Rapid catabolism of tyrosine-*O*-sulphated proteins and the formation of free tyrosine *O*-sulphate as an end product in rat embryo fibroblasts

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Rat embryo fibroblasts, line 3Y1, were prelabelled for 24 h with [³⁵S]sulphate and incubated in fresh medium without [³⁵S]sulphate. A rapid efflux of the overall ³⁵S-labelled compounds from the cells into the medium was observed. After 9 h of incubation, about 50% of the total ³⁵S radioactivity appeared in the medium and up to 84.3% did so at the end of a 48 h incubation. Determination of [³⁵S]sulphated macromolecules present in both the cell-associated and the incubation-medium fractions at different time points during incubation indicated that the majority of the ³⁵S-labelled compounds released from the cells were low-*M_r* products derived from digestion of the [³⁵S]sulphated macromolecules. Further analysis for tyrosine-*O*-[³⁵S]sulphated proteins, which constituted only a small fraction of the overall [³⁵S]sulphated macromolecules, showed that, after 9 h of incubation, there was a 65% decrease in the cell-associated fraction, and only 16.4% remained after 48 h. During that time, an amount equivalent to 20.7% of the cell-associated tyrosine-*O*-[³⁵S]sulphated proteins originally present was released into the medium. Free tyrosine *O*-[³⁵S]sulphate was generated in the cells and excreted into the incubation medium. Its rate of increase with time, however, was slow, and could account for only 12.4% of the tyrosine-*O*-[³⁵S]sulphated proteins catabolized at the end of the 48 h incubation.

INTRODUCTION

Post-translational protein modification by tyrosine sulphation was first discovered in bovine fibrinogen over 30 years ago [1]. Although only a few proteins and some biologically active peptides have been found to be tyrosine-*O*-sulphated [2–10], labelling *in vivo* with [³⁵S]sulphate followed by the analysis of protein-bound radioactive tyrosine *O*-sulphate (TyrS) had shown that this type of covalent modification occurred in proteins of diverse molecular size [11,12]. On the other hand, free TyrS was detected in human [13] and other mammalian [14] urine, and it is now considered to be the primary degradation product of tyrosine-*O*-sulphated proteins *in vivo*. In our previous work, we detected a drastic decrease of protein tyrosine sulphation in avian sarcoma virus-transformed rat embryo fibroblasts as compared with their normal counterparts [12]. As part of the effort to elucidate the mechanism of that decrease after oncogenic transformation, we decided to study the metabolism of tyrosine-*O*-sulphated proteins.

In the present study, we metabolically labelled rat embryo fibroblasts, line 3Y1, with [³⁵S]sulphate. Cells were then incubated in fresh medium without [³⁵S]sulphate and the fate of [³⁵S]sulphated compounds was followed at different periods of time thereafter. Amounts of tyrosine-*O*-[³⁵S]sulphated proteins associated with the cells and those released into the medium were measured at each time point. A high rate of

catabolism of cell-associated, tyrosine-*O*-[³⁵S]sulphated proteins during the early phase of incubation together with a concomitant accumulation, at a lower level, of tyrosine-*O*-[³⁵S]sulphated proteins in the incubation medium were observed. Free Tyr[³⁵S] appeared to be generated in the cells and quickly released into the medium. Its net formation, however, was low compared with the amount of tyrosine-*O*-[³⁵S]sulphated proteins catabolized during the same period of time. Possible reasons for the discrepancy are discussed.

EXPERIMENTAL PROCEDURES

Materials

Protein *M_r* standards were obtained from Bethesda Research Laboratories. Pre-coated cellulose t.l.c. plates were purchased from Brinkmann; carrier-free [³⁵S]sulphuric acid was from New England Nuclear. Tyrosine *O*-sulphate standard was synthesized by the procedure of Jevons [15]. Pronase was from Calbiochem–Behring. All other reagents were of the highest grade commercially available.

Cell culture and radioactive labelling

Rat embryo fibroblasts, line 3Y1, were obtained from Dr H. Hanafusa (The Rockefeller University). Cells were grown at 37 °C in DMEM containing 10% (v/v) calf serum (Flow Laboratories). For radioactive labelling,

Abbreviations used: TyrS, tyrosine *O*-sulphate; Tyr[³⁵S], tyrosine *O*-[³⁵S]sulphate; DMEM, Dulbecco's modified Eagle's medium; P_i/NaCl, phosphate-buffered saline.

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five 65 mm dishes of newly confluent cells were each incubated in 2.5 ml of sulphate-free (prepared by replacing MgSO_4 with MgCl_2) DMEM containing 10% (v/v) dialysed calf serum and [^{35}S]sulphate (0.15 mCi/ml). After 24 h, the labelling medium was removed, the dishes were rinsed three times with fresh DMEM and each placed under 4 ml of DMEM without serum. The five dishes of cells were then incubated for 0, 3, 9, 24 and 48 h, respectively, before the incubation medium was separated. Cells in each dish, after rinsing three times with P_i/NaCl , were lysed in 1 ml of Laemmli's SDS sample buffer [16]. Incubation medium fractions separated were dialysed extensively against P_i/NaCl at 4°C, lyophilized and each redissolved in 0.25 ml of Laemmli's SDS sample buffer.

Determination of [^{35}S]sulphated macromolecules in the cell-associated and the incubation-medium fractions

To avoid confusion, in the following sections a distinction will be made between '[^{35}S]sulphated macromolecules', which consist of large, non-dialysable molecules, and '[^{35}S]compounds', which include both the [^{35}S]sulphated macromolecules and the small, dialysable ^{35}S -labelled compounds. Samples of the cell lysates and the dialysed incubation medium fractions prepared were subjected to SDS gel electrophoresis [16]. After the electrophoresis, the gel was stained with Coomassie Brilliant Blue in methanol/acetic acid/water (5:1:4, by vol.), destained with methanol/acetic acid/water (10:3:27, by vol.) and dried under vacuum at room temperature. Through the electrophoresis and the following staining and destaining procedures, [^{35}S]sulphated macromolecules present in the cell lysates and the incubation medium fractions were fixed in the gel and separated from the small, dialysable ^{35}S -labelled compounds. Gel electrophoretic lanes corresponding to different samples of cell lysates and incubation medium fractions were excised and each was sliced into small pieces before being placed in separate tubes. Portions (10 ml) of a solution containing Pronase (150 $\mu\text{g}/\text{ml}$) and NH_4HCO_3 (50 mM) were individually added and the tubes were incubated at 37°C with constant shaking. [^{35}S]sulphated macromolecules in the gel pieces were, under these conditions, hydrolysed by Pronase and eluted from the gel. After 24 h incubation, the gel eluates were separated for the determination of ^{35}S radioactivity.

Determination of tyrosine-*O*-[^{35}S]sulphated proteins in the cell-associated and the incubation-medium fractions

For the determination of tyrosine-*O*-[^{35}S]sulphated proteins, a second stage Pronase hydrolysis was performed on the gel eluates separated in the previous step. Samples (5 ml) of the gel eluates were taken and 0.5 ml of Pronase (1.5 mg/ml) in 50 mM- NH_4HCO_3 solution was individually added, followed by 24 h incubation at 37°C. (This procedure, as we have used previously [4,12] and now also by others [27,28], allows the nearly quantitative release of protein-bound TyrS.) The Pronase hydrolysates were then lyophilized and each of the lyophilized residues was carefully redissolved in 100 μl of TyrS standard solution (1 mg/ml). Redissolved samples (5 μl) were spotted on cellulose t.l.c. plates and analysed by a two-dimensional thin-layer separation procedure combining high-voltage electrophoresis and t.l.c. [4,12]. After the separation, TyrS spots visualized

by ninhydrin coloration were scraped off for determination of ^{35}S radioactivity.

Quantification of free Tyr[^{35}S] in the cell-associated and the incubation-medium fractions

Aliquots of cell lysates and incubation medium fractions (without dialysis) prepared immediately after the incubations were diluted 1:1 with TyrS solution (1 mg/ml). A 5 μl portion of each of the samples was, without Pronase hydrolysis, directly spotted on to cellulose t.l.c. plates. The plates were subjected to the same two-dimensional separation procedure [12] with a slight modification, i.e. the conditions for the high-voltage thin-layer electrophoresis were changed to be 15 min at 250 V, followed by 90 min at 500 V. TyrS spots revealed after ninhydrin coloration were scraped off for the determination of ^{35}S radioactivity.

RESULTS

Amounts of [^{35}S]compounds present in the cell-associated and the incubation-medium fractions at different time points following incubation

As shown in Table 1, there was a rapid efflux of the overall [^{35}S]compounds from the labelled cells into the incubation medium. Almost 50% of the original ^{35}S radioactivity appeared in the medium after 9 h of incubation. At the end of a 48 h incubation, 84.3% was released from the cells.

The cell-associated and the incubation-medium fractions prepared at different time points during incubation were subjected to SDS gel electrophoresis and visualized by Coomassie Brilliant Blue staining (Fig. 1a). The autoradiograph taken from the dried gel (Fig. 1b) revealed that, in the cell-associated fractions (lanes 1–5), there was a rapid decrease of ^{35}S radioactivity intensity upon incubation. In the incubation-medium fractions (lanes 7–10), the increase in ^{35}S radioactive intensity was considerably slower compared with the rate of decrease in the cell-associated fractions. [^{35}S]sulphated macromolecules retained in the gel were eluted and determined as described in the Experimental procedures section. The results showed that, in the cell-associated fractions, those large, non-dialysable [^{35}S]sulphated macromolecules constituted a large portion (from 55.3% slowly decreasing to 39%) of the overall [^{35}S]compounds throughout the incubation time (Table 2), while in the incubation medium, the proportion remained low (from 5.29% slowly decreasing to 3.28%). These data indicated that the rapid efflux of the [^{35}S]compounds from the cells was

Table 1. Distribution of total [^{35}S]compounds in cell-associated and incubation-medium fractions at different periods of time following incubation

Time (h)	$10^{-3} \times ^{35}\text{S}$ radioactivity (c.p.m.) (% of total)		
	Total	Cell-associated	Incubation medium
0	119 500	119 500 (100%)	—
3	119 515	92 814 (77.6%)	26 701 (22.4%)
9	121 410	62 854 (51.8%)	58 556 (48.2%)
24	119 518	32 933 (27.6%)	86 525 (72.4%)
48	124 346	19 466 (15.7%)	104 880 (84.3%)

Fig. 1. Electrophoretic pattern of [^{35}S]sulphated macromolecules (visualized by Coomassie Brilliant Blue staining) in the cell-associated and the incubation-medium fractions prepared at different time points following incubation (a) and the corresponding autoradiograph (b)

Samples 1–5 were cell lysates prepared after 0, 3, 9, 24 and 48 h of incubation. Lane 6 shows the positions of protein M_r standards used: cytochrome *c* (M_r 12300), β -lactoglobulin (M_r 18400), α -chymotrypsinogen (M_r 25700), ovalbumin (M_r 43000), bovine serum albumin (M_r 68000), phosphorylase *b* (M_r 92500) and myosin H-chain (M_r 200000). Samples 7–10 were incubation medium fractions separated at 3, 9, 24 and 48 h following incubation. It is to be noted that one-twentieth (50 μl from 1 ml) of cell lysates and one-fifth (50 μl from 0.25 ml) of concentrated incubation medium fractions were used for the electrophoresis.

mainly due to the small [^{35}S]compounds derived from the catabolism of the large [^{35}S]sulphated macromolecules present at the beginning of the incubation.

Fig. 2. Amounts of tyrosine- O -[^{35}S]sulphated proteins in the cell-associated and the incubation-medium fractions at different time points following incubation

—, Cell-associated, ----, incubation medium.

Table 2. Proportions of large non-dialysable [^{35}S]sulphated macromolecules among the overall [^{35}S]compounds in the cell-associated and the incubation-medium fractions at different periods of time following incubation

Time (h)	Macromolecules as % of ^{35}S -labelled compounds	
	Cell-associated	Incubation medium
0	55.3	—
3	53.6	5.29
9	51.4	3.89
24	45.0	3.65
48	39.0	3.28

Amounts of tyrosine- O -[^{35}S]sulphated proteins in the cell-associated and the incubation-medium fractions following incubation

Quantification of tyrosine- O -[^{35}S]sulphated proteins among the overall [^{35}S]sulphated macromolecules, in both the cell-associated and the incubation-medium fractions, also revealed that there was a rapid decrease in the cells upon incubation (Fig. 2). Within the first 9 h, a 65% reduction of cell-associated, tyrosine- O -[^{35}S]sulphated proteins was observed. The decrease thereafter slowed down and, at the end of the 48 h incubation, approx. 16.4% of the original tyrosine- O -[^{35}S]sulphated proteins remained. The accumulation of tyrosine- O -[^{35}S]sulphated proteins in the incubation medium was also slow compared with the rate of decrease in the cells. More than 11% was released into the medium after 9 h of incubation, and equivalent to 20.7% of the original tyrosine- O -[^{35}S]sulphated proteins appeared at the end of the 48 h incubation. It is to be noted that, in both the cell-associated and the incubation-medium fractions, tyrosine- O -[^{35}S]sulphated proteins constituted only a

Table 3. Amounts of free Tyr[³⁵S] present in 3Y1 cells and in incubation medium at different periods of time following incubation

Time (h)	³⁵ S radioactivity (c.p.m.) (% of total)		
	Total	Cell lysate	Incubation medium
0	189081	189081 (100%)	— —
3	199172	126744 (63.6%)	72428 (36.4%)
9	221171	89373 (40.4%)	131798 (59.6%)
24	221526	37629 (17.0%)	183897 (83.0%)
48	268009	24128 (9.0%)	243881 (91.0%)

small proportion of the large, non-dialysable [³⁵S]sulphated macromolecules. In the cell-associated fractions, the percentage of tyrosine-*O*-[³⁵S]sulphated proteins among the [³⁵S]sulphated macromolecules varied in the range 1.09–2.19%, and that in the incubation-medium fractions was between 5.08 and 6.79%.

Amounts of free Tyr[³⁵S] in the cell-associated and the incubation-medium fractions during incubation

To follow the fate of those tyrosine-*O*-[³⁵S]sulphated proteins which were rapidly catabolized in the cells during the incubation, the presence of free Tyr[³⁵S] was tested. In both the cell-associated and the incubation-medium fractions, free Tyr[³⁵S] was detected. Table 3 shows the amounts of free Tyr[³⁵S] in the cell-associated and the incubation-medium fractions present at different time points. The data indicated that there was a rapid excretion of free Tyr[³⁵S] from the cells into the medium. To assess the amount of free Tyr[³⁵S] generated during the incubation, free Tyr[³⁵S] in both the cell-associated and the incubation-medium fractions should be considered. The net formation of free Tyr[³⁵S] at different time points following incubation can be calculated as the total amount of Tyr[³⁵S] in the two fractions minus the amount of Tyr[³⁵S] present intracellularly prior to the incubation. It was found that, at the end of the 48 h incubation, there was a 41.7% increase from the zero-time value of the 'total free Tyr[³⁵S]' (that in the cells plus that in the incubation medium). Based on their ³⁵S radioactivity values, this amount of increase could account quantitatively for only 12.4% of the cell-associated, tyrosine-*O*-[³⁵S]sulphated proteins catabolized during the same time. The low yield of free Tyr[³⁵S] could be in part due to the incomplete degradation of tyrosine-*O*-[³⁵S]sulphated proteins. In that case, at the end of the 48 h incubation period, some of the Tyr[³⁵S] was still bound in the form of short peptides which slipped by in our procedure.

DISCUSSION

Despite the fact that tyrosine sulphation has been known for over 30 years, the functional implications of this type of covalent modification are still not clear. In a number of cases, it had been shown to exert some modulatory effects on the activities of biologically active peptides such as gastrin [17], cholecystokinin [18] and caerulein [17]. In another report [5], it was proposed that tyrosine sulphation might serve as a signal for protein

secretion. Our earlier work [12] led to the discovery that, in avian sarcoma virus-transformed rat embryo fibroblasts, there was a remarkable decrease of tyrosine-*O*-sulphated proteins. The question that thus arises is: what role does protein tyrosine sulphation play in the overall transformation process? With all these points in mind, it is evident that some basic information on the metabolism of tyrosine-*O*-sulphated proteins is necessary for the correct evaluation of their functional relevance.

The current study attempted to follow the metabolic fate of sulphated macromolecules with emphasis on the tyrosine-*O*-sulphated proteins in normal rat embryo fibroblasts. Rapid efflux of [³⁵S]compounds from [³⁵S]sulphate-labelled cells (Table 1) may indicate the rapid release of intact [³⁵S]sulphated macromolecules, or a rapid intracellular catabolism of these molecules followed by the excretion of the catabolic products, or, most likely, a combination of these two factors. A similar kinetic study which also showed a rapid release of [³⁵S]compounds from [³⁵S]sulphate-labelled hamster fibroblasts [19] has been reported. The data shown in Table 2 indicate that the rapid accumulation of ³⁵S-labelled compounds in the incubation medium is due quantitatively more to the catabolism of [³⁵S]sulphated macromolecules followed by the release of their small catabolic products, than to the release of intact [³⁵S]sulphated macromolecules. A similar observation has been reported in a study on the proteoglycan heparan sulphate [20]. In that case, the heparan sulphate in the pericellular matrix was said to be taken up by the cells and rapidly catabolized. Whether this is also the situation in our study will need to be clarified. It should be remembered that we made no distinction between [³⁵S]sulphated macromolecules present intracellularly or in the extracellular matrix. The 'cell-associated [³⁵S]sulphated macromolecules' comprised those in both fractions.

Tyrosine-*O*-[³⁵S]sulphated proteins among the overall [³⁵S]sulphated macromolecules also showed the same trend, with a high catabolic rate. The considerable amount of tyrosine-*O*-[³⁵S]sulphated proteins released into the medium (almost 21% of the amount originally associated with the cells), however, deserves some attention. At the end of the 48 h incubation, approx. 16.4% remained with the cells, and by then was decreasing at a much slower rate compared with that in the early phase of incubation. This could indicate that those cell-associated tyrosine-*O*-[³⁵S]sulphated proteins still present toward the end of incubation were located in the extracellular matrix, fibronectin being an example [4,21], and were out of the reach of intracellular catabolism unless endocytosed.

The metabolic fate of tyrosine-*O*-sulphated proteins has been an interesting issue. Soon after tyrosine sulphation was discovered in bovine fibrinogen [1], free TyrS was detected in human urine [13]. On the other hand, intensive efforts to look for arylsulphatases that can hydrolyse TyrS in the mammalian system were without much success [22,23]. Of the three mammalian arylsulphatases (A, B and C), only arylsulphatase A revealed a weak activity toward TyrS [23,24]. It was further shown that a patient with metachromatic leukodystrophy (a disease caused by arylsulphatase A deficiency) excreted free TyrS in the urine in quantities comparable with the normal control subjects [25]. Furthermore, attempts to achieve biological sulphation

of free L-tyrosine have also been unsuccessful [32]. These were thought to be indications of TyrS being an end product of the catabolism of tyrosine-*O*-sulphated proteins. Indeed, when free Tyr[³⁵S] or [³⁵S]sulphate-labelled rabbit fibrinopeptide B, in which TyrS is labelled with [³⁵S]sulphate, was injected into the rabbit, free Tyr[³⁵S] was detected as one of the two metabolites excreted in the urine, the other one being a deaminated derivative of Tyr[³⁵S], 4-hydroxyphenylacetic acid *O*-[³⁵S]sulphate [29]. In our study, we detected the presence in the cells of TyrS, which was rapidly excreted into the incubation medium. Based on the ³⁵S radioactivity values, the net formation of free Tyr[³⁵S] (total amount of Tyr[³⁵S] in both the cell-associated and the incubation-medium fractions minus the amount originally present in the zero-time cell lysate) could account for only 12.4% of the tyrosine-*O*-[³⁵S]sulphated proteins catabolized at the end of the 48 h incubation. The low yield could be partly due to the longer time needed for the complete degradation of tyrosine-*O*-[³⁵S]sulphated proteins than the 48 h incubation period monitored in the present study. The abrupt increase of the total free Tyr[³⁵S] in the 48 h sample (41.7%) as compared with the increase in the first 24 h (17.1%) is indicative of such a situation. Other potential factors which may contribute to the quantitative discrepancy include (i) further deamination of the free Tyr[³⁵S] generated, forming 4-hydroxyphenylpyruvic acid *O*-[³⁵S]sulphate which, in turn, produces 4-hydroxyphenylacetic acid *O*-[³⁵S]sulphate spontaneously, and (ii) desulphation of either tyrosine-*O*-[³⁵S]sulphated proteins or the free Tyr[³⁵S] and its metabolites. Studies both *in vivo* and *in vitro* [29,30,31] had established that Tyr[³⁵S] could undergo deamination, forming 4-hydroxyphenylpyruvic acid *O*-[³⁵S]sulphate and 4-hydroxyphenylacetic acid *O*-[³⁵S]sulphate. Examination of the presence of these compounds in the cell metabolites is necessary, as is a further investigation into the presence in the cells of other arylsulphatases, in addition to arylsulphatase A [23,24], that are capable of desulphating tyrosine-*O*-sulphated proteins and free TyrS (and/or its further metabolites). If these desulphation reactions indeed occur, the sulphate ultimately released could be recycled into the cellular metabolism through the sulphate-activation mechanism. However, only a small fraction of the 'active' sulphate, adenosine 3'-phosphate 5'-phosphosulphate, thus formed will eventually appear in the tyrosine-*O*-sulphated proteins, since this compound serves as the sulphate donor for other sulphonation reactions as well, and protein-bound TyrS constitutes less than 2% of the total bound sulphate in the cells [12].

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