Increased stability of human growth hormone with reduced lactogenic potency

Alexey A. Schulga[a], Alexander A. Makarov[b,*], Iliya V. Levichkin[a], Yuliya V. Belousova[c],
Vladimir M. Lobachov[b], Irina I. Protasevich[b], C. Nick Pace[d], Mikhail P. Kirpichnikov[a]

[a]*Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Miklukho-Maklaya St. 17/10, Moscow 117997, Russia*
[b]*Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Vavilov St. 32, Moscow 119991, Russia*
[c]*Research Center of Molecular Diagnostics and Therapy, Simpheropolsky Blvd. 8, Moscow 113149, Russia*
[d]*Department of Medical Biochemistry and Genetics, Texas A&M University, College Station, TX 77843, USA*

Abstract Human growth hormone (hGH), whose main function is the somatic growth stimulation, induces diverse effects including lactation. We examined the possibility of hGH stabilization by elimination of its lactogenic activity. Chimeric GHs were constructed by replacement of different segments of hGH with sequences derived from non-lactogenic porcine GH. As was observed in the rat Nb2-11C lymphoma cell test, lactogenic activity of some chimeric hormones was seriously destroyed. This kind of hormones displayed the substantial increase in thermal and guanidine hydrochloride stability. The more stable hGH variants were found to be more soluble in *Escherichia coli* cells. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

1. Introduction

Human growth hormone (hGH, somatotropin) is a single chain polypeptide hormone consisting of 191 residues with two disulfide bonds. Its main function is the stimulation of somatic and bone growth, as well as an increase in the size and mass of organs and tissues. In addition, it influences protein, carbohydrate, and lipid metabolism. A unique peculiarity of human and primate growth hormones (GHs) is the broad species specificity and the capability of a direct effect on the mammary gland cells by increasing RNA and protein synthesis (lactogenic activity) [1]. Practically all of the recombinant hGH produced by bacteria at high temperature (37°C) is localized in large insoluble aggregates, the so-called inclusion bodies [2], which pose a serious obstacle to efficient hGH production for biomedical use. One of the ways of solving the problem of hGH aggregation in vivo is to increase its intrinsic stability so as to compensate for the lack of disulfide bonds in bacterial cytoplasm.

Numerous effects caused by hGH are based on its ability to bind to specific receptors on the target cell surfaces. Evidently, the hGH binding interfaces have evolved to make favorable intermolecular contacts with the receptors. This may be at the expense of intramolecular interactions and burying hydrophobic residues, and so lower the hGH conformational stability [3]. Indeed, there are many examples showing that the replacement of the ligand binding residues results in increased protein stability [4,5]. Then, attenuation of one of the hGH minor activities might lead to a stabilization of its structure. To check such a possibility, we have constructed a series of chimeric GHs with homologous replacements in hGH based on the structure of porcine growth hormone (pGH). Since the latter is devoid of lactogenic activity, we expect that the activity will be lost in some of the chimeric hormones. The primary structures of human and porcine GHs are highly homologous (67%) and their tertiary structures are similar. Therefore, it may be anticipated that the mutations introduced into the hGH molecule will not significantly change its conformation. This work reports a study of lactogenic activity and stability of chimeric hormones '14–33', '41–73', '14–95', and '78–95'. Figures designating chimeric GHs correspond to the residues from the pGH sequence that are inserted into hGH; the numbering follows the hGH sequence. In accordance with this, variant '14–33' is a hGH in which the region flanked by residues 14 and 33 is replaced with a similar region of pGH. We show that the elimination of lactogenic activity results in stabilization of hGH molecule and that intracellular hormone solubility is determined by its stability.

2. Material and methods

2.1. Gene expression and protein purification

Genes for chimeric GHs were constructed by the 'homolog recombination' method [6]. The nucleotide sequences of GHs genes were determined in both directions. Expression plasmids for GHs were constructed by replacement of gene 10 in pGEMEX1 (Promega) with a GH gene [2]. Proteins were purified from the *Escherichia coli* strain BL21(DE3) harboring the corresponding expression plasmid [7].

2.2. Nb2-11C cell proliferation assay

Synchronization of Nb2-11C cells in the G_0/G_1 phase and monitoring of cell proliferation were performed as described earlier [8]. The doubling rates were calculated after 72 h from the following equation: No. of doublings = log [(no. of cells in the presence of a hormone)/(no. of cells in the absence of a hormone)]/log2. The initial number of cells/ml was 250 000.

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*Corresponding author. Fax: +7-095-135 14 05.
E-mail address: aamakarov@genome.eimb.relarn.ru (A.A. Makarov).

2.3. Differential scanning calorimetry (DSC)

Microcalorimetric measurements were carried out on a DASM-4 microcalorimeter (NPO Biopribor, Pushchino, Russia) in 0.48 ml cells at a heating rate of 1 K/min on 0.4–0.8 mg/ml proteins in 10 mM Na_2HPO_4, pH 7.5. The partial molar heat capacity of the protein (C_p), denaturation temperature (T_d) and calorimetric denaturation enthalpy (ΔH_{cal}) were determined as described elsewhere [9]. The accuracy of the calorimetric enthalpy was 6%, that of T_d within 0.2°C.

2.4. Circular dichroism (CD)

Far-UV CD spectra were recorded on a Jasco J-715 spectropolarimeter equipped with Neslab RTE-111 water bath in a 0.02 cm cell on 0.3 mg/ml proteins in 10 mM Na_2HPO_4, pH 7.5. Protein melting was carried out at the same heating rate as in microcalorimetry (1 K/min). Denaturation temperatures were determined from the peaks in the first derivatives of the melting profiles (accuracy 0.3°C). The results were expressed as molar ellipticity, [Θ] (deg cm^2 dmol^{-1}), based on a mean amino acid residue weight of 115.7 for hGH, 113.1 for variant '14–95', 115.1 for variant '14–33', 114.3 for variant '41–73' and 115.5 for variant '78–95'.

2.5. Equilibrium guanidine hydrochloride (GdnHCl)-induced denaturation

Denaturation experiments were performed at 25°C in 10 mM Na_2HPO_4, pH 7.5, at a final protein concentration of 0.2 mg/ml. The GdnHCl concentrations were determined by refractometry. The unfolding transition was monitored by CD at 222 nm.

3. Results and discussion

3.1. hGH and chimeric GHs lactogenic activity

Lactogenic activity of GHs was determined by an increase in the amount of preliminarily synchronized cells of the rat lymphoma line Nb2-11C in response to the preparation under study. Hormones devoid of lactogenic activity are not able to stimulate proliferation of this cell line [10]. The results of measurements of proliferative activity are shown in Fig. 1. It is seen that activity of GHs '14–33' and '78–95' do not differ from that of hGH. The '14–95' GH is devoid of ability to stimulate cell proliferation in the whole interval of concentrations from 0 to 500 pM. The '41–73' GH activity is markedly decreased. Thus, cells begin to proliferate only at the hormone concentration of 8 pM (such a 'threshold' concen-
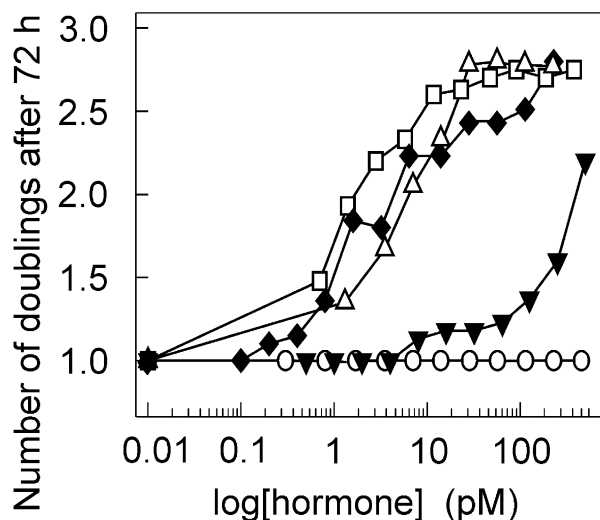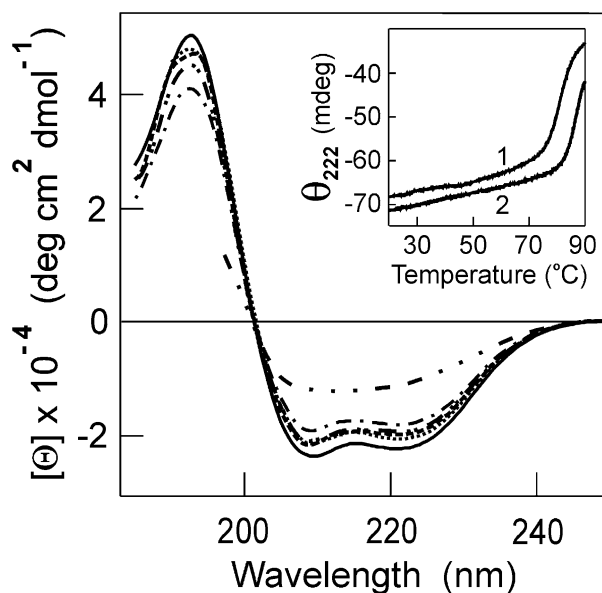

Fig. 2. CD spectra of hGH (double chain line – 20°C, triple chain line – 90°C) and chimeric GHs '14–33' (solid line), '14–95' (dotted line), '41–73' (dashed line) and '78–95' (chain line) at pH 7.5, 20°C. Inset: Temperature dependence of CD at 222 nm for hGH (1) and chimeric GH '14–33' (2).

tration for hGH is 0.2 pM), whereas cell doubling in 72 h is observed at the '41–73' GH concentration of 440 pM (and at 2 pM hGH). It is quite obvious that such a drastic decrease in the hormone activity is due to mutations in the 41–73 region.

The '14–33' variant is equipotent to hGH in Nb2-11C bioassay (Fig. 1). Moreover, substitution of the segment 8–19 in hGH for homologous segment derived from pGH affects neither the proliferative activity in Nb2-11C bioassay, nor the binding affinity for the Nb2 prolactin receptors [8]. Thus, the homologous substitutions in the hGH segment 8–33 do not influence the hGH binding to Nb2 rat prolactin receptors. In contrast, according to [11], the hGH mutant containing segment 11–33 of pGH causes tremendous disruption in binding affinity for the human prolactin receptor. Similarly, introduction of Lys168Ala/Glu174Ala mutations into hGH caused more than 6000 times decrease in its activity in the test of FDC-P1 cells transfected with the full-length human prolactin receptor and only 12 times decrease in the Nb2-11C test if the half-maximal concentrations for stimulation of cell proliferation (EC$_{50}$) were compared [12]. Altogether, this suggests that the functional determinants for the hGH binding to the human and rat prolactin receptors do not coincide. The complete loss of the '14–95' GH activity and preservation of that of '14–33' and '78–95' GHs in the rat lymphoma Nb2 test mean that the region 34–77 contains amino acid residues that are critical for the interaction of hGH with the rat prolactin receptors.

3.2. Secondary structure and thermostability of hGH and chimeric hormones

To study conformational changes in chimeric proteins, CD in the far-UV region was used. The CD spectrum of hGH (Fig. 2) is typical of a protein with a high content of α-helices and coincides with the previously published data [13]. All spectra of hybrid proteins are practically identical in shape

Fig. 1. The effect of various concentrations of hGH (□) or chimeric GHs '14–95' (○), '14–33' (△), '41–73' (▼) and '78–95' (◆) on the proliferation rate of Nb2-11C lymphoma cells.
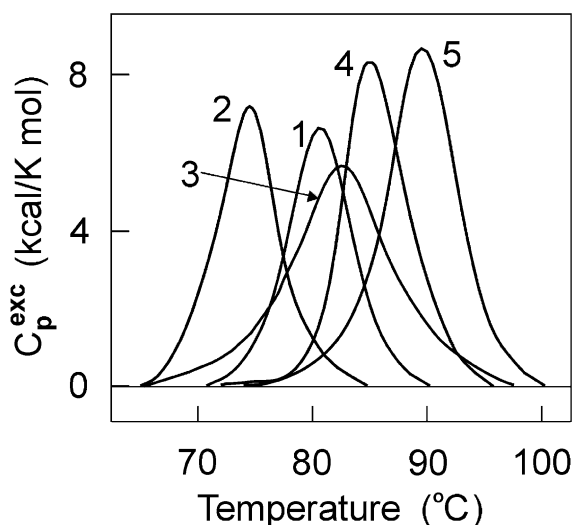
Fig. 3. Temperature dependence of the excess heat capacity of hGH (1) and chimeric GHs '78–95' (2), '41–73' (3), '14–95' (4) and '14–33' (5) at pH 7.5.

to that of hGH. However, the spectral amplitudes in characteristic minima and maximum are different, i.e. chimeric hormones and hGH are characterized by a different helical content (Table 1).

The effect of mutations on the parameters characterizing the thermal denaturation of the hormone was studied using DSC. Fig. 3 shows the temperature dependence of the excess heat capacity of hGH and chimeric hormones at pH 7.5. In all cases denaturation was irreversible. Partial heat capacities of hGH and hybrids at 25°C are the same and equal to (0.34 ± 0.02) cal/K·g. This value is typical of compact globular proteins [9]. The calorimetric enthalpy of hGH denaturation (Fig. 3, curve 1) is 48 kcal/mol that is more than two times lower than mean values of denaturation enthalpy corresponding to full unfolding of the near-size proteins [9]. A similar result was obtained for hGH in the acid pH interval [14]. The midpoint of the hGH thermal denaturation was equal to 81°C. For GHs '41–73', '14–95' and '14–33', denaturation temperature increases for 2.7, 4.0 and 8.3°C, respectively, whereas for '78–95' it decreases for 6°C (Table 1).

To collate the thermodynamic and structural data for hGH and '14–95', the temperature dependence of the CD at 222 nm was measured. hGH undergoes a cooperative transition at 81.2°C, whereas it occurs at 86.6°C for '14–95' (Fig. 2, inset). These values are very close to the corresponding T_d values obtained using scanning microcalorimetry. The CD amplitude

Table 1
Content of α-helices, temperature of heat denaturation and GdnHCl concentration at $D_{50\%}$ for hGH and chimeric hormones at pH 7.5

Hormone	f_H[a]	T_d (°C)[b]	$D_{50\%}$ (M)[c]
'14–33'	0.66	89.3	4.77
'14–95'	0.60	85.0	4.85
'41–73'	0.58	83.7	4.54
hGH	0.56	81.0	4.54
'78–95'	0.52	75.0	4.33

[a]Calculation of α-helix content (f_H) was based on equation $f_H = -([\Theta]_{222}+2340)/30\,300$ [21]. The estimated uncertainty is 3%.
[b]The estimated uncertainty is 0.2°C.
[c]The estimated uncertainty is 1%.

for hGH at 90°C made up about 50% of initial level (Fig. 2). Similar results were obtained in [14] at acid pH. This is indicative of preservation of a significant part of secondary structure in the denatured protein and supports calorimetric data also pointing to a partial unfolding of hGH during heat denaturation due to aggregation.

3.3. Equilibrium denaturation of hGH and chimeric hormones by GdnHCl

The GdnHCl denaturation of GHs was extensively studied [15–17]. Non-human species of GH were characterized by the presence of the unfolding intermediates having a tendency to self-associate. At low protein concentrations (≤ 0.2 mg/ml), the hGH denaturation, studied by various techniques, was shown to be completely reversible and consistent with the two-state mechanism. However, at higher protein concentrations there is the effect of protein concentration on the free energy of unfolding due to the presence of self-associating unfolding intermediates [18].

The GdnHCl denaturation data for hGH and chimeric hormones are presented in Fig. 4. The S-shaped dependences of CD intensities on GdnHCl at 222 nm are indicative of a highly cooperative melting process [9]. Mutations in hGH resulted in an increase of the GdnHCl concentration at 50% protein unfolding ($D_{50\%}$) for GHs '14–33' and '14–95' (Table 1).

3.4. Relationship between inclusion bodies formation in E. coli cells and GHs stability

It is well known that the reducing milieu of bacterial cytoplasm prevents disulfide bond formation in proteins [19]. At the same time, reduction of disulfide bridges in hGH significantly lowers its stability and results in an increased tendency for self-association as compared with the disulfide-containing hGH molecules [20]. This may have relevance for the formation of inclusion bodies during the heterologous expression of hGH gene in E. coli.
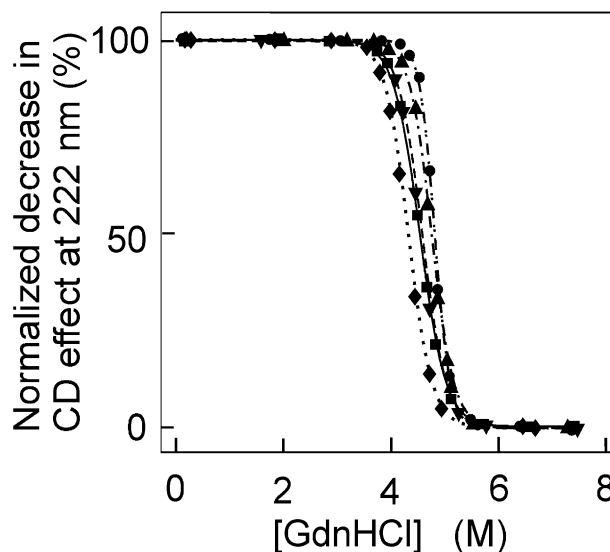

Fig. 4. The GdnHCl-induced equilibrium denaturation of hGH (■, solid line) and chimeric GHs '78–95' (♦, dotted line), '41–73' (▼, dashed line), '14–33' (▲, chain line) and '14–95' (●, double chain line) at 25°C, pH 7.5, as detected by CD at 222 nm. Protein concentration was 0.2 mg/ml.

Table 2
The proportion (%) of soluble GHs at different growth temperatures, determined from SDS–PAGE of native lysis supernatants and total cell protein

Hormone	24°C	30°C	37°C	42°C
'14–33'	100	100	7	nd
'14–95'	100	100	100	20
'41–73'	100	100	30	5
hGH	100	25	5	nd
'78–95'	30	20	0	nd

nd – not determined.

We compared the tendency of the wild-type and chimeric GHs to form inclusion bodies in bacteria. Soluble and total cellular fractions were prepared and analyzed by SDS–PAGE (Table 2). All the proteins were produced at the same level (about 300 mg/l). At 37°C about 95% hGH is localized within a cell as insoluble aggregates. As growth temperature decreases, a soluble protein fraction is increased, and at 24°C hGH is mainly accumulated in a soluble form in the cytoplasm. On the contrary, pGH is found in inclusion bodies in the whole temperature interval from 37 to 9°C (data not shown). Substitutions in the region 14–95 as a rule improve intracellular solubility of hGH. Thus, already at 30°C (vs. 24°C for hGH) chimeric GHs '14–33' and '41–73' are detectable mainly in a soluble fraction. '14–95' GH is fully soluble even at 37°C. Moreover, about 20% of this protein is soluble even at 42°C. This markedly distinguishes it both from hGH and pGH. '78–95' GH resembles hGH by its properties.

The comparison of intracellular solubility of chimeric hormones and corresponding values of $D_{50\%}$ reveals a regularity – the more resistant the protein to GdnHCl denaturation, the higher its solubility. The most stable variant, '14–95' GH (Table 1), has the highest solubility in the series of the hormones studied (Table 2), and the least stable hormones, '78–95' GH and hGH, have the lowest solubility (only 20% of '78–95' GH and 25% of hGH are soluble at 30°C). '14–33' GH with intermediate value of $D_{50\%}$ was found entirely in the soluble fraction at 30°C. A similar relationship between the protein solubility and its stability was found for a single chain antibody fragment [19].

The cytoplasmic expression is preferable for protein production because it provides maximal product yield. The main obstacle to this route is the protein deposition in inclusion bodies as an insoluble and inactive material. The data described in this work demonstrate the possibility to increase intracellular solubility of a protein by increasing its intrinsic stability.

3.5. Elimination of lactogenic activity results in stabilization of hGH

Our results indicate an inverse relationship between the lactogenic activity of hGH and its stability. Demolishing lactogenic activity made it possible to construct the hGH variants ('41–73' and '14–95') exhibiting higher conformational stability and lower propensity to form inclusion bodies in bacterial cytoplasm. At the first glance, the mutant '14–33'

is an exception to the rule. Being most stable to the thermal denaturation it is equipotent to hGH in bioactivity determined in Nb2 test. But it appears to be the exception that proves the rule. Really, as was discussed above, the functional hGH determinants for binding to the human and rat prolactin receptors do not coincide completely. The homologous substitution of the segment 11–33 in hGH for porcine segment causes dramatic decrease in binding affinity for the human prolactin receptor [11]. Thus, although the variant '14–33' is fully active in the Nb2-lymphoma bioassay, it most likely lacks the lactogenic potency in humans.

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