Tyrosine sulfation: a post-translational modification of proteins destined for secretion?

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Received 21 August 1984

Protein sulfation was studied in germ-free rats by prolonged in vivo labeling with [^{35}S]sulfate. Specific sets of sulfated proteins were observed in all tissues examined, in leucocytes, and in blood plasma. No protein sulfation was detected in erythrocytes. Analysis of the type of sulfate linkage showed that sulfated proteins secreted into the plasma contained predominantly tyrosine sulfate, whereas sulfated proteins found in tissues contained largely carbohydrate sulfate. This implies some kind of selection concerning the intracellular processing, secretion, turnover or re-uptake of sulfated proteins which is responsible for the enrichment of tyrosine-sulfated proteins in the plasma.

Tyrosine sulfate *Protein sulfation* *Protein secretion* *Plasma protein* *Germ-free rat*

1. INTRODUCTION

Sulfation of tyrosine residues is a widespread post-translational modification of proteins [1]. Tyrosine-sulfated proteins have been observed not only in many tissues in mammals [1], but also in all vertebrate and invertebrate metazoa studied so far, and in simple multicellular organisms, e.g., the green alga *Volvox carteri* (unpublished).

The functions of most tyrosine-sulfated proteins are not yet known. The few functionally identified tyrosine-sulfated proteins include: fibrinogen [2], complement C4 [3], vitellogenin of *Drosophila melanogaster* (submitted), IgG2a [4], IgM (unpublished), fibronectin [5], hirudin [6] and several peptide hormones and neuropeptides such as gastrin [7,8], cholecystokinin [9] and leu-enkephalin [10]. It is interesting to note that these proteins, despite their different functions, share one common property: they are all secretory proteins.

Abbreviation: SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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Here we have used prolonged [^{35}S]sulfate labeling of germ-free rats to analyze the tyrosine sulfate content of proteins in various tissues and in the blood plasma, a body fluid known to consist almost exclusively of secretory proteins. We found the highest level of tyrosine sulfate in plasma proteins. Our results are consistent with the hypothesis that tyrosine sulfation occurs predominantly, if not exclusively, in proteins destined for secretion.

2. METHODS

2.1. *[^{35}S]Sulfate labeling*

Female and male germ-free rats (100 g) (Zentralinstitut für Versuchstiere, Hannover) were injected intraperitoneally under sterile conditions at 0, 4 and 12 h with carrier-free [^{35}S]sulfate (17 mCi/injection) in saline containing 0.8 mM methionine and 1.8 mM cysteine. At 15 h, the whole animals were perfused under ether anaesthesia through the left heart ventricle with a solution of 150 mM NaCl, 5 g/l procain-HCl and 64000 units/l heparin for 10 min at a flow rate of 25 ml/min [11]. Immediately after starting the perfusion, the right atrium was cut open, and blood was collected and mixed with 10 vols of perfusion medium. Red

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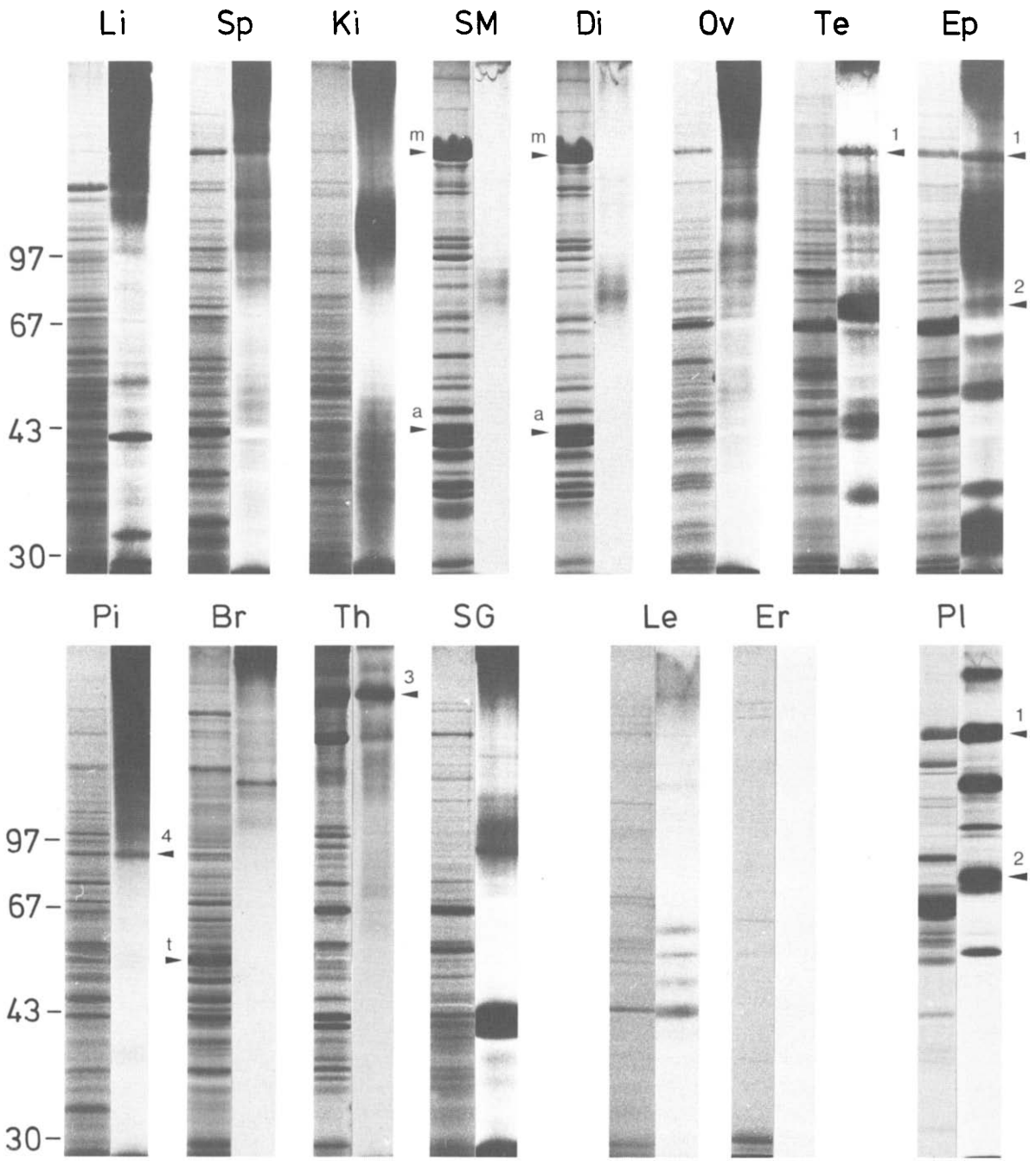

Fig.1. Specific sets of sulfated proteins and proteoglycans are found in various tissues, blood cells and the blood plasma of the rat. Proteins (50 μg/lane) were separated in 10% SDS-polyacrylamide gels under reducing conditions, followed by staining with Coomassie blue (left lanes) and fluorography (right lanes). The exposure time of the fluorograms shown varied from 2 weeks (Li, Sp, Ki, Ov, Pi, Pl) to 4 weeks (SM, Di, Te, Ep, Br, Th, SG, Le, Er) to obtain adequate resolution for each sample. Li, liver; Sp, spleen; Ki, kidney; SM, skeletal muscle; Di, diaphragma; Ov, ovary; Te, testis; Ep, epididymis; Pi, anterior pituitary; Br, brain; Th, thyroid; SG, salivary gland; Le, leucocyte-enriched fraction of blood cells (note the substantial proportion of erythrocyte protein); Er, erythrocyte; Pl, blood plasma. Arrowheads indicate proteins that are specifically discussed in the text: a, actin; m, myosin; t, tubulin. The positions of molecular mass standards (given in kDa) are shown on the left.

cells essentially free of white cells and platelets, a fraction enriched in white cells, and the soluble plasma protein fraction were prepared from the diluted blood by differential centrifugation as in [12] with minor modifications. The perfused tissues were dissected, frozen in liquid nitrogen, crushed, and homogenized in ice-cold 20 mM Tris-HCl (pH 7.5), 10 mM EDTA, 5 mM β-mercaptoethanol prior to storage (-70°C).

2.2. *Analytical procedures*

Proteins present in the various tissues, the blood cell fractions, and the blood plasma were analyzed by SDS-PAGE [13] and two-dimensional PAGE [14]. Analysis of sulfated proteins by acid treatment of polyacrylamide gels and tyrosine sulfate analysis of proteins eluted from polyacrylamide gels were performed as in [15]. SDS-solubilized proteins of tissues, blood cells and blood plasma were precipitated with acetone [15], the precipitate was washed with acetone and the ^{35}S-radioactivity in the precipitate, referred to as total sulfate incorporation (TSI), was determined by liquid scintillation counting. The precipitate was analyzed for protein-bound tyrosine-*O*-[^{35}S]sulfate (TOS) as in [15]. Protein was determined as in [16] after precipitation in 10% trichloroacetic acid.

3. RESULTS

3.1. *Tissue-specific occurrence of sulfated proteins in the rat*

Germ-free rats were used for [^{35}S]sulfate labeling in vivo to avoid the synthesis of [^{35}S]-methionine and [^{35}S]cysteine from [^{35}S]sulfate by microorganisms. This ensured that only [^{35}S]sulfate-labeled proteins and proteoglycans were detected by fluorography of polyacrylamide gels (fig.1). Each tissue yielded a characteristic pattern of sulfated proteins and proteoglycans. Related tissues, e.g., skeletal muscle and diaphragm (fig.1, SM, Di), showed similar patterns of sulfated macromolecules. In the tissues analyzed, major cytoplasmic proteins such as actin, myosin and tubulin (fig.1, arrowheads a,m,t) did not incorporate sulfate. In contrast, some of the secretory proteins that could be identified in the tissue homogenates were found to be sulfated, e.g., secretogranin of the anterior pituitary ([17], submitted) (fig.1, arrowhead 4) and thyroglobulin (fig.1, arrowhead 3). Moreover, many of the proteins secreted into the blood plasma were sulfated. In erythrocytes, which neither synthesize nor secrete proteins, no sulfate incorporation into proteins was detectable (fig.1, Er) whereas leucocytes contained several sulfated bands (fig.1, Le).

3.2. *Tyrosine sulfate is found predominantly in secretory proteins*

The total [^{35}S]sulfate incorporation into acetone-precipitated macromolecules, and the proportion of incorporated [^{35}S]sulfate that was present as tyrosine-[^{35}S]sulfate are quantitated in fig.2. The total sulfate incorporation showed large differences between tissues (fig.2A). It was high for tissues containing large amounts of sulfated proteoglycans, e.g., kidney and ovary (cf fig.1).

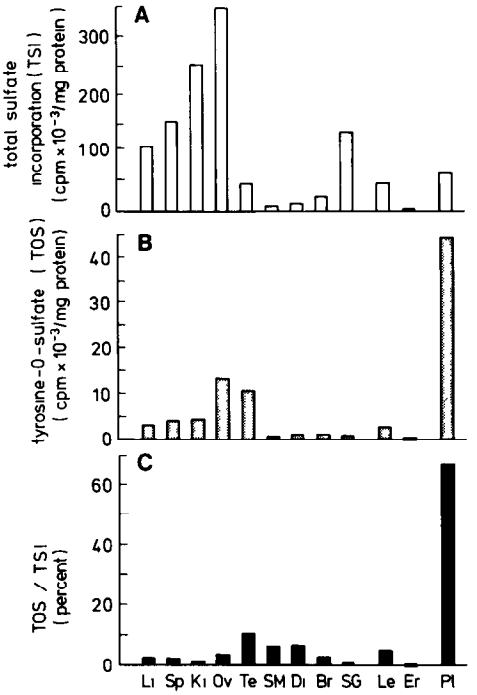

Fig.2. Comparison of the sulfate and tyrosine sulfate contents of protein and proteoglycans from various tissues, blood cells and the blood plasma, showing the highest level of tyrosine sulfate in plasma proteins. (A) Total [^{35}S]sulfate incorporation (TSI) into acetone-precipitated macromolecules. (B) Tyrosine-*O*-[^{35}S]sulfate of acetone-precipitated macromolecules. (C) Proportion of the total incorporated [^{35}S]sulfate recovered as tyrosine-*O*-[^{35}S]sulfate (TOS/TSI). Samples are abbreviated as in fig.1.

Consistent with the results shown in fig.1, no [^{35}S]-sulfate incorporation was detected in erythrocytes, indicating that free and non-covalently bound [^{35}S]sulfate was removed by the acetone precipitation.

A moderate level of total sulfate incorporation was observed in plasma proteins, yet plasma protein showed the highest amount of tyrosine sulfate, both when expressed per mg protein (fig.2B) and when expressed as a percentage of the total sulfate incorporation (fig.2C). The various tissues and the leucocytes contained significantly less protein-bound tyrosine sulfate than plasma. In tissues and leucocytes, most of the incorporated [^{35}S]sulfate was hydrolyzed and precipitated as $BaSO_4$ upon alkaline hydrolysis, suggesting that the sulfate was bound to carbohydrate residues [15].

Plasma proteins were separated by SDS–PAGE and individually analyzed for tyrosine sulfate by two different methods. First, tyrosine sulfate analysis of proteins eluted from the gel showed that for most sulfated proteins, the major portion of the incorporated [^{35}S]sulfate could be recovered as tyrosine sulfate (fig.3, right). Second, treatment of gels with HCl, a procedure known to hydrolyze the tyrosine sulfate ester bond [15], removed almost all the [^{35}S]sulfate label in most plasma proteins (fig.3, Pl). In contrast to plasma, most of the [^{35}S]sulfate label of tissue proteins, e.g., of epididymal proteins (fig.3, Ep), was not removed by the acid treatment. This was consistent with the apparent linkage of most of the sulfate incorporated into tissue protein to carbohydrate residues (see fig.2). The few acid-sensitive bands seen in certain tissues, e.g., epididymis (arrowheads 1,2 in figs 1,3), apparently corresponded to major tyrosine-sulfated proteins of the plasma (cf fig.3). Their presence did not seem to result from incomplete perfusion of tissues and thus incomplete removal of the blood since only certain sulfated plasma proteins were observed in these tissues.

Plasma proteins were separated by two-dimensional PAGE to allow better identification (fig.4). Most plasma proteins with isoelectric points lower than that of serum albumin and with apparent molecular masses greater than 65 kDa were found to be tyrosine-sulfated. Almost all tyrosine-sulfated proteins of the plasma were present in quantities sufficient to be detected by

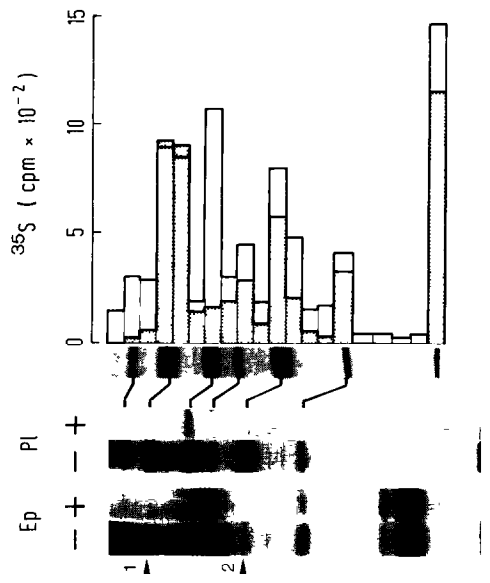

Fig.3. Tyrosine sulfate analysis of proteins separated by SDS–PAGE under reducing conditions. Left, fluorograms of 10% gels containing 50 μg per lane of epididymal (Ep) and blood plasma (Pl) protein. After electrophoresis, the gels were treated with acid (−, control; +, acid treatment), a procedure known to hydrolyze the tyrosine sulfate ester bond [14]. Right, fluorogram of a 7.5% gel containing plasma proteins as lane Pl− on the left. Lines connect corresponding bands. The gel was cut into 20 equal sections which were individually analyzed for protein-incorporated [^{35}S]sulfate (expressed by the overall length of the horizontal columns) and tyrosine sulfate (expressed by the shaded portion of the columns). The slight over-recovery of tyrosine sulfate of 3.5% and 7% in the 4th and 5th column from the top, respectively, was within the range of experimental error.

Coomassie blue-staining. The most abundant tyrosine-sulfated protein, a 185-kDa protein (fig.4, arrowhead 1) was most likely α_2-macroglobulin, as determined from its mobility in reducing and non-reducing gels (not shown) (cf [18,19]). [^{35}S]Sulfate incorporation was found in the α-, β- and γ-chains of fibrinogen (fig.4, arrowheads 6–8) identified by immunoprecipitation (not shown). The two most abundant plasma proteins of germ-free rats, albumin and transferrin (the latter one identified by immunoprecipitation, not shown) did not appear to contain [^{35}S]sulfate. The apparent lack of sulfate incorporation was not due to a slow rate of

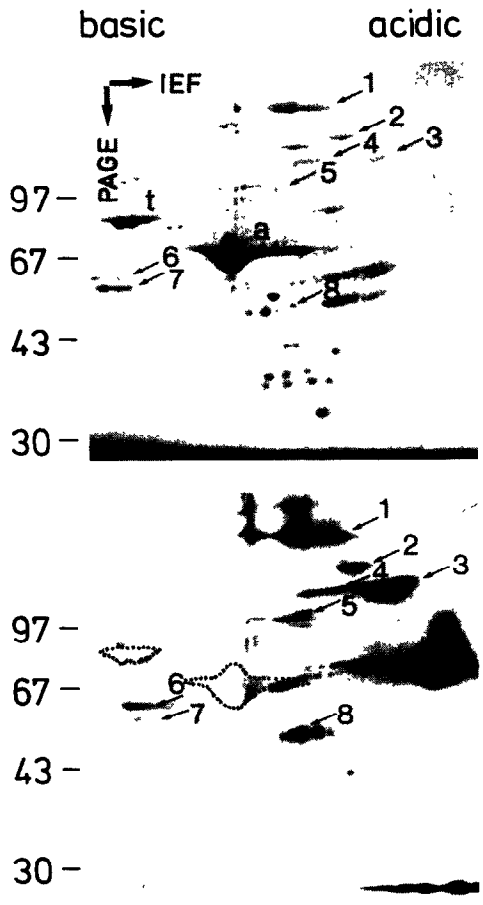

Fig.4. Two-dimensional PAGE under reducing conditions of blood plasma proteins followed by Coomassie blue staining (top) and fluorography (bottom). Numbered arrows indicate proteins (top) that are tyrosine-sulfated (bottom) (cf fig.3). The a and t (top) and the dotted lines (bottom) indicate albumin and transferrin, respectively. Note the small amount of immunoglobulins in the germ-free rats. The positions of molecular mass standards (given in kDa) are shown on the left.

synthesis, since these proteins incorporated [^{35}S]-methionine and [^{35}S]cysteine during a similar period of labeling (not shown).

4. DISCUSSION

Here we chose prolonged labeling with [^{35}S]-sulfate, the most sensitive approach for the detection of sulfated macromolecules, to investigate tyrosine sulfation of proteins in rats in vivo. The most striking result was the value of tyrosine [^{35}S]-sulfate in proteins of the plasma, which was considerably higher than that in proteins of various tissues (fig.2). This was not inconsistent with the result that liver (which produces most of the major plasma proteins) contained only low values of tyrosine-[^{35}S]sulfate in proteins since plasma proteins synthesized in the liver are known to be secreted rapidly without storage in the liver cell [20].

Several lines of evidence indicated that the higher value of radioactive tyrosine sulfate in plasma proteins than in tissue proteins reflected in fact a higher content of tyrosine sulfate. First, it is very unlikely that the [^{35}S]sulfate incorporated into proteins found in the plasma had a higher specific activity than that incorporated into tissue proteins. The sulfation of the proteins found in the plasma almost certainly took place within tissues from which these proteins were thereafter released into the plasma. Consistent with this assumption, no [^{35}S]sulfate incorporation into plasma proteins was observed when plasma was incubated with [^{35}S]-sulfate in vitro (not shown). The sulfation of proteins found in the plasma therefore utilized as co-substrate the same ^{35}S-labeled 3′-phosphoadenosine 5′-phosphosulfate as the sulfation of the tissue proteins. Second, the higher value of tyrosine [^{35}S]sulfate in plasma proteins than tissue proteins was observed both when tyrosine sulfate was expressed per mg protein (fig.2B) and when expressed as a percentage of the total sulfate incorporation (fig.2C). Two conclusions can be drawn from this result:

(i) With the tyrosine sulfate values expressed per mg protein, the tyrosine sulfate content of plasma proteins may have been overestimated due to a possibly higher rate of synthesis (and thus labeling) of these proteins than of tissue proteins. This, however, did not appear to be the case since only tyrosine sulfate, but not the total sulfate incorporation, was higher in plasma proteins than in tissue proteins.

(ii) With the tyrosine sulfate values expressed per total sulfate incorporation, the tyrosine sulfate content of tissue proteins may have been underestimated because of the abundance of sulfated proteoglycans in tissues. This, however, was not

the case since the tyrosine sulfate content of tissue proteins was lower than that of plasma proteins also when expressed per mg protein. The values illustrated in fig.2B,C therefore supported each other and indicated that the tyrosine sulfate content of plasma proteins was higher than that of tissue proteins.

It is interesting to note that only sulfate linked to tyrosine residues, but not sulfate linked in an alkali-labile form (presumably carbohydrate-linked sulfate), was enriched in plasma proteins compared with tissue proteins. This enrichment was not simply caused by the occurrence of one very abundant and highly tyrosine-sulfated protein in the plasma. Rather, it resulted from the fact that many plasma proteins were sulfated (fig.4) and contained the sulfate predominantly as tyrosine sulfate (fig.3), and that most sulfated tissue proteins contained the sulfate largely as carbohydrate sulfate (fig.3, left). In topological terms, the most obvious difference between proteins found in tissues and proteins found in the blood plasma is that the former comprise proteins of all subcellular compartments whereas the latter consist almost exclusively of secretory proteins. The enrichment of tyrosine sulfate in proteins secreted from various tissues into the blood may therefore imply some kind of a selection process. For example, proteins which are destined to be secreted may become more frequently tyrosine-sulfated than proteins with other destinations. Alternatively, proteins which are appropriately tyrosine-sulfated may become secreted. These assumptions are also consistent with previous data which indicate that all tyrosine-sulfated proteins identified so far are secretory, and that tyrosylprotein sulfotransferase [21] appears to be localized in the Golgi complex (in preparation).

ACKNOWLEDGEMENTS

We wish to thank Professor H. Thoenen for his generous support, Dr M. Schwab for help with the perfusion, P.A. Baeuerle and Dr R.W.H. Lee for their helpful comments on the manuscript and H. Macher for typing the manuscript. W.B.H. was supported by grants from the Deutsche Forschungsgemeinschaft (Hu 275/3-1, Hu 275/3-2).

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