Vol. 138, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1986 Pages 230-237

ENZYMATIC DEGLYCOSYLATION OF THYROID-STIMULATING HORMONE WITH PEPTIDE N-GLYCOSIDASE F AND ENDO-β-N-ACETYLGLUCOSAMINIDASE F

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Received May 23, 1986

SUMMARY: We investigated the ability of two enzymes, peptide N-glycosidase F (PNGase F) and endo-β-N-acetylglucosaminidase F (Endo F), to deglycosylate microgram quantities of bovine TSH and its subunits under nondenaturing conditions. One oligosaccharide chain could be selectively removed from the α subunit by PNGase F, and all the oligosaccharide chains from both subunits could be removed by Endo F. These methods of enzymatic deglycosylation should permit study of the functional role of each N-linked carbohydrate chain of various glycoprotein hormones. © 1986 Academic Press, Inc.

The functional role of the carbohydrate moieties of most glycoproteins remains poorly understood (1). However, several studies have shown that deglycosylation of the glycoprotein hormones, LH, FSH, hCG, and TSH, virtually abolishes their biological activities (2-5). In fact, it has been suggested recently (6) that the carbohydrate of the common α subunit of these related hormones is functionally more important than that of the unique β subunits.

Enzymatic deglycosylation with sequential exoglycosidases was the initial method used in the study of hCG, since it contains terminal sialic acid

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Abbreviations: TSH, thyroid-stimulating hormone; bTSH, bovine TSH; LH, luteinizing hormone; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; PNGase F, peptide N-glycosidase F; Endo F, endo-β-N-acetylglucosaminidase F; HF, hydrogen fluoride; TFMSA, trifluoromethane sulfonic acid; β-ME, β-mercaptoethanol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; NP-40, Nonidet P-40; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Octyl-Pyran, octyl β-D-pyranoside.

0006-291X/86 $1.50
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residues which were readily removed by neuraminidase (7, 8). In contrast, the presence of terminal sulfate groups in most pituitary glycoprotein hormones has made them resistant to the hitherto available endo- and exoglycosidases (9, 10). As a result, chemical methods of deglycosylation, namely anhydrous HF and TFMSA, have been used (11). Although useful, these chemical methods have important disadvantages : the deglycosylation is incomplete and gives heterogeneous products; special equipment is required; the use of HF is hazardous; and alterations could occur in the protein chains.

We investigated the abilities of two recently described enzymes (12), endo-β-N-acetylglucosaminidase F (E.C.37278-88-9) and peptide N-glycosidase F (E.C.83534-9-8), to deglycosylate bTSH under nondenaturing conditions. We show that under appropriate conditions, deglycosylation of bTSH and its subunits can be achieved. In addition, we are able to remove selectively one oligosaccharide chain from bTSH-α.

MATERIALS AND METHODS

Highly purified bTSH, bTSH-α and bTSH-β were a gift from Dr J G Pierce. Other materials were purchased (sources in parentheses, all U.S.A.) : PNGase F (Genzyme, Boston, MA), Endo F (Grade II, Boehringer Mannheim, New York, NY), all other chemical reagents (Sigma, St Louis, MO and Fisher, Fairlawn, NJ). Enzymatic reactions were carried out as follows : 20 µg of glycoprotein (2 mg/ml) were added to 10 µl 0.5M buffer at the designated pH (sodium phosphate, pH 6.1 or 8.6; sodium acetate, pH 4.0) and enzyme was added at the appropriate concentrations. Phenanthroline dihydrate (Sigma) was added at a concentration of 2 mM to inhibit protease activity. Reactions proceeded overnight for 15 h at 37°C. In some experiments, various detergents were added to give a final detergent concentration of 0.5%. Where stated, the glycoprotein hormones were denatured by boiling for five minutes in 100 mM β-ME and 0.2% SDS (w/v) prior to the addition of enzyme. When SDS was present, Nonidet P-40 was added to a final concentration of 1% to stabilize the enzymes.

Discontinuous slab gel SDS-PAGE was carried out using a 12 - 20% gradient separating gel and a 5% stacking gel. All samples were boiled with 2% SDS and 5% β-ME for 5 minutes and the gels were run at a constant current of 30mA. Staining was with Coomassie blue.

Assay for proteolytic activity (13) was carried out using hide powder azure (Calbiochem, La Jolla, Ca). HPLC purification of the subunits was carried out using two 60 cm TSK G2000SW columns and 0.1 M pH 7 ammonium acetate buffer. After acid hydrolysis, amino sugar and amino acid analyses were carried out using a Beckman amino acid analyzer model 121MB as previously described (14).

RESULTS

We have shown previously that the two dissimilar subunits of TSH, α and β, migrate under reducing conditions on SDS-PAGE with apparent mol wts of 21K

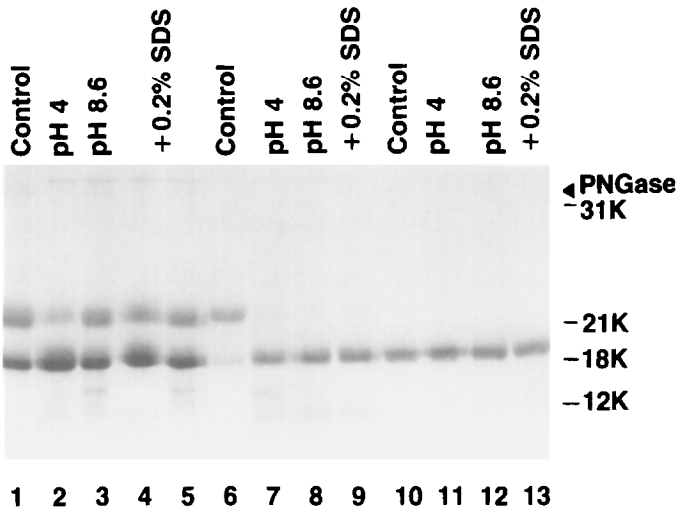

FIG. 1. Effect of pH and SDS on the deglycosylation of bTSH and its subunits by PNGase F. bTSH (lanes 1-5), bTSH-α (lanes 6-9) and bTSH-β (lanes 10-13) were incubated at designated pH's and SDS concentrations for 15 h with PNGase F (0.15 IU/ml) and the products then analyzed by SDS-PAGE. Staining was with Coomassie blue. bTSH-α migrated with apparent mol wt 21K, bTSH-β 18K, and fully deglycosylated subunits (α and β) 12K. bTSH-α with one oligosaccharide chain removed migrated with an apparent mol wt of 18K. PNGase F has an apparent mol wt of 33K and can be seen faintly at the top of the gel.

and 18K respectively (15). The α subunit has two N-linked oligosaccharide chains and the β subunit has only one. When both subunits are unglycosylated (by inhibition with tunicamycin), they co-migrate at 12K (15). Figure 1 shows the SDS-PAGE migration of untreated and PNGase F treated glycoproteins. Lane 1 shows intact bTSH which migrated as α and β subunits. When bTSH was treated with PNGase F (lanes 2-5), deglycosylation of the α subunit occurred most notably at pH 4 (lane 2). If only one oligosaccharide chain had been removed, the change in migration of the α subunit from 21K to 18K would be masked by the β subunit. Indeed, when α subunit alone (lane 6) was treated with PNGase F, it was apparent that only one oligosaccharide chain had been removed (lanes 7-9), and the migration on SDS-PAGE was now identical to the β subunit. Interestingly, PNGase F was able to remove one oligosaccharide chain from the uncombined α subunit completely (lanes 7-9), but only partially from intact bTSH (lanes 2-5). PNGase F was unable to deglycosylate the β subunit (lanes 2-5, 11-13).

PNGase F has been reported to have greatest activity at pH 8.6 (12). However, the above results show that deglycosylation of the susceptible α

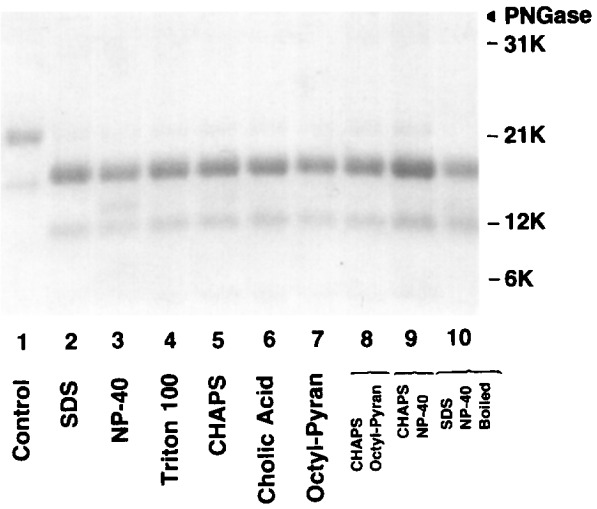

FIG. 2. Effect of detergents on the ability of PNGase F to deglycosylate the bTSH-α subunit. bTSH-α was incubated with PNGase F (0.1 IU/ml) and appropriate detergent(s) for 15 h. bTSH-α migrated with an apparent mol wt of 21K, bTSH-α with one oligosaccharide chain 18K, and deglycosylated bTSH-α 12K.

chain in intact bTSH was most efficient at pH 4. There was little effect of pH on the susceptibility of the resistant chains to enzymatic deglycosylation.

The efficiency of enzymatic deglycosylation has been reported to be increased by detergents (16). Figure 2 shows treatment of the α subunit with PNGase F in the presence of various detergents. Although a small amount of deglycosylation occurred (12K bands), no particular detergent was superior, and the remaining oligosaccharide chain on the α subunit was >80% resistant to PNGase F in all cases. Incubation time was varied from 4 to 15 h and did not change the efficiency of removal of the remaining oligosaccharide chain.

We investigated the enzyme preparations for proteolytic activity. PNGase F had protease activity just detectable at 18 hours equivalent to 12 pg/ml of pronase and therefore all incubations with PNGase F were restricted to a maximum of 15 h. No protease activity was detectable with Endo F at 18 h.

Figure 3 shows the results of deglycosylation of bTSH and subunits by Endo F and PNGase F and the effect of prior denaturation on the efficiency of the deglycosylation. With Endo F, there is significant deglycosylation of intact bTSH and subunits, resulting in the appearance of a 12K band (lanes 2-7). Denaturation increased the efficiency of deglycosylation of intact bTSH

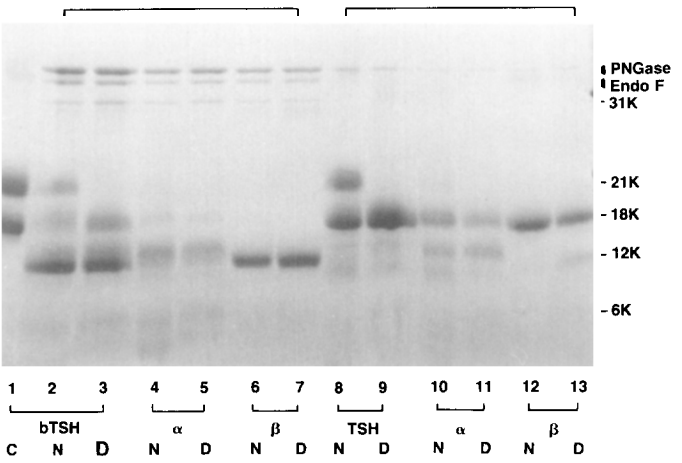

FIG. 3. Effect of prior substrate denaturation on the efficiency of enzymatic deglycosylation. bTSH, bTSH-α, and bTSH-β were incubated with endo F (0.3 IU/ml, lanes 2-7) or PNGase F (0.1 IU/ml, lanes 8-13) either under non-denaturing (N) or denaturing (D) conditions and products were characterized on SDS-PAGE. Lane 1 (control) shows bTSH-α as a 21K and bTSH-β as an 18K band. PNGase F (33K band) and Endo F (31K band) are seen at the top of the gel; enzyme activity was not related to the amount visualized on the gel.

(lanes 2-3, 8-9) by both enzymes, but had only a minor effect on deglycosylation of uncombined subunits (lanes 4-7, 10-13). Thus the β subunit and the second α subunit oligosaccharide chains were resistant to the action of PNGase F even after denaturation. The staining in Fig 3 suggested a higher concentration of enzymes in the Endo F preparation; however, when the PNGase F: substrate ratio was increased 100 times above that required for removal of the one susceptible oligosaccharide chain on the α subunit, the resistance to deglycosylation of the second α subunit oligosaccharide chain and the β subunit persisted (data not shown).

The presence of PNGase F activity has been reported in the Endo F preparation used (12, 17). To ascertain which enzyme was responsible for the deglycosylation, the products were purified by gel permeation HPLC, and amino sugar analysis was performed (14). Amino acid analysis was performed to quantify and normalize the carbohydrate moiety to the protein core. The results of amino sugar analysis of the untreated subunits (Table 1) are in close agreement with previous reports (18). They also confirmed that it was Endo F which was active since it leaves the innermost N-acetylglucosamine

Table 1: Amino-sugar, in moles per subunit, calculated and normalized from amino-acid analysis

	Glucosamine	Galactosamine
α-subunit	5.7	2.7
β-subunit	3.1	1.7
α PNGase F	4.2	2.0
α Endo F	2.8	0.0
β Endo F	1.4	0.0

attached to the protein core. Therefore the α subunit would have two moles, and the β subunit one mole of N-acetylglucosamine remaining, which is consistent with our data (Table 1). PNGase F, a peptide N-glycosidase, would completely remove all the carbohydrate (17). Amino sugar analysis also confirmed that PNGase F removed one oligosaccharide chain from the α subunit. There was no detectable change in the amino acid composition of the treated and untreated hormones.

DISCUSSION

The study of the biological properties of deglycosylated glycoproteins has been hindered by the lack of a suitably specific and efficient method which could be applied to microgram quantities. We have described in this study the use of PNGase F to remove selectively one oligosaccharide chain from bTSH-α, and of Endo F to deglycosylate bTSH and its subunits. Since the discovery of these two enzymes (12) there has been only one previous report on the use of these enzymes under nondenaturing conditions (16) and no report for the glycoprotein hormones. This study is also the first to evaluate the action of the two enzymes by direct amino sugar analysis of the products.

Our study suggests that the resistance to PNGase F in the β subunit and the remaining oligosaccharide chain of the α subunit is due to a limitation on the enzyme by the peptide structure. This resistance to deglycosylation persisted despite denaturation, the presence of various detergents, and an enzyme:substrate ratio 100 times greater than that required for removal of the susceptible oligosaccharide chain in the α subunit. This is consistent with a

recent report showing that although PNGase F was unable to deglycosylate a glycoprotein even after denaturation, the glycopeptides from the same protein could be deglycosylated (19). Thus the size and shape of the protein core exert a significant influence on the action of PNGase F. It is conceivable that with longer incubation times and replenishment with larger quantities of enzyme that complete deglycosylation could be achieved, as has been suggested by Chu (19). However, the expense of enzyme required and the risk of protease damage with the longer incubation times would be prohibitive. In any case our data clearly show different accessibility of the two carbohydrate chains of the α subunit, which may have functional implications.

Recently, there have been several reports on the effects of chemical deglycosylation of bovine and human TSH (20, 21, 22). Although all showed decreased bioactivity after deglycosylation, there were major differences in the receptor binding properties in each study. Most surprisingly, Berman et al (22) reported a major decrease in receptor binding of deglycosylated bTSH and suggested that this may have been artifactual in nature. The availability of specific enzymatic deglycosylation should now resolve these discrepancies, and in addition, permit study of the role of the different carbohydrate moieties in glycoprotein hormone function.

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