 min before being used in the assays.

Analysis of [³⁵S]sulphated dopa/tyrosine, *p*-nitrophenol and dopamine

A previously developed [21] two-dimensional TLC separation procedure was employed for the separation of L-dopa[³⁵S], D-dopa[³⁵S], L-*m*-Tyr[³⁵S], D-*m*-Tyr[³⁵S], L-*p*-Tyr[³⁵S] and D-*p*-Tyr[³⁵S] generated during the dopa/tyrosine sulphotransferase assays. Aliquots (10 μ l) of the reaction mixtures, cleared of precipitated material, were each mixed with 1 μ l of a 10 mg/ml synthetic L-dopaS, D-dopaS, L-*m*-TyrS, D-*m*-TyrS, L-*p*-TyrS or D-*p*-TyrS solution, plus 1 μ l of a 10 mg/ml synthetic dopamine *O*-sulphate (dopamineS) or tyramine *O*-sulphate (tyramineS) solution, and spotted on to 20 cm $\times$ 20 cm cellulose TLC plates for the analysis of L-dopa[³⁵S], D-dopa[³⁵S], L-*m*-Tyr[³⁵S], D-*m*-Tyr[³⁵S], L-*p*-Tyr[³⁵S] or D-*p*-Tyr[³⁵S]. After the separation, autoradiographs were taken from the TLC plates used for the analysis. To determine quantitatively the amounts of L-dopa[³⁵S], D-dopa[³⁵S], L-*m*-Tyr[³⁵S], D-*m*-Tyr[³⁵S], L-*p*-Tyr[³⁵S] or D-*p*-Tyr[³⁵S] produced, the ninhydrin-stained spots of L-dopaS, D-dopaS, L-*m*-TyrS, D-*m*-TyrS, L-*p*-TyrS or D-*p*-TyrS were scraped off, suspended in 0.5 ml aliquots of water, mixed with 4 ml aliquots of scintillation cocktail (Ecolume; ICN Radiochemicals) and counted for the radioactivity associated with Tyr[³⁵S] or dopa[³⁵S]. For the analysis of *p*-nitrophenyl *O*-[³⁵S]sulphate (*p*-nitrophenyl[³⁵S]) or dopamine[³⁵S] generated during the assays of P-form or M-form phenol sulphotransferase, 10 μ l aliquots of the reaction mixtures, cleared of precipitated material, were spotted on to 20 cm $\times$ 20 cm cellulose TLC plates and subjected to TLC in butan-1-ol/88% formic acid/propan-2-ol/water (3:1:1:1, by vol.). On completion of the chromatography, the plates were air-dried and autoradiographed to reveal the positions of radioactive *p*-nitrophenyl[³⁵S] and dopamine[³⁵S]. The radioactive spots were scraped off, suspended in 0.5 ml aliquots of

water, mixed with 4 ml aliquots of Ecolume, and counted for radioactivity associated with *p*-nitrophenyl[³⁵S] or dopamine[³⁵S].

Miscellaneous methods

L-*p*-TyrS, D-*p*-TyrS, DL-*m*-TyrS, dopamineS, tyramineS, a mixture of L-3,4-dihydroxyphenylalanine 3-*O*-sulphate and L-3,4-dihydroxyphenylalanine 4-*O*-sulphate (collectively abbreviated to L-dopaS) and a mixture of D-3,4-dihydroxyphenylalanine 3-*O*-sulphate and D-3,4-dihydroxyphenylalanine 4-*O*-sulphate (collectively abbreviated to D-dopaS) were synthesized by the procedure developed by Jevons [22]. PAP[³⁵S] (15 Ci/mmol) was synthesized from ATP and [³⁵S]sulphate using the sulphate-activating enzymes, ATP sulphurylase and adenosine 5'-phosphosulphate kinase, from *Bacillus stearothermophilus* as described previously [23]. L-*m*-Tyrosine and D-*m*-tyrosine in the DL-*m*-tyrosine commercially obtained were separated by HPLC using a CROWNPAK CR (+) chiral column (0.46 cm $\times$ 15 cm) (Daicel Chemical), followed by HPLC using a Vydac C₁₈ reverse-phase column (0.46 cm $\times$ 25 cm) (Rainin Instrument Co., Emeryville, CA, U.S.A.). Protein determination was based on the method of Lowry et al. [24] with BSA as the standard.

RESULTS

Generation of [³⁵S]sulphated dopa and tyrosine isomers by HepG2 cells metabolically labelled with [³⁵S]sulphate

As shown in Figures 2(A)–2(C), when HepG2 cells were metabolically labelled with [³⁵S]sulphate in media containing respectively L-dopa, D-dopa and DL-*m*-tyrosine, L-dopa[³⁵S], D-dopa[³⁵S] and DL-*m*-Tyr[³⁵S] were produced and released into the labelling medium. Both the synthetic L-dopaS and D-dopaS revealed two closely migrating ninhydrin-stained spots, presumably corresponding to dopa 3-*O*-sulphate and dopa 4-*O*-sulphate. In both cases, L-dopa[³⁵S] and D-dopa[³⁵S] detected in the labelling media co-migrated, during the high-voltage electrophoresis, with the slower moving (less acidic) dopaS species (Figures 2A and 2B). Based on these results, it appears that the enzymic sulphation of either L-dopa or D-dopa occurred specifically on only one of the two ring hydroxyl groups. Whether the product is the 3-*O*-sulphate or 4-*O*-sulphate, however, remains to be clarified. It was noted that, when the cells were labelled in the absence of *m*-hydroxybenzylhydrazine, an aromatic amino acid decarboxylase inhibitor [19], only a small amount of L-dopa[³⁵S] was generated, with a large amount of dopamine[³⁵S] concomitantly produced (Figure 2A). In the presence of *m*-hydroxybenzylhydrazine, the production of L-dopa[³⁵S] was increased and the formation of dopamine[³⁵S] was decreased (Figure 2D). For the labelling medium containing D-dopa (Figures 2B and 2E), the formation of dopamine[³⁵S] also diminished in the presence of *m*-hydroxybenzylhydrazine. Whether the decarboxylation took place before or after the sulphation of L-dopa and D-dopa remains to be clarified. In the case of DL-*m*-tyrosine (Figures 2C and 2F), a dramatic increase in the amount of DL-*m*-Tyr[³⁵S] produced was also observed in the presence of *m*-hydroxybenzylhydrazine. An additional radioactive species migrating slightly toward the anode direction during the high-voltage electrophoresis was detected when the metabolic labelling was performed in the absence of *m*-hydroxybenzylhydrazine (Figure 2C). When the labelling was performed in the presence of *m*-hydroxybenzylhydrazine, the amount of this radioactive species decreased dramatically (Figure 2F), suggesting its identity to be *m*-tyramine *O*-[³⁵S]sulphate. When D-*p*-tyrosine was included in the labelling medium, D-*p*-Tyr[³⁵S] was also produced and released by the cells (Figures

Figure 2 Generation of [^{35}S]sulphated forms of L-dopa, D-dopa, DL-*m*-tyrosine and D-*p*-tyrosine by [^{35}S]sulphate-labelled HepG2 human hepatoma cells incubated in the presence or absence of *m*-hydroxybenzylhydrazine in media containing L-dopa, D-dopa, DL-*m*-tyrosine, D-*p*-tyrosine or DL-*o*-tyrosine

Autoradiographs taken from TLC plates used for two-dimensional TLC analysis of spent labelling media are shown. (A), (B), (C), (G) and (I) correspond to medium samples containing respectively L-dopa, D-dopa, DL-*m*-tyrosine, D-*p*-tyrosine and DL-*o*-tyrosine, without the addition of *m*-hydroxybenzylhydrazine. (D), (E), (F) and (H) correspond to medium samples containing respectively L-dopa, D-dopa, DL-*m*-tyrosine and D-*p*-tyrosine, with the addition of *m*-hydroxybenzylhydrazine. The upper dashed-line circles indicate the positions of synthetic L-dopaS, D-dopaS, DL-*m*-TyrS or D-*p*-TyrS standards detected by ninhydrin staining. The lower dashed-line circles in (A), (B), (D) and (E) and (C) and (F) indicate respectively the positions of synthetic dopamineS and tyramineS standards detected by ninhydrin staining.

2G and 2H). Virtually no D-*p*-tyramine O- ^{35}S sulphate was generated (Figure 2G), indicating stereoselectivity of the aromatic amino acid decarboxylase for the L-form of the substrate. When DL-*o*-tyrosine was included in the labelling medium, no radioactive spot located at the position expected for its sulphated form was observed (Figure 2I). This latter finding indicated the inability of the HepG2-cell enzyme to catalyse the sulphation of *o*-tyrosine, possibly because of the proximity of the *o*-hydroxy group to the α -carbon that carries both the α -carboxyl group and α -amino group.

Enzymic sulphation of dopa and tyrosine isomers

Even though the metabolic labelling experiments described above showed clearly the generation of the sulphated forms of D-dopa, L-dopa and DL-*m*-tyrosine by HepG2 cells, it can still be argued that these sulphated compounds might have been secondary

sulphated products derived from other metabolic reactions. To obtain evidence for the direct sulphation of D-dopa, L-dopa and DL-*m*-tyrosine, radioactive PAP ^{35}S was used as the sulphate donor to examine the presence, in the cytosolic fraction prepared from HepG2 cells, of enzyme(s) catalysing the sulphation of these compounds. As shown in Figure 3, the autoradiographs taken from the cellulose TLC plates used for the analysis of the reaction mixtures indeed revealed the formation of L-dopa ^{35}S , D-dopa ^{35}S and DL-*m*-Tyr ^{35}S . It was noted that, in the presence of the aromatic amino acid decarboxylase inhibitor, *m*-hydroxybenzylhydrazine, the ^{35}S sulphated dopamine or *m*-tyramine, derived from decarboxylation of dopa or DL-*m*-tyrosine, was virtually undetectable (Figures 3D, 3E and 3F). The additional minor radioactive spots observed probably belonged to the ^{35}S sulphated form of *m*-hydroxybenzylhydrazine included in the assay mixtures, since the same radioactive spots were found when the reaction was carried out without dopa or

Figure 3 Enzymic sulphation of L-dopa, D-dopa and DL-m-tyrosine using PAP[³⁵S] as the sulphate donor

Autoradiographs taken from the TLC plates used for two-dimensional TLC analysis of the reaction mixtures with or without *m*-hydroxybenzylhydrazine are shown. (A), (B) and (C) correspond to reaction mixtures containing L-dopa, D-dopa or DL-*m*-tyrosine, without the addition of *m*-hydroxybenzylhydrazine. (D), (E) and (F) correspond to medium samples containing L-dopa, D-dopa or DL-*m*-tyrosine, with the addition of *m*-hydroxybenzylhydrazine. The upper dashed-line circles indicate the positions of synthetic L-dopaS, D-dopaS or DL-*m*-TyrS standards detected by ninhydrin staining. The lower dashed-line circles in (A), (B), (D) and (E) and (C) and (F) indicate respectively the positions of synthetic dopamineS and tyramineS standards detected by ninhydrin staining.

Table 1 Summary of data on the production of the sulphated forms of dopa and tyrosine isomers on metabolic labelling and enzymic assays

+ and - indicate only qualitatively the presence and absence of the sulphated dopa or tyrosine isomers produced during the metabolic labelling or enzymic assays.

	L-DopaS	D-DopaS	D- <i>p</i> -TyrS	DL- <i>m</i> -TyrS	DL-D- <i>p</i> -TyrS
Metabolic labelling	+	+	+	+	-
Enzymic assay	+	+	+	+	-

tyrosine as substrate (not shown). Under the same assay conditions, the sulphation of L-*m*-tyrosine, D-*m*-tyrosine, L-*p*-tyrosine and D-*p*-tyrosine was also observed (not shown).

Data in Figures 1 and 2 are qualitatively summarized in Table 1.

Table 2 Specific activities of dopa/tyrosine sulphotransferase in the cytosolic fraction prepared from HepG2 human hepatoma cells

Specific activity refers to the activity per mg of protein present in HepG2-cell cytosolic fraction. Data represent calculated means $\pm$ S.D. derived from three experiments.

Substrate	Specific activity of dopa/tyrosine sulphotransferase (pmol/min per mg)			
	Without MnCl ₂	With MnCl ₂	With EDTA	With MnCl ₂ + EDTA
L- <i>p</i> -Tyrosine	0.074 $\pm$ 0.009	0.566 $\pm$ 0.010	0.068 $\pm$ 0.004	0.085 $\pm$ 0.002
D- <i>p</i> -Tyrosine	0.189 $\pm$ 0.001	50.606 $\pm$ 0.950	0.174 $\pm$ 0.005	1.626 $\pm$ 0.021
L- <i>m</i> -Tyrosine	0.067 $\pm$ 0.002	0.934 $\pm$ 0.016	0.071 $\pm$ 0.002	0.125 $\pm$ 0.008
D- <i>m</i> -Tyrosine	1.080 $\pm$ 0.013	88.592 $\pm$ 1.599	1.002 $\pm$ 0.018	3.566 $\pm$ 0.062
L-Dopa	0.732 $\pm$ 0.064	2.361 $\pm$ 0.392	0.581 $\pm$ 0.055	1.818 $\pm$ 0.350
D-Dopa	24.874 $\pm$ 1.419	56.019 $\pm$ 1.746	24.005 $\pm$ 0.571	42.001 $\pm$ 2.213

Basal activities of the dopa/tyrosine sulphotransferase with different dopa and tyrosine isomers as substrates

It is at present unknown whether, in the HepG2-cell cytosol, there is a single enzyme catalysing the sulphation of all dopa and tyrosine isomers, or, instead, there are multiple sulphotransferases responsible for the sulphation of individual dopa and tyrosine isomers. For the convenience of data presentation, the term dopa/tyrosine sulphotransferase will be referred to in a general sense regarding the enzyme(s) catalysing the sulphation of dopa and tyrosine isomers. Data on the quantitative analysis of the basal dopa/tyrosine sulphotransferase activities with different dopa and tyrosine isomers as substrates are compiled in Table 2. Of the three pairs of stereoisomers assayed in the absence of added bivalent cation, the D-isomers were found to serve as better substrates than their L-form counterparts. Dopa/tyrosine sulphotransferase activity with the three D-form substrates were in the order: D-dopa > D-*m*-tyrosine > D-*p*-tyrosine. Of the three L-isomers, L-dopa gave an activity approximately one order of magnitude higher than that detected for either L-*m*-tyrosine or L-*p*-tyrosine. Addition of EDTA, aimed at sequestering residual bivalent cations present in the dialysed HepG2-cell cytosol used in the assay mixture, exerted no significant changes in the basal activities of the dopa/tyrosine sulphotransferase.

Effects of Mn²⁺ ions on dopa/tyrosine sulphotransferase activity

In an earlier study [14], we observed a bivalent-cation-dependence of the HepG2 dopa/tyrosine sulphotransferase activity with L-*p*-tyrosine as substrate. Of the bivalent cations tested, Mn²⁺ displayed the strongest stimulatory effects, followed by Co²⁺ and Mg²⁺. Based on these results, we decided to examine the effects of Mn²⁺ on dopa/tyrosine sulphotransferase activity with different dopa and tyrosine isomers as substrates. As shown in Figure 4, within the concentration range tested, optimal activities were detected at 10 mM MnCl₂ with D-*p*-tyrosine or D-*m*-tyrosine as substrate. Data on the stimulation of dopa/tyrosine sulphotransferase activity by 10 mM MnCl₂ with different dopa and tyrosine isomers as substrates are compiled in Table 2. A greater than 267 times stimulation was observed with D-*p*-tyrosine as substrate, followed by 82 times for D-*m*-tyrosine, 13.9 times for L-*m*-tyrosine and 7.6 times for L-*p*-tyrosine. For L-dopa and D-dopa, smaller, yet significant (3.2 times for L-dopa and 2.25 times for D-dopa), stimulatory effects were still observed. Incorporation of an equimolar amount of EDTA in the assay

Figure 4 Effects of different concentrations of MnCl_2 on dopa/tyrosine sulphotransferase activity

Dopa/tyrosine sulphotransferase assays were carried out under standard assay conditions as described in the Materials and methods section except that different concentrations of MnCl_2 were used. Data represent calculated mean values derived from three experiments.

Figure 5 pH-dependence of dopa/tyrosine sulphotransferase activity

Dopa/tyrosine sulphotransferase assays were carried out under standard assay conditions as described in the Materials and methods section except for the different buffers used. The substrate used was *D-p*-tyrosine. Data shown represent calculated mean values derived from three experiments.

mixture abolished to a large extent the stimulatory effects of Mn^{2+} on dopa/tyrosine sulphotransferase activity. These results show clearly a remarkable stimulatory effect of Mn^{2+} ions on the activity of dopa/tyrosine sulphotransferase.

pH-dependence of dopa/tyrosine sulphotransferase activity

As shown in Figure 5, in the presence of 10 mM MnCl_2 , dopa/tyrosine sulphotransferase activity displayed a pH optimum of approx. 8.75 with *D-p*-tyrosine as the substrate. With L-dopa or D-dopa as the substrate, similar optimum pH values were observed.

Figure 6 Fractionation of the HepG2-cell dopa/tyrosine sulphotransferase (D/TST) and the P-Form (P-PST) and M-Form (M-PST) phenol sulphotransferases during DEAE Bio-Gel A column chromatography

Relationship of dopa/tyrosine sulphotransferase to other human sulphotransferases

The following studies were carried out to investigate whether the dopa/tyrosine sulphotransferase activities are associated with new enzyme or enzymes or known sulphotransferase or sulphotransferases previously identified in human tissues [17,18].

Fractionation using DEAE and hydroxyapatite column chromatography

As shown in Figure 6, when the HepG2-cell cytosol was subjected to DEAE Bio-Gel column chromatography, dopa/tyrosine sulphotransferase activity, with *D-p*-tyrosine as the substrate, appeared to be co-eluted with the M-form phenol sulphotransferase activity, but not the P-form phenol sulphotransferase activity. When the dopa/tyrosine sulphotransferase-containing fractions were combined and subjected to Bio-Gel HTP hydroxyapatite column chromatography, the enzyme was again co-eluted closely with the M-form phenol sulphotransferase (results not shown).

Thermostability and sensitivity to DCNP

Previous studies have revealed distinct differences in thermostability between the P-form and M-form phenol sulphotransferases [17,18]. When dopa/tyrosine sulphotransferase activity, in comparison with those of the P-form and M-form phenol sulphotransferases, was tested for thermostability at 43 °C, it was found that it displayed a thermostability similar to that of the M-form phenol sulphotransferase (Figure 7). The inhibitory effects of DCNP on the three sulphotransferase activities were examined with *D-p*-tyrosine, *p*-nitrophenol and dopamine as substrate. The IC_{50} values calculated from the data obtained were 2×10^{-3} M, 8×10^{-7} M and 9.5×10^{-4} M respectively for dopa/tyrosine sulphotransferase and the P-form and M-form phenol sulphotransferases.

Presence of the sulphotransferase activity in platelets

To distinguish dopa/tyrosine sulphotransferase activity from dehydroepiandrosterone sulphotransferase and oestrogen sulphotransferase, extracts of human platelets that lack the latter two enzymes were prepared and assayed for dopa/tyrosine sulphotransferase activity. The results show clearly the presence

Figure 7 Thermostability of the HepG2-cell dopa/tyrosine sulphotransferase (D/TST) and the P-Form (P-PST) and M-Form (M-PST) phenol sulphotransferases

Data presented indicate the percentage of activity remaining after heat-treatment at 43 °C for different lengths of time.

of sulphotransferase activity catalysing the sulphation of all dopa and tyrosine isomers (except DL-*o*-tyrosine), as well as *p*-nitrophenol and dopamine.

DISCUSSION

The current study is a continuation of our previous finding on the sulphation of the free amino acid form of L-*p*-tyrosine [14,15]. As mentioned above, a controversial issue with regard to the previous finding is why do mammalian cells perform the sulphation of L-*p*-tyrosine, an amino acid universally needed for protein synthesis? Being unable to provide a plausible reason for this interesting phenomenon except to suggest the possibility that sulphation is a means of removing excess L-*p*-tyrosine, we have grown increasingly curious about whether L-*p*-tyrosine represents the true physiological substrate of the enzyme, initially designated tyrosine sulphotransferase. In searching for the real (physiological) substrate(s) of the enzyme, we realized that, for the HepG2-cell enzyme (now designated dopa/tyrosine sulphotransferase), D- and L-dopa, D- and L-*m*-tyrosine and D-*p*-tyrosine, in addition to L-*p*-tyrosine which we originally tested, could also be used as substrates for sulphation. The question once again is why do mammalian cells sulphate these dopa and tyrosine isomers? Although the functional relevance of the sulphation of these newly identified substrates still remains obscure, some possibilities can be proposed by first taking into account the metabolic roles of these compounds.

L-Dopa is generally thought to be a precursor for the biosynthesis of catecholamines including dopamine, noradrenaline and adrenaline [25]. Although there have been no reports on the existence of D-dopa *in vivo*, it is worth while pointing out that decarboxylation of D-dopa yields the same compound, dopamine, as that derived from decarboxylation of L-dopa. Similarly, the presence *in vivo* of D-*m*-tyrosine is unknown. However, if D-*m*-tyrosine could be hydroxylated to form D-dopa, the same compound, dopamine, could be generated on further decarboxylation. L-*m*-Tyrosine, on the other hand, has been shown to be present *in vivo* [26] and is capable of crossing the blood–brain barrier [27]. Quantitative analysis has shown that, although L-*p*-tyrosine represents the predominant species, L-*m*-tyrosine constitutes approximately 2.8% of the total tyrosine circulating in blood [26]. Using bovine adrenal medulla extract or rat brain homogenate, it has been demonstrated that L-*m*-

tyrosine could be produced through the *meta*-hydroxylation of L-phenylalanine [28,29]. Furthermore, studies *in vivo* have shown that L-*m*-tyrosine could be converted into L-dopa [17,30,31] or *m*-tyramine [27,32], a decarboxylated product of L-*m*-tyrosine with neurotransmitter activity [32]. Considering that dopa and *m*-tyrosine are both involved in the biosynthesis of neurotransmitters, it may be important to regulate the concentration of these compounds *in vivo*. A working hypothesis we have thus proposed with regard to the functional relevance of the sulphation of dopa and tyrosine isomers is that, under normal circumstances, sulphation may be employed as a safeguard against the overproduction of dopa and *m*-tyrosine which, if not prevented, may lead to overproduction of catecholamines and, consequently, various neurological problems. When dopa or *m*-tyrosine is in excess, the sulphation reaction may kick in to convert them into the sulphated forms which can then be readily excreted. In this regard, the dopa/tyrosine sulphotransferase may occupy an important position controlling neurotransmitter metabolism. D-*p*-Tyrosine, another tyrosine isomer that can be used as a substrate by dopa/tyrosine sulphotransferase, has recently been shown to be present *in vivo*. For example, it has been demonstrated that tyrosine residues of the human lens protein may gradually undergo racemization forming the D-isomer during the aging process [33]. Since D-tyrosine cannot be used for protein synthesis, it is likely that sulphation may be used as a means of facilitating the excretion of the D-*p*-tyrosine generated from the turnover of D-tyrosine-containing proteins.

Having demonstrated enzymic sulphation of dopa and tyrosine isomers, an important issue that needs to be clarified is whether, in the HepG2-cell cytosol, there is only one enzyme catalysing the sulphation of all dopa and tyrosine isomers or, instead, there are multiple sulphotransferases responsible for the sulphation of individual dopa and tyrosine isomers. Furthermore, is (are) the(se) enzyme(s) identical with or distinct from the sulphotransferases previously reported? Earlier studies revealed the presence in human tissues, among other enzymes, of two distinct aryl sulphotransferases [17,18], a thermostable P-form, which catalyses the sulphation of simple phenols, and a thermolabile M-form, which catalyses the sulphation of dopamine and other catechol and phenol amines, and two steroid sulphotransferases [34–36], oestrogen sulphotransferase and dehydroepiandrosterone sulphotransferase. To investigate the relationship between dopa/tyrosine sulphotransferase and these known sulphotransferases, several studies were carried out. Although the presence of dopa/tyrosine sulphotransferase activity in platelets precludes its relationship to oestrogen sulphotransferase and dehydroepiandrosterone sulphotransferase, the enzyme was co-eluted with the M-form phenol sulphotransferase during both DEAE and hydroxyapatite column chromatography steps, and displayed thermostability and sensitivity to DCNP similar to the M-form phenol sulphotransferase. Additional studies, employing protein purification or molecular-cloning techniques, are needed to clarify whether the M-form phenol sulphotransferase is indeed (solely) responsible for the dopa/tyrosine sulphotransferase activity discovered in HepG2 cells. In a separate study, we have purified to electrophoretic homogeneity a single species of dopa/tyrosine sulphotransferase from rat liver. The purified enzyme was found to be capable of catalysing the sulphation of all dopa and tyrosine isomers tested, except DL-*o*-tyrosine. Partial amino acid sequences obtained for the purified enzyme indicated unequivocally its identity as a new type of sulphotransferase, being distinct from all known aryl sulphotransferases (Y. Sakakibara, Y. Takami, C. Zwieb, T. Nakayama, M. Masahito, H. Nakajima and M.-C. Lin, unpublished work).

In examining the activities of the HepG2-cell dopa/tyrosine sulphotransferase with different dopa and tyrosine isomers as substrates, it was intriguing to find stereoselectivity of the HepG2-cell enzyme for the D-enantiomers. In this regard, it has previously been reported that HepG2 cells also display a higher sulphate-conjugation activity with the (+)-enantiomers of several chiral adrenergic drugs [37]. Of the three pairs of substrates tested in the current study, dopa/tyrosine sulphotransferase activities with L- and D-dopa as substrates were more than one order of magnitude higher than those with L- and D-*m*-tyrosine or L- and D-*p*-tyrosine as substrates. These results imply that dopa is the major target of the HepG2-cell enzyme. Our recent studies on purified rat liver dopa/tyrosine sulphotransferase also revealed L- and D-dopa to be better substrates than L- and D-*m*-tyrosine or L- and D-*p*-tyrosine (Y. Sakakibara, Y. Takami, C. Zwieb, T. Nakayama, M. Masahito, H. Nakajima and M.-C. Liu, unpublished work). Whether dopa truly represents the primary substrate of dopa/tyrosine sulphotransferase under physiological conditions, however, remains to be clarified. In this regard, it should be pointed out that the co-elution with synthetic L-*p*-TyrS standard on ion-exchange column chromatography was the major, if not exclusive, criterion used for the identification of free TyrS excreted in mammalian urine [4–6]. This procedure, however, is unlikely to provide enough resolution to distinguish between different sulphated tyrosine and/or dopa isomers. It is important to investigate, using more reliable methods, the true identity (or identities) of the TyrS excreted in mammalian urine. Such information will be valuable in delineating the real substrate(s) of dopa/tyrosine sulphotransferase *in vivo*.

Earlier studies showed that some aryl sulphotransferases, e.g. tyrosylprotein sulphotransferase, may require Mn²⁺ or Mg²⁺ for optimal activity [38–40]. Our previous study on the sulphation of L-*p*-tyrosine by the HepG2-cell enzyme revealed a bivalent-cation-dependence in the order: Mn²⁺ > Co²⁺ > Mg²⁺. Using Mn²⁺ as the metal ion cofactor, we have detected in the current study even more remarkable stimulatory effects on dopa/tyrosine sulphotransferase with different dopa and tyrosine isomers as substrates. It should be pointed out that, although the Mn²⁺-stimulation experiments were carried out at pH 8.75 (the optimum pH determined for the enzyme), a similar experiment [14] previously carried out at pH 8.0 showed comparable stimulatory effects. It was interesting to note in the current study that stimulation by Mn²⁺ was more dramatic with D-isomers of tyrosine as substrates for sulphation, although significant stimulatory effects were also found for L-tyrosine isomers and both L- and D-dopa. Although the physiological relevance of this Mn²⁺-dependence of dopa/tyrosine sulphotransferase activity is currently not known, it is tempting to speculate on a possible link with manganese poisoning or manganism previously reported to occur frequently among miners [41]. In this regard, it could be speculated that, for those afflicted with manganese poisoning, the dopa/tyrosine sulphotransferase may be activated *in vivo*. Increasing amounts of L-dopa, L-*p*-tyrosine and L-*m*-tyrosine, which serve directly or indirectly as biosynthetic precursors for catecholamines, may become sulphated and excreted. The lowering of the levels of these dopa and tyrosine isomers in turn may result in the decreased production of dopamine and other catecholamines. In line with these hypothetical events, individuals afflicted with manganese poisoning have been reported to display symptoms resembling those of Parkinson's disease [42], and reduction or even elimination of some of these symptoms has been observed in those receiving treatment with L-dopa [42,43].

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