0026-895X/89/040647-07$02.00/0

MOLECULAR PHARMACOLOGY, 36: 647-653

Sulfation of *tert*-Butoxycarbonylcholecystokinin and Other Peptides by Rat Liver Tyrosylprotein Sulfotransferase

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Received April 17, 1989; Accepted July 24, 1989

SUMMARY

The sulfoconjugation of tyrosyl residues is a widespread post-translational modification of biologically active proteins and peptides. The rat liver Golgi enzyme tyrosylprotein sulfotransferase has previously been shown to catalyze the transfer of a sulfate moiety from 5′-phosphoadenosine-3′-phosphosulfate to the synthetic acidic polymer poly(Glu_6, Ala_3, Tyr_1) (EAY). In this study, we further characterized the biochemical properties and the substrate specificity of rat liver tyrosylprotein sulfotransferase using a variety of synthetic peptides, including EAY, *tert*-butoxycarbonylcholecystokinin-8 (Boc-CCK), and two carboxy terminal peptide fragments of complement component C4. The data demonstrate that all substrates can be sulfated by an isolated Golgi membrane fraction. In addition, Boc-CCK was also found to be sulfated by a cytosolic sulfotransferase. Using an enriched Golgi fraction, rat liver tyrosylprotein sulfotransferase sulfation of Boc-CCK displayed a pH optimum between 6.0 and 6.5, was stimulated by 50–150 mM NaCl, and required either Mn^{2+} or Mg^{2+} for maximal activity. In contrast, EAY sulfation displayed a pH optimum of 6.7, was not stimulated by NaCl, and required Mn^{2+} for maximal activity. Both Boc-CCK and EAY sulfation were similarly decreased when the enriched Golgi preparation was preincubated at 45° for varying lengths of time. The particulate tyrosylprotein sulfotransferase could be effectively solubilized by 1% Triton X-100 in the presence of 0.2 M NaCl and anion exchange chromatography of the solubilized enzyme yielded a single peak of Boc-CCK- and EAY-sulfating activities. Rat liver tyrosylprotein sulfotransferase was also shown to sulfate a variety of other acidic and basic synthetic tyrosine-containing polymers, as well as two synthetic peptides of 10 and 16 amino acid residues that corresponding to the C-terminal portion of C4.

Tyrosine sulfation has been shown to be a ubiquitous post-translational modification of a variety of diverse biologically active proteins and peptides such as CCK (1), gastrin (2), C4 (3), secretogranin I and II (4), thyroglobulin (5), and α_2-antiplasmin (6). Tyrosine sulfation has been reported to promote optimal biological activity of the peptides CCK (7), gastrin (7), and C4 (8) and has been suggested to play an integral role in regulating the proteolytic processing (9) and secretion (10) of other proteins. However, the physiological significance of this reaction for the majority of the known tyrosine-sulfated proteins and peptides is not well understood (11).

Tyrosine sulfation of proteins and peptides is catalyzed by the membrane-bound Golgi enzyme tyrosylprotein sulfotransferase. Prior studies have partially characterized this enzyme, using the synthetic substrate EAY, in a Golgi membrane preparation from bovine adrenal medulla (12) and rat liver (13). Tyrosylprotein sulfotransferase activity has also been detected in a microsomal subfraction of rat cerebral cortex using the Boc derivative of CCK and other structurally related peptides as acceptor substrates (14, 15). A recent report from our laboratory has revealed several differences in the properties of these enzymes (13). For example, the pH optima, subcellular location, detergent activation, and metal ion cofactor requirements were shown to vary between the three enzymes. These differences in the biochemical properties of the membrane-bound sulfotransferases from different tissue sources could be reflective of the existence of more than one enzyme catalyzing the *in vivo* sulfation of tyrosyl residues. However, it is equally possible, because the apparent discrepancies were obtained using different acceptor substrates, that some of the variance could be related to the physical properties of the substrates themselves.

The possibility that Boc-CCK and EAY are sulfated by a single or multiple forms of tyrosylprotein sulfotransferase was

This work was supported by National Institutes of Health Grant NS 20530.
[1] Supported by a Pharmaceutical Manufacturers Association Foundation Predoctoral Fellowship.
[2] Supported by National Institutes of Health Grant GM 38280 and by the Washington University/Monsanto Biomedical Research Group.

ABBREVIATIONS: CCK, cholecystokinin-8; Boc, *tert*-butoxycarbonyl; C4, fourth component of complement; EAY, poly(Glu_6, Ala_3, Tyr_1); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-[*N*-morpholino]ethane sulfonic acid; PAPS, 5′-phosphoadenosine-3′-phosphosulfate; TFA, trifluoroacetic acid.

previously investigated by Vargas and Schwartz (16), using rat cerebral cortex as the enzyme source. Based on their results, these investigators suggested that only a single membrane-bound sulfotransferase exists in rat cerebellar cortex, which is capable of sulfating the tyrosine residues of both Boc-CCK and EAY, although they do not rule out the possibility of the existence of multiple forms of this enzyme. Because prior studies in our laboratory have reported distinct biochemical differences between the rat liver and cerebral cortex tyrosylprotein sulfotransferases (13), it was of interest to further characterize the rat liver enzyme and compare the properties of rat liver tyrosylprotein sulfotransferase, using Boc-CCK and EAY as the sulfate acceptors.

Experimental Procedures

Materials. EAY (average M_r 47,000) and other tyrosine-containing polymers, MES and HEPES buffers, Triton X-100, Lubrol PX, and unlabeled PAPS were purchased from Sigma Chemical Corp. (St. Louis, MO). Trichloroacetic acid, glycerol, and Na_2SO_4 were obtained from J. T. Baker (Phillipsburgh, NJ), bicinchoninic acid protein assay solution from Pierce (Rockford, IL), and ultrapure sucrose from Schwartz/Mann (Boston, MA). Porapak Q (100–200 mesh) and C18 Sep-Pak cartridges were purchased from Waters (Milford, MA). Boc-CCK was obtained from Bachem (Torrance, CA) and [^{35}S]3′,5′-PAPS (0.6–1.0 Ci/mmol) from New England Nuclear/Dupont (Wilmington, DE).

Tissue preparation. Livers from male Holtzman rats (150–300 g) were removed after decapitation and homogenized in 5 volumes of 0.5 M sucrose solution containing 10 mM HEPES buffer, pH 7.0, using a Potter-Elvehjem motorized homogenizer (clearance, 0.66 mm). The crude homogenate was then filtered over four layers of gauze. Twenty milliliters of the filtrate were placed over 18 ml of 1.25 M sucrose solution containing 10 mM HEPES, pH 7.0, and centrifuged at 82,700 × *g* for 60 min (SW-28 rotor). After centrifugation, four fractions were collected, the 0.5 M sucrose layer, the 0.5/1.25 M sucrose interface, the 1.25 M sucrose layer, and a pellet, which was subsequently resuspended in homogenization buffer. For the preparation of an enriched Golgi fraction, the 0.5/1.25 M sucrose interface was diluted 1:2 with 10 mM HEPES buffer, pH 7.0, containing 5 mM EDTA, and was recentrifuged at 111,000 × *g* for 60 min. The resulting enriched Golgi pellet was resuspended in 25% glycerol and stored at −80° until further use. To separate soluble and membrane-bound sulfotransferase activities in our rat liver preparations, the 0.5 M sucrose layer was centrifuged at 111,000 × *g* for 60 min, the supernatant was removed, and the pellet was resuspended in homogenization buffer.

Solubilization. For the solubilization of rat liver tyrosylprotein sulfotransferase, the EDTA-treated enriched Golgi pellet was resuspended in 10 mM HEPES buffer, pH 7.0, containing 1 M NaCl, 1 mM dithiothreitol, and 12.5% glycerol, and the resulting membrane preparation was recentrifuged at 134,038 × *g* for 60 min (50 Ti rotor). The pelleted material was then gently resuspended in a 10 mM HEPES buffer, pH 7.0, containing 0.2 M NaCl, 1% Triton X-100, 1 mM dithiothreitol, and 25% glycerol, and the resulting solution was recentrifuged at 134,038 × *g* for 60 min. As will be reported in Results, the supernatant solution from this latter spin contained the solubilized rat liver tyrosylprotein sulfotransferase.

Anion exchange chromatography. The solubilized tyrosylprotein sulfotransferase was subjected to anion exchange chromatography using a Pharmacia FPLC system. A Mono Q 5/5 anion exchange column was equilibrated in 10 mM HEPES buffer, pH 7.0, containing 1% Triton X-100, 10% glycerol, and 0.1 M NaCl. Solubilized rat liver tyrosylprotein sulfotransferase (6.5 mg of protein), in 5 ml of the equilibration buffer, was applied to the column at a flow rate of 0.5 ml/min using a Superloop 10 injector. The column was eluted with a 20-ml linear salt gradient of 0.1–0.4 M NaCl, in the buffered solution, described above, at a flow rate of 0.5 ml/min. Forty fractions of 0.5 ml each were collected and assayed for Boc-CCK- and EAY-sulfating activities.

Polymer sulfation assay. The standard assay for the measurement of the sulfation of the synthetic polymers, including EAY, was accomplished by a modification of the method of Rens-Domiano and Roth (13). The incubation mixture routinely consisted of 20 mM $MnCl_2$, 50 mM NaF, 1 mM 5′-AMP, 0.5% (w/v) Triton X-100, 2 μM [^{35}S]PAPS (~1100 dpm/pmol), 2 μM EAY, and enzyme, all in a final volume of 100 μl of 40 mM MES buffer, pH 6.5. The reaction was incubated at 30° for 30 min, after which a 75-μl aliquot of the reaction mixture was applied to a 2.0 × 2.5 cm 3 MM Whatman filter paper. The filter paper was then placed in a 10% trichloroacetic acid/10 mM Na_2SO_4 bath to precipitate the proteins onto the filter paper. After three 15-min washes, the filter paper, containing the precipitated proteins, was rinsed with 95% ethanol, dried, and placed in 15 ml of Omnifluor/toluene scintillation cocktail. The ^{35}S-radiolabeled product precipitated on the filter paper was measured using a Beckman LS-9800 liquid scintillation counter. Some assays, as indicated, were performed in the presence of Lubrol-PX in place of Triton X-100. No differences were observed between the properties of the enzyme when assayed in the presence of Triton X-100 or Lubrol-PX.

Boc-CCK sulfation assay. The assay mixture for the sulfation of Boc-CCK was the same as that for polymer sulfation, except that the acceptor substrate was 0.3 mM Boc-CCK (dissolved in 0.05 N NH_4OH). After incubating the reaction mixture for 30 min at 30°, the assay was stopped by the addition of 400 μl of cold 75 mM EDTA, pH 6.5, and the assay tubes were placed on ice. The radioactive sulfated product was separated from [^{35}S]PAPS using a modification of the method of Vogel and Altstein (17). Briefly, 450-μl aliquots of the assay mixtures were placed over 0.5 × 1 cm Porapak Q columns (preswollen in 95% ethanol for at least 1 h before use) packed in Pasteur pipettes. The columns were then sequentially eluted with two successive 4-ml washes with MES buffer, pH 6.5, to remove [^{35}S]PAPS, followed by a 6-ml 50% ethanol wash to remove [^{35}S]Boc-CCK-SO_4. The radiolabeled product was then measured by liquid scintillation counting.

Other synthetic peptides assay. Synthetic peptides (see Table 1) corresponding to the site of sulfation in the α-chain of C4 (18) were made with an Applied Biosystems 430A peptide synthesizer, using phenylacetamidomethyl resin, as previously described (19). Peptides were cleaved from the resin with anhydrous HF and purified by reverse phase high pressure liquid chromatography. Identities of the purified peptides were confirmed by compositional analysis. Kinetic constants were determined for these synthetic substrates using the reaction conditions described previously for the sulfation of EAY, except that the PAPS concentration was increased to 10 μM. After the reactions were incubated for 30 min at 30°, the assays were terminated by the addition of 400 μl of cold 75 mM EDTA, pH 6.5, and the reaction tubes were subsequently placed on ice. The radioactive sulfated product was separated from [^{35}S]PAPS by reverse phase chromatography using C18 Sep Pak cartridges. The Sep-Paks were prewashed with 4 ml of methanol/0.05% TFA and 8 ml of water/0.05% TFA before use. Aliquots (450 μl) of the reaction mixtures were placed over the Sep-Pak cartridges and the [^{35}S]PAPS and radiolabeled sulfated peptides were eluted from the cartridges with 20 ml of water/0.05% TFA and 10 ml of methanol/0.05% TFA, respectively. Sulfation of Boc-CCK was also measured by this assay procedure and gave results comparable to those obtained with the Porapak Q method described above.

TABLE 1

Synthetic peptides sulfated by rat liver tyrosylprotein sulfotransferase

Boc-CCK
Boc-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2
C-terminal segment of the α-chain of C4
Glu-Asp-Tyr-Glu-Tyr-Asp-Glu-Leu-Pro-Ala(COOH)
Glu-Ala-Asn-Glu-Asp-Tyr-Glu-Asp-Tyr-Glu-Tyr-Asp-Glu-Leu-Pro-Ala(COOH)

Protein measurement. Proteins were determined by the bicinchoninic acid protein assay method, using bovine serum albumin as the protein standard (20).

Results

Distribution of Boc-CCK and EAY sulfation by rat liver tyrosylprotein sulfotransferase. It has been reported previously that tyrosylprotein sulfotransferase in rat cerebral cortex was localized predominantly to a microsomal subfraction using either Boc-CCK or EAY as substrates (16), whereas the rat liver enzyme using only EAY as the sulfate acceptor was primarily localized to the Golgi apparatus (13). Accordingly, it was necessary to determine whether rat liver tyrosine sulfation of Boc-CCK also occurs in the same subcellular compartment as that of EAY. As shown in Table 2, the distributions of Boc-CCK and EAY sulfation over the sucrose gradient were markedly different. Whereas EAY sulfation was predominantly observed at the 0.5/1.25 M sucrose interface (65.5%), Boc-CCK-sulfating activity was distributed in both the 0.5 M sucrose layer (52.4%) and the 0.5/1.25 M sucrose interface (21.0%). Recentrifugation of these two fractions at 111,000 × *g* for 60 min demonstrated that the majority of the Boc-CCK-sulfating activity associated with the 0.5 M sucrose layer was in the soluble fraction, whereas the activity in the 0.5/1.25 M sucrose interface was predominantly associated with a membrane-bound cellular component.

Because prior studies have demonstrated that rat liver Golgi apparatus membranes primarily sediment at the 0.5/1.25 M sucrose interface of the sucrose gradient described above (13), the data in Table 2 suggest that sulfation of both Boc-CCK and EAY by a membrane-bound sulfotransferase most likely occurs within the Golgi apparatus. This fraction was treated with 2.5 mM EDTA and centrifuged at 111,000 × *g* for 60 min to obtain an enriched Golgi membrane fraction, which was used for subsequent experiments described in this paper. In different rat liver tissue preparations, the average recovery of tyrosylprotein sulfotransferase activity in the EDTA-treated Golgi pellet, as measured by EAY sulfation, was approximately 70%.

Sulfation of Boc-CCK and EAY by rat liver tyrosylprotein sulfotransferase. The effect of pH and ionic strength on Boc-CCK activity was measured in the EDTA-treated Golgi membrane preparation described above. As illustrated in Fig. 1, the pH optimum using Boc-CCK as the acceptor substrate was very broad and lies between 6.0 and 6.5. For comparison, the pH optimum for EAY sulfation by rat liver tyrosylprotein sulfotransferase was previously reported to be approximately 6.7 (13). The effect of ionic strength on both Boc-CCK and EAY activity is illustrated in Fig. 2. The data indicate that rat liver tyrosylprotein sulfotransferase activity was greatly influenced by ionic strength and that this effect on activity was substrate dependent. In this regard, Boc-CCK sulfation was stimulated by NaCl concentrations between 50 and 150 mM, whereas EAY sulfation was relatively unaffected at these NaCl concentrations. However, both substrates were similarly inhibited when the NaCl concentration was raised above 200 mM.

An earlier study from this laboratory demonstrated that EAY sulfation by rat liver tyrosylprotein sulfotransferase was stimulated maximally by 20 mM Mn^{2+} but not by Mg^{2+} (13). This finding was in conflict with prior studies that showed that rat cerebral cortex (14–16) and bovine adrenal medulla (12) tyrosylprotein sulfotransferases were stimulated by either Mn^{2+} or Mg^{2+}. In our previous studies, 50 mM NaF was used to prevent PAPS degradation and it is possible that MgF_2, an insoluble salt, could have been formed in our assay, thus interfering with our results. It, therefore, was necessary to determine whether high NaF concentrations, in the presence of Mg^{2+}, could affect the *in vitro* sulfation of EAY, as well as Boc-CCK. As indicated by the data in Table 3, in the presence of 50 mM NaF, Boc-CCK sulfation was indeed stimulated by Mn^{2+} as well as Mg^{2+}. However, consistent with previous studies, EAY sulfoconjugation was not stimulated by Mg^{2+} under these conditions. In contrast, when 10 mM NaF was used in the assay, Mg^{2+} was able to stimulate sulfation of both EAY and Boc-CCK, al-

TABLE 2

Distribution of Boc-CCK and EAY sulfation over a sucrose gradient

Rat liver tyrosylprotein sulfotransferase activity was measured for 30 min at 30° in the presence of 2 μM [^{35}S]PAPS and either 0.3 mM Boc-CCK or 2 μM EAY. The data shown are mean values obtained from a single experiment performed in duplicate. ND, not detected.

Fraction	Total protein	Boc-CCK		EAY	
		Specific activity	Total activity	Specific activity	Total activity
	mg	*pmol/min/mg of protein*	*pmol/min*	*pmol/min/mg of protein*	*pmol/min*
Homogenate	1138	1.07	1217	0.28	318
0.5 M Sucrose layer	419	1.52	638	0.08	33.6
Pellet	35.3	ND	ND	0.09	3.2
Soluble	377	1.56	558	0.06	22.6
0.5/1.25 M Sucrose layer	111	2.29	255	1.83	204
Pellet	57.6	5.65	325.4	2.81	161
Soluble	52.3	ND	ND	0.14	7.3
1.25 M Sucrose layer	80.8	0.44	35.6	0.15	12.1
Gradient pellet	395	0.02	7.9	0.09	35.6

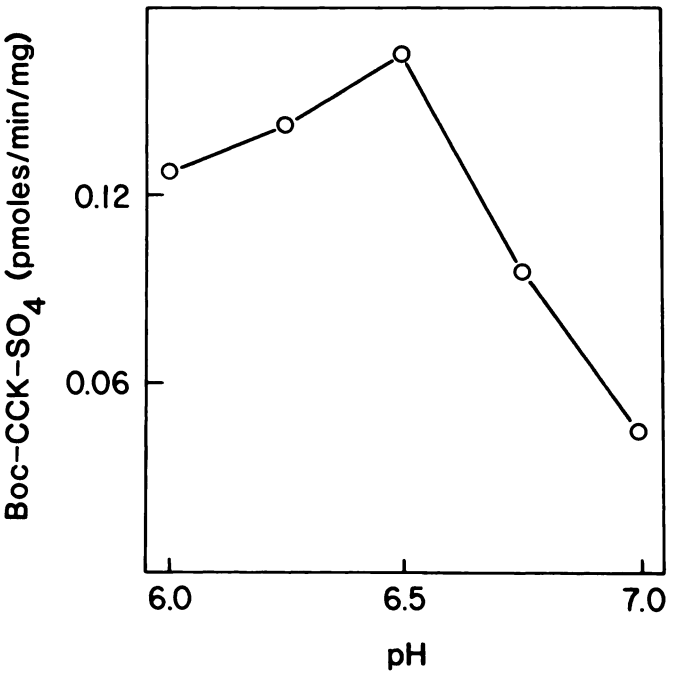

Fig. 1. Effect of pH on the sulfation of Boc-CCK by rat liver tyrosylprotein sulfotransferase. Tyrosylprotein sulfotransferase activity was measured for 30 min at 30°, using a rat liver enriched Golgi preparation (46 μg of protein) in the presence of 2 μM [^{35}S]PAPS, 0.3 mM Boc-CCK, 20 mM $MnCl_2$, 50 mM NaF, 1 mM 5′-AMP, and 0.5% Lubrol-PX, in a total volume of 100 μl of MES buffer at the pH values indicated.

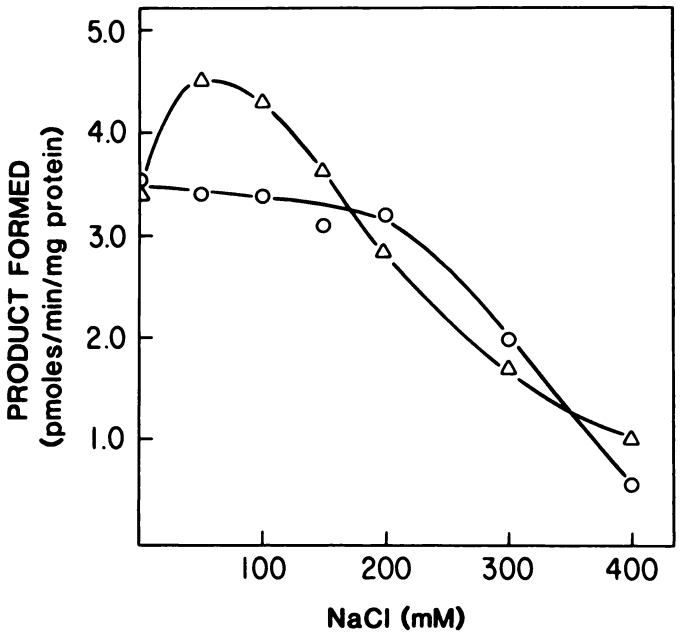

Fig. 2. Effect of ionic strength on the sulfation of Boc-CCK and EAY by rat liver tyrosylprotein sulfotransferase. Tyrosylprotein sulfotransferase activity was measured for 30 min at 30°, using a rat liver enriched Golgi preparation (30 μg of protein), in a total volume of 100 μl of MES buffer, pH 6.5, containing 2 μM [^{35}S]PAPS, either 0.3 mM Boc-CCK (△) or 2 μM EAY (○), 20 mM $MnCl_2$, 50 mM NaF, 1 mM 5′-AMP, 0.5% Triton X-100, and varying concentrations of NaCl, as indicated.

TABLE 3

Effect of Mn^{2+} and Mg^{2+} on rat liver tyrosylprotein sulfotransferase activity

Tyrosylprotein sulfotransferase activity was measured for 30 min at 30° using 2 μM EAY or 0.3 mM Boc-CCK as the sulfate acceptor, 2 μM [^{35}S]PAPS, and an EDTA-treated enriched Golgi preparation (35 μg of protein) in the presence of 20 mM $MnCl_2$ or 20 mM $MgCl_2$, at either 10 or 50 mM NaF. Each value represents the mean of two experiments, each performed in triplicate.

Metal Cofactor	NaF	Boc-CCK	EAY
	mM	*pmol/min/mg*	*pmol/min/mg*
Mn^{2+}	10	2.86	2.48
	50	4.87	3.28
Mg^{2+}	10	3.70	2.80
	50	4.48	0.16

though, in general, the activities were lower than when 50 mM NaF was used. The lower activity observed in the presence of 10 mM NaF was not caused by an increase in PAPS degradation, because both 10 and 50 mM NaF were found to be equally effective in preventing PAPS metabolism during incubations with a rat liver enriched Golgi fraction.[3]

Effect of temperature on rat liver tyrosylprotein sulfotransferase. Differences in the properties of the sulfation of EAY and Boc-CCK by rat liver tyrosylprotein sulfotransferase, reported above, possibly suggest the presence of more than one form of this enzyme. To determine whether EAY and Boc-CCK were sulfated by the same or different forms of membrane-bound sulfotransferases, the effect of preincubation of the Golgi preparation at 45° for various lengths of time was examined. Results of this experiment revealed that both EAY and Boc-CCK sulfation decreased identically, displaying first-order kinetics (correlation coefficient = 0.997 and 0.991, respectively). The first-order rate constant for loss of sulfotransferase activity at 45° was approximately 0.11 min^{-1}.

Substrate specificity of rat liver tyrosylprotein sulfotransferase. Several investigators have suggested that the sulfoconjugation of tyrosyl residues occurs within strongly acidic amino acid domains of the protein (12, 14, 15, 19). Accordingly, the synthetic acidic polymer EAY was used previously as a model substrate for characterization of rat liver tyrosylprotein sulfotransferase (13). Because little is known about the substrate specificity of this enzyme, we examined a variety of other commercially available tyrosine-containing random polymers to determine whether they also can serve as substrates for rat liver tyrosylprotein sulfotransferase. The data shown in Table 4 demonstrate that all of the polymers examined were able to undergo tyrosine sulfation. At equimolar tyrosine concentrations (0.06 mM Tyr), poly(Glu_6, Lys_3, Tyr_1) had a 2-fold higher specific activity than the model acidic substrate EAY. The activities of the other polymers ranged from 20 to 80% of the activity of EAY. It is important to note that the polymers containing the basic amino acid residues lysine and ornithine were also capable of being sulfated by rat liver tyrosylprotein sulfotransferase.

To further characterize the structural requirements for tyrosine sulfation by rat liver tyrosylprotein sulfotransferase, three synthetic peptide substrates, Boc-CCK and two peptides corresponding to the carboxy-terminal end of C4 (see Table 1 for structures), were examined. The K_{mapp} and $V_{max_{app}}$ values reported in Table 5 were determined from Lineweaver-Burke plots. Boc-CCK had a K_{mapp} of 298 μM and a $V_{max_{app}}$ of 29.0 pmol/min/mg of protein. The two synthetic peptide segments of 10 and 16 amino acid residues, corresponding to the tyrosine-sulfated region of the α-chain of C4, displayed a considerably higher affinity for the sulfotransferase than Boc-CCK, with K_{mapp} values of 17.4 and 0.410 μM, respectively. The $V_{max_{app}}$ values for the sulfation of these two synthetic C4 peptides were 69.7 and 25.6 pmol/min/mg of protein for the 10- and 16-amino acid peptide fragments, respectively. Unlike that previously reported for EAY and Boc-CCK, substrate inhibition was not observed with these synthetic peptides at the concentrations tested.

Solubilization and ion exchange chromatography of rat liver tyrosylprotein sulfotransferase. Lee and Huttner

TABLE 4

Sulfation of polyamino acids by rat liver tyrosylprotein sulfotransferase

Polyamino acid sulfation by rat liver tyrosylprotein sulfotransferase was measured for 30 min at 30°, using an enriched Golgi fraction (58 μg of protein), in the presence of 2 μM [^{35}S]PAPS and the appropriate polymer at an equimolar tyrosine concentration (0.056 mM Tyr). Each value represents the mean ± standard error of three to five experiments, each performed in duplicate. Relative specific activity is based on the sulfation of EAY (Glu,Ala,Tyr; 6:3:1), which was 3.35 ± 0.30 pmol/min/mg of protein.

Polyamino acid	Ratio	Relative specific activity
Glu,Ala,Tyr	6:3:1	1.00
Glu,Lys,Tyr	6:3:1	2.02 ± 0.22
Glu,Ala,Tyr	1:1:1	0.53 ± 0.05
Glu,Tyr	4:1	0.61 ± 0.08
Glu,Tyr	1:1	0.80 ± 0.07
Lys,Tyr	4:1	0.43 ± 0.04
Lys,Tyr	1:1	0.18 ± 0.04
Orn,Tyr	4:1	0.45 ± 0.08
Lys,Ala,Glu,Tyr	5:6:2:1	0.44 ± 0.07

[3] Unpublished observations.

TABLE 5

Sulfation of synthetic peptides by rat liver tyrosylprotein sulfotransferase

Sulfation of the synthetic peptides by a rat liver enriched Golgi fraction (30 μg of protein) was measured in the presence of 10 μM [^{35}S]PAPS for 30 min at 30°. Kinetic constants were calculated from Lineweaver-Burke plots and the values represent the mean of two separate experiments.

Peptide	$K_{m_{app}}$	$V_{max_{app}}$
	μM	pmol/min/mg
Boc-CCK	298	29.0
C4 peptide		
10 amino acids	17.4	69.7
16 amino acids	0.410	25.6

TABLE 6

Solubilization of rat liver tyrosylprotein sulfotransferase activity

Rat liver tyrosylprotein sulfotransferase activity was measured for 30 min at 30° in the presence of 2 μM [^{35}S]PAPS and either 0.3 mM Boc-CCK or 2 μM EAY. The data shown are mean values obtained from a single experiment performed in duplicate and are representative of similar experiments.

Treatment	Total protein	Boc-CCK		EAY		Ratio CCK/EAY
		Specific activity	Total activity	Specific activity	Total activity	
	mg	pmol/min/mg	pmol/min	pmol/min/mg	pmol/min	
2.5 mM EDTA pellet	57.6	5.65	325	2.81	161	2.0
1 M NaCl						
Pellet	42.8	7.62	326	4.10	175	1.9
Soluble	8.7	0.34	3.0	0.22	1.9	1.6
1% Triton X-100 + 0.2 M NaCl						
Pellet	2.1	8.18	17.2	4.30	9.0	1.9
Soluble	38.3	8.54	327	4.40	168	1.9

(12) have previously demonstrated that bovine adrenal medulla tyrosylprotein sulfotransferase is a tightly bound integral membrane protein. Consistent with this observation, rat liver tyrosylprotein sulfotransferase was not able to be extracted from the Golgi membrane by treatment with either 2.5 mM EDTA (Table 2) or 1 M NaCl (Table 6). As demonstrated in Table 6, tyrosylprotein sulfotransferase can, however, be effectively solubilized with essentially no loss of Boc-CCK- or EAY-sulfating activity when treated with 1% Triton X-100 in the presence of 0.2 M NaCl. During the solubilization procedure, the ratio of total Boc-CCK-sulfating activity to EAY-sulfating activity remained constant. Although not shown, neither 1% Triton X-100, 1% Lubrol-PX, nor 1% Lubrol-PX in the presence of 0.2 M NaCl was as effective as 1% Triton X-100 plus 0.2 M NaCl in solubilizing rat liver tyrosylprotein sulfotransferase when either Boc-CCK or EAY was used as substrate.

The properties of the solubilized rat liver tyrosylprotein sulfotransferase were further characterized by ion exchange chromatography. The elution profile of Boc-CCK and EAY sulfation from an anion exchange column (Mono Q) was examined. The data in Fig. 3 illustrate that rat liver tyrosylprotein sulfotransferase activity bound to the anion exchange column and that both Boc-CCK- and EAY-sulfating activities eluted from the column as a single peak between 140 and 280 mM NaCl. Approximately 50% of the total protein applied to the column was retained, and of this bound protein about 74% eluted as a broad peak during the same portion of the NaCl gradient as Boc-CCK- and EAY-sulfating activities.

Discussion

As previously reported (13), there are a number of differences between the properties of rat liver tyrosylprotein sulfotransferase and those of similar membrane-bound sulfotransferases characterized from bovine adrenal medulla (12) and rat cerebral cortex (14, 15). These differences suggest the possibility that there is more than one enzyme that catalyzes the *in vivo* sulfation of tyrosine residues. Accordingly, the present study was undertaken to further characterize rat liver tyrosylprotein sulfotransferase and to provide evidence for the existence of one or more enzymes that catalyze the sulfation of tyrosyl residues *in vivo*.

Tyrosylprotein sulfotransferase has previously been localized to the Golgi apparatus using the model acidic substrate EAY in bovine adrenal medulla (12) and rat liver (13), whereas Boc-CCK- and EAY-sulfating activities were shown to be present in the microsomal fraction of rat cerebral cortex (16). In neither bovine adrenal medulla nor rat cerebral cortex was there any discernible sulfation of either Boc-CCK or EAY by the soluble cell fraction, although it has been suggested that a soluble sulfotransferase may be loosely associated with the microsomal membrane of rat cerebral cortex (16). The data presented herein suggest that, in rat liver, both EAY and Boc-CCK are sulfated within the Golgi apparatus and that Boc-CCK is also sulfated by a soluble sulfotransferase. In rat liver, the soluble enzyme, arylsulfotransferase IV, has previously been reported to be capable of sulfating a number of small peptides containing an *N*-terminal tyrosyl residue, such as CCK-7 and enkephalin (21). Our data reveal that rat liver also contains a soluble sulfotransferase that is capable of sulfating Boc-CCK, a peptide that contains an endotyrosyl group. Whether this is the same enzyme as aryl sulfotransferase IV has not been determined. Interestingly, human brain has been reported to contain a soluble sulfotransferase that is capable of sulfating both endo- and exotyrosyl residues of a number of small peptides (22).

The tyrosylprotein sulfotransferases that have been identified and partially characterized from bovine adrenal medulla, rat cerebral cortex, and rat liver appear to display differences in their biochemical properties, such as pH optimum and response to varying ionic strengths. Some of these observed differences may be attributed to structural differences in the substrates used to characterize these enzymes or to differences in the lipid environment of the enzymes. The substrates, EAY and Boc-CCK, possess very different physical properties, such as ionic charge, hydrophobicity, size, and secondary structure. Accordingly, a change in assay conditions, such as detergent to lipid ratios, pH, or ionic strength, could affect the physical state of the substrate or the Golgi membranes and, thus, lead to the apparent differences in the enzymatic properties measured.

In general, the data reported in this paper are consistent with the hypothesis that in rat liver a single enzyme species is responsible for the sulfation of both EAY and Boc-CCK. For example, loss of tyrosylprotein sulfotransferase activity during temperature inactivation with either substrate was essentially identical and demonstrated first-order kinetics. In addition, membrane-bound sulfotransferase activities, using both EAY and Boc-CCK as substrates, were solubilized to the same extent by the procedures described in Results and these solubilized sulfotransferase activities eluted from an anion exchange column with overlapping peaks of activity. Although it is possible that the sulfotransferase activities eluting from the column were contained within mixed micelles, the similarities of the net ionic charge and elution profile properties of Boc-CCK-

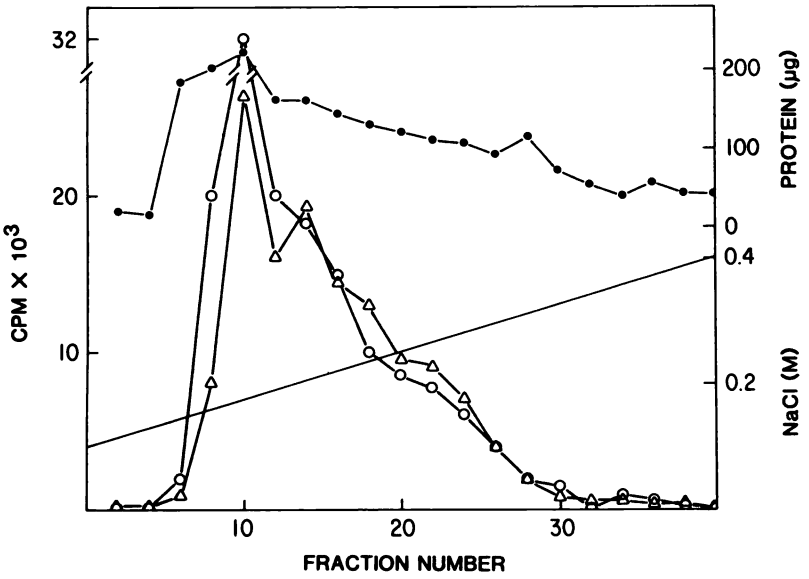

Fig. 3. Anion exchange chromatography of solubilized rat liver tyrosylprotein sulfotransferase. Solubilized rat liver tyrosylprotein sulfotransferase was eluted from a Mono Q 5/5 column, using a 0.1–0.4 M NaCl gradient in HEPES buffer, pH 7.0, containing 1% Triton X-100 and 10% glycerol. Tyrosylprotein sulfotransferase activity was measured for 30 min at 30°, using a solubilized rat liver enriched Golgi preparation, using 2 μM [^{35}S]PAPS, either 0.3 mM Boc-CCK or 2 μM EAY, 20 mM $MnCl_2$, 50 mM NaF, 1 mM 5′-AMP, and 1.0% Triton X-100, in a total volume of MES buffer, pH 6.5. △, Boc-CCK sulfation; ○, EAY sulfation; ●, total protein; ——, salt concentration.

and EAY-sulfating activities from the anion exchange column tend to suggest that only a single sulfotransferase is eluted from the anion exchange column.

Tyrosine sulfation appears to be a highly specific and selective posttranslational modification. Hortin *et al.* (19) have proposed that the major determinant directing sulfation appears to be an abundance of acidic amino acids in close proximity to the tyrosyl residue that is sulfated. Other structural features facilitating tyrosine sulfation include the lack of basic amino acids near the site of sulfoconjugation. The data reported herein demonstrating that the basic random polymers such as Glu_6, Lys_3, Tyr_1, Lys_4, Tyr_1, and Orn_4, Tyr_1 are substrates for this enzyme appear to be inconsistent with the criteria proposed by Hortin *et al.* (19). It is possible that the broad substrate specificity observed *in vitro* is an artifact of either the enzyme preparation procedure or the assay method and that tyrosylprotein sulfotransferase actually possesses a more rigid structural specificity *in vivo* when present in its natural environment within the Golgi apparatus.

To further characterize the binding specificities of rat liver tyrosylprotein sulfotransferase, the K_m and V_{max} values for three peptides corresponding to the naturally occurring sites of sulfation of CCK and C4 were measured. Considerable differences were observed in all cases, with the most striking difference being a 100-fold lower apparent K_m value for the 16-amino acid peptide fragment of the C-terminus of the α-chain of C4, as compared with the corresponding 10-amino acid peptide fragment. The reasons for this change in specificity are not known, although differences in peptide size, secondary structure, number and position of sulfatable tyrosine residues, and number of acidic amino acid residues may contribute to the increase in the binding affinity of the larger C4 peptide fragment.

In this paper, we describe several new assay methods by which tyrosylprotein sulfotransferase can be measured *in vitro* and that can be employed for a number of structurally different tyrosine-containing peptides. In addition, new information is presented regarding the biochemical properties and substrate specificity of rat liver tyrosylprotein sulfotransferase. The data presented are also consistent with a single sulfotransferase species within the rat liver Golgi apparatus, which is responsible for catalyzing the transfer of a sulfate moiety from PAPS to a variety of biologically active peptides and proteins.

Acknowledgments

The authors wish to thank Ms. Maureen Adolf for her expert technical assistance.

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