Characterization of dimeric forms of human pituitary growth hormone by bioassay, radioreceptor assay, and radioimmunoassay

Peter Brostedt*, Marguerite Luthman2, Leif Wide3, Sigbritt Werner2 and Paul Roos1

Department of Biochemistry1, Biomedical Centre, University of Uppsala; Department of Endocrinology2, Karolinska Institute, Stockholm, and Department of Clinical Chemistry3, University Hospital, Uppsala, Sweden

Abstract  Seven highly purified dimeric forms of human pituitary growth hormone, composed of the monomeric forms 20 K hGH, 22 K hGH and 24 K hGH linked together by noncovalent or covalent bonds, have been characterized by an in vitro bioassay (the Nb2 assay), a radioreceptor assay and a radioimmunoassay. Considerable differences in the ability to displace labelled recombinant hGH were observed in the radioreceptor assay. The seven dimeric forms varied over a range between 22 K hGH (most effective) and 20 K hGH. The three covalently-linked dimeric forms had nearly identical affinity constants. The mitogenic action of all but one of the hGH dimers in the Nb2 assay was in the same mutual order as the receptor binding activity in the radioreceptor assay. In the RIA, all dose-response curves were parallel except for those obtained with 20 K hGH and with the dimeric form (20 K-20 K)hGH. In this assay, dimeric variants of the constituents 22 K hGH and 24 K hGH were approximately twice as active as 22 K hGH on a molar basis, suggesting about the same affinity between the antibodies and each of the monomeric forms. Determination of the amino acid compositions of the dimeric forms provided support for their content of monomeric constituents as established earlier by electrophoretic analysis.

The heterogeneity of human growth hormone has been studied during the last three decades and both size and charge pleomorphism have been documented. The major monomeric form consists of 191 amino acids in a single polypeptide chain, with two loops formed by intrachain disulfide bridges (1). It has a molecular mass of 22 000 daltons (22 K hGH) and an isoelectric point of 4.9 (2). Besides direct growth promoting properties, hGH affects lipid and carbohydrate metabolism (both diabetogenic and insulin-like activities), stimulates DNA, RNA, and protein synthesis, and moreover has an intrinsic lactogenic effect (3,4). The 20 000 dalton variant of hGH (20 K hGH) is a product of an alternative mRNA processing (5,6) leading to the deletion of amino acid residues 32-46. Deamidated isomers of both 22 K hGH and 20 K hGH have been isolated (7,8) and the occurrence of a cleaved monomeric form (24 K hGH) has been reported (9). In addition to these monomeric variants, a covalently-linked interchain disulfide dimer has been prepared (10) utilizing methods including a denaturing agent (urea).

Seven highly purified dimeric forms of pituitary hGH (11) were isolated recently, using mild separation conditions to avoid splitting of labile structures and to minimize conformational changes which might influence the activities measured by different assays. In this paper, these seven dimers (four noncovalently-linked and three covalently-linked isomers) are characterized by in vitro bioassay, radioreceptor test, and radioimmunoassay. The bioassay is based on the observation that a rat lymphoma cell line (Nb2) proliferates in the presence of lactogens such as different prolactins and hGH (12,13). The radioreceptor test utilized solubilized bovine liver receptors (14), and in the radioimmunoassay (15) rabbit antiserum to 22 K hGH was used.

*This work was included as part of a doctoral dissertation at Uppsala University, 1989.
The results indicated that the activities varied among the dimers. However, the differences seem to be explainable in terms of a general pattern considering the composition of the dimers and the behaviours of their respective monomeric constituents (20 K hGH and 22 K hGH) in the three assays.

Material and Methods

Isolation of different variants of hGH
22 K hGH was freshly prepared from a lyophilized preparation (7) (clinical grade hGH) by molecular-sieve chromatography (Sephadex G-100, Pharmacia LKB Biotechnology AB, Sweden, equilibrated in 0.04 mol/l ammonium acetate, pH 6.9). From crude dimeric material (7) obtained as a side-fraction during the preparation of Crescormon® (KABI, Sweden), the major form of 20 K hGH was isolated by anion-exchange chromatography and zone electrophoresis in agarose suspension, both done in the presence of urea (16). Prior to the assays the material was dialyzed against 0.001 mol/l NH₄HCO₃, lyophilized and redissolved in 0.1 mol/l potassium phosphate buffer, pH 7.0. The same crude starting material was utilized for the isolation of the different dimeric forms of hGH. However, the purification was, in this case, performed under conditions mild enough to avoid splitting of dimers into monomers. The isolation was achieved by two consecutive chromatographies on anion exchangers followed by zone electrophoresis in agarose suspension and molecular-sieve chromatography (the last step utilizing 0.1 mol/l potassium phosphate buffer, pH 7.0) (11).

Bioassay (Nb2 assay)
Nb2-lymphoma cells were routinely cultured in 75 cm² tissue-culture flasks (Nunc, Kamstrup, Denmark) essentially under the conditions described by Gout et al. (12) (Fischer's medium supplemented with fetal bovine serum (FBS), horse serum and 2-mercaptoethanol). The Nb2 cells were transferred to medium without FBS on the day before assay to slow down the rate of cell division.

The Nb2 assay was performed in 24 multiwell plates (Nunc) following the method of Tanaka et al. (13), except for the use of serum-free medium according to Walker et al. (17). The different samples of hGH to be assayed were diluted to a concentration of 1 mg/l using 0.06 mol/l potassium phosphate buffer, pH 7.0, containing 0.1% bovine serum albumin. Five dilutions of Fischer's medium of each sample were added into the wells in triplicate. The plates were incubated at 37°C in an atmosphere of 5% CO₂ and 95% air for one hour. Approximately 24 h after the change to the FBS-free medium, the Nb2 cells were washed twice with the serum-free medium, diluted to a concentration of 1-2 × 10⁶ cells/l and pipetted into the wells. The final volume in the wells was 2.0 ml and the final hormone concentrations were in the range of 5-165 ng/l.

The number of Nb2 cells, after three days incubation, was counted in a Coulter Counter (Coulter Electronics Limited, Luton, England).

Radioceptor assay
Prior to the radioreceptor assay (RRA), the different hGH samples were desalted on PD-10 columns (Pharmacia LKB Biotechnology AB) equilibrated with 0.025 mol/l TRIS-HCl buffer, pH 7.4, containing 0.01 mol/l magnesium chloride. The RRA was performed with solubilized bovine liver receptors (14). A comparative displacement procedure was used (8,14) wherein increasing amounts (ranging between 0.1 μg-10 mg/l) of the different variants of hGH were allowed to compete with iodine-labelled recombinant hGH (25-I-rhGH, Genotropin®, KABI, Sweden). The labelling was performed using the iodogen method (18), which gave a specific activity of 80 μCi/μg. The specific activity of the radioligand was controlled according to Bürgisser (19) using the approximation of Cheng & Prusoff (20), whereby the molar concentration of the non-labelled form of hGH at 50% inhibition of labelled rhGH shall be equal to the sum of the dissociation constant and the molar concentration of labelled rhGH. Each variant was assayed in 10 concentrations (triplicates). Three independent assays were performed. The incubation time was 16 h at 24°C. The affinity constants were calculated using a computer programme (21).

Radioimmunoassay
The different variants to be assayed were initially diluted with 0.06 mol/l potassium phosphate buffer, pH 7.0, containing 0.2% bovine serum albumin to obtain a hormone concentration of 1 mg/l. Further dilutions were made with 0.05 mol/l sodium phosphate buffer, pH 7.4, supplemented according to Wide et al. (22), and the assays were performed at a level of 0.5-10 μg/l. A competitive solid-phase radioimmunoassay (15) was used whereby the samples were measured using hGH of clinical grade (7) labelled with [125I] and rabbit anti-hGH. The results were expressed in mIU/l using the WHO 1st International Reference Preparation (IRP) of GH, human, 66/217, as a reference. The results were calculated using a computer programme based on a four-parameter logit-log dose-response relationship. Tests of validity (linearity and parallelism) were calculated according to Gaddum (23).

Amino acid analysis
Before analyses, the samples were desalted on PD-10 columns equilibrated with 0.05 mol/l NH₄HCO₃ and lyophilized. Amino acid analyses were performed on an LKB 4151 Alpha Plus amino acid analyzer after hydrolysis in 6 mol/l HCl at 110°C for 24 and 72 h (24). Cystine and methionine were determined after a performic acid oxidation according to Moore (25). About 75 μg of protein
was used for a complete analysis. The tryptophan content was estimated utilizing the fact that the tyrosine and tryptophan residues are the cause for the absorbance at 280 nm, except for an addition from the tertiary structure of the sample. Assuming one tryptophan residue in 22 K hGH (1), the contribution to the absorption from the tertiary structure was calculated to be 12%, which also was used for the dimeric forms, with the exception of the two forms containing 24 K hGH, where the addition was 15% because these forms have a slightly different tertiary structure (9). The absorption coefficient was obtained as the ratio between the absorption at 280 nm (see below) of the sample analyzed and its concentration (including tryptophan) determined from the amino acid analysis data.

**Protein determination**

Ultraviolet absorption spectra of all the variants were measured in the interval 240-340 nm and the light scattering at 280 nm was corrected by linear extrapolation. Corrected values at 280 nm and absorption coefficients determined from the amino acid analysis data were utilized to express concentrations of samples to be assayed on a weight basis (g/l). Conversion to molar basis was obtained using the values 22,000 daltons for 22 K hGH and 24 K hGH and 20,000 daltons for 20 K hGH (1,26).

**Results**

**Amino acid analysis**

The results of the amino acid analyses are presented in Table 1. The compositions are expressed as integral values obtained by normalization of the data to the total number of residues present in the monomeric forms (1,6) and the corresponding numbers for the dimeric forms (Table 2, Constituents). The data in Table 1 agree well with the values given in the literature (1,26) for 22 K hGH and 20 K hGH (these data are given within parentheses). Furthermore, the data support the dimeric combinations (Table 2) established by the electrophoretic analysis.

**Bioassay**

The mutual order of the mitogenic action of the variants of hGH was the same in the three separate experiments and the dose-response curves were parallel within the error of the test (Fig. 1). The dimeric forms composed of two 22 K hGH (No. 3 and 5, Fig. 1 and Table 2, Bioactivity) as well as one of the two (22 K-24 K)hGH forms (No. 6) behaved equivalently to 22 K hGH on a molar basis. The mitogenic actions on the Nb2 cells initiated by 20 K hGH and the variant composed of two 20 K hGH (No. 1, Fig. 1) were only 10% and 30%, respectively, of that observed with 22 K hGH. The highest ability to initiate mitogenic action, about twice that of 22 K hGH, was exhibited by the least acidic of the (20 K-22 K)hGH forms (No. 2, Fig. 1 and Table 2). In contrast, the most acidic of these isomers (No. 4) was only about half as active as 22 K hGH.

**Radioceptor assay**

The abilities of the seven different dimeric forms to displace the labelled hGH varied considerably from 22 K hGH (the most effective displacer) to 20 K hGH (Fig. 2 and Table 2, K<sub>a</sub>). All the displacement curves were parallel. With the exception of the noncovalently-linked (22 K-22 K)hGH form (No. 3, Fig. 2) and the least acidic of the (20 K-22 K)hGH isomers (No. 2), they were all significantly (Student's t-test) less effective than 22 K hGH in inhibiting the binding of labelled hGH. The three covalently-linked dimeric forms (No. 5-7) behaved almost identically with about 20% displacement of the value for 22 K hGH on a molar basis, or 10% on a weight basis.

**Table 1.**

<table>
<thead>
<tr>
<th>Residue</th>
<th>22 K- hGH&lt;sup&gt;b&lt;/sup&gt;</th>
<th>20 K- hGH&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Dimer&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>ASX</td>
<td>20(20)</td>
<td>20(20)</td>
<td>39</td>
</tr>
<tr>
<td>THR</td>
<td>10(10)</td>
<td>10(10)</td>
<td>20</td>
</tr>
<tr>
<td>SER</td>
<td>17(18)</td>
<td>17(18)</td>
<td>32</td>
</tr>
<tr>
<td>GLX</td>
<td>27(27)</td>
<td>22(22)</td>
<td>43</td>
</tr>
<tr>
<td>PRO</td>
<td>9(8)</td>
<td>8(7)</td>
<td>15</td>
</tr>
<tr>
<td>GLY</td>
<td>9(8)</td>
<td>9(8)</td>
<td>22</td>
</tr>
<tr>
<td>ALA</td>
<td>8(7)</td>
<td>7(6)</td>
<td>13</td>
</tr>
<tr>
<td>½-CYS</td>
<td>4(4)</td>
<td>4(4)</td>
<td>8</td>
</tr>
<tr>
<td>VAL</td>
<td>7(7)</td>
<td>7(7)</td>
<td>15</td>
</tr>
<tr>
<td>MET</td>
<td>3(3)</td>
<td>3(3)</td>
<td>6</td>
</tr>
<tr>
<td>ILE</td>
<td>8(8)</td>
<td>7(7)</td>
<td>14</td>
</tr>
<tr>
<td>LEU</td>
<td>24(26)</td>
<td>23(25)</td>
<td>48</td>
</tr>
<tr>
<td>TYR</td>
<td>7(8)</td>
<td>5(6)</td>
<td>11</td>
</tr>
<tr>
<td>PHE</td>
<td>12(13)</td>
<td>11(12)</td>
<td>23</td>
</tr>
<tr>
<td>HIS</td>
<td>3(3)</td>
<td>3(3)</td>
<td>6</td>
</tr>
<tr>
<td>LYS</td>
<td>9(9)</td>
<td>8(7)</td>
<td>14</td>
</tr>
<tr>
<td>ARG</td>
<td>11(11)</td>
<td>10(11)</td>
<td>21</td>
</tr>
<tr>
<td>TRP</td>
<td>1(1)</td>
<td>1(1)</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> See Table 2 for identification of the dimeric forms. <sup>b</sup> The values within parentheses are from the literature (1,26).
Table 2.
Parameters determined for the hGH variants.

<table>
<thead>
<tr>
<th>hGH variant (number)</th>
<th>Constituents</th>
<th>Type of binding</th>
<th>pIa</th>
<th>kb</th>
<th>Bio-activity</th>
<th>k_d/mol ± SEMb</th>
<th>Immuno-activity IU mol⁻¹ · 10⁻⁸</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(20 K-20 K) hGH</td>
<td>noncovalent</td>
<td>5.5</td>
<td>0.70</td>
<td>0.34</td>
<td>1.5 × 10⁸ ± 0.11 × 10⁸</td>
<td>(0.64)</td>
</tr>
<tr>
<td>2</td>
<td>(20 K-22 K) hGH</td>
<td>noncovalent</td>
<td>5.2</td>
<td>0.73</td>
<td>2.5</td>
<td>2.6 × 10⁸ ± 1.2 × 10⁸</td>
<td>0.71</td>
</tr>
<tr>
<td>3</td>
<td>(22 K-22 K) hGH</td>
<td>noncovalent</td>
<td>0.76</td>
<td>1.2</td>
<td>3.7 × 10⁹ ± 1.8 × 10⁹</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>(20 K-22 K) hGH</td>
<td>noncovalent</td>
<td>0.73</td>
<td>0.45</td>
<td>4.6 × 10⁸ ± 0.85 × 10⁸</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>(22 K-22 K) hGH</td>
<td>covalent</td>
<td>0.76</td>
<td>1.2</td>
<td>1.1 × 10⁹ ± 0.22 × 10⁹</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>(22 K-24 K) hGH</td>
<td>covalent</td>
<td>4.9</td>
<td>0.99</td>
<td>1.1 × 10⁹ ± 0.20 × 10⁹</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>(22 K-24 K) hGH</td>
<td>covalent</td>
<td>0.76</td>
<td>0.27</td>
<td>1.1 × 10⁹ ± 0.26 × 10⁹</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>22 K hGH</td>
<td>covalent</td>
<td>4.9</td>
<td>0.76</td>
<td>1.0</td>
<td>5.7 × 10⁹ ± 0.81 × 10⁹</td>
<td>0.52</td>
</tr>
<tr>
<td>9</td>
<td>20 K hGH</td>
<td>noncovalent</td>
<td>5.5</td>
<td>0.70</td>
<td>0.13</td>
<td>4.6 × 10⁷ ± 0.39 × 10⁷</td>
<td>(0.29)</td>
</tr>
</tbody>
</table>

a Isoelectric point; decreasing values in the direction of the arrow. The values estimated from analytical isoelectric focusing in agarose gel with standard proteins (Pharmacia LKB Biotechnology AB) as markers. b Absorption coefficient (A₂₈₀, 0.1% (w/v) solution, 1 cm) obtained from amino acid analysis. c Relative bioactivity, the rate of proliferation of Nb2 cells/mol of 22 K hGH was arbitrarily defined as 1. d Based on 3 independent observations.

Fig. 1.
Proliferation of Nb2 cells stimulated by nine variants of hGH (dimer 1 □, dimer 2 ○, dimer 3 ●, dimer 4 ◊, dimer 5 ◇, dimer 6 ▲, dimer 7 △, 22 K hGH ★, 20 K hGH □; see Table 2 for compositions). Each point represents the mean of triplicates. The figure illustrates one of three separate experiments.
Fig. 2.
Radioreceptor assay. Comparative displacement experiments using bovine liver receptors wherein 125I-rhGH were displaced by nine variants of hGH (dimer 1 □, dimer 2 ●, dimer 3 ○, dimer 4 ◦, dimer 5 ◆, dimer 6 ▲, dimer 7 △, 22 K hGH ★, 20 K hGH □; see Table 2 for compositions). The figure illustrates the mean of three separate experiments.

Radioimmunoassay
The dose-response relationships for the reference standard (1st IRP of hGH, 66/217) and the nine hGH preparations plotted on a logit-log scale are shown in Fig. 3. The dose-response curves for two preparations, 20 K hGH and (20 K-20 K)hGH (Dimer 1), were not parallel (p<0.001) to that of the standard. The immunoactivities of the hGH preparations expressed as IU · mol⁻¹ · 10⁻⁸ are given in Table 2. The immunoactivities of the preparations 20 K hGH and (20 K-20 K)hGH could not be determined accurately and the values given within parentheses are estimates at 50% inhibition in the RIA.

Discussion
Based on the observation that the rat lymphoma cell line (Nb2) proliferates in the presence of prolactin (12), Tanaka et al. (13) developed a specific and sensitive bioassay for lactogens, including hGH, which is known to have an intrinsic lactogenic activity. The hormones initiate the mitogenic action on the Nb2 cells by binding to a lactogen type of receptor (27). Recently, a serum-free medium was designed which supported the growth of the Nb2 cells in the presence of hGH (17). By using this medium unwanted interference by serum factors was avoided. In the present study the mitogenic action on the Nb2 cells of variants of hGH was investigated using serum-free medium. The ability of the Nb2 cells to proliferate in the presence of lactogens has been used previously in studies on some variants of hGH; the 20 K hGH form, a subtilisin-cleaved two-chain form, and different fragments of hGH (28). The sensitivity of the Nb2 assay in serum-free medium, where concentrations as low as 5 ng/l (about 0.2 pmol/l 22 K hGH) were measurable (Fig. 1), was fully comparable to the results reported by Tanaka et al. (13) using medium supplemented with horse serum.

It has been suggested that bovine liver receptors
are mainly of somatogenic character, since both rat and bovine GH displaced labelled hGH from these receptors more easily than from rat and rabbit liver receptors, which have both somatogenic and lactogenic properties (29,30). The receptor material (14) used in the present study (14) has not been characterized with regard to different types of binding sites.

Comparative displacement data in the RRA showed (No. 8 and 9, Table 2) that the difference in effectiveness between 22 K hGH and 20 K hGH to displace $^{125}$I-rhGH was about 100-fold. This is in the same range as reported earlier with bovine liver receptors (14). On the contrary, the biological potencies of these two variants are reported to be similar in the hypophysectomized rat weight-gain and tibial bioassays (16), and in the Nb2 assay an approximately 10-fold difference was obtained (Table 2). This latter value for 20 K hGH is in the same range as the value reported by Emoto et al. (28), where the response in the Nb2 assay was expressed in terms of DNA synthesis. Furthermore, the interchain disulfide dimer isolated by Lewis et al. (10) had low growth-promoting activity (about 20% of the activity of 22 K hGH on a molar basis) determined by the weight-gain bioassay, and the activities of equivalent dimeric forms in the present study (Table 2, $K_a$, No. 5-7) were also close to 20% of the value for 22 K hGH. The relationship between biological responses (in vivo and in vitro) and binding to hepatic receptors is obviously not simple.
Taken together, the results indicate (in all three assays) that the presence of 22 K hGH in the variant tested means that the response can be classified as high, whereas the presence of 20 K hGH means a low response. (This generalisation has a few exceptions which will be discussed).

In the Nb2-assay variants No. 2, 3, 5, 6, and 8 (Table 2) follow the primary rule, whereas variant No. 4 ((20 K-22 K)hGH), is an exception where the influence of 20 K hGH seems to be predominant. Further variants with low activity are consistently variants No. 1 and 9 (Table 2). The difference in activity between the isomeric variants No. 2 and 4 might be due to the charge differences (different pI-values) and/or conformational differences. Variant No. 7 does not fit this scheme, which might depend on an error in the concentration determination because the absorbancy of this sample was the lowest among the variants studied (A290=0.045).

Inspection of the values in Table 2 reveals that the primary rule holds regardless of whether the monomers are covalently- or noncovalently-linked or whether or not the cleaved form, 24 K hGH, is involved.

In principle, the results of RRA (Table 2) agree fully with those obtained in the Nb2 assay. The only exception is that the value obtained for variant No. 7 here is in accordance with the main rule.

The general rule is valid also in the RIA (Table 2 and Fig. 3). The antibodies utilized were primarily directed towards 22 K hGH and therefore the variants 20 K hGH and (20 K-20 K)hGH could not be accurately evaluated. There was, however, obvious cross-reactivity with 20 K hGH with a modified slope of the dose-response curve (Fig. 3). DimERIC variants of the composition (20 K-22 K)hGH (No. 2 and 4) did not deviate in behaviour, but showed comparatively low activities. On a molar basis, the dimeric variants (No. 3, 5, 6, 7) consisting of (22 K-22 K)hGH or (22 K-24 K)hGH were approximately twice as active as 22 K hGH, suggesting equal affinity of the antibodies toward each of the monomeric constituents.

A large number of monomeric and dimeric variants of hGH have been isolated and a diversity of biological effects (3,4) have been attributed to monomeric hGH, whereas dimeric variants have been studied only slightly (10) owing to shortage of purified material. As some variants of hGH occur in the pituitary predominantly in dimeric forms and it were split during the isolation procedures (8,9,26) seems consistent to attach more importance to in vivo bioassays of dimeric hGH.

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References

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Dr Paul Roos,
Department of Biochemistry,
Biomedical Centre,
University of Uppsala,
Box 576,
S-753 21 Uppsala,
Sweden.