Affinity chromatographic separation of gonadotropic hormone agonist and antagonist antibodies

Implications for structure, immunological and biological properties of glycoproteins

M. R. Sairam and L. G. Jiang

Reproduction Research Laboratory, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Quebec H2W 1R7 (Canada)

ABSTRACT

Gonadotropic hormones which have lost peripheral sugar residues in their oligosaccharide chains display antagonistic properties and produce antibodies that fail to recognize the fully glycosylated hormone (agonist form). These polyclonal antibodies were separated by successive affinity chromatography on divinylsulfonyl-Sepharose coupled agonist and antagonist columns. The immunoglobulin G fraction from the agonist affinity columns recognizes both free agonist and antagonist in solution radioimmunoassays and also when these hormone forms are bound to receptors on gonadal cells. However, antagonist-specific antibodies recognize only the free antagonist in solution but not when it is receptor bound, implying that the conformation of the receptor-bound antagonist is different from that of the agonist. Affinity-purified antibodies against the different forms are useful in analyzing immunological and biological properties of the hormones. The studies with these glycosylated hormones serve as a useful model for other glycoproteins of pharmaceutical value.

INTRODUCTION

Receptor–ligand interactions are widely used to design compounds with desired pharmacological properties with either cellular activation (agonistic) or inhibitory (antagonistic) characteristics. For simple ligands such as amino acid derivatives, steroids or even peptides of moderate length, to indicate only a few, the task of designing structural variants is now explored in a systematic manner. This is facilitated by advances in organic synthesis and computer modeling so that appropriate conformations can be selected beforehand for detailed study. For complicated structures such as larger peptides, simple

protein hormones and even more complex glycoprotein hormones such as the gonadotropins, the usual and classical methods of structure–function study and careful analysis in various assays have been the established avenues of choice and are likely to remain so for some time. More and more revelations of the crystal structure of pharmacologically active compounds such as hormones, enzymes and receptors and site-directed mutagenesis approaches offer exciting avenues for rapid exploration in this direction.

Among hormones are many examples in which selected deletions of amino acids by either natural occurrence or deliberate design have produced molecules with altered biological properties. Some examples of these are corticotropin inhibitory peptide and analogues of ACTH and β-endorphin [1,2], recombinant growth hormones [3–5], different forms

---

Correspondence to: Dr. M. R. Sairam, Reproduction Research Laboratory, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Quebec H2W 1R7, Canada.

of thyrotropin in tumor extracts, sera and pituitary extracts [6] and recombinant [7,8] and chemically modified gonadotropins [9,10].

Extensive studies have been conducted in our laboratory emphasizing the hormonal antagonistic properties of deglycosylated gonadotropins [9,11]. Together with reports from other laboratories [10,12–14], these studies have shown that the deglycosylated gonadotropins bind to the cell membrane receptors on target cells but fail to convey the message into the interior of the cell. Post-receptor events, such as second messenger production (viz., cyclic AMP) or end responses such as steroid accumulation in gonadal cells are greatly compromised. Because of their discordant behavior, these variants have displayed antagonistic properties under various experimental conditions. Many biophysical investigations [9,10] have provided suggestive evidence but not proof of significant changes in hormone conformation following loss of peripheral sugars. Immunological analyses of hydrofluoride treated gonadotropins in which the only documented change is the reduction in peripheral sugars with an intact quaternary structure have offered the best evidence of alteration in conformation [15–20]. In this regard, affinity chromatographic separation of agonist and antagonist-specific antibodies from polyclonal antisera have been very valuable in exploring the differences in receptor–ligand interactions and events leading to biological response. These results are highlighted here with selected examples.

EXPERIMENTAL

Highly purified gonadotropin preparations, human choriogonadotropin (hCG), ovine luteinizing hormone (oLH) and ovine follide-stimulating hormone (oFSH), prepared in our laboratory were subjected to one-step chemical deglycosylation with anhydrous HF treatment and further purified by exclusion chromatography on concanavalin A-Sepharose and gel filtration [21]. These steps remove traces of remaining glycosylated hormone. All three deglycosylated hormones (DG-hCG, DG-oLH and DG-oFSH) were used for immunization of male mice, rats and rabbits [17–19]. Radioimmunoassays using the respective ^{125}I-labeled agonist and antagonist variety of hormones revealed differences in

immunological cross-reactivities, which showed the presence of two classes of antibodies in all three species [17–19]. This then led to experiments designed to separate the two sets of antibodies. Affinity chromatography was performed using ligands immobilized on divinylsulfonyl (DVS)-Sepharose according to methods which we have used on previous occasions [22]. This involved coupling the agonist and antagonist forms of each hormone (except for DG-oFSH) to the affinity matrix at pH 9.3. Excellent coupling (80–90%) was achieved within 2–4 h as determined by measuring the absorbance at 280 nm. The columns were extensively washed with 0.025 M Tris–HCl buffer (pH 7.5). Rabbit antisera (5 ml per run) were diluted and fractionated batchwise first on DVS–hormone (agonist) columns and then on DVS–hormone (antagonist) columns. In each instance after eliminating non-volatile buffer salts with 0.05 M ammonium hydrogencarbonate bound immunoglobulin Gs (IgGs) were eluted with 0.1 M ammonia solution at room temperature. The eluted fractions were diluted and lyophilized for storage in powder form at 4°C. Whenever needed, they were weighed accurately on an electrobalance and dissolved in appropriate buffers for immunoassay, bioassays or radiolabeling to examine post-receptor binding phenomena (see Results and Discussion).

RESULTS AND DISCUSSION

Because of their availability, rabbit antisera to DG-hCG and DG-oLH have been studied extensively to obtain the two kinds of antibodies of different specificity. According to the scheme depicted in Fig. 1, two successive affinity chromatographies produced IgG populations designated B and A2. A quantitative evaluation performed using the respective ligands and fractionated antibodies (Table I) reveals a clear separation. In general, the B fraction in each instance could react to the same extent with labeled agonist and antagonists, but the A2 fraction was clearly more discriminatory, showing preferential binding of labeled antagonists.

The antibodies were highly purified, as revealed by protein staining (not shown), and specific when examined by Western blot analysis (Fig. 2). For comparison, in lanes 1 and 2 are shown two oLH antibodies (1 μg) derived from animals immunized

```
            Polyclonal rabbit antiserum
            to DG-oLH, DG-oFSH, DG-hCG
                       |
                       |         First chromatography
                       |         DVS-Sepharose coupled
                       |         oLH, oFSH or hCG
                       |
          +------------+----------------------------+
          |                                         |
          ▼                                         ▼
     Unadsorbed A                        Adsorbed and eluted by NH₃
                                               Fraction B
  Second chromatography |                      Agonist/Antagonist
  DVS-Sepharose coupled |
  DG-LH or DG-hCG       |
          +-------------+----------------+
          |                              |
          ▼                              ▼
     Unadsorbed            Adsorbed and eluted by NH₃
      Discard                    Fraction A2
                               DG-LH specific or
                               DG-hCG specific
```
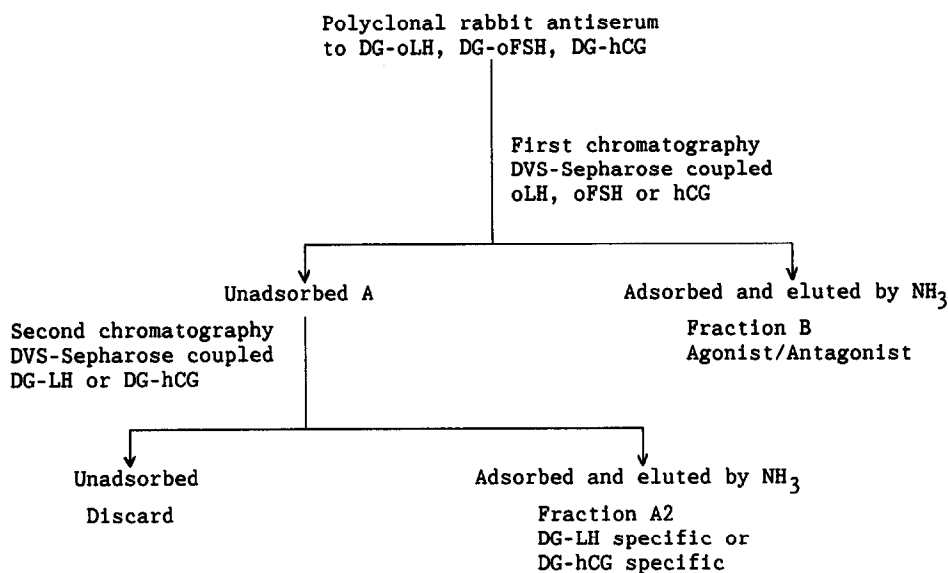
Fig. 1. Affinity chromatographic separation of agonist and antagonist gonadotropin antibodies by successive fractionation of DG-hormone polyclonal antisera on DVS-linked columns of native or DG-hormone. For DG-oFSH antiserum, the IgG in fraction A was processed by ammonium sulfate precipitation. In each example antibody in fraction B shows binding to both [^{125}I]native or -DG-hormone, but antibody in fraction A2 shows preferential binding of [^{125}I]DG-hormone.

TABLE I

BINDING OF LABELED GONADOTROPIN AGONISTS (oLH, oFSH AND hCG) AND ANTAGONISTS (DG-oLH, DG-oFSH, DG-hCG) TO AFFINITY-PURIFIED ANTIBODIES

For determination of binding 100 ng of purified lyophilized antibody (except in experiment 2, where the asterisk denotes the ammonium sulfate fraction) were incubated with the appropriate labeled ligand. In the three experiments the antisera fractionated were of DG-oLH, DG-oFSH and DG-hCG, respectively.

Experiment No.	Fraction	Binding (%)	
		[^{125}I]oLH	[^{125}I]DG-oLH
1	Fraction B	56	36
	Fraction A2	4	36
	Ratio (A2/B)	1:0.07	1:1
		[^{125}I]oFSH	[^{125}I]DG-oFSH
2	Fraction B	34	38
	Fraction A*	2	46
	Ratio (A/B)	1:0.06	1:1.2
		[^{125}I]hCG	[^{125}I]DG-hCG
3	Fraction B	40	41
	Fraction A2	5	50
	Ratio (A2/B)	1:0.125	1:1.2

with oLH. In lanes 3 and 4, containing 1 μg of fractions B and A2, the latter representing DG-oLH antibody reacted very weakly with [^{125}I]oLH compared with the intense reaction in the other lanes. Similar results were obtained with fractions derived from DG-hCG antiserum.

Thus, successive affinity chromatographic steps effected the separation of antibodies that could differentiate between agonist (oLH, hCG and oFSH) and antagonistic forms (DG-LH, DG-hCG and DG-oFSH) of the three hormones. Based on detailed competitive displacement studies we could calculate the percentage cross-reactivities using labeled agonist and antagonists and the respective purified antibodies (e.g., oLH–DG-oLH) (Table II). As similar results were obtained with hCG–DG-hCG and their antibodies also, we are able to generalize that sites in the protein core of the subunits in the quaternary structure of the hormones play a major role in contributing to the distinctive epitopes. We can also conclude that some new epitopes must have arisen after removal of antennary sugars in the N-glycosylation sites of the hormones. This conclusion is reasonable because there has been no documented evidence of nicks in polypeptide structure [15,21] of the two subunits in the hormones. Based on other detailed studies with deglycosylated
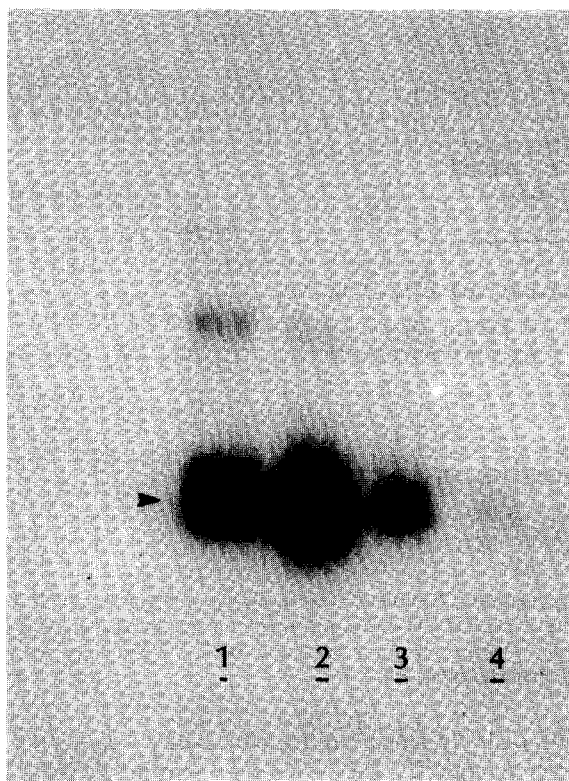
Fig. 2. Western blotting of affinity-purified oLH and DG-oLH antibodies. A 1-μg amount of the lyophilized IgG fraction was subjected to 5–15% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose sheets. Following saturation with 1% skim milk powder, the sheet was allowed to react with $2 \cdot 10^6$ cpm of [^{125}I]oLH in 25 ml of phosphate-buffered saline (pH 7.5) for 16 h. The sheet was then washed and exposed to Kodak X-ray film for 72 h. Lanes 1 and 2 contain affinity-purified oLH IgG, lane 3 IgG of fraction B from DG-LH antiserum (Fig. 1) and lane 4 IgG of fraction A2 of DG-LH antiserum (Fig. 1). Densitometric analysis (with lane 1 as 100%) showed 181% for lane 2, 60% for lane 3 and 5% for lane 4. Arrows show migration of M_r 150 000.

subunits and preferential recombinant hormones [18], we have argued against the direct participation of peripheral sugars in generating new determinants. However, their absence has had the net effect of generating new determinants not previously present in the mature fully glycosylated hormone.

Affinity-purified antibodies are valuable in probing differences in the manner in which agonists and antagonists react with the same receptor. A striking example of such differences is shown in Table III, where we examined antigenic sites that may be available for reaction once the agonist or antagonist has already reacted with the receptor. Rat testicular membranes containing receptors for hCG/LH were first incubated with the unlabeled hormone (100 ng of hCG or DG-hCG) in separate tubes. After removal of the free hormone by centrifugation and washing, the washed H–R complex was incubated a second time with excess of purified antibodies (B or A2 in Fig. 1). A third incubation with ^{125}I-labeled protein A facilitates detection of the bound IgG in the form of an R–H–Ab–protein A* complex. In the example shown, antibody to native hCG (B) reacts with receptor-bound hCG or DG-hCG; however, DG-hCG antibodies (fraction A2) fail to recognize receptor-bound DG-hCG. We interpret these data to mean that occupation of receptor by native hormone (agonist) leaves antigenic sites available to be recognized by B-type antibodies, but when antagonist binds these same antigenic sites are still available but others, which can be recognized by antagonist antibodies, are all masked. Hence, there are distinct differences in agonist–receptor and antagonist–receptor interactions and this may be one of the underlying causes of the loss of signal transduction.

TABLE II

IMMUNOLOGICAL CROSS-REACTIVITY OF AGONISTS AND ANTAGONISTS USING AFFINITY PURIFIED IgGs

These % cross-reactivity calculations are derived from competitive displacement analyses and calculating concentration required for 50% displacement and compared to agonist or antagonist. Purified antibodies were used at 50 ng per tube.

Hormone	Assay type and % cross-reaction			
	[^{125}I]DG-LH/fraction A2	[^{125}I]DG-FSH/fraction A	[^{125}I]LH/fraction B	[^{125}I]oFSH/fraction B
oLH	0		100	
DG-LH	100		100	
oFSH		7.1		100
DG-oFSH		100		82

TABLE III

REACTION OF AFFINITY-PURIFIED ANTIBODIES WITH RECEPTOR-BOUND AGONIST OR ANTAGONIST

This experiment using rat testicular membranes as receptor (R) was performed in three parts. In the first incubation, hormone (H) binding occurs for 16 h at 22°C. During the second incubation, available antigenic sites of the receptor bound hormone react with added antibody (Ab) (10 μg) to form an R–H–Ab complex. In the third incubation, the added ^{125}I-labeled protein A detects the complex via its liaison with Ab to form R–H–Ab–protein A*. For each set, the net radioactivity found in the presence of the native hormone Ab (fraction B) was set as 100% for comparison. The experiment shows that antigenic sites of antagonist are not available (for reaction with fraction A2) once it has reacted with the receptor. (Adapted from ref. 11.)

First incubation, 16 h, 22°C	Second incubation + antibody, 2 h, 22°C	Third incubation, [125I]protein A (cpm ± S.E.M.)[a]	%
R + 100 ng hCG	Fraction B	9955 ± 694	100
R + 100 ng hCG	Fraction A2	–	0
R + 100 ng DG-hCG	Fraction B	10 753 ± 792	100
R + 100 ng DG-hCG	Fraction A2	189 ± 20	1.8

[a] S.E.M. = standard error of the mean (n = 3).

We used the fractionated antibodies in additional studies to show that only B-type IgGs are able to revive signal transduction in DG-hormones bound to cells (Table IV). In these experiments done with mouse Leydig tumor cells, progesterone production is indicative of hormone response. As in Table III, these also include multiple incubations, but in a different format. Following agonist or antagonist binding, the respective antibodies are included in the incubation and after allowing time for interaction at 4°C metabolic activity is resumed at 37°C, a temperature at which steroidogenesis will proceed. As seen in Table IV, only agonist antibodies are able to restore full steroidogenic response in DG-hCG exposed cells. It should be noted that cells incubated with DG-hCG alone or those with added

TABLE IV

ABILITY OF AFFINITY-PURIFIED ANTIBODIES TO REVIVE CELLULAR RESPONSE BY HORMONE ANTAGONIST

These experiments were done in mouse Leydig tumor cells (MA-10) growing in 24-well plates [11] with 10⁵ cells per well. As in Table III, the incubations were performed in three parts. In the first the added hormone (agonist or antagonist) binds to cell membrane receptor for 1 h at 37°C. In the second incubation, the added IgG (normal or affinity-purified fraction, 10 μg each, see Fig. 1) from DG-hCG antiserum reacts for 2 h at 4°C. After a third incubation at 37°C for 2 h (total 5 h), the amount of progesterone accumulated in the medium is determined and represents a measure of response. Note that DG-hCG by itself is inactive (only 3%), but in the presence of fraction B antibody almost complete revival occurs. In treatment 5, when hCG has already initiated action, the addition of fraction B has no diminishing effect. (Adapted from ref. 11.)

Control	Progesterone per well in 5 h (ng)[a]	Response (%) (revival)
(1) Basal (no hormone)	0.15 ± 0.02	
(2) Antibody fraction	0.20 ± 0.03	
(3) 10 ng hCG + 10 μg normal IgG	134.50 ± 4.1	100
(4) 10 ng DG-hCG + 10 μg normal IgG	4.5 ± 0.20	3
(5) 10 ng hCG + fraction B IgG	110.4 ± 3.30	82
(6) 10 ng DG-hCG + fraction B IgG	111.40 ± 4.10	(83)
(7) 10 ng DG-hCG + fraction A2 IgG	2.30 ± 0.30	(1.7)

[a] Mean values ± S.E.M. (n = 3).

normal non-immune IgG also do not cause proges-
terone accumulation in the medium. We infer that
the anchoring of agonist antibodies on the receptor-
bound antagonist (DG-hCG) has substituted for
the loss of antennary sugars and that this in turn
altered the orientation of the hormone–receptor
complex for productive coupling to the effector sys-
tem inside the cell [11]. Others also have made simi-
lar if not identical observations [16,23].

In conclusion, the exercise of separating gona-
dotropin agonist and antagonist antibodies by af-
finity chromatography has shown that peripheral
glycosylation has an effect on the conformation of
biologically active proteins. As many substances of
therapeutic value produced by biotechnology are
glycosylated, due consideration must be given to
the extent of glycosylation of the product produced
in different cellular systems. For example, a chosen
mammalian cell for the production of a therapeu-
tically active substance such as erythropoietin pro-
duced molecules which differed in their antennary
oligosaccharides with biological activity being pro-
portional to the ratios of the different structures
[24]. Because the presence or absence of peripheral
sugar residues could also alter the antigenicity of
the protein, it is best to choose a system which
yields antennary carbohydrates as close as possible
to the natural product. Even in normal physiology,
as shown in the example of glycoprotein hormones,
glycosylation patterns could vary, reflecting differ-
ences in biological potency [11,25].

ACKNOWLEDGEMENTS

REFERENCES

1 C. H. Li, D. Chung, D. Yamashiro and C. Y. Lee, *Proc. Natl. Acad. Sci. U.S.A.*, 75 (1978) 4306.
2 C. Y. Lee, M. McPherson, V. Licko and J. Ramachandran, *Arch. Biochem. Biophys.*, 201 (1980) 411.
3 A. Gertler, A. Shamay, N. Cohen, A. Ashkenazi, H. G. Frie-sen, A. Levanon, M. Gorecki, H. Aviv, D. Hadary and T. Vogel, *Endocrinology*, 118 (1986) 720.
4 A. Ashkenazi, T. Vogel, I. Barash, D. Hadari, A. Levanon, M. Gorecki and A. Gertler, *Endocrinology*, 121 (1987) 414.
5 U. J. Lewis, L. J. Lewis, M. A. M. Salem, N. R. Stalen, S. S. Galosy and G. G. Krivi, *Mol. Cell. Endocrinol.*, 78 (1991) 45.
6 L. Joshi and B. D. Weintraub, *Endocrinology*, 113 (1983) 2145.
7 M. Matzuk and I. Boime, *Biol. Reprod.*, 40 (1989) 48.
8 R. K. Campbell, D. M. Dean-Emig and W. R. Moyle, *Proc. Natl. Acad. Sci. U.S.A.*, 88 (1991) 760.
9 M. R. Sairam, in C. H. Li (Editor), *Hormonal Proteins and Peptides*, Vol. 11, Academic Press, New York, 1983, p. 1.
10 R. J. Ryan, M. C. Charlesworth, D. J. McGormick, R. P. Milius and H. T. Keutmann, *FASEB J.*, 2 (1988) 2661.
11 M. R. Sairam, *FASEB J.*, 3 (1989) 1915.
12 N. K. Kalyan and O. P. Bahl, *J. Biol. Chem.*, 258 (1983) 67.
13 M. Matzuk, J. L. Keene and I. Boime, *J. Biol. Chem.*, 264 (1989) 2409.
14 W. K. Liu, J. D. Young and D. N. Ward, *Mol. Cell. Endocri-nol.*, 37 (1984) 29.
15 H. T. Keutmann, L. Johnson and R. J. Ryan, *FEBS Lett.*, 185 (1985) 333.
16 R. V. Rebois and M. T. Liss, *J. Biol. Chem.*, 262 (1987) 3891.
17 M. R. Sairam, J. Linggen and G. N. Bhargavi, *Biosci. Rep.*, 8 (1988) 271.
18 M. R. Sairam, J. Linggen, J. Sairam and G. N. Bhargavi, *Biochem. Cell. Biol.*, 68 (1990) 889.
19 L. Lamarre and M. R. Sairam, *Mol. Cell. Endocrinol.*, 66 (1989) 181.
20 M. J. Papandreou, I. Sergi, G. Medri, C. L. Jullie, J. M. Braun, C. Canonne and C. Ronin, *Mol. Cell. Endocrinol.*, 78 (1991) 137.
21 P. Manjunath and M. R. Sairam, *Methods Enzymol.*, 109 (1985) 725.
22 M. R. Sairam and J. Porath, *Biochem. Biophys. Res. Com-mun.*, 69 (1976) 190.
23 M. Hattori, T. Hachisu, Y. Shimohigashi and K. Waka-bayashi, *Mol. Cell. Endocrinol.*, 57 (1988) 17.
24 A. Kobata, in *15th International Congress of Biochemistry, Jerusalem, Israel, 1991*, p. 307.
25 C. A. Wilson, A. J. Leigh and A. J. Chapman, *J. Endocrinol.*, 125 (1990) 3.