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Efficient preparation of glycoprotein hormones lacking an α-subunit oligosaccharide

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Abstract

The oligosaccharide on α-subunit loop 2 (α2) is needed for full glycoprotein hormone efficacy. Efforts to prepare glycoprotein hormone antagonists usually involve removing the α2 oligosaccharide and are hampered by its requirement for efficient heterodimer secretion from mammalian cells. Here we show that hormones lacking this oligosaccharide can be produced by treating them at low pH to dissociate the heterodimer and permitting the subunits to re-associate in the presence of peptide *N*-glycosidase F (PNGase F). Re-assembly of human choriogonadotropin, human follitropin, and bovine lutropin occurred rapidly and efficiently following removal of the α2 oligosaccharide by PNGase F. Consequently, virtually all heterodimers formed in the presence of this enzyme lacked this oligosaccharide. These findings support the notion that heterodimer assembly in vitro occurs by a threading mechanism that is impeded by the presence of the α2 oligosaccharide. This procedure should facilitate the study of glycoprotein hormone structure and function.

Keywords: Human choriogonadotropin (hCG); Human follicle stimulating hormone (hFSH); Bovine lutropin (bLH); α2 oligosaccharide; PNGase F

Choriogonadotropin (CG), lutropin (LH), follitropin (FSH), and thyrotropin (TSH) are heterodimeric glycoprotein hormones that control reproduction and thyroid function. Each hormone contains an α-subunit derived from the same gene and a hormone-specific β-subunit [1]. The crystal structures of human CG (hCG) [2] and human FSH (hFSH) [3] revealed that these heterodimers are stabilized by a strand of their β-subunit known as the seatbelt that is wrapped-around glycosylated loop 2 of their α-subunits (α2). The position of the seatbelt is stabilized by a disulfide that "latches" its end to loop 1 of the β-subunit core. Previous studies have shown that assembly of the heterodimer in vitro occurs by a threading mechanism that involves passage of α2 beneath the seatbelt and through the hole in the β-subunit [4]. Since the N-linked oligosaccharide attached to α2 must also traverse this hole during heterodimer dissociation and re-association, its presence would be expected to retard these processes significantly. In fact, this oligosaccharide has been shown to inhibit the formation of heterodimers that contain hyperglycosylated α-subunits [5]. This oligosaccharide can be removed from α2 by PNGase F digestion of the free subunit but not the heterodimer [6,7]. Based on the possibility that α-subunits lacking the α2 oligosaccharide would outcompete the native α-subunit for reassembly of the heterodimer, we tested the notion that α2 deglycosylated heterodimer could be prepared by dissociating the native heterodimer at low pH [8] and treating the free subunits with PNGase F under conditions that permitted heterodimer assembly in vitro. Here we show that this strategy works efficiently and should be useful for removing the α2 oligosaccharide to produce analogs such as those that have only 40% of the efficacy of hCG [9].

Materials and methods

Materials. Pure recombinant hCG and hFSH were obtained from Dr. Robert Campbell, Serono Reproductive Biology Institute, Rockland, MA. Bovine LH (bLH) was obtained from Dr. John Pierce,

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Department of Biochemistry, UCLA, Los Angeles, CA. PNGase F was purchased from New England BioLabs, Beverly, MA. Antibodies A113, B112, and B603 were obtained from Dr. William Munroe, Hybritech, a division of Beckman Coulter, San Diego, CA. Antibody B110 was prepared in this laboratory [10]. Antibody A113 recognizes an epitope on human α-subunit loop 1 and binds a similar region of the bovine α-subunit, albeit with much lower affinity. Antibody B110 binds an epitope on hCG and bLH β-subunits on the convex surfaces of loops 1 and 3. Antibodies B603 and B112 recognize an epitope on β-subunit loop 3 of hFSH and hCG, respectively.

Heterodimer disassembly and recombination in the presence of PNGase F. Glycoprotein hormone heterodimers were disassembled by treating them at pH 2 for 30 min at 37 °C. Deglycosylation of α2 and assembly of the partially deglycosylated heterodimer were initiated by diluting the resulting subunits with 50 mM phosphate buffer, pH 7.5 (G7 buffer, provided by New England Biolabs) containing 5 units of PNGase F per microliter. This elevated the pH to 7.5 and provided appropriate conditions for the activity of PNGase F and assembly of the heterodimer. The extent of hCG and bLH dimer formation was monitored by sandwich immunoassays [11] using antibody A113 for capture and radiolabeled β-subunit antibodies ^{125}I-B110 for detection. FSH assembly was monitored using A113 and ^{125}I-B603.

Analysis of carbohydrate deglycosylation during assembly in the presence of PNGase F. Removal of the α2 oligosaccharide was examined by SDS–PAGE. The sizes of the α-subunits were determined following disassembly of the heterodimers in 10 M urea, pH 8.0, at 37 °C for 1 h [8] and separation of intact and deglycosylated subunits by electrophoresis through 12% polyacrylamide gels containing 1% sodium dodecyl sulfate. Proteins electroeluted from the gels onto nitrocellulose films were blotted with ^{125}I-A113 for the detection of the α-subunits and ^{125}I-B112 for the detection of the hCG β-subunit. The extent of deglycosylation was also examined by MALDI-TOF mass spectrometry using the Voyager DE/PRO instruments (PE Biosystems, Framingham, MA) operated in linear mode using delayed extraction.

Results

Assembly of fully glycosylated glycoprotein hormone heterodimers occurs only at high subunit concentrations [4,8,12]. Initial studies were designed to learn if deglycosylation of α2 influenced the rate and extent of assembly. PNGase F treatment resulted in nearly complete heterodimer reassembly following 2 h incubation at 37 °C (Fig. 1A). Less than 10–20% of the untreated α-subunit combined with the β-subunit under these conditions. Assembly of heterodimers containing PNGase F treated α-subunit was nearly complete well before this, however, particularly when the concentration of starting material was high (Fig. 1B and C). Thus, when the subunit concentration was sufficient to form 100 ng hCG/μl, nearly 50% of the starting material reformed in the presence of PNGase F but none had formed in its absence. Approximately 70% of the heterodimer reformed within 5 min in the presence of PNGase F when a high concentration of hCG (1 μg/μl) was used as the starting material. Only 20% of the heterodimer reformed following a 2 h incubation in the absence of PNGase F, however. PNGase F treatment had a similar effect on the assembly of hFSH and bLH heterodimers (Figs. 2A and B). Assembly of these deglycosylated heterodimers was at least 70% of the amount of starting materials.

Heterodimer assembly in the presence of PNGase F was accompanied by complete removal of the α2 oligosaccharide (Fig. 3). Assembly of hCG in the absence of PNGase F did not influence the mobility of the α-subunits on SDS–polyacrylamide gels. In marked contrast, the α-subunit isolated from the PNGase F treated samples migrated more rapidly than the native α-subunit, but slower than that expected for completely deglycosylated α-subunit, indicating that one of its oligosaccharides had been removed. The mobility of the β-subunit was not changed, showing that the two N-linked carbohydrates in hCG β-subunits were not accessible to PNGase F under these conditions. Surprisingly, much of the heterodimer remained intact during electrophoresis and its mobility was essentially unchanged, even though it lacked the α2 oligosaccharide. Western blot analysis of hFSH also suggested that one α-subunit oligosaccharide, most likely that on α2, was completely removed during hFSH assembly catalyzed by PNGase F (Fig. 3). Due to the low affinity of A113 for the bovine α-subunit, we were unable to detect the bovine α-subunit by Western blot.

Analysis of the reformed heterodimer by MALDI-TOF mass spectrometry confirmed that only one oligo-

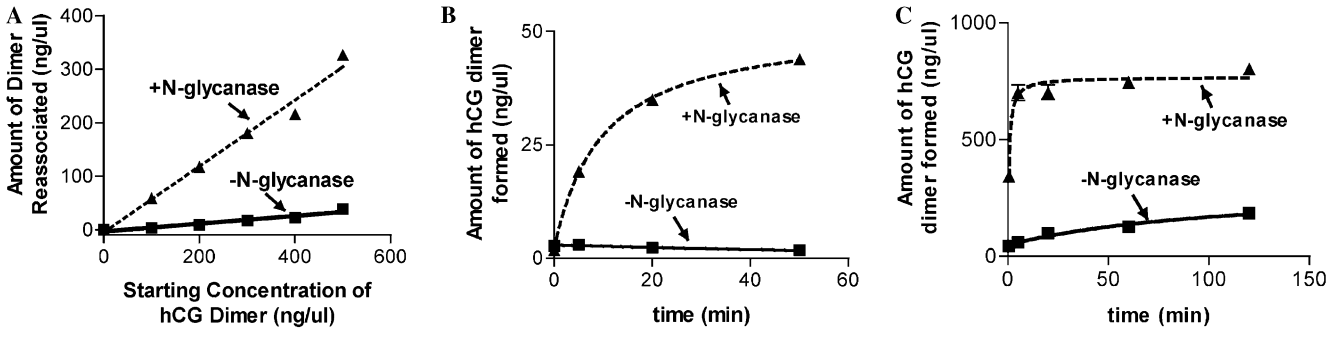

Fig. 1. hCG subunit assembly in the presence and absence of PNGase F was tested at different protein concentrations in a 2 h incubation period (A). Time course of hCG subunit assembly was studied at a protein concentration of 100 ng/μl (B) as well as 1000 ng/μl (C) in the presence and absence of PNGase F.

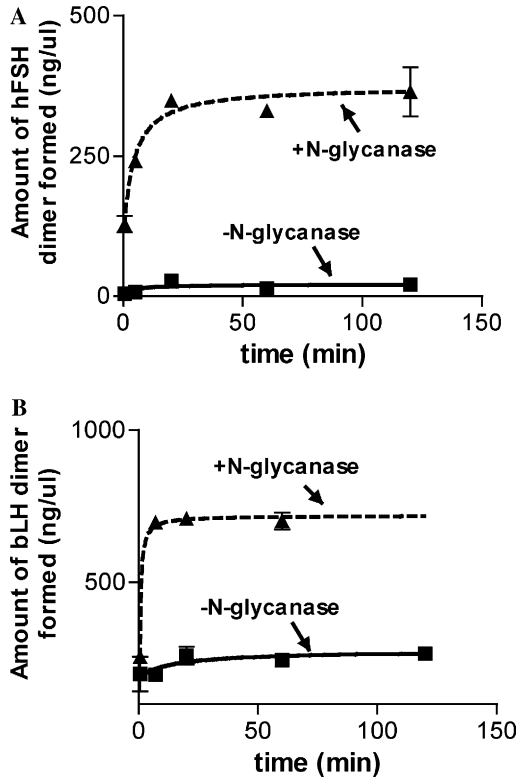

Fig. 2. Time course study of hFSH (A) and bLH (B) subunit assembly in the presence and absence of PNGase F at a protein concentration of 500 and 1000 ng/μl, respectively.

saccharide was removed from the α-subunit (Figs. 4–6). Prior to PNGase F treatment the molecular weight of the α-subunits was roughly 14 kDa and had the wide variation typical of glycoproteins. PNGase F treatment reduced the molecular weights of all three α-subunits by roughly 1600 Da (Figs. 4–6), making the bovine α- and β-subunits readily distinguishable (Fig. 6). The α-subunit isolated from the PNGase F treated heterodimers also had the size distribution typical of that in glycosylated

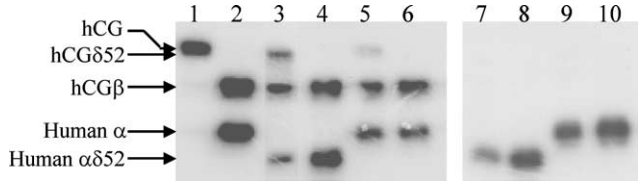

Fig. 3. Western blot analysis of samples from hCG and hFSH subunit assembly in the presence and absence of PNGase F. Lane 1, untreated hCG control; lanes 3 and 5, hCG subunit assembly in the presence and absence of PNGase F; lanes 7 and 9, hFSH subunit assembly in the presence and absence of PNGase F. Lanes 2, 4, 6, 8, and 10 are the corresponding samples for lanes 1, 3, 5, 7, and 9 that had been treated with 10 M urea. The positions of hCG, hCGδ52, hCGβ, human α, and human αδ52 following electrophoresis are indicated. Lanes 1–6 were blotted with radiolabeled α-subunit antibody ^{125}I-A113 and hCG β-subunit antibody ^{125}I-B112. Lanes 7–10 were blotted with ^{125}I-A113.

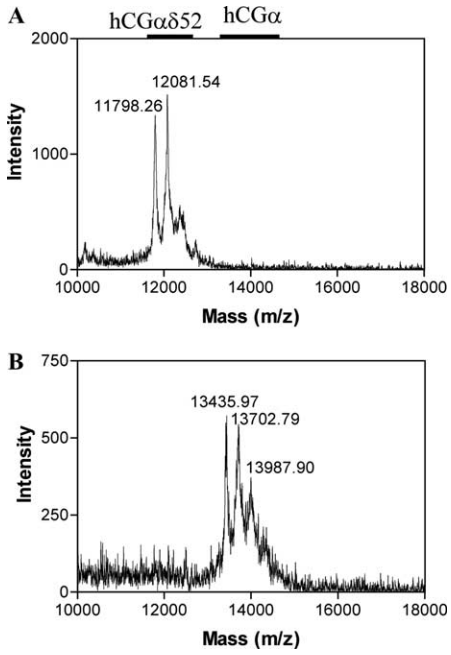

Fig. 4. MALDI-TOF mass spectrometry analysis of samples from hCG subunit assembly in the presence (A) and absence (B) of PNGase F.

proteins, showing that only one of the two α-subunit oligosaccharides had been removed (Figs. 4–6). Indeed, we failed to find the mass peak corresponding to the fully deglycosylated human (10,206) or bovine (10,792) α-subunits.

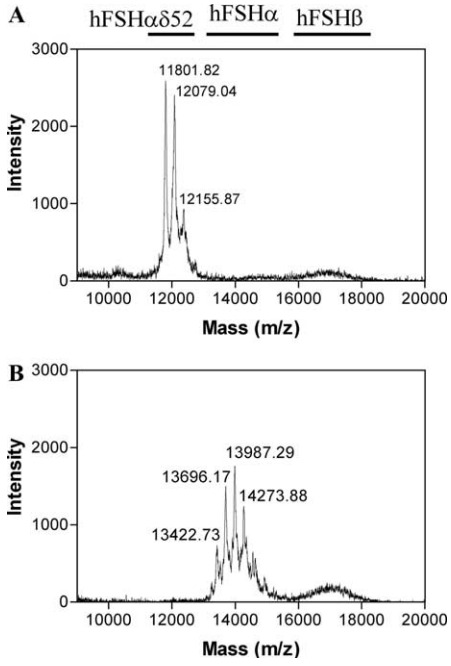

Fig. 5. MALDI-TOF mass spectrometry analysis of samples from hFSH subunit assembly in the presence (A) and absence (B) of PNGase F.

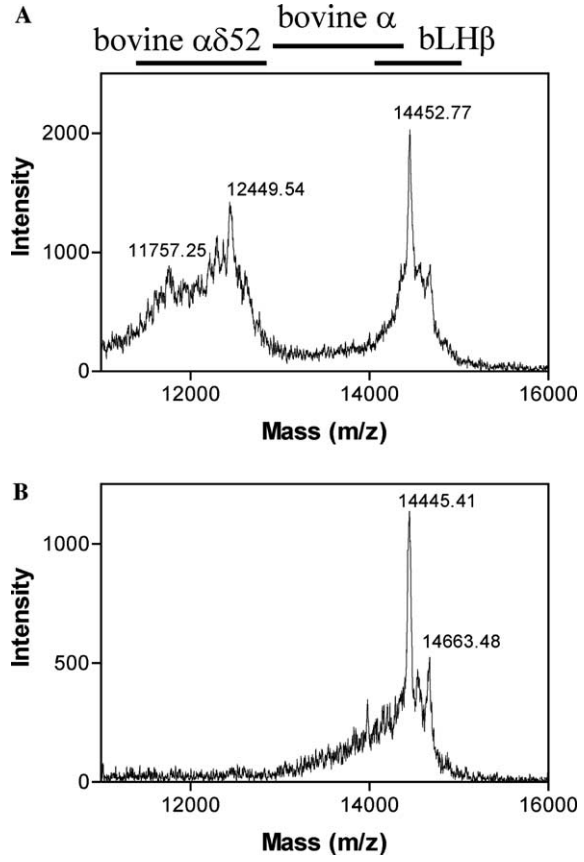

Fig. 6. MALDI-TOF mass spectrometry analysis of samples from bLH subunit assembly in the presence (A) and absence (B) of PNGase F.

Discussion

These observations show that glycoprotein hormone reassembly in the presence of PNGase F can be used to prepare heterodimers that lack one α-subunit oligosaccharide. While we did not attempt to determine which of the two α-subunit oligosaccharides had been removed by PNGase F treatment, it seems almost certain that it was that on α2. Indeed, studies from Bousfield's laboratory have demonstrated the high susceptibility of this oligosaccharide to PNGase F treatment [6]. Since this oligosaccharide must pass beneath the seatbelt and through the hole in the β-subunit during assembly of the heterodimer in vitro, its removal would explain the acceleration of heterodimer assembly we observed. The finding that the β-subunit had the same molecular weight following PNGase F treatment showed that its oligosaccharides had not been altered during the recombination process.

All glycoprotein hormone α-subunits contain an N-linked glycosylation site in loops 2 and 3. In contrast the β-subunits contain N-linked oligosaccharides at one or two sites on the convex surface of the β-subunit loops 1 and 3. While several N-linked oligosaccharides contribute to full hormone efficacy, that in α2 exhibited the greatest influence [13–18] and its removal is likely to be required for preparation of hormone antagonists [19]. Efforts to produce analogs lacking this oligosaccharide in mammalian cells have been hindered by the finding that it has an influence on heterodimer secretion [20]. The observation that digestion of the free α-subunit with PNGase F removes only the α2 oligosaccharide [6,7] suggested that it might be possible to prepare heterodimers lacking this oligosaccharide by dissociating natural heterodimers and treating the resulting subunits with PNGase F to remove the α2 oligosaccharide. The earlier finding that the α2 oligosaccharide must pass beneath the seatbelt during recombination [4] suggested that it may impede the assembly process. Differences in the rates of assembly of the native and α2 deglycosylated α-subunits were expected to favor production of the deglycosylated heterodimer. Consistent with this idea, recombination of dissociated subunits in the presence of PNGase F resulted in heterodimers lacking essentially all the α2 oligosaccharide. As shown here, this simple procedure can be used successfully with as little as 1 μg of starting material. Thus, it should be effective in treating small amounts of analogs that are produced readily in cell culture and thereby facilitate studies of hormone structure and function.

Acknowledgments

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