Biochemical and Biophysical Research Communications **262,** 193–197 (1999)
Article ID bbrc.1999.1173, available online at http://www.idealibrary.com on IDEAL®

Sulfated Tyrosines of Thyroglobulin Are Involved in Thyroid Hormone Synthesis

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Received July 14, 1999

Thyroid hormone synthesis is under the control of thyrotropin (TSH), which also regulates the sulfation of tyrosines in thyroglobulin (Tg). We hypothesized that sulfated tyrosine (Tyr[S]) might be involved in the hormonogenic process, since the consensus sequence required for tyrosine sulfation to occur was observed at the hormonogenic sites. Porcine thyrocytes, cultured with TSH but without iodide in the presence of [^{35}S]sulfate, secreted Tg which was subjected to *in vitro* hormonosynthesis with increasing concentrations of iodide. A 63% consumption of Tyr[S] (1 residue) was observed at 40 atoms of iodine incorporated into Tg, corresponding to a 40% hormonosynthesis efficiency. In addition, hyposulfated Tg secreted by cells incubated with sodium chlorate was subjected to *in vitro* hormonosynthesis. With 0.5 Tyr[S] residue (31% of the initial content), the efficiency of the hormonosynthesis was 29%. In comparison, when hormonosynthesis was performed by cells, with only 0.25 Tyr[S] residue (16% of the initial content), the hormonosynthesis efficiency fell to 18%. These results show that there exists a close correlation between the sulfated tyrosine content of Tg and the production of thyroid hormones. © 1999 Academic Press

Key Words: **sulfated tyrosine; thyroid hormonosynthesis; sulfation inhibition; chlorate.**

The biosynthesis of thyroid hormones (thyroxine (T_4) and triiodothyronine (T_3)) occurring in thyroglobulin (Tg) involves two steps: the iodination of a limited number of tyrosine residues, followed by the coupling of two iodotyrosines (one acceptor and one donor). The acceptor, diiodotyrosine residue (DIT), will remain attached to the peptide chain, while the donor, monoiodotyrosine residue (MIT) or some other DIT, will be released from the peptide chain. These reactions are catalyzed by thyroid peroxydase (TPO), *via* mechanisms which have not yet been completely elucidated (for a review see (1)). Thyrotropin (TSH) has been previously reported to control the process of hormonosynthesis by modulating several partners (2), and to alter the utilization of Tg hormonogenic sites (3). We recently observed that TSH also regulates the sulfation of tyrosyl residues in Tg (4). The consensus sequence (Asp/Glu-Tyr) has been previously described in the case of tyrosyl residues involved in hormonosynthesis (5–8). This sequence has also been found to be a prerequisite for tyrosine sulfation to occur (9, 10). Based on these findings, we postulated that some of the sulfated tyrosines present in Tg might be involved in the hormonosynthesis reaction. We first examined the changes in the Tyr[S] content of Tg which occur with increasing levels of *in vitro* Tg-iodination and coupling. Secondly, the sulfation process was inhibited by sodium chlorate, which is a potent inhibitor of protein sulfation (11–15), with a view to investigating the effects of changes in the Tg-sulfation rates on the hormonosynthesis. This study was carried out on Tg secreted by porcine thyrocyte monolayers cultured on porous filters and on Tg secreted into the colloid of follicles. We demonstrated that there exists a correlation between Tyr[S] utilization and hormonosynthesis; this finding was confirmed by the fact that decreasing the Tyr[S] content of Tg resulted in a decrease in the thyroid hormonosynthesis efficiency.

MATERIALS AND METHODS

Cell culture. Porcine thyrocytes were isolated from thyroid glands and cultured on collagen-coated filters as previously described (16), in the presence of TSH with or without iodide (KI) added daily to the basal medium (17). On day 15, the apical medium and basal medium contained 0.4 mM sulfate, and the iodide content, when present, was 10 μM in the apical medium and 0.6 μM in the basal medium (4). Various concentrations of sodium chlorate, a protein-sulfation inhibitor (13), were added to the basal media to give final concentrations of 1 mM, 3 mM, and 5 mM with or without the radiolabeled precursor [^{35}S]sulfate (120 μCi/ml) (Amersham Corp., England, 1000 Ci/mmol). The cell culture was maintained for 24 hours and apical media were then collected. Tg was purified on a Bio-Gel A-5m (Bio-Rad, Ivry-sur-Seine, France) (4). For the sake of comparison, cells were cultured as follicles in the presence of TSH

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Abbreviations: Tg, thyroglobulin; TPO, thyroperoxidase; LPO, lactoperoxidase; TSH, thyrotropin; Tyr[S], sulfated tyrosine.

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(18) and incubated for 24 hours with or without various concentrations of sodium chlorate as above, in the presence of either [^{35}S]sulfate or Na^{125}I (0.5 μCi/ml, 0.5 μM NEN CIS Biointernational, Gif sur Yvette, France). To release the colloid content, the follicles were incubated for 10 min at 37°C with Splittix 0.14 M (Biomedia, Boussens, France). The colloid was recovered after gentle centrifugation. Tg was then purified as described above.

In vitro hormonosynthesis. Tg, secreted by control or chlorate-treated cells cultured on filters in the absence of iodide was subjected to *in vitro* hormonosynthesis by incubating it with unlabelled KI, in a standard incubation medium (lactoperoxidase + glucose-glucose oxidase) to which Na^{125}I was added or not, as previously described (17).

Gel electrophoresis. Aliquots of apical media or colloids were analyzed by 5% SDS-PAGE. Gels were stained with Coomassie blue and dried, and Tg was quantitatively determined (4); after exposing the gels to an imaging plate and analysing the [^{35}S]sulfate labelled compounds with the TINA 2.09 image software program (Raytest Isotopenmeßgeräte GmbH, Raytest SARL Courbevoie, France), the specific radioactivity (dpm/pmole) was calculated (4).

Enzymatic digestions. In vitro ^{125}I-labelled Tg or ^{125}I-Tg secreted by control or chlorate-treated cells, was dissolved in a 0.05 M phosphate buffer pH 7.4, and the samples were digested for 48 h at 37°C by 20% (w/w) pronase (Boehringer Mannheim, Germany) before being subjected for 48 h at 37°C to digestion by 20% (w/w) leucine aminopeptidase, LAP (Cytosol Type V from Porcine kidney, Sigma). The iodoamino acids were separated by HPLC (19) and analyzed by performing thin-layer chromatography (TLC) (4). The efficiency of hormonosynthesis, which reflected the coupling of the iodotyrosines, was assessed by determining the ^{125}I-DIT content of the iodothyronines (T_3 + T_4) versus the total ^{125}I-DIT (DIT + T_3 + T_4) content; while the rate of hormonosynthesis was determined from the ratio of the hormonosynthesis efficiency obtained with hyposulfated Tg to that of the control Tg.

Determination of sulfated-tyrosine levels. To quantify the sulfated tyrosines and other Tg sulfated compounds, [^{35}S]sulfate labelled Tg purified from control and chlorate-treated cells was hydrolyzed with 0.2 M NaOH for 24 h at 110°C and analyzed by performing thin-layer chromatography (TLC) (4). The proportion of Tyr[S] was expressed as a percentage of the specific radioactivity of Tg, and the number of residues was calculated as previously described (4).

RESULTS

Decrease in the Tyr[S] Levels, Depending on the Number of Iodine Atoms Bound to Tg

To investigate the relationship between tyrosine sulfation of Tg and hormonosynthesis, [^{35}S]sulfate-Tg secreted by cells cultured without iodide was incubated with increasing concentrations of iodide with a view to performing *in vitro* hormonosynthesis. The [^{35}S]sulfate-Tyr[S] content corresponding to various levels of Tg iodination was then determined after alkaline hydrolysis. A decrease in the amount of [^{35}S]sulfate-Tyr[S] was already observed at 3 atoms of iodide bound to Tg (Fig. 1); the decrease reached 63% and corresponded to a consumption of 1 residue of Tyr[S] at 40 atoms of iodine bound. The decrease tended towards a plateau at 70%, which suggested that about 30% Tyr[S] was always left after maximum hormonosynthesis. A possible explanation for these data is that Tyr[S] may have been consumed by the iodination-coupling reaction.

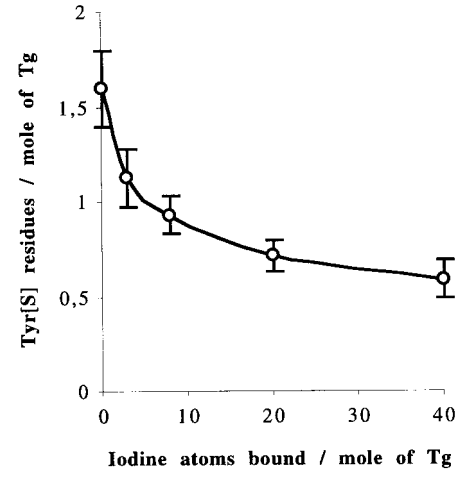

FIG. 1. Decrease in the Tyr[S] levels, depending on the number of iodine atoms bound to Tg. Standard *in vitro* hormonosynthesis was performed with increasing concentrations of KI, on purified [^{35}S]sulfate-Tg secreted by porcine thyrocytes cultured on filters without any iodide. The number of Tyr[S] residues/mole of Tg was determined after alkaline hydrolysis and TLC analysis. Values are means ± S.D. (n = 4), based on 2 experiments.

Preparation and Characterization of Hyposulfated Tg

We prepared hyposulfated Tg by incubating thyrocytes with increasing concentrations of sodium chlorate. The rate of [^{35}S]sulfate incorporation into Tg decreased with increasing concentrations of sodium chlorate, whereas the amount of Tg secreted remained unchanged (SDS-PAGE not shown). Specific radioactivities were calculated (dpm/pmole of Tg) from the SDS-PAGE data. [^{35}S]sulfate incorporation into Tg decreased by 80% and 97% at a chlorate concentration of 3 mM in the monolayer and follicle culture medium, respectively (Fig. 2, Table I). As this concentration was sufficiently high for significant inhibition to occur, it was chosen for the subsequent investigations.

To determine the proportion of sulfate residues remaining on the tyrosines, Tg were subjected to an alkaline hydrolysis. In Tg secreted by monolayers, the average rate of residual sulfated tyrosines was 36% versus 15% in Tg secreted by follicles; and very low levels of sulfated oligosaccharides were recovered (Table I).

Effects of the Decrease in the Sulfated Tyrosine Content on the Efficiency of Hormonosynthesis

To determine whether tyrosine sulfation is essential to hormonosynthesis, we studied this process in Tg in which the rate of sulfation had greatly decreased. For this purpose, the Tg secreted by control or chlorate-treated cell monolayers cultured without any iodide was subjected to *in vitro* hormonosynthesis. In addition, cultured follicular thyrocytes were incubated so that hormonosynthesis could occur. All the Tg were

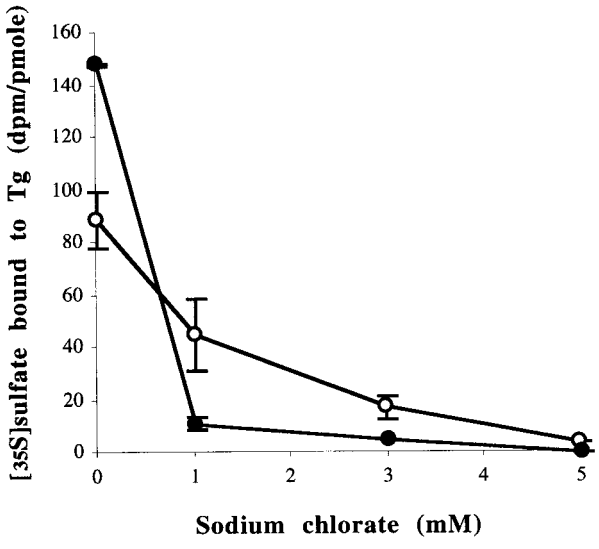

FIG. 2. [^{35}S]sulfate incorporated into Tg at increasing concentrations of sodium chlorate. The specific radioactivity was calculated from unshown results of SDS-PAGE (dpm measured per pmole of Tg. Monolayers (-○-) Follicles (-●-). Values are means ± S.D.: Monolayers (n = 12), based on 4 experiments; Follicles (n = 6), based on 2 experiments.

digested by enzymatic hydrolysis and the ^{125}I-iodoamino acids were quantified. Hormones were produced with both control and hyposulfated Tg, but the rates of hormonosynthesis decreased following Tyr[S] depletion (Fig. 3a). The decrease of hormonosynthesis performed *in vitro* was in range with the decrease of hormonosynthesis performed by cells. Subsequently, the hormonosynthesis efficiency was correlated to the Tyr[S] content remaining after sulfate depletion (Fig. 3b). Indeed, it was observed that the hormonosynthesis efficiency reached a 29% value when ~0.5 Tyr[S] residue was consumed (Fig. 3b) and this corresponded to 4 atoms of iodine bound/mole of Tg (Fig. 1), while we obtained a 40% hormonosynthesis efficiency when ~1 Tyr[S] residue was consumed (Fig. 3b) which corresponded to 40 atoms of iodine bound to Tg (Fig. 1). The relationship that appeared between Tyr[S] content and hormonosynthesis efficiency did not obviously depend on the number of iodine atoms bound to Tg.

DISCUSSION

The aim of the present study was to investigate the role of sulfated tyrosine in thyroid hormone synthesis. The results obtained here by performing *in vitro* hormonosynthesis on Tg with increasing concentrations of incorporated iodine show the existence of a clear-cut correlation between the consumption of sulfated tyrosine and the iodination-coupling reaction. In the case of a highly iodinated-Tg, approximately 70% of the total Tyr[S] was consumed. This finding is consistent with the results of previous studies on a fully-iodinated Tg obtained from calf gland (20), in which Tyr[S] was thought to be present at levels of less than 1 residue/mole of Tg; it is worth noting that this value is in line with that of 0.6 residue of Tyr[S]/mole of Tg remaining in the present study after high levels of *in vitro* hormonosynthesis. Upon incubating porcine cell monolayers and follicles with sodium chlorate, it was observed that chlorate treatment up to 3 mM did not affect either the cells' integrity or the Tg secretion process, as described in the case of other proteins (11). Here the rate of sulfate incorporation into Tg greatly decreased, resulting in a decrease in both the Tyr[S] levels, and the oligosaccharide sulfation, as previously described in the case of other proteins (14, 15). The decrease in tyrosine sulfation was more pronounced in follicles than in monolayers, but parallel decreases in the rate of hormonosynthesis were obtained when hormonosynthesis was performed with hyposulfated Tg, either *in vitro* or by follicles which closely matches the *in vivo* conditions. One hypothesis which comes to mind in the light of the results (Table I), is that hyposulfation of oligosaccharides might alter the conformation of Tg molecules, and a change in the three-dimensional structure might therefore interfere with the hormonosynthesis process. This hypothesis can be ruled out,

TABLE I

Distribution of Sulfated Compounds: Tyr[S] and Sulfated Oligosaccharides in Hyposulfated Tg Compared to Control Tg

Cell culture	dpm/pmole of Tg	dpm bound to (Tyr[S])	dpm bound to (oligosacch[S])	content (%) Tyr[S]	content (%) oligosacch[S]
M	88.7 ± 9.2	7.3 ± 0.4	81.5 ± 9.4	100	100
M + ClO_3^-	17.3 ± 3.9	2.8 ± 0.6	14.7 ± 4.1	**35.9 ± 4.4**	18.4 ± 1.4
F	147.9 ± 29.0	8.5 ± 4.4	139.5 ± 5.1	100	100
F + ClO_3^-	4.8 ± 1.5	0.9 ± 0.2	3.9 ± 0.2	**14.5 ± 0.9**	2.8 ± 0.2

Note. [^{35}S]sulfate labelled compounds of Tg: Tyr[S] and oligosaccharides[S] were evaluated after alkaline hydrolysis and TLC analysis. The proportions obtained with each compound were referred to the specific radioactivity of Tg and expressed as dpm bound to Tyr[S] or dpm bound to oligosacch[S]. The percentage of the residual sulfate content was deduced from these values for both compounds. Control (M) and Chlorate-treated monolayers (M+ ClO_3^-). Control (F) and Chlorate-treated follicles (F + ClO_3^-). Values are means ± S.D.: Monolayers (n = 12), based on 4 experiments; Follicles (n = 6), based on 2 experiments.

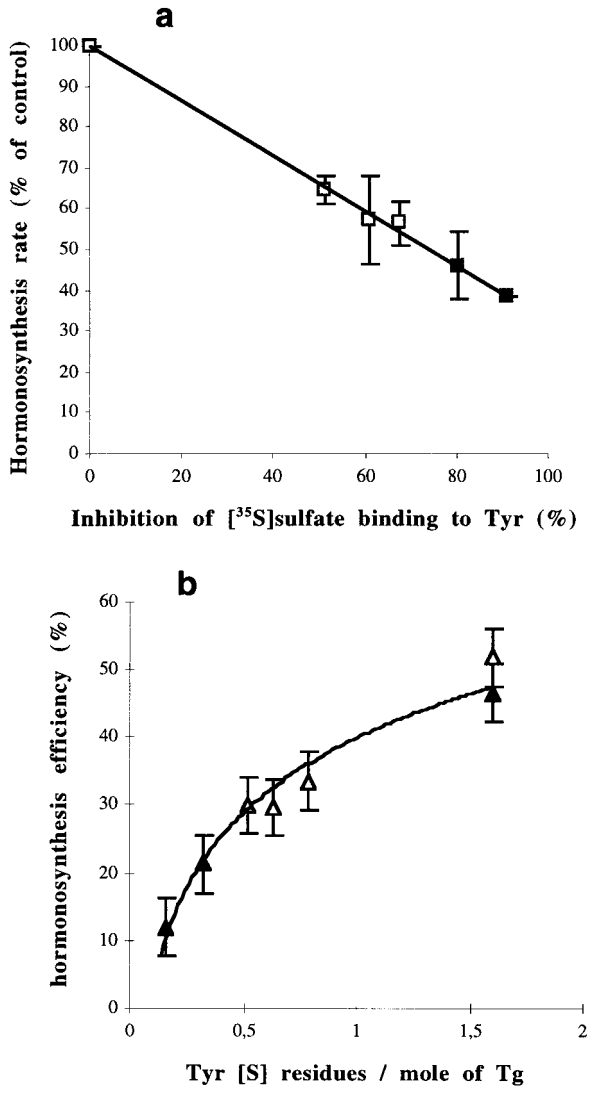

FIG. 3. Hormonosynthesis efficiency depending on the Tyr[S] content of Tg. Standard *in vitro* hormonosynthesis was performed with $Na^{125}I$ on non-iodinated Tg purified from apical media of cell monolayers. Comparatively ^{125}I-Tg was purified from colloids of control or chlorate-treated follicles cultured with $Na^{125}I$. These ^{125}I-Tg were subjected to enzymatic hydrolysis. ^{125}I-iodoamino acids: MIT, DIT, T_3 and T_4 were separated by HPLC and analyzed by TLC. (a) Hormonosynthesis rates determined as described in Materials and Methods was plotted against the inhibition of tyrosine sulfation (-□-) *in vitro*, (-■-) by cells. (b) The efficiency of *in vitro* and cellular hormonosynthesis determined as described in Materials and Methods and was plotted against Tyr[S] content of Tg. (-△-) *in vitro* (-▲-) by cells. Values are means ± S.D.: *in vitro* (n = 8), based on 4 experiments; by cells (n = 4), based on 2 experiments.

however, in view of the consumption of sulfated tyrosine which occurred during *in vitro* hormonosynthesis using Tg in which no alteration in oligosaccharide sulfation had taken place. On the other hand, the efficiency of the hormonosynthesis was clearly found to depend on the Tyr[S] content. Since the consensus sequence (Asp/Glu-Tyr) is the same for both thyroid hormone synthesis and the sulfation of tyrosyl residues (5, 10), it can be proposed that sulfate residues bound to tyrosines might act either as a signal for iodination or in the coupling reaction. However, the sulfation of tyrosine is not a prerequisite for tyrosine iodination to occur (21). In addition, the most striking outset of our results is that, with very few amount of iodine bound to Tg, we recorded a relevant hormonosynthesis, which was showed to consume half of the Tyr[S] usable for this process. This indicated that Tyr[S] were more likely involved in the coupling reaction than in the iodination step. Since we detected only about 2 moles of Tyr[S]/mole of Tg (4), we hypothesized that sulfate residues may modify some of the chemical characteristics of tyrosines so as to make them suitable for the coupling reaction. This reaction involves two iodotyrosines, where DIT is always the acceptor, resulting in the formation of thyroid hormones (1). Tyr[S] might become the DIT[S] acceptor and may act either as a chemical partner for the iodotyrosine donor (MIT or DIT) or as the bioactivating signal for the key enzyme (TPO) in the process of hormonosynthesis. Tyr[S] has been reported to play roles of this kind in some protein-protein interactions, such as those occurring in the case of cholecystokinin A (CCKA), where a Tyr[S] is needed to promote a high affinity state to the CCKA receptor (22). It is therefore possible that sulfate groups linked to tyrosine at Tg hormonogenic sites may activate the interaction between TPO and Tg.

It can therefore be concluded that there exists a close relationship between Tyr[S] utilization and hormonosynthesis efficiency. This point is worth investigating in the near future if some identified hormonogenic tyrosines are sulfated. In fact, Tyr 5 the preferential hormonogenic site, seems to be the best candidate for this purpose.

ACKNOWLEDGMENTS

This study was supported by INSERM (U 38), CNRS (SDI 401038), and the Université de la Méditerranée. The authors thank Professor P. Carayon for reading the manuscript. Marie-Christine Nlend is recipient of a research grant from ADEREM.

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