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Characterization of a Tyrosine Sulfotransferase in Rat Brain Using Cholecystokinin Derivatives as Acceptors

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ABSTRACT: An apparently novel tyrosyl sulfotransferase activity was detected in a crude microsomal fraction from rat cerebral cortex by using 3′-phosphoadenosine 5′-phospho[^{35}S]sulfate ([^{35}S]PAPS) as the sulfate donor and various cholecystokinin (CCK) fragments or derivatives as acceptors. Among the latter, the shortest substrate was *tert*-butoxycarbonylaspartyltyrosine (Boc-Asp-Tyr), but the reaction was optimized by increasing the length of the peptide sequence on the C-terminal side up to *tert*-butoxycarbonylcholecystokinin octapeptide (Boc-CCK-8) as well as by the presence of acidic amino acid residues at the N-terminal side. Peptides with an N-terminal Tyr residue (e.g., CCK-7 or enkephalins) were not sulfated. With Boc-CCK-8 the optimum pH was 5.8, and apparent K_M values were 0.14 ± 0.02 mM for the peptide (0.5 μM PAPS) and 0.12 ± 0.01 μM for PAPS (0.25 mM Boc-CCK-8). In the presence of 0.2 mM $MnCl_2$ the V_{max} of the reaction was enhanced without change of apparent affinities of the two substrates. The possible role of this sulfotransferase activity in posttranslational modification of CCK and other secretory proteins is suggested.

Various posttranslational covalent modifications of proteins like phosphorylation of Ser, Thr, or Tyr residues are now recognized as important cell regulation processes and therefore widely studied (Uy & Wold, 1977; Cohen, 1982). In contrast, protein sulfation on Tyr residues was long considered a rare modification and has attracted little attention: the presence of an *O*-sulfate ester group on this amino acid, first detected in fibrinopeptide B (Bettelheim, 1954), was thought to occur only in a few peptides like fibrinogens (Jevons, 1963), gastrin (Gregory et al., 1964), or cholecystokinin (Mutt & Jorpes, 1968). However, recent studies have shown that Tyr-*O*-sulfate residues occur in a large number of secretory proteins, only some of which, like immunoglobin G or fibronectin, are as yet identified (Huttner, 1982; Lee & Huttner, 1983; Baeuerle & Huttner, 1984; Lin & Lippmann, 1985). The role of Tyr sulfation is generally not yet understood, but in the case of cholecystokinin, it appears essential for its recognition by receptors mediating its various hormonal or neuronal actions (Rehfeld, 1981; Dockray, 1982; Morley, 1982). Also, very little is known about the enzyme(s) responsible for this posttranslational modification, which has been described to occur in a cell-free extract from a rat pheochromocytoma with unidentified proteins as acceptors (Lee & Huttner, 1983). Among known sulfotransferases (Mulder et al., 1982) only aryl sulfotransferase IV (EC 2.8.2.1) purified from the cytosol of rat liver has the ability to sulfate Tyr residues (Sekura & Jacoby, 1981; Sekûra et al., 1981). However, this transfer only occurs onto peptides like CCK-7[1] in which the Tyr is in the N-terminal position, indicating that it is presumably not responsible for the sulfation of proteins in which Tyr residues are otherwise located (such as CCK-8 or its precursors).

We describe here the properties of an apparently novel tyrosyl sulfotransferase activity evidenced in a crude microsomal fraction from rat brain, using a simple assay system with synthetic CCK derivatives as acceptors and [^{35}S]PAPS as the sulfate donor.

Materials and Methods

The materials used were obtained from the following sources: nonradioactive PAPS, adenosine 5′-triphosphate, tyrosine methyl ester, 2,6-dichloro-4-nitrophenol, *p*-(chloro-

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[1] Abbreviations: Boc, *tert*-butoxycarbonyl; CCK, cholecystokinin; CCK-7, cholecystokinin heptapeptide; CCK-8, cholecystokinin octapeptide; CCK-8 (ns), cholecystokinin octapeptide (nonsulfated); PAPS, 3′-phosphoadenosine 5′-phosphosulfate; Tris, tris(hydroxymethyl)-aminomethane.

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mercuri)benzenesulfonic acid, and ECTEOLA from Sigma Chemical Co.; Boc-CCK-8 and other peptides and derivatives from Bachem, Bubendorf, Switzerland; polystyrene beads, 200–400 mesh, Porapak Q, from Waters Associates; organic solvents and other analytical grade chemicals from Prolabo, Paris, France.

[^{35}S]PAPS (1–5 Ci/mmol) was purchased from New England Nuclear Chemicals and ACS scintillation mixture from Radiochemical Center (Amersham). Wistar male rats (180–220 g) were obtained from Iffa-Credo (L'Arbresle, France).

Microsomal Fraction from Rat Brain. Immediately after decapitation, the whole cerebral cortex was dissected out and homogenized in 20 volumes of ice-cold 0.32 M sucrose by using a Teflon–glass Potter (0.15-mm clearance, eight up and down strokes). The microsomal fraction was obtained at 3–5 °C essentially as described (Appel et al., 1972). The homogenate was centrifuged (Lourdes Model A, Instrument Corp., Brooklyn, NY) at (4×10^5)g·min and the resulting supernatant then centrifuged (Beckman Ultracentrifuge Model L) at (6×10^6)g·min. The pellet was rinsed, resuspended in 10 mM potassium phosphate buffer, pH 7.0, and recentrifuged at (3×10^6)g·min. Washed microsomes were resuspended in buffer of the same composition. This suspension was used within 1–3 h for tyrosyl sulfotransferase activity assays.

Assays of Tyrosyl Sulfotransferase Activity. The optimal assay conditions were selected after a series of trials described under Results. The incubations were usually performed for 30 min at 37 °C in 0.2 mL of a mixture containing 75 mM potassium phosphate buffer, pH 5.8, 125 mM NaCl, 25 mM NaF, and, where indicated, 0.2 mM $MnCl_2$. The two substrates, [^{35}S]PAPS (usually at a 0.3 μM concentration) and Boc-CCK-8 (ns) (usually at a 0.25 mM concentration), were added to start the incubations, after a 3-min preincubation of the microsomal fraction (usually 80 μg of protein). The reaction was stopped by heat denaturation (95 °C, 4 min); tubes were chilled and then centrifuged for 30 s in a table microfuge. The ^{35}S-sulfated peptide formed was isolated from an aliquot of the supernatant by chromatography on a polystyrene bead column (0.4 × 3 cm). The column was washed with 3 × 2 mL of potassium phosphate buffer (75 mM, pH 5.8), then with 2 × 2 mL of doubly distillated water, and finally eluted into a liquid scintillation vial with 1 mL of pure ethanol. The eluted radioactivity was determined by liquid scintillation spectrometry using ACS scintillation mixture. Samples were counted on a Packard Tri-Carb. The recovery of Boc-CCK-8, as evaluated spectrophotometrically (λ = 295 nm) with a known standard, was 90%. In contrast, contamination by [^{35}S]PAPS (or $^{35}SO_4$) in the final eluate was less than 0.1%. Blanks, obtained in parallel incubations in the absence or presence of [^{35}S]PAPS but in the absence of Boc-CCK-8 (ns), represented about 5–7% of control values obtained under optimal conditions and were subtracted. In a typical experiment (80 μg of microsomal protein, 30-min incubation in the presence of 0.2 mM $MnCl_2$) [^{35}S]Boc-CCK-8 formation represented 4400 ± 150 disintegrations per minute (dpm) whereas the blank value was 300 ± 20 dpm.

When substrates other than Boc-CCK-8 (ns) were used in the sulfotransferase assay, their recoveries in the polystyrene bead column chromatographic procedure were evaluated as follows. Standards of the substrates were passed onto the column, and their concentration in the final eluate was estimated spectrophotometrically either by UV absorption or by using Lowry's reagent (Lowry et al., 1951). In most cases an 80–90% recovery was obtained, except for Boc-Asp-Tyr (70%). The recoveries of ^{35}S-sulfated peptides were similar to those of nonsulfated peptides as shown by submitting the eluted radioactivity to column chromatography.

In some cases, i.e., when the hexapeptide Asp-Arg-Asp-Tyr-Met-Gly or its Boc derivative was used as substrate, a different isolation procedure was utilized. Aliquots of the supernatant of the heated reaction mixture were incubated for 60 min at 37 °C in the presence of 0.2 M HCl in order to hydrolyze the excess of [^{35}S]PAPS (it was checked that complete hydrolysis occurred in less than 30 min). The sample was then neutralized with an equal volume of 0.2 M NaOH, diluted 30-fold with 20 mM ammonium bicarbonate, and applied onto an ECTEOLA minicolumn (0.6 mL of wet resin suspended in 20 mM ammonium bicarbonate). The inorganic [^{35}S]sulfate was washed out from the column with 20 mL of 20 mM ammonium bicarbonate and the ^{35}S-labeled peptide finally eluted with 10 mL of 0.5 M ammonium bicarbonate. The recoveries of sulfated peptides were higher than 90%.

Assay of Soluble Phenol Sulfotransferase Activity. A soluble fraction from rat cerebral cortex was obtained by homogenization in 10 volumes of ice-cold 10 mM potassium phosphate buffer (pH 7.0) followed by centrifugation at 100000*g* for 30 min. The supernatant was used as an enzyme source without further purification. An aliquot (corresponding to about 0.1 mg of protein) was incubated in 0.2 mL of potassium phosphate buffer (75 mM, pH 7.4) containing 125 mM NaCl and 25 mM NaF in the presence of 2 mM tyrosine methyl ester as the acceptor and 0.3 μM [^{35}S]PAPS as the donor. After 30 min at 37 °C, incubations were stopped by heating (4 min at 95 °C), and formed ^{35}S-sulfated tyrosine methyl ester was isolated on the polystyrene bead column as described above for Boc-CCK-8. The recovery of a standard of O-sulfated tyrosine methyl ester was 80%.

Results

Identification of [^{35}S]Boc-CCK-8 Formed by Microsomal Fraction. Preliminary studies with various subcellular fractions from rat brain using the standard procedure described under Materials and Methods indicated that no detectable sulfotransferase activity could be found in soluble fractions. In contrast, incubation of several particulate fractions, among which the microsomal fraction displayed the highest specific activity, resulted in the formation of significant amounts of a ^{35}S-labeled material that could be separated from [^{35}S]PAPS by the polystyrene bead column chromatographic method. The identity of the compound eluted from the columns by EtOH was first checked by high-performance liquid chromatography (HPLC) after concentration under reduced pressure. A single main peak representing 84% of the total radioactivity was eluted with a retention time of 21–23 min, closely corresponding to that of a standard of Boc-CCK-8 (in sulfated form) (Figure 1A). When the microsomal fraction was boiled before incubation, no significant formation of [^{35}S]Boc-CCK-8 was observed. In addition, a possible degradation of formed [^{35}S]Boc-CCK-8 during the incubation with the microsomal fraction was assessed as follows. After a typical assay performed under standard conditions the EtOH eluate of the polystyrene bead column was dried under reduced pressure, and the residue was dissolved in buffer and reincubated for 30 min in the presence of a new aliquot of the microsomal fraction. Passage of the reaction medium over a polystyrene bead column resulted in a 95% recovery of the applied radioactivity in the EtOH eluate, indicating that no detectable hydrolysis occurred during the incubation.

In a slightly different HPLC system, using the same μBondapak C_{18} column but 32% acetonitrile, 1% trifluoroacetic

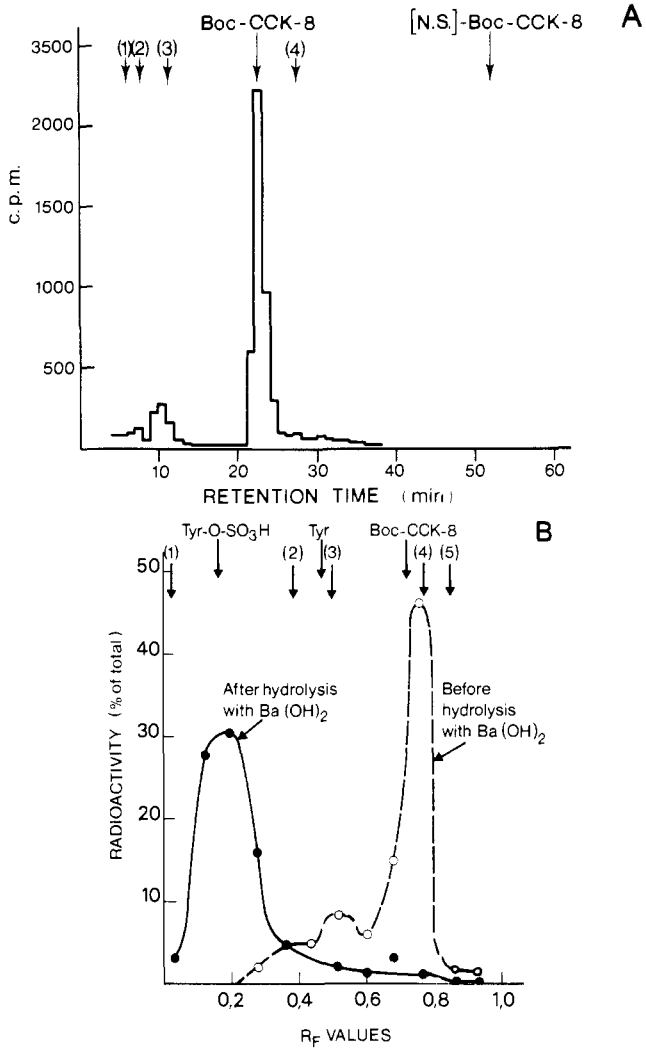

FIGURE 1: Sulfation of Boc-CCK-8 (ns) by a microsomal fraction of rat cerebral cortex: chromatographic identification of [^{35}S]Boc-CCK-8. The microsomal fraction (0.1 mg of protein) was incubated for 30 min in 0.2 mL of 75 mM potassium phosphate buffer, pH 5.8, containing 125 mM NaCl and 25 mM NaF in the presence of 0.12 μM [^{35}S]PAPS and 0.2 mM Boc-8 (ns); the peptide was subsequently isolated by passage onto a polystyrene bead column. The ^{35}S-labeled material eluted from this column by EtOH was identified by HPLC and TLC. (A) Reverse-phase HPLC on a μBondapak column with 30% acetonitrile and 1% w/v trifluoroacetic acid buffered with diethylamine to pH 2.5 (70%). The retention times of standards were determined by their absorbance at 254 nm and fractions collected every minute for radioactivity measurements. The main radioactivity peak (fractions 22–25) represented 84% of the injected radioactivity. Arrows correspond to the following standards: (1) Boc-Asp-Tyr-Met-Gly, (2) CCK-8, (3) CCK-8 (ns), and (4) Boc-Asp-Tyr-Met-Gly-Trp-Met. (B) Tlc before and after hydrolysis by $Ba(OH)_2$ (0.2 M, 95 °C, 24 h). The samples were applied to a silica gel plate and developed with 1-butanol/acetic acid/water (125:30:125). Standards were localized by Ehrlich's reagent and α-nitroso-β-naphthol or by their radioactivity (PAPS and inorganic sulfate). Arrows correspond to the following standards: (1) [^{35}S]PAPS or inorganic sulfate, (2) CCK-8, (3) CCK-8 (ns), (4) Boc-Asp-Tyr-Met-Gly, and (5) Boc-CCK-8 (ns).

acid, and 68% diethylamine, pH 2.5, as solvent, the retention time of the radioactivity peak was 15 min, again identical with that of a Boc-CCK-8 standard (not shown).

Following thin-layer chromatography (TLC) analysis of a similar sample the radioactivity was found to migrate as a single main peak with a R_f value corresponding to that of Boc-CCK-8 (sulfated) (Figure 1B). When the sample was previously hydrolyzed for 24 h at 95 °C with 0.2 M $Ba(OH)_2$ (Jevons, 1963), the radioactivity was found to migrate at the

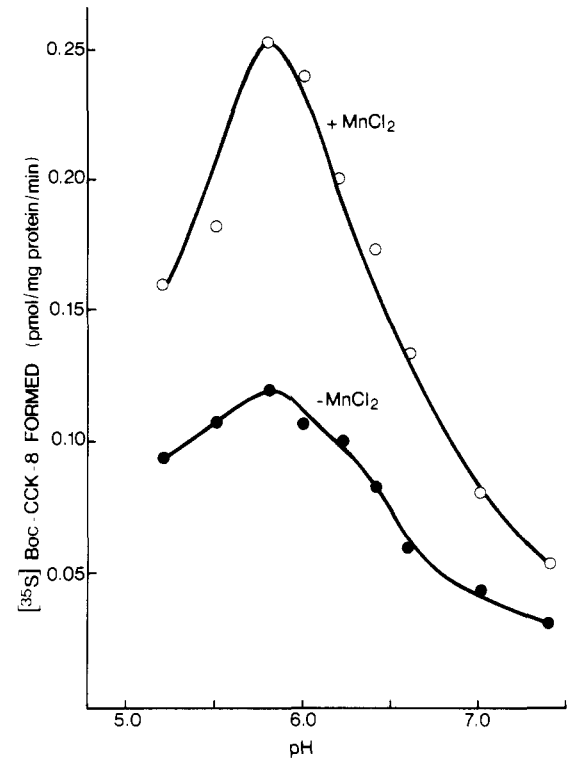

FIGURE 2: pH dependence of tyrosyl sulfotransferase activity of the microsomal fraction. Activities were evaluated in 30-min incubations in 75 mM phosphate buffer, 125 mM NaCl, and 25 mM NaF in the presence or absence of 0.2 mM $MnCl_2$.

same R_f value (0.2) as a standard of Tyr-OSO_3H (Figure 1B). Finally, when the polystyrene bead column eluate was concentrated and treated with an antibody raised in rabbits against the C-terminal end of CCK-8 (Zuzel et al., 1985), a significant immunoprecipitation of the radioactivity was observed (not shown).

Properties of Tyrosyl Sulfotransferase Activity in the Microsomal Fraction Evaluated with Boc-CCK-8 (ns) as Acceptor. The sulfotransferase activity as measured in a 30-min incubation was optimally enhanced (by about 70%) in the presence of 0.1–0.2 mM $MnCl_2$ whereas further increasing this salt concentration resulted in a slightly decreased activity. Boc-CCK-8 formation in the absence or the presence of 0.2 mM $MnCl_2$ increased linearly with the incubation time up to 60 min, and a 30-min incubation time was therefore selected in all further experiments. The sulfotransferase activity in the absence as well as in the presence of 0.2 mM $MnCl_2$ increased linearly with the amount of microsomal protein in the assay at least up to 750 μg of protein/mL. Further assays were performed with about 500 μg of microsomal protein/mL.

Varying the pH of the phosphate buffer in the incubation medium indicated that the optimal pH value was around 5.8 in the presence as well as in the absence of $MnCl_2$ with, in both cases, a strongly decreased activity at pH above 7.0 (Figure 2). Similar results were obtained with Tris–maleate as buffer instead of phosphate buffer (not shown).

Increasing the [^{35}S]PAPS concentration in the presence of a fixed Boc-CCK-8 (ns) concentration (0.25 mM) led to typical Michaelis–Menten saturation kinetics in the absence as well as in the presence of $MnCl_2$ (Figure 3). In both cases the apparent K_M value of the sulfate donnor was 120 nM with apparent V_{max} values of 0.22 ± 0.01 and 0.48 ± 0.02 pmol (mg of protein)$^{-1}$ min^{-1}, respectively. Increasing the concentration of the acceptor Boc-CCK-8 (ns) in the presence of [^{35}S]PAPS

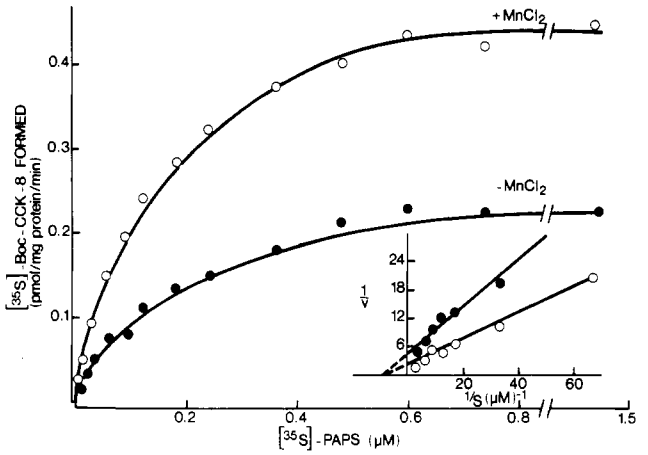

FIGURE 3: Tyrosyl sulfotransferase activity in the presence of [^{35}S]PAPS in increasing concentrations. Activities were evaluated in 30-min incubations in the presence of 0.25 mM Boc-CCK-8 (ns) and in the presence (or absence) of 0.2 mM $MnCl_2$. Inset: Double-reciprocal plots of the data. The apparent K_M values of the sulfate donor were 125 ± 8 nM and 118 ± 9 nM in the absence or presence of $MnCl_2$, respectively. V_{max} values were 0.22 ± 0.01 and 0.48 ± 0.02 pmol (mg of protein)$^{-1}$ min^{-1} in the absence and presence of $MnCl_2$, respectively.

at fixed concentration (0.5 μM) led to a progressive saturation with some inhibition occurring at acceptor concentrations above 0.3 mM, both in the absence and in the presence of 0.2 mM $MnCl_2$ (not shown). The double-reciprocal plot analysis of data led to similar apparent K_M values (100 ± 16 and 143 ± 22 μM) in both cases whereas the apparent V_{max} value was about 3-fold higher in the presence of $MnCl_2$ than in its absence, 0.23 ± 0.04 and 0.67 ± 0.11 pmol (mg of protein)$^{-1}$ min^{-1}, respectively.

Effects of Various Acceptors and Inhibitors on Sulfotransferase Activity of the Microsomal Fraction. In addition to Tyr (methyl ester), various Tyr-containing peptides (0.3–0.5 mM) were tested as potential acceptors in the standard assay system under conditions optimal for Boc-CCK-8 (pH 5.8, 0.2 mM $MnCl_2$, 0.4 μM [^{35}S]PAPS). In each case their recovery (70–90%) from the chromatographic columns (either polystyrene beads or ECTEOLA, see Materials and Methods) was evaluated and, data were accordingly corrected. Whereas no activity above blanks could be detected with Tyr (methyl ester), angiotensin I (Asp-Arg-Val-Tyr-Val-Tyr-Val-His-Pro-Phe-His-Leu), enkephalin (Tyr-Gly-Gly-Phe-Met), or gastrin 1–17 (pGlu-Gly-Pro-Trp-Met-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2), significant activities were found with various fragments of the CCK molecule (except CCK-7) as well as with nonsulfated caerulein (pGlu-Glu-Asp-Tyr-Thr-Gly-Trp-Met-Asp-Phe-NH_2) (Table I).

The microsomal sulfotransferase activity measured with 0.3 mM Boc-CCK-8 (ns) as an acceptor was inhibited by the thiol reagent *p*-(chloromercuri)benzenesulfonic acid, ATP, various metal ions, and the phenol sulfotransferase inhibitor (Mulder & Scholtens, 1977; Rein et al., 1982; Giorgo & Meek, 1985) 2,6-dichloro-4-nitrophenol (Table II). However, in the latter case, whereas soluble phenol sulfotransferase activity from rat brain was inhibited with an IC_{50} value of about 10 μM, approximately 100-fold higher concentration were required for the microsomal tyrosyl sulfotransferase (Figure 4).

Table I: Sulfotransferase Activity of Microsomal Fraction from Rat Cerebral Cortex Evaluated with Various Acceptors[a]

compounds	sulfotransferase activity [pmol (mg of protein)$^{-1}$ h^{-1}]
Tyr (methyl ester)	nd[b]
Boc-Asp-Tyr	1.38 ± 0.20
Boc-Asp-Tyr-Met	3.82 ± 0.11
Boc-Asp-Tyr-Met-Gly	5.21 ± 0.37
Boc-Asp-Tyr-Met-Gly-Trp	9.50 ± 0.64
Boc-Asp-Tyr-Met-Gly-Trp-Met	10.42 ± 0.55
Boc-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2 (Boc-CCK-8, ns)	23.14 ± 1.07
CCK-8 (nonsulfated)	0.87 ± 0.13
CCK-7 (nonsulfated)	nd[b]
Boc-Asp-Arg-Asp-Tyr-Met-Gly	5.20 ± 0.80
Asp-Arg-Asp-Tyr-Met-Gly	4.80 ± 0.66
caerulein (nonsulfated)	3.10 ± 0.38
angiotensin I	nd[b]
[Met^5]enkephalin	nd[b]
gastrin 1–17 (nonsulfated)	nd[b]

[a] Sulfotransferase activity determined in 30-min incubations in the presence of 0.2 mM $MnCl_2$, 0.4 μM [^{35}S]PAPS, and the various acceptors at 0.3 mM concentration (except for Tyr methyl ester, CCK-8, Asp-Arg-Asp-Tyr-Met-Gly, and its Boc derivative, which were used at 0.5 mM). The ^{35}S-sulfated compounds formed were isolated by polystyrene bead or ECTEOLA column chromatography as described under Materials and Methods. Values given are ± SEM from 3–5 determinations. [b] nd, not detectable.

Table II: Inhibition of Microsomal Tyrosyl Sulfotransferase Activity by Various Compounds[a]

compounds	IC_{50} value
2,6-dichloro-4-nitrophenol	650 μM
p-(chloromercuri)benzenesulfonic acid	22 μM
EDTA	>1 mM
adenosine 5′-triphosphate	8 μM
Triton X-100	0.04%
$FeCl_3$	830 μM
$CuCl_2$	22 μM
$ZnCl_2$	>1 mM
$MgCl_2$, $CaCl_2$, V_2O_5, $CoCl_2$	>1 mM

[a] The tyrosyl sulfotransferase activity of the microsomal fraction was measured in the presence of 0.3 mM Boc-CCK-8 (ns), 0.3 μM [^{35}S]-PAPS, and the various compounds in increasing concentrations. The activity was completely abolished in the presence of compounds with IC_{50} values indicated as <1000 μM.

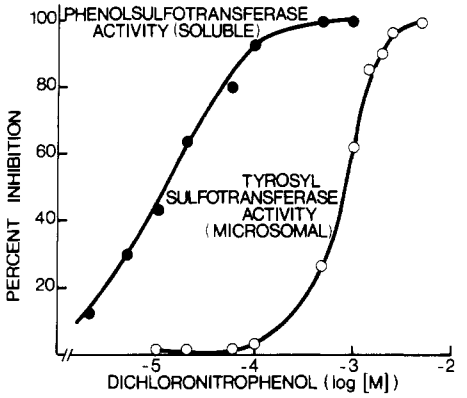

FIGURE 4: Inhibition of microsomal tyrosyl sulfotransferase and soluble phenol sulfotransferase activities from rat cerebral cortex by 2,6-dichloro-4-nitrophenol. The activities in the microsomal and soluble fractions were evaluated in 30-min incubations with 0.25 mM Boc-CCK-8 (ns) and 2 mM tyrosine methyl ester, respectively, as acceptors and 0.3 μM [^{35}S]PAPS as donor.

Discussion

This work characterizes an apparently novel sulfotransferase activity, present in the microsomal fraction of rat brain, that transfers sulfate groups from the universal donor PAPS (Lipmann, 1958) to Tyr residues of synthetic peptides derived from the CCK molecule. Several of its properties suggest that

it may be responsible for the posttranslational covalent modification of CCK and, possibly, other Tyr-containing polypeptides.

In spite of various attempts consisting of widely varying incubation conditions (buffers, pH, incubation time, concentrations of substrates, addition of peptidase inhibitors), no soluble tyrosyl sulfotransferase activity could be found in brain extracts. This indicates that neither the phenol sulfotransferase acting on, e.g., phenolic monoamines (EC 2.8.2.1) nor any of the other sulfotransferases acting on a variety of molecules like steroids (EC 2.8.2.2) that are all cytosolic (Sekura et al., 1981; Sandler & Usdin, 1981) are responsible for Boc-CCK-8 (ns) sulfation. In addition, purified aryl sulfotransferase IV is able to transfer sulfate groups on Tyr methyl ester or on des-Asp^1-CCK-8 (ns) (Sekura & Jakoby, 1981), i.e., on Tyr residues with a free NH_2 group, whereas these compounds were not sulfated in a detectable manner by the microsomal enzyme activity (Table I). Also, the soluble phenol sulfotransferase was much more sensitive to inhibition by 2,6-dichloro-4-nitrophenol (Mulder & Scholtens, 1977; Rein et al., 1982; Giorgi & Meek, 1985) than the microsomal tyrosyl sulfotransferase (Figure 4). On the other hand, sulfotransferases responsible for the formation of sulfated glycosaminoglycans and sulfolipids are associated with Golgi apparatus membranes (Silbert & Freilich, 1980) known to be present in the microsomal fraction presently used (Farrell & McKhann, 1971). However, besides their very different substrate specificity, a variety of differential properties indicate that these enzymes were not responsible for sulfation of Boc-CCK-8 (ns) since (i) their optimal pH is 7.0–7.8 (Balasbramanian & Bachhawat, 1964), which contrasts with the optimal pH of 5.8 for the tyrosyl sulfotransferase (Figure 2), (ii) they are activated by detergents like Triton X-100 or ATP, which, in low concentrations, abolish the tyrosyl sulfotransferase activity (Table II), and (iii) the effects of divalent ions like Mn^{2+}, Mg^{2+}, and Ca^{2+} markedly differ on these enzyme activities (Mulder et al., 1982).

Recently, a membrane-bound enzyme activity able to transfer [^{35}S]sulfate residues from PAPS to several as yet unidentified proteins of a rat pheochromocytoma cell line was described (Lee & Huttner, 1983). This "tyrosyl protein sulfotransferase" (or related enzymes) might also be responsible for the sulfation of a variety of secretory proteins like immunoglobulin G (Baeuerle & Huttner, 1984) and fibronectin (Liu & Lippmann, 1985). It is also possibly related to the presently evidenced enzyme, but this remains to be established since its properties and substrate specificity were not characterized.

Among a variety of possible acceptors, Boc derivatives of several fragments of the CCK molecule, starting from the dipeptide Asp-Tyr, were sulfated by the microsomal enzyme activity at a rate that markedly increased with the length of the amino acid sequence and culminated with the full CCK-8 (ns) sequence (Table I). Sulfation of CCK-8 (ns) itself (in nonprotected form) occurred at a rate 25-fold lower than that of its Boc derivative whereas the nonprotected hexapeptide Asp-Arg-Asp-Tyr-Met-Gly (corresponding to sequence 24–29 of CCK-33) was a relatively good substrate. The strong favorable effect of the Boc protecting group, not found with the latter acceptor, might be attributable to its binding to a subsite of the enzyme recognizing the Asp-Arg sequence (the latter corresponding to the N-terminal extension of CCK-8 in its precursor). Caerulein, in which the Tyr residue is preceded by three dicarboxylic amino acid residues, was also a relatively good acceptor. Interestingly, fibrinogens, like CCK, caerulein, and gastrin, contain several dicarboxylic amino acid residues in the vicinity of their sulfated Tyr residues, suggesting that a strongly electronegative domain may contribute to recognition of these molecules by tyrosyl sulfotransferase(s). In a parallel way phosphorylation of Tyr residues by tyrosine protein kinases seems to require the presence of one or more acidic amino acid residues at their N-terminal side (Hunter, 1982), which may indicate some analogies between the enzymes transferring sulfate and phosphate groups on Tyr within proteins. The fact that gastrin 1–17 was not detectably sulfated by the microsomal enzyme (Table I) does not rule out a participation of the latter in the sulfation of the gastrin precursor: conceivably this failure may reflect secondary and tertiary structure requirements not satisfied in our cell-free assay system.

Some features of the sulfotransferase indicate that it satisfies several criteria (Docherty & Steinn, 1982) for it to be considered responsible for the maturation of CCK and other secretory polypeptides. Among various subcellular fractions from rat brain, the microsomal and vesicular fractions displayed the highest relative specific activities (Vargas et al., unpublished results), which agrees with the idea that such a process mostly occurs in Golgi-derived elements, particularly the secretory granules (Loh & Gainer, 1983). Furthermore, the optimum pH value of the sulfotransferase (Figure 2) corresponds to the pH value of 5–6 inside many secretory granules. This low pH being reached under the action of a membrane proton pump that starts to work when the granule seals, this is consistent with the hypothesis that the secretory granule is the major site of maturation of secretory polypeptides (Pollard et al., 1977). Nevertheless, it should be remarked that several sulfotransferases, probably not involved in maturation processes, also display optimum pH values in the acidic range (Sekura et al., 1981).

Finally, although the tyrosyl sulfotransferase was mainly studied on peptides derived from CCK, it should be underlined that, if it is responsible for the maturation of this neurohormonal peptide, its function might be much larger since its distribution among cerebral regions or peripheral tissues does not match that of CCK immunoreactivity (Vargas et al., unpublished results). Hence the specificity of Tyr sulfation by this enzyme would not result from its selective cellular localization but from its selective recognition of some amino acid sequence features, as already suggested by this work.

Registry No. Boc-CCK-8, 25687-34-7; CCK-8 (nonsulfated), 25679-24-7; CCK-7 (nonsulfated), 47910-79-2; PAPS, 482-67-7; Boc-Asp-Tyr, 97826-78-3; Boc-Asp-Tyr-Met, 97826-79-4; Boc-Asp-Tyr-Met-Gly, 97826-80-7; Boc-Asp-Tyr-Met-Gly-Trp, 97826-81-8; Boc-Asp-Tyr-Met-Gly-Trp-Met, 97826-82-9; Boc-Asp-Arg-Asp-Tyr-Met-Gly, 97826-83-0; caerulein (nonsulfated), 20994-83-6; manganese, 7439-96-5; cholecystokinin, 9011-97-6; tyrosine sulfotransferase, 9055-56-5.

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Papain Fragmentation of the (Na^+,K^+)-ATPase β Subunit Reveals Multiple Membrane-Bound Domains†

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ABSTRACT: Purified dog kidney (Na^+,K^+)-ATPase was reacted with tritiated sodium borohydride after treatment with neuraminidase and galactose oxidase. This procedure did not affect the ATPase activity of the enzyme, and all of the covalently bound radioactivity was found in the β subunit (M_r 54 000). Papain digestion of the tritiated enzyme produced two labeled fragments of M_r 40 000 and 16 000. Further proteolysis generated an M_r 31 000 peptide from the larger fragment. Unlike the tryptic and chymotryptic sites of the α subunit, the sites of papain hydrolysis were insensitive to conformations of the (Na^+,K^+)-ATPase. Determination of the NH_2-terminal sequences was used to arrange the fragments within the linear map of the β chain. Finally, none of the labeled peptides was released from the membrane under nondenaturing conditions. These results are consistent with a model of the β subunit containing a 40 000-dalton NH_2-terminal piece and a 16 000-dalton COOH-terminal piece. Both fragments have extracellularly exposed carbohydrate and at least one membrane-bound domain.

The (Na^+,K^+)-ATPase couples the hydrolysis of ATP to the active transport of Na^+ and K^+ across the plasma membrane [for reviews, see Forgac & Chin (1984), Jorgensen (1982), and Cantley (1981)]. It consists of two polypeptide chains, α of M_r 100 000 and β of M_r 60 000.[1] Proteolytic enzymes and chemical labeling reagents have been used to identify cytoplasmic, extracellular, and membrane-embedded regions of the protein. The α chain traverses the lipid bilayer many times and extends into both aqueous compartments (Chin & Forgac, 1983; Jorgensen et al., 1982; Farley et al., 1980).

Less is known about the disposition of the β chain with respect to the lipid bilayer. The small subunit contains covalently bound carbohydrate, and the mass of the protein portion has been estimated at 30 000–50 000 daltons (Fambrough & Bayne, 1983; Craig & Kyte, 1980). Lipid-soluble reagents and a digitoxin derivative have been shown to react with the β subunit (Jorgensen & Brunner, 1983; Jorgensen et al., 1982; Montecucco et al., 1981; Farley et al., 1980; Hall & Ruoho, 1980). Giradet et al. (1981) have described an antiserum apparently directed toward cytoplasmic determinants of the β subunit. All of these studies have dealt only with the intact β polypeptide. To obtain greater resolution of the protein structure, as has been achieved with the α chain, it would be advantageous to be able to examine smaller regions whose locations within the native molecule are known.

In this work, papain is used to fragment the membrane-bound dog kidney (Na^+,K^+)-ATPase. The sites of proteolysis and the papain fragments are characterized. These results and the data presented by other workers are incorporated into a model of the structure of the β subunit with respect to the membrane.

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[1] Apparent molecular weights are based on mobilities in SDS–polyacrylamide gel electrophoresis.

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