Volume 211, number 2, 234–238 FEB 04389 January 1987

Apparent identity of cerebral tyrosylsulfotransferase activities using either a cholecystokinin derivative or an acidic amino acid polymer as substrate

Froylan Vargas and Jean-Charles Schwartz

Laboratoire de Physiologie, Université René-Descartes, 4 Avenue de l'Observatoire, 75006 Paris and *Unité 109 de Neurobiologie et Pharmacologie, Centre Paul Broca de l'INSERM, 2 ter rue d'Alésia, 75014 Paris, France*

Received 15 November 1986

The tyrosylsulfotransferase activities of rat cerebral fractions transferring [^{35}S]sulfate groups from 3'-phosphoadenosine 5'-[^{35}S]phosphosulfate to either Boc-cholecystokinin-8 (in non-sulfated form) or the acidic amino acid polymer (Glu, Ala, Tyr)$_n$ (6:3:1) were compared. They appear similar regarding subcellular distribution (both being enriched in the microsomal fraction) and inhibition by an excess of the acidic amino acid polymer, NaCl or 2,6-dichloro 4-nitrophenol. These results obtained with artificial substrates suggest that identical (or closely similar) tyrosylsulfotransferases are responsible for sulfation of tyrosine residues of several secretory proteins from various tissues.

Tyrosylsulfotransferase; Cholecystokinin

1. INTRODUCTION

As compared to the various post-translational covalent modifications of proteins, *O*-sulfation on tyrosine residues has for a long time attracted relatively little attention mainly because it was considered a rare modification. The presence of a sulfated tyrosine residue was first detected in fibrinopeptide B [1,2], then on gastrin [3] and cholecystokinin (CCK) [4] and was more recently shown to occur in a rather large number of secretory proteins, only some of which, such as immunoglobulin G [5], fibronectin [6] or procollagens [7], have been identified.

Until recently very little was known about the enzyme(s) responsible for this post-translational modification. A membrane-bound enzyme activity transferring sulfate groups from the universal donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to synthetic peptides derived from the CCK molecule was first characterized in brain [8,9]. Its presence in Golgi-enriched fractions of a variety of peripheral tissues which are not known to secrete CCK suggested that this tyrosylsulfotransferase may be involved in the sulfation of many different proteins. This view was further substantiated by structure-activity studies among synthetic acceptors showing the importance for recognition by the tyrosylsulfotransferase of acidic amino acid residues [9] which are known to be abundant in the vicinity of the sulfated tyrosine residue of natural proteins [10].

At about the same time a tyrosylsulfotransferase activity was independently characterized in Golgi-enriched fractions of bovine adrenal medulla using PAPS as the donor and an acidic amino acid polymer (Glu^{62}, Ala^{30}, Tyr^{8})$_n$ as acceptor [11]. The two sulfotransferase activities appeared to share not only similar subcellular localizations but also

Correspondence address: J.-C. Schwartz, Unité 109 de Neurobiologie et Pharmacologie, Centre Paul Broca de l'INSERM, 2 ter rue d'Alésia, 75014 Paris, France

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

similar catalytic properties such as acidic optimum pH or activation by Mn^{2+} and Mg^{2+}.

To explore further the hypothesis that the two sulfotransferase activities might correspond to a single enzyme we have compared sulfation reactions in rat brain fractions using either the acidic amino acid polymer (poly EAY) or Boc-cholecystokinin-8 in non-sulfated form (Boc-CCK-8, n.s.) as substrates.

2. MATERIALS AND METHODS

2.1. *Materials*

Boc-CCK-8 (n.s.) was obtained from Bachem (Bubendorf, Switzerland); the random polymers $(Glu, Ala, Tyr)_n$ (6:3:1, average size 25 kDa) (poly EAY) or $(Glu,Tyr)_n$ (4:1) (poly EY), 2,6-dichloro 4-nitrophenol (DCNP) and 2-mercaptoethanol were purchased from Sigma (St. Louis, MO). All other reagents (analytical grade) were from Prolabo (Paris).

[^{35}S]PAPS (1–3 Ci/mmol) was purchased from NEN Chemicals (Dupont, France) and ACS scintillation mixture from The Radiochemical Center (Amersham, England). Male Wistar rats (180–220 g) were obtained from Iffa-Credo (St Germain l'Arbresle, France).

2.2. *Subcellular fractionation*

Rats were killed by stunning and decapitation. Subcellular fractions were prepared as described [12]. Briefly, the brain was carefully removed and homogenized in 20 vols of 0.32 M sucrose with 10 up and down strokes at 700 rpm in a teflon-glass homogenizer (clearance 0.12 mm). The homogenate was centrifuged at 1000 × *g* for 10 min and the pellet resuspended in 10 vols of 0.32 M sucrose and recentrifuged. The resulting pellet (P1) was resuspended in 5 ml of 10 mM Tris-maleate buffer (pH 7.0) and kept at 4°C. The combined supernatants were centrifuged at 20000 × *g* for 20 min to give a crude mitochondrial pellet (P2). The supernatant was then centrifuged at 100000 × *g* for 60 min to give the microsomal fraction (P3). The particulate fractions were resuspended in 10 mM Tris-maleate buffer (pH 7.0) at about 10 mg protein per ml.

A high-speed supernatant fraction (cytosol) from rat brain was obtained by homogenization in 10 vols of 10 mM Tris-maleate buffer, pH 7, followed by centrifugation at 100000 × *g* for 30 min.

2.3. *Solubilization of the microsomal fraction*

The microsomal (P3) fraction was resuspended in 10 mM Tris-maleate buffer, pH 7.0 (10 ml/g tissue), kept at 4°C for 10 min, then NaCl (0.1 M, final concentration) was added. After 10 min at 4°C, the suspension was centrifuged at 100000 × *g* for 60 min, the supernatant discarded and the pellet resuspended (4 mg protein/ml) in a buffer containing 10 mM Tris-HCl, pH 7.4, 25% glycerol, 5 mM 2-mercaptoethanol and 0.05% Triton X-100. After 30 min at 4°C, the suspension was centrifuged (100000 × *g*, 60 min) the supernatant discarded and the pellet treated with a solubilization buffer of similar composition except for Triton X-100 (1% instead of 0.05%). After gentle shaking for 30 min at 4°C, the solubilized fraction was separated by centrifugation (100000 × *g*, 60 min) and used as an enzyme source either on the same day or within 3 days of storage at −40°C, since no significant loss of activity could be detected under these conditions.

2.4. *Assays of tyrosylsulfotransferase activity*

The tyrosylsulfotransferase activity of particulate fractions was assayed using 0.15 mM Boc-CCK-8 (n.s.) as the acceptor and 0.5 μM [^{35}S]PAPS as donor, the [^{35}S]sulfated peptide being isolated by chromatography on polystyrene bead microcolumns as described [9].

The tyrosylsulfotransferase activity of solubilized fractions was assayed using either Boc-CCK-8 (n.s.) or poly EAY as the acceptor in a reaction medium derived from that described by Lee and Huttner [11]. In this case, the standard reaction mixture (100 μl) was composed of (final concentrations) 50 mM Tris-maleate buffer, pH 6.6, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 12.5% glycerol, 2.5 mM 2-mercaptoethanol, 25 mM NaF, 0.2 mM ATP, 0.5% Triton X-100, 0.5 μM [^{35}S]PAPS, 2.5 μM poly EAY (or 1.5 mM Boc-CCK-8, n.s.) and 100–250 μg protein. Blanks consisted of the same reaction medium without poly EAY (or Boc-CCK-8, n.s.). Reactions were stopped by the addition of 50 μl of a solution containing 4 mM ATP and 10 mM Na_2SO_4 followed by heating at 90°C for 3 min. The [^{35}S]sulfated Boc-CCK-8 formed was isolated and determined as in [9] whereas

[^{35}S]sulfated poly EAY was isolated by trichloroacetic acid precipitation. Briefly 50 μl bovine serum albumin solution (10 mg/ml) was added as a carrier to the cooled reaction mixture, followed by 3 ml cold trichloroacetic acid (10% final concentration). After 10 min at 5°C precipitated proteins were pelleted by centrifugation (5000 rpm, 10 min) then resuspended in 0.3 ml of an alkaline solution containing 0.1 M NaOH, 5 mM Na_2SO_4 and 0.2% Triton X-100 and precipitated again with trichloroacetic acid. The final pellet was resuspended in 0.5 ml of the alkaline solution and counted in 5 ml liquid scintillation solution (ACS).

3. RESULTS AND DISCUSSION

Using poly EAY as the acceptor a major part of the tyrosylsulfotransferase activity of the homogenate of rat cerebral cortex was associated with the microsomal (P3) fraction (table 1) which primary subcellular fractionation studies had also shown to contain the highest CCK-sulfotransferase activity [8].

The tyrosylsulfotransferase activity of bovine adrenal medulla, evaluated with poly EAY under conditions similar to those used here, is also the highest in Golgi-enriched subcellular fractions [11]. Furthermore, sulfation of another substrate of the adrenal sulfotransferase, the acidic amino acid polymer poly EY [11], was observed with the cerebral enzyme (not shown). These observations are consistent with the hypothesis that the same sulfotransferase activity in Golgi membranes was responsible for the sulfation of tyrosine residues in the two exogenous substrates Boc-CCK-8 (n.s.) and poly EAY.

However, sulfation of these two substrates by the microsomal fraction from cerebral cortex differed in various aspects. Firstly, in agreement with data obtained with the adrenal medulla fractions [11], sulfation of poly EAY could not be observed in the absence of detergent whereas this was not the case for Boc-CCK-8, a difference which might be related to the more difficult access of the large M_r acidic substrate to the active site of the membrane-bound sulfotransferase. It has been suggested that the catalytic site of the latter is oriented toward the Golgi lumen [11], a localization which would be consistent with the absence of any detectable PAPS-synthesizing activity [13] together with the presence of a transmembrane carrier system for PAPS in Golgi vesicles [14]. Secondly, an activity loosely associated with microsomal membranes (i.e. solubilized with only 0.05% Triton X-100) and transferring [^{35}S]sulfate residues from [^{35}S]PAPS to Boc-CCK-8 (n.s.) but not poly EAY was detected (not shown). This activity, inhibited by 2,4-dinitrophenol at low concentrations (IC_{50} ~15 μM), was presumably attributable to cytosolic phenolsulfotransferase(s) (EC 2.8.2.1 and EC 2.8.2.9) adsorbed to the membranes and was not further studied.

When solubilized in the presence of 1% Triton X-100 the tightly bound microsomal sulfotransferase activity displayed quite similar properties when studied with either poly EAY or Boc-CCK-8 as acceptors. With both substrates, the sulfotransferase activity had a pH profile with a

Table 1

Subcellular distribution of tyrosylsulfotransferase activity in rat cerebral cortex using poly EAY as compared to Boc-CCK-8 (n.s.) as acceptor

Fraction	Tyrosylsulfotransferase activity (relative specific activity)	
	Poly EAY	Boc-CCK-8 (n.s.)[a]
Homogenate	1.00	1.00
Nuclear (P1)	0.6 ± 0.3	0.5 ± 0.1
Crude mitochondrial (P2)	0.7 ± 0.2	0.9 ± 0.3
Microsomal (P3)	2.9 ± 0.4	3.3 ± 0.7
Soluble (cytosol)	not detectable	

[a] Values taken from Vargas et al. [8]

The homogenate or subcellular fractions were suspended in 10 mM Tris-HCl buffer, pH 7.4, containing 25% glycerol, 5 mM 2-mercaptoethanol and 1% Triton X-100 to a final concentration of about 5 mg protein/ml. After 30 min at 4°C, the enzyme activity was measured by incubating for 30 min at 30°C 10–50 μl aliquots of the suspension (containing 0.10–0.25 mg protein) in the presence of 2.5 μM poly EAY as acceptor and 0.5 μM [^{35}S]PAPS as sulfate donor. Blanks were obtained from incubations in the absence of poly EAY. The tyrosylsulfotransferase activity of the homogenate was 3.6 ± 0.40 pmol/mg protein per h

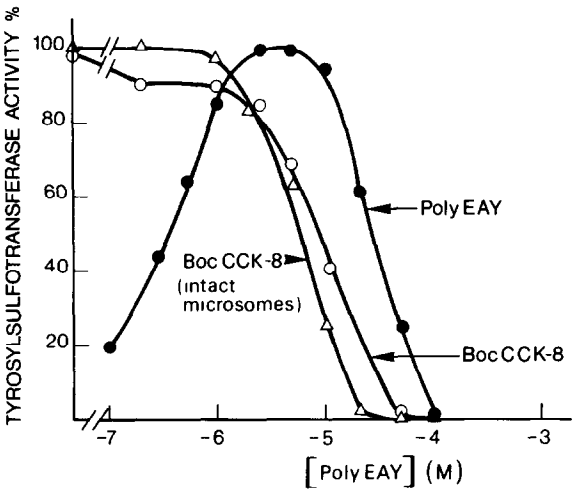

Fig.1. Inhibitory effects of poly EAY on microsomal tyrosylsulfotransferase activity of rat cerebral cortex measured with either Boc-CCK-8 (n.s.) or poly EAY as acceptor. The microsomal fraction used for enzyme activity assays was either simply resuspended in 10 mM Tris-maleate buffer pH 7 (intact microsomes) or previously solubilized as described in section 2. Tyrosylsulfotransferase activity of intact microsomes was evaluated in the presence of 0.5 μM [^{35}S]PAPS, 0.15 mM Boc-CCK-8 (n.s.) and poly EAY in increasing concentrations; control activity (in the absence of poly EAY) was 0.32 ± 0.04 pmol/mg protein per min. Tyrosylsulfotransferase activity of the solubilized preparation was evaluated in the presence of 0.5 μM [^{35}S]PAPS either using 1.5 mM Boc-CCK-8 (n.s.) (in the presence of poly EAY in increasing concentrations) or poly EAY in increasing concentrations as the acceptors. Control activities were 8.2 ± 0.5 pmol/mg protein per h (Boc-CCK-8 in the absence of poly EAY) and 11.2 ± 1.4 (10 μM poly EAY), respectively. Values derived from 30 min incubations at 30°C. Means ± SE of 12 determinations.

peak between 6.4 and 6.6, similar to that of the solubilized bovine adrenal medulla activity [11] and resembling that of the non-solubilized microsomal fraction of rat cerebral cortex [8]. Furthermore, poly EAY was not only a substrate but, at concentrations above 10 μM, inhibited its own sulfation or, at similar concentrations, that of Boc-CCK-8 by either intact or solubilized microsomes (fig.1). Sulfation of poly EAY by the Golgi-enriched fraction of the adrenal medulla occurred with an apparent K_m of 0.3 μM (consistent with our data) and the effects of poly EAY at concentrations above 10 μM were found to be inhibitory.

Finally in the presence of DCNP, an inhibitor of several sulfotransferases [15,16] or of NaCl at concentration above 200 mM, the tyrosylsulfotransferase activity of the solubilized microsomal fraction was similarly inhibited when measured

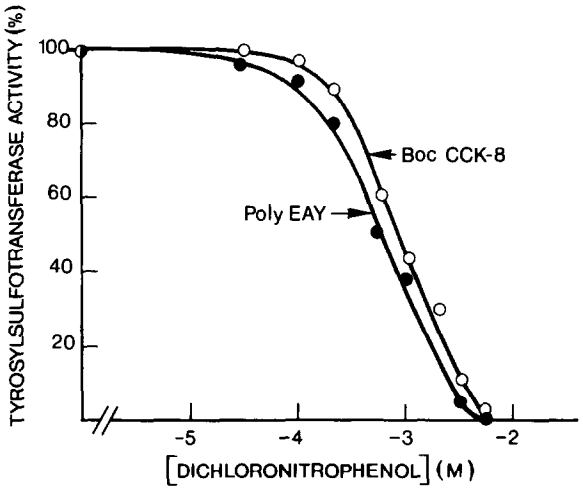

Fig.2. Inhibition of the solubilized tyrosylsulfotransferase from rat brain microsomes by 2,6-dichloro-4-nitrophenol. The enzyme activities were evaluated in 30 min incubations at 30°C using either 1.5 mM Boc-CCK-8 (n.s.) or 2.5 μM poly EAY as acceptors and 0.5 μM [^{35}S]PAPS as sulfate donor. Control activities (100%) were 10.8 ± 1.1 and 7.9 ± 0.6 pmol/mg protein per h with poly EAY and Boc-CCK-8 (n.s.), respectively.

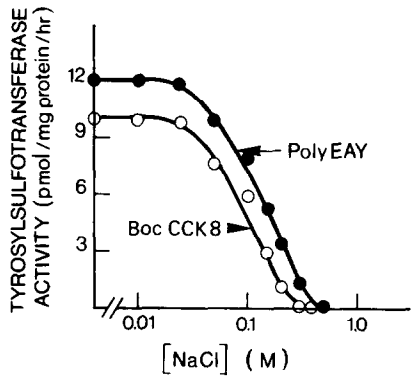

Fig.3. Effect of NaCl on the solubilized tyrosylsulfotransferase activity from rat brain microsomes. Boc-CCK-8 (n.s.) or poly EAY sulfation was evaluated as described in the legend to fig.2.

with either poly EAY or Boc-CCK-8 (figs 2,3). The inhibitory activity of DCNP is similar to that previously described with intact cerebral microsomes [9,17], whereas that of NaCl is closely similar to that observed with the adrenal medulla preparation [11].

Taken together, all our observations are consistent with the idea that poly EAY and Boc-CCK-8 (n.s.) are sulfated by the same (or closely similar) tyrosylsulfotransferase activity(ies) found in Golgi-enriched fractions of a large variety of tissues. This hypothesis will be fully verified with the purification of the corresponding enzyme(s).

REFERENCES

[1] Bettelheim, F.R. (1954) J. Am. Chem. Soc. 76, 2838.
[2] Jevons, F.R. (1963) Biochem. J. 89, 621.
[3] Gregory, H., Hardy, P.M., Jones, D.S., Kenner, G.H. and Sheppard, R.C. (1964) Nature 204, 931.
[4] Mutt, V. and Jorpes, J.E. (1968) Eur. J. Biochem. 6, 156.
[5] Baeuerle, P.A. and Huttner, W.B. (1984) EMBO J. 3, 2209.
[6] Liu, M.C. and Lipmann, F. (1985) Proc. Natl. Acad. Sci. USA 82, 34.
[7] Fessler, L.I., Chapin, S., Brosh, S. and Fessler, J.H. (1986) Eur. J. Biochem. 158, 511.
[8] Vargas, F., Frerot, O., Trung-Tuong, M.D., Zuzel, K., Rose, C. and Schwartz, J.-C. (1985) Ann. NY Acad. Sci. 448, 110.
[9] Vargas, F., Frerot, O., Trung-Tuong, M.D. and Schwartz, J.-C. (1985) Biochemistry 24, 5938.
[10] Huttner, W.B. (1984) Methods Enzymol. 107, 200.
[11] Lee, R.W.H. and Huttner, W.B. (1985) Proc. Natl. Acad. Sci. USA 82, 6143.
[12] Appel, S.H., Day, E.D. and Mickey, D.D. (1972) in: Basic Neurochemistry (Albers, R.W. et al. eds) pp.425–448, Little Brown, Boston.
[13] Brion, F., Schwartz, J.-C. and Vargas, F. (1986) J. Neurochem., in press.
[14] Schwarz, J.K., Capasso, J.M. and Hirschberg, C.B. (1984) J. Biol. Chem. 259, 3554.
[15] Mulder, G.J. and Scholtens, E. (1977) Biochem. J. 172, 247.
[16] Mulder, G.J. (1984) Prog. Drug Metabolism 8, 35.
[17] Vargas, F., Trung-Tuong, M.D. and Schwartz, J.-C. (1986) J. Enzyme Inhibition 1, 105.