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1 Undissociable chemically cross-linked and single-chain gonadotropins

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5

6 Abstract

7 Undissociable gonadotropins can be obtained either by chemical cross-linking of the natural heterodimeric
8 hormones or by expressing recombinant single-chain molecules through the fusion of their α and β polypeptide
9 sequences. These undissociable hormones are not more active than their natural heterodimeric counterparts
10 indicating that the β -subunit seatbelt embracing the α -subunit ensures the $\alpha\beta$ heterodimer stability in
11 physiological conditions. The main interests of single-chain gonadotropins are that 1/ only one single plasmid is
12 required to produce an active recombinant hormone, 2/ the two subunits' domains are constantly present in equal
13 amounts and 3/ they remain in close proximity even at low concentration for forming the hormone bioactive 3D
14 structure. These undissociable gonadotropins have been shown to exhibit excellent stability and activity but they
15 have not yet been commercialized probably because of immunogenicity risks and cost of production.
16 Nevertheless, they might be used as a basis for the development of chemically simpler and cheaper ligands of
17 LH and FSH receptors.

18

19 Introduction

20 It has been now known for almost sixty years that ovine Luteinizing Hormone (oLH), also previously named
21 Interstitial Cell Stimulating Hormone (ICSH) in some old papers, is a ~30kD dimer consisting of two non-
22 covalently associated 15kD « monomers » [1]. These monomers were later found to be two dissimilar subunits
23 [2], initially named C1 and C2 and later α and β . Both α and β subunits are devoid of biological activity when
24 alone but recover it when recombined [3]. It was then demonstrated that one of the subunits was common to
25 Thyroid-Stimulating Hormone (TSH) and LH in the bovine species [4, 5]. However, the common human α -
26 subunit from human Chorionic Gonadotropin (hCG α) was found to be able to recombine with either the bovine
27 TSH β -subunit or the bovine LH β -subunit to give active TSH or LH heterodimers respectively [6]. Subsequent
28 studies in 1970-72 showed that the pituitary and chorionic glycoprotein hormone α and β subunits from different
29 mammalian species can recombine into potent chimeric heterodimers [7-11].

30 In the present short review, we will present the quaternary structure of gonadotropins, and address the
31 consequences of the chemical linkage of their subunits or, more recently, the covalent association of their
32 subunits in single-chain recombinant molecules. Finally, we will consider the present and potential advantages
33 and drawbacks of these gonadotropins for clinical or veterinary usage.

34 Quaternary structure of glycoprotein hormones (GPHs)

35 The quaternary structure of glycoprotein hormones is accompanied by changes in the secondary and tertiary
36 structures of their constitutive subunits. The changes in their secondary structures have been initially followed by
37 circular dichroism (CD) [12-18]. Only ~50% of the sequences in glycoprotein hormone were found by CD to

38 adopt a secondary periodic structure (essentially β -sheet and a mere 4% as α -helix), the rest of it being in a non-
39 periodic structure. Their tertiary structures were studied essentially by specific group chemical modifications (for
40 example [19-24]) or by analyzing their epitopes at their surface (for example [25-29]).

41 The dynamic of the three-dimensional structure of glycoprotein hormones has been studied using nuclear
42 magnetic resonance (NMR) [30-33] and isotopic hydrogen exchange (HX) [34]. Only much later, the crystal 3D
43 structure of hCG was disclosed by two independent groups [35, 36]. The most unexpected feature in this
44 structure was the β -subunit peptide (β CTP) embracing its α -subunit counterpart in the heterodimer, forming a
45 « seatbelt » that is fastened by a single intra- β disulfide bridge between Cys β 26 and Cys β 110. Another
46 outstanding feature of these crystal structures is the presence of one three-bridge cystine knot in the α and β
47 subunits [37]. Interestingly, these knots already exist in the α - and β -subunit's glycoprotein ancestors,
48 respectively, named GPA2 and GPB5 [38]. Moreover, the GPB5 possesses a sequence shorter than the
49 β -subunits and, most importantly, does not possess the seatbelt sequence, so it does not naturally form a stable
50 heterodimer with GPA2 [39, 40].

51 The $\alpha\beta$ heterodimeric non-covalent association is mandatory for allowing the expression of the bioactivity
52 specified by the identity of the β -subunit [3, 6, 8-10, 41, 42]. Nevertheless, the specific β -subunits do not express
53 any biological activity when isolated, and their association with the α -subunit, common to all glycoprotein
54 hormones in each species, is indispensable for expressing gonadotropic (LH, FSH) or thyrotropic (TSH) activity.
55 Likewise, the isolated α -subunit which is synthesized in excess relative to the β -subunit does not exhibit any
56 gonadotropic or thyrotropic activity. Nevertheless, it has been shown to exert lactogen activity during embryonic
57 pituitary development [43, 44] as well as in the human placenta [45]. In contrast to the α -subunits combined with
58 β -subunits, the free α -subunit has been shown to bear an O-glycan chain at Thr43 in the bovine pituitary [46]. It
59 is unclear whether this modification is the cause or the consequence of its non-association with a β -subunit [47].
60 Moreover, it is still unclear whether this O-glycan plays a role in the free alpha lactogenic activity [44].

61 **Consequences of chemical linkage of glycoprotein hormone subunits**

62 Since glycoprotein hormones are heterodimeric, it is interesting to question the functional importance of the non-
63 covalent association of their subunits. Our first guess in the 1970s' was that these hormones could act like the
64 cholera toxin, with one subunit allowing membrane binding and the other alone being transferred into the target
65 cells [48]. In such a case, the subunits must dissociate for acting. It was thus interesting to test whether an
66 undissociable glycoprotein hormone lost its bioactivity. Alternatively, considering that the hormone dissociation
67 could play a role in its elimination from blood, it was interesting to test whether an undissociable glycoprotein
68 hormone exhibited enhanced *in vivo* bioactivity. We cross-linked the porcine LH subunits to answer these
69 questions using water-soluble carbodiimide EDC treatment [49]. A porcine LH derivative that was undissociable
70 under urea, guanidinium, low pH, or high pH treatments was obtained and analyzed. Its *in vivo* bioactivity was
71 unchanged, indicating that it does not act through a cholera toxin-like mechanism and does not exhibit a longer
72 half-life in the blood. The same EDC treatment applied to ovine LH led to multiple cross-links and a substantial
73 loss of bioactivity, probably because of the derivatization of multiple NH_3^+ and COO^- groups and/or
74 rigidification of the hormone structure [50]. The difference between porcine and ovine LH is due to the absence
75 of Lys residues in pLH β so that only α -Lys ϵ NH-CO-Glu/Asp- β covalent amide bridge(s) can be formed and not

76 the reverse as in ovine LH. This procedure was also used for bLH, bTSH, and hCG with 0 to 80% activity loss.
77 These losses were due to COO⁻ and/or NH₃⁺ group derivatization and not to the undissociability of hormone
78 structures [51].

79 Are heterodimeric quaternary structures already present in subunits' ancestors GPA2 and GPB5? Recombinant
80 GPA2 and GPB5 form heterodimers only upon reaction with a bifunctional crosslinker and not in its absence
81 [38, 40]. Nevertheless, the recombinant GPB5 expressed alone in a baculovirus/insect cell system has been
82 shown to form homodimers [40]. Thus, it is likely that these molecules do not form stable heterodimers in
83 contrast to glycoprotein hormone subunits because of the absence of the seatbelt sequence in GPB5.

84 Artificial cross-linked GPA2/GPB5 heterodimers have been shown to exhibit TSH-like activity, and the
85 molecule has been named « thyrostimulin » [39]. The reality of GPA2/GPB5 heterodimer formation *in vivo* is
86 still a matter of debate in all animal species. Considering its weak stability, it is likely that GPA2 and GPB exert
87 their still-unknown functions mainly as monomers or maybe as a heterodimer at a short range, i.e., as a paracrine
88 factor [52].

89

90 **The emergence of recombinant single-chain glycoprotein hormones**

91 Although chemically cross-linked glycoprotein hormones provided interesting information, they were not easy to
92 prepare. Most importantly, they were found not to be more or less active than the unmodified natural hormones.

93 The idea of synthesizing recombinant single-chain glycoprotein hormones emerged from studies dealing with the
94 enhancement of the *in vivo* FSH half-life through the fusion of the hCG β CTP at the FSH β C-terminus [53-55].
95 It was later shown that fusion of the hCG β CTP at the h α subunit N-terminus also increased the *in vivo*
96 bioactivity of heterodimers comprising various β -subunits but was deleterious when fused at the h α subunit
97 C-terminus [56]. On this basis, it appeared reasonable to fuse the h α sequence downstream of the glycoprotein
98 hormone β -subunits to get single-chain $\beta\alpha$ hormones. This was first done using the hCG subunits [57-59] and the
99 hCG β subunit CTP extension could act as a hinge between the two. Indeed, with hFSH subunits, introducing a
100 hCG β CTP linker between the β - and α -subunit led to a 3-fold increase in its expression with retention of its *in*
101 *vitro* biological activity [60]. Likewise, with hLH or hTSH subunits, the presence of the hCG β CTP as a linker
102 between the β and α subunits had a favorable effect on their expression and did not alter their *in vitro*
103 bioactivities [61, 62]. Various single-chain glycoprotein hormones (LH, FSH, TSH, CG) have now been
104 obtained in many species and characterized [63-71]. It has been shown that the hCG β CTP linker in hFSH can be
105 replaced by a different sequence including two N-glycan chains. This single-chain « hFSH2N » exhibited *in vivo*
106 activity thanks to its prolonged half-life in blood circulation [72]. Nevertheless, in this paper, the authors have
107 not tested a similar single-chain hFSH with no N-glycans in the linker. It is therefore not possible to distinguish
108 the importance of covalent subunits linkage relative to that of the supplementary N-glycans.

109 Multidomain single-chain molecules comprising several β -subunit sequences fused with one α sequence were
110 also produced and characterized [63, 73, 74]. In this case, the two activities determined by the two β -subunits are
111 expressed by this complex molecule. This dual activity can be due to either a static or dynamic sharing of the

112 sole α -subunit by two fused β -subunits (figure 1). A dynamic interconversion would be dependent on the redox
113 potential which affects the $\text{CysS-SCys} \leftrightarrow 2 \text{CysSH}$ equilibrium controlling the seatbelt opening/fastening [75].

114 Moreover, single-chain molecules comprising the $\beta\alpha$ sequence fused to that of the corresponding receptor were
115 also expressed [76-78], leading to the receptor's sustained stimulation. Further studies in this direction allowed
116 new insights into the structure-function relationships of glycoprotein hormones [79-83] but are outside the scope
117 of the present review.

118

119 **Scaling-up of single-chain glycoprotein hormones production**

120 *In vitro* cell culture production. Different CTPs have been used to optimize the heterodimeric and single-chain
121 hormone secretions [84-86]. The human hCG β CTP has been the most frequently used linker, but the equine
122 LH/CG β CTP was also used in some instances [87]. It is interesting to point out that the pseudo-CTP sequence,
123 downstream of the bovine LH β stop codon also enhanced by about 3-fold the rate and level of secretion from the
124 transfected cells [84]. This bovine pseudo-CTP β or cryptic-CTP β did so despite it contains no O-glycosylated
125 Thr and/or Ser in contrast to the genuine hCG β or eLH/CG β CTPs. Such a linker without glycosylation might be
126 modified to introduce N-glycosylation sites since N-glycosylations are more manageable to synthesize in
127 classical CHO or HEK cells than O-glycosylations. The choice of cells should also be primordial to get the
128 highest possible quantities of secreted glycoprotein hormones. Cell lines such as expiCHO and expiHEK
129 harboring an episomal expression system for recombinant protein production are well suited for this purpose [88,
130 89].

131 Production in rabbit milk. Recombinant single-chain eLH/CG could be expressed at a concentration of 21.7
132 mg/L in rabbit milk [83] and exhibited both *in vitro* LH and FSH bioactivities as expected. However, it showed
133 an extremely rapid clearance ($t_{1/2} \sim 10$ min), which explains its lack of *in vivo* bioactivities. Likely, the glycosyl
134 sidechains introduced by the mammary gland in the $\beta\alpha$ polypeptide chain are recognized by the recipients'
135 macrophages, which rapidly eliminate the hormone from blood circulation [90]. Another difficulty is that if the
136 hormone could leak toward blood, it would alter reproduction and consequently impede milk production in the
137 rabbit. At present, this approach does not seem the most promising for the production of single-chain
138 glycoprotein hormones.

139

140 Production in *Pichia/Spodoptera* system Several groups have produced single-chain glycoprotein hormones
141 [64, 65, 70, 71] in insect cells such as the *Spodoptera frugiperda* cell line using recombinant *Pichia pastoris*
142 baculovirus for infection. Although these recombinant single-chain hormones were active *in vitro*, they were not
143 *in vivo*, most probably because of their rapid removal from blood.

144

145 ***In vivo* biological properties of recombinant single-chain glycoprotein hormones**

146 Although sc-gonadotropins have been shown to be biologically active *in vitro*, it remains a great challenge to use
147 them *in vivo*.

148 Clinical uses: Recombinant FSHs with hCG CTP at their β -subunit C-terminus or as a linker between their β and
149 α have been tested in humans and animals [91-95]. Nevertheless, single-chain gonadotropins have not yet been
150 commercialized for clinical use in humans [96] probably because of potential immunogenicity of these
151 molecules. Indeed, although the hCG CTP is of human origin, it cannot be excluded that the junctions
152 respectively between hFSH β and hCG β -CTP and/or between hCG β -CTP and h α can be immunogenic as they do
153 not exist in the natural hormones (figure 2). Alternative flexible, non-immunogenic sequences [97, 98] might be
154 used in the future to link the two glycoprotein hormone subunits.

155 Another limitation of the development of single-chain gonadotropins in Humans is that they do not offer superior
156 biological properties relative to their heterodimeric counterparts. Their advantage lies in their production: The
157 use of a single plasmid allows 1/ strictly balanced 1 :1 synthesis of the α and β domains (avoiding the useless
158 synthesis of a free subunit in excess) and 2/ their close proximity in the single-chain molecule (allowing their
159 highly efficient association).

160 Veterinary uses. For the time being, there are only a few available commercial single-chain glycoprotein
161 hormones for animals [93, 99, 100], as it is very expensive to develop recombinant gonadotropins specifically
162 for the veterinary market. Nevertheless, a patent for single-chain equine choriogonadotropin derivatives has been
163 recently published [101]. These molecules comprise either one or several additional eCG β carboxy-terminal
164 peptide(s) (CTP) including 12 O-glycosylation sites and/or one or several amino-terminal peptide(s) (NTP) each
165 including four N-glycosylation sites. One of these recombinant single-chain eCG derivatives is expected to be
166 commercialized soon in Argentina and other Latin American countries.

167 The human and animal undissociable gonadotropins have thus been shown to exhibit excellent stability and
168 activity but their commercialization is still very limited probably because of immunogenicity risks and cost of
169 production.

170

171 **Conclusion**

172 Recombinant single-chain gonadotropins or chemically-linked undissociable gonadotropins are highly active but
173 not more than their natural heterodimeric counterparts. This indicates that the β -subunit seatbelt embracing the α -
174 subunit in natural gonadotropins ensures the $\alpha\beta$ heterodimer stability in physiological conditions. The main
175 interests of single-chain gonadotropins are that 1/ only one single plasmid is required to produce an active
176 recombinant hormone, 2/ the two subunits' domains are constantly present in equal amounts and 3/ they remain
177 in close proximity even at low concentration for forming the hormone bioactive 3D structure.

178

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182

183 **Declaration of interest**

184 The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of
185 the research reported.

186

187

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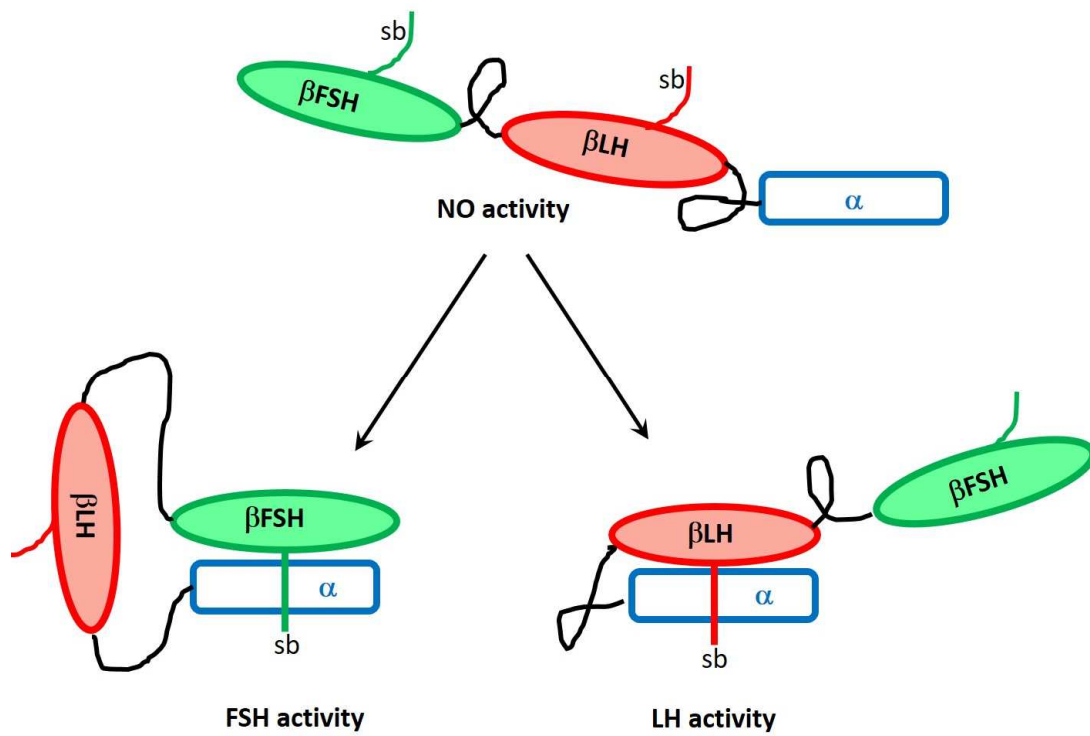
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432 Figures

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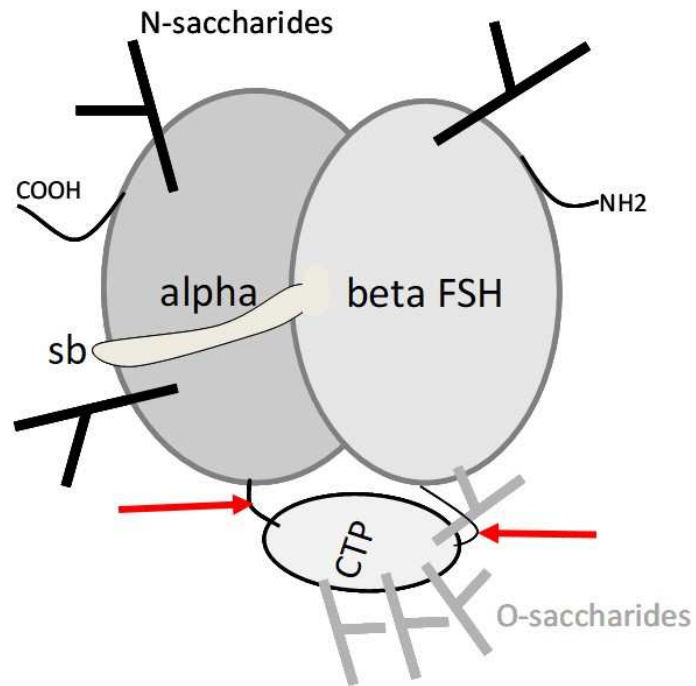


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436 Figure 1 : **Hypothetical structure of multidomain LH + FSH single-chain glycoprotein**

437 Either the β FSH or the β LH domain embraces the α -domain with its « seatbelt » (sb) sequence to form the structures with
 438 FSH or LH activity, respectively. Depending on the physicochemical conditions, particularly the redox potential (allowing
 439 opening and fastening of the « seatbelt latch » formed by the disulfide bridge between Cys β 26 and Cys β 110). The two
 440 bioactive conformations are expected to be either static or dynamically inter-convertible.



441

442 **Figure 2: Schematic structure of single-chain hFSH and potential immunogenic sites (red arrows)**

443 The fusion sequences between the β FSH and CTP and between the CTP and α (red arrows) do not exist naturally and are
 444 thus potentially immunogenic. The β -subunit's « seatbelt » (sb) sequence embracing the α -subunit, present in the natural
 445 hormone, is conserved in the single-chain gonadotropin structures.

446 sb = seatbelt.