Volume 242, number 1, 139–143 FEB 06613 December 1988

Presence of tyrosine-*O*-sulfate in sheep pituitary prolactin

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Received 5 September 1988; revised version received 7 October 1988

When the metabolically obtained ^{35}S-labelled sheep pituitary prolactin-rich fraction was subjected to chemical deglycosylation the radioactivity was retained in the immunoprecipitable prolactin. ^{35}S-labelled prolactin-rich pituitary extract was fractionated on SDS-PAGE and protein was extracted from prolactin positive bands. When the extracted ^{35}S-labelled prolactin was hydrolysed by alkali and then chromatographed on a thin layer of silica, it showed the presence of a radioactive compound which had an R_f value identical to the standard Tyr-*O*-SO_4 synthesized and characterized in our laboratory.

Prolactin; Tyrosine-*O*-SO_4; Immunoprecipitation; (Sheep pituitary)

1. INTRODUCTION

Prolactin has been known to occur in variant molecular forms differing from one another either in molecular size or in net charge. In recent years much attention has been given to the characterization [1,2] and possible biological and immunological properties of these variant forms [3–6].

We have previously reported that sheep and buffalo pituitary PRLs are sulfated [7] and possibly glycosylated too [7]. The major source of evidence came from metabolic labelling experiments where ^{35}S-labelled pituitary proteins subjected to SDS-PAGE followed by immunoblotting on nitrocellulose showed two radioactive, immunoreactive bands around 25 kDa. We had suggested a number of possibilities regarding the physico-chemical nature of these two bands on SDS-PAGE. These were (i) bands on SDS-PAGE could be two forms of tyrosine-sulfated PRL, the larger protein having been modified (glycosylation); (ii) the larger protein was a sugar sulfate containing PRL while the smaller protein was a Tyr-*O*-SO_4 containing PRL. In addition, the Con A-Sepharose unbound fraction of the ^{35}S-labelled pituitary proteins was both radioactive and PRL immunoreactive [7], leading us to suspect that there could be a non-sugar bound sulfate. Further studies reported in this paper reveal the presence of both tyrosine as well as carbohydrate-linked sulfate in ovine pituitary PRL.

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Abbreviations: SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PRL, prolactin; PBS, phosphate-buffered saline; TLC, thin-layer chromatography; TFMS, trifluoromethane sulfonic acid; [^{14}C]Man, ^{14}C-labelled D-mannose; dpm, disintegrations per minute

2. MATERIAL AND METHODS

2.1. *Chemicals*

TFMS was purchased from Sigma (St. Louis, MO, USA). All other chemicals were purchased from the sources indicated previously [7].

2.2. *Deglycosylation by TFMS*

$^{35}SO_4^{2-}$ and [^{14}C]Man-labelled HCl-pellet (PRL-rich fraction) glycoproteins were deglycosylated using TFMS as described in [11] with slight modifications. 1 ml anisole and 2 ml TFMS were mixed in a glass tube with a Teflon-lined screw cap and cooled to 0°C. Samples (3–10 mg) of $^{35}SO_4^{2-}$ and [^{14}C]Man-labelled proteins were dissolved in 1 ml of this mixture in a 2 ml Reactivial. Nitrogen gas was bubbled through the solution for 30 s followed by flushing for 30 s. The tubes were then capped under N_2 stream and allowed to stand with magnetic stirring at 0°C (ice-water bath) for 3 h. Controls were performed for each

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00145793/88/$3.50

treatment without TFMS. The deglycosylated proteins were freed of reagents and low molecular mass sugars by treating them with a 50-fold excess of diethyl ether containing 10% (v/v) *n*-hexane. The mixture was allowed to stand at −20°C for 1 h. The proteins were then pelleted by centrifugation. The resultant pellet was resuspended in ice-cold 95% ethanol and recentrifuged. The pellet was insoluble in aqueous buffer and therefore was dissolved in 4 M guanidine hydrochloride and dialysed against water. The dialysed protein solution was lyophilized and then dissolved in 0.01 M PBS and kept for immunoprecipitations against anti-PRL serum as described previously [7]. Immunoprecipitates were pelleted down by centrifugation (3000 rpm/15 min) and pellets were dissolved in 0.01 N NaOH. Aliquots of these were taken for radioactive counting in a Packard model 2000 CA, TRI-CARB liquid scintillation analyser.

2.3. *Synthesis of standard Tyr-O-$^{35}SO_4$*

The standard Tyr-*O*-$^{35}SO_4$ was synthesized from L-tyrosine and concentrated sulfuric acid according to the procedure of Jevons [9] with the following modifications, 200 mg L-tyrosine and 1 ml concentrated H_2SO_4 containing 200 μCi of $H_2{}^{35}SO_4$ (carrier free) were separately cooled on an ice-salt mixture. Solid L-tyrosine was then added to the chilled sulfuric acid and the mixture stirred for 30 min at −15°C. The reaction was stopped by the addition of ice (made from distilled water) and solid $Ba(OH)_2$ until the pH of the suspension was 9. The suspension was centrifuged at 10000 rpm for 30 min in a Sorvall RC-2B refrigerated centrifuge. The supernatant was passed through a column of Dowex-50-X8 (100–200 mesh, H^+ form). The unbound fraction was immediately adjusted to pH 7.5 with NaOH. Estimation of tyrosine was done with the Folin and Ciocalteu's phenol reagent [10] and from a UV spectrum.

2.4. *Preparation of ^{35}S-labelled PRL-rich fraction*

Incubations of sheep pituitary tissue with radioactive sulfate ($H_2{}^{35}SO_4$) and [^{14}C]Man, purification of PRL up to the acid precipitation step, immunoprecipitations of radiolabelled products and SDS-PAGE were performed as described previously [7]. The two PRL positive bands on polyacrylamide gels were extracted from the gel slices by homogenizing them in 0.2 M NH_4HCO_3 and allowing them to stand overnight at 4°C. The homogenates were centrifuged at 5000 rpm for 20 min, the supernatants collected and then lyophilized. The lyophilized powders were dissolved in 1 N NaOH and subjected to alkaline hydrolysis for 24 h at 110°C in sealed evacuated tubes [8]. After hydrolysis the solutions were neutralized and lyophilized. The lyophilized hydrolysates were dissolved in 0.01 N HCl (100 μl) and aliquots loaded on silica gel-G TLC plates. The solvent system used was butanol/acetic acid/water (4:1:1).

3. RESULTS AND DISCUSSION

Treatment of the PRL-rich fraction (acid pellet) with TFMS resulted in about 75% deglycosylation in the case of [^{14}C]Man-labelled proteins (table 1). After treatment with TFMS there was a 5-fold increase in the $^{35}SO_4^{2-}$ radioactivity in immunoprecipitable PRL expressed as percentage of the initial radioactivity taken for immunoprecipitation (table 2). In contrast, the percentage of radioactivity due to [^{14}C]Man remains almost the same in immunoprecipitable PRL before and after treatment with TFMS (table 1). The significant increase in $^{35}SO_4$-PRL specific radioactivity after deglycosylation leads us to propose (i) that the $^{35}SO_4$ moiety is attached to a site other than carbohydrate and that this site may be tyrosine, which is known to be another potential site for sulfation; (ii) that in the PRL-rich fraction, PRL is one of the most abundant tyrosine-sulfated proteins.

To get additional clues to the presence of tyrosine-*O*-sulfate in PRL, we analysed the amino acid sequence of standard ovine PRL, especially in the regions surrounding the seven tyrosines present in this hormone, by following the method of Hortin et al. [12] who developed certain criteria for

Table 1

Effect of TFMS on [^{14}C]Man-labelled glycoproteins of ovine pituitaries

	Radioactivity (dpm)[a]		
	Total	PRL-specific[b]	$\frac{\text{PRL-specific}}{\text{total}} \times 100$
Before TFMS treatment	1668	304	18
After TFMS treatment	408	83	20
% removal by TFMS	75		

[a] Average of 2 experiments only
[b] Immunoprecipitable by a specific antiserum

Table 2

Effect of TFMS on ^{35}S-labelled glycoproteins of ovine pituitaries

	Radioactivity (dpm)[a]		
	Total	PRL-specific[b]	$\frac{\text{PRL-specific}}{\text{total}} \times 100$
Before TFMS treatment	3228	183	5.66
After TFMS treatment	336	85	25.3
% removal by TFMS	90	-	

[a] Average of 2 experiments only
[b] Immunoprecipitable by a specific antiserum

tyrosine sulfation based on the analysis of amino acid sequences surrounding known tyrosine sulfation sites in a number of proteins. The amino acid sequence of ovine PRL shows that out of seven tyrosines only one, present at position 96, satisfies their criteria, except for the presence of an acidic residue at position −1 or −2. There are three other known examples i.e. canine fabrinopeptide B, bovine gastrin and feline gastrin in which the site of sulfation conforms to their criteria [13], except that acidic residues are absent from −1 and −2 positions.

To study the presence of Tyr-*O*-$^{35}SO_4$ in PRL, Tyr-*O*-$^{35}SO_4$ was synthesized by the method of Jevons [9] with a few modifications. Based on the estimation of tyrosine content using the Folin and Ciocalteu's phenol reaction as well as a UV spectral analysis before and after acid hydrolysis, we have calculated that there was a 40–50% conversion of tyrosine to Tyr-*O*-SO_4. In NaOH, tyrosine exhibited absorption maxima at 240 nm and 295 nm, whereas Tyr-*O*-SO_4 in the same medium exhibited maxima at 245 nm and 310 nm, hence there was a red shift. After acid hydrolysis, the spectral peaks of free tyrosine were reestablished (fig.1). On Dowex 50, Tyr-*O*-SO_4 was found to be unadsorbed while tyrosine was bound to the resin thus effecting separation and hence purification. Purified Tyr-*O*-$^{35}SO_4$ after silica gel TLC followed by ninhydrin and/or α-nitroso-β-naphthol staining yielded a single spot (fig.2) and it also showed the presence of a significant amount of radioactivity. After acid hydrolysis, the R_f value of Tyr-*O*-SO_4 changes to that of free tyrosine. The R_f values of tyrosine and Tyr-*O*-SO_4 on silica gel TLC under our conditions were 0.53 and 0.20, respectively.

Alkaline hydrolysis of the two PRL positive bands from preparative SDS-PAGE gels followed by TLC of the hydrolysate on silica gel plates showed the presence of significant radioactivity at the position corresponding to that of Tyr-*O*-SO_4 in the case of the lower band of SDS-PAGE (table 3). These data clearly show that PRL exists in a form in which tyrosine is *O*-sulfated. A previous study by Lewis et al. [1] has shown that of the two PRL bands on SDS-PAGE around 25 kDa, the upper one is glycosylated and the lower one is not glycosylated. This was confirmed by our studies. We have also found in our labelling experiments that the upper ^{35}S-labelled PRL band (in SDS-PAGE) on alkaline hydrolysis did not yield any Tyr-*O*-$^{35}SO_4$ (Kohli, R. et al., unpublished). Therefore, it is interesting to note that sheep pituitary PRL is one of the rare proteins having both sugar and tyrosine-bound sulfate. The significance of these two post-translational sulfations is not clear at present. A sulfate moiety attached to a carbohydrate chain might play a protective role by conferring a longer plasma life on that particular hormone, as in the case of other pituitary hormones [14], and in the storage phenomenon of the hormone (Kohli, R. et al., unpublished).

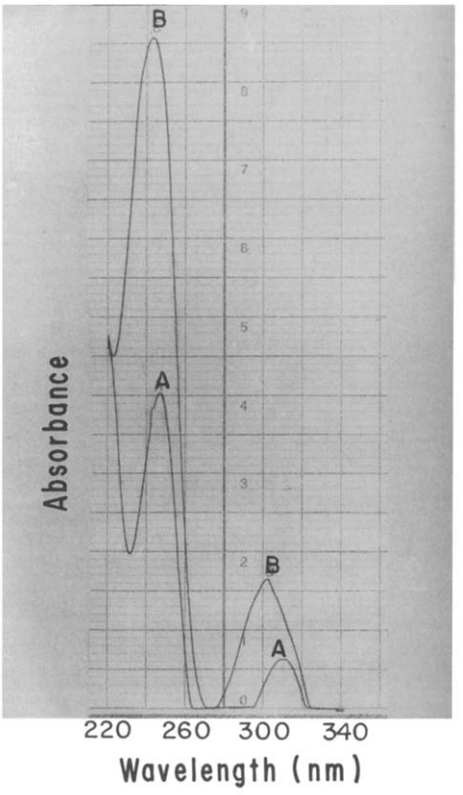

Fig.1. A UV absorption spectrum of the purified standard Tyr-*O*-SO_4: (A) before and (B) after hydrolysis in 4 N HCl/boiling H_2O for 15 min.

The presence of Tyr-*O*-SO_4 in proteins was first noted in bovine fibrinogen by F.R. Bettelheim in 1954 [15] but till today the physiological role for tyrosine sulfation has not been clearly established. However, its possible role in intracellular traffic signalling and as a protective mechanism against oxidation of tyrosine has been suggested [16,17]. In

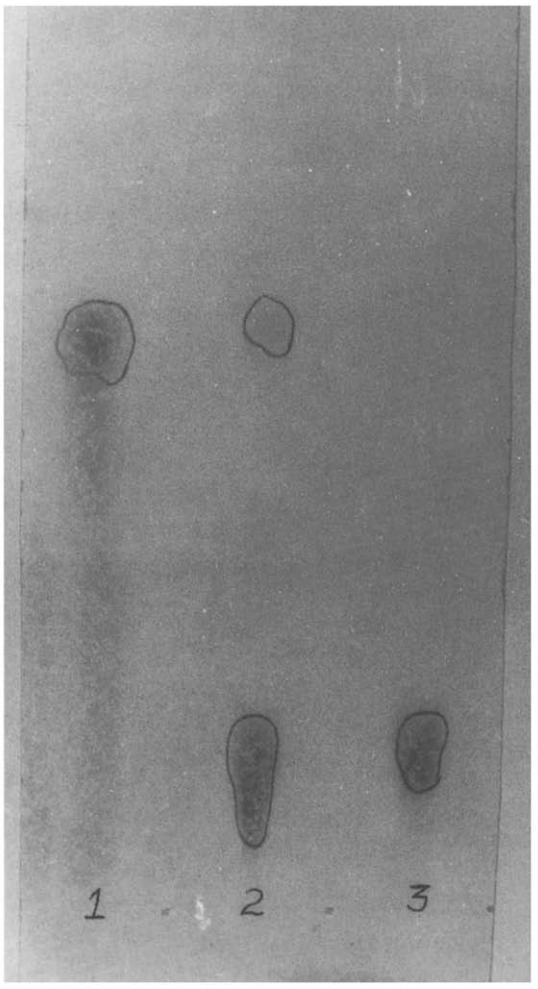

Fig.2. Thin-layer chromatographic separation of tyrosine and Tyr-*O*-SO_4. Lane 1 is tyrosine, lane 2 is crude Tyr-*O*-SO_4 and lane 3 is Dowex-50 unbound Tyr-*O*-SO_4 (purified). For details see section 2.

the case of some peptides such as cholecystokinin [18], phyllokinin [19] and leucosulfakinin [20], sulfation of specific tyrosine residue(s) was found to augment dramatically the bioactivity of the peptide. Sulfation of tyrosine residues has been shown to make gastrin a pancreatic secretogog [21].

On the basis of the existing knowledge on more than 80 biological effects exhibited by PRL it is possible that the molecular variants of PRL have specific individual biological effects in addition to providing structural diversity [6]. Further studies are being pursued to shed more light on the physiological importance of tyrosine sulfation vis-a-vis sugar sulfation in PRL.

Table 3

Tyr-*O*-$^{35}SO_4$ in PRL

Strip no.	Radioactivity (dpm)	
	Alkaline hydrolysate	Standard Tyr-*O*-$^{35}SO_4$
1	-	-
2	-	-
3	-	-
4	-	-
5	-	-
6	192	-
7	200	113
8	79	-
9	-	-
10–12	-	-

Sheep pituitaries were incubated with $^{35}SO_4^{2-}$. The semi purified ^{35}S-labelled proteins were fractionated on SDS-PAGE. The lower of the two bands corresponding to PRL [7] was extracted and then hydrolysed in alkali. The alkaline hydrolysate was subjected to TLC in butanol/acetic acid/H_2O (4:1:1) along with standard Tyr-*O*-$^{35}SO_4$ and tyrosine

Acknowledgements: Work reported here was supported by grants from the Council of Scientific and Industrial Research (CSIR) India. R.K. and N.C. are Senior Research Fellows of CSIR.

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