Vol. 170, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1990 Pages 104-110

SYNTHESIS OF A NEW HELICAL PROTEIN: THE EFFECT OF SECONDARY STRUCTURE REARRANGEMENT ON STRUCTURE FORMATION

Toshiaki Tanaka, Yasuhiko Shiratori, Shin Saitoh, Yoshio Yamamoto, Atuko Kohara, Haruki Nakamura, Toshiki Tanaka*, Masakazu Kikuchi and Morio Ikehara

Protein Engineering Research Institute
6-2-3, Furuedai, Suita, Osaka 565, Japan

Received May 14, 1990

A new helical protein was designed and synthesized to alter the sequential connectivity of the 4 helices in human growth hormone and to delete the long surface loop structures. The protein accumulated as an insoluble form in E.coli was solubilized and purified to apparent homogeneity in the presence of 7M urea, and refolded by the aid of 1% n-octyl-β-D-glucopyranoside. The circular dichroism spectrum was typical of a highly helical protein. The molecular weight estimated by gel permeation chromatography and the red-shift of the fluorescence maximum by urea-induced denaturation suggest that the protein folds into a compact globular form. The new protein obtained, however, was destabilized relative to the original human growth hormone.

One of the goals of protein engineering is to design and to construct new functional proteins (1). To achieve this, it is necessary to build up specified tertiary structures from a combination of secondary structural units and functional units, arranged in the primary amino acid sequence. Information about amino acid sequences which direct the formation of the specified secondary structures is now being accumulated (2). If the formation of secondary structure is an early event in protein folding (3), a specific tertiary structure may be generated by combining these structural units.

Recently, Regan and DeGrado (4) reported that incremental joining of designed amphiphilic helices yielded a 4-helix bundle protein. Luger et al (5) have also reported that circularly permuted variants of an α/β barrel enzyme folded into active forms. They obtained folded molecules just by considering the interaction between the contiguous structural units. These observations

* To whom correspondence should be addressed.

Abbreviations: CD, circular dichroism; CHAPS, 3-[(3-cholamidoporopyl) dimethylammonio]-1-propanesulfonate; hGH, human growth hormone; hGH-hv, helix-reshuffled variant of hGH made by this work; OG, n-octyl-β-D-glucopyranoside; pGH, porcine growth hormone; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

0006-291X/90 $1.50

prompted us to make a new protein with a thoroughly reshuffled sequential connectivity of natural protein's secondary structural units. This approach may answer the importance of interaction between structural units and the influence of their sequential connectivity on a protein structure formation.

In this paper, a new protein with an altered sequential connectivity of hGH's 4 α-helices was designed and synthesized. Spectroscopic measurements and molecular size estimation showed that the protein folded into a compact globular form.

MATERIALS AND METHODS

Preparation of hGH-hv and hGH: The DNA fragment encoding hGH-hv (Fig. 2) was assembled from DNA oligomers essentially by the method previously described (6), and inserted between the ClaI and SalI sites of pGH-L9 (7) to give the expression plasmid pHGC-hv1. E.coli HB101 transformed by this plasmid was cultured at 30°C in M9 medium (8) containing 2 fold strength of the salts and 0.5% casamino acids. Protein synthesis was induced by adding 10 μg/ml of 3-β-indoleacrylic acid at the Klett unit between 120 to 200. After 20 hr of the induction, cells were harvested and disrupted by passing through a French Press at 8000 psi. The insoluble material was collected by centrifugation in 1M sucrose, and dissolved in 50 mM Tris-HCl(pH8.0) containing 7M urea. After removing insoluble materials by centrifugation, proteins were applied to a column of Asahipak 502NP (21.5 x 100 mm, Asahi Chemical Industry) at a flow rate of 1.5 ml/min. The column was washed with 25 mM Tris-HCl (pH8.0) containing 7M urea and protein was eluted by a linear gradient of 0 to 0.3 M NaCl in the same buffer. hGH-hv was identified by immunoblotting (9) using anti-hGH polyclonal antibody. The fractions containing hGH-hv were collected, concentrated and applied to a column of TSK gel G3000SW (21.5 x 600 mm, Tosoh). The column was developed isocratically by 50 mM Tris-HCl (pH7.1) containing 7M urea at a flow rate of 1.5 ml/min. OG was added to the peak fractions containing hGH-hv to 1% and dialyzed against 25 mM Tris-HCl (pH8.0) containing 1% OG. The dialyzed material was concentrated and used for further studies. NH_2-terminal sequence of purified hGH-hv was determined with a gas-phase protein sequencer and HPLC system (Model 477A and 120A, Applied Biosystems). Amino acid composition was analyzed with amino acid analyzer (Model 835, Hitachi), using 4 nmoles of purified hGH-hv, after hydrolysis at 110°C for 22 hr in 6M HCl. Natural hGH was prepared as described previously (10).

Molecular size estimation by gel permeation chromatography: hGH-hv was dissolved in 25 mM Tris-HCl (pH8.0) containing OG and/or CHAPS . Each sample was applied to a column of TSK gel G3000SW (7.5 x 600 mm, Tosoh) preequilibrated by the same buffer of the sample solution, and developed isocratically at 0.5 ml/min. The molecular weights were determined from the calibration curve obtained by the proteins; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; ribonuclease A, 13.7 kDa, under 25 mM Tris-HCl (pH 8.0) containing 1% OG. The retention times of these standards were little affected by the detergents added.

Spectroscopic measurements: CD spectrum and the ellipticity at 220 nm were measured by using JASCO J-600 spectropolarimeter using 0.2 cm path length cuvette. The fluorescence spectrum was measured on Hitachi F4000 Fluorescence Spectrophotometer, using an excitation wavelength of 295 nm and a slit width of 5 nm. All the measurements were carried out at 25°C.

RESULTS AND DISCUSSION

Design of a new helical protein

The objective of new helical protein design was to reshuffle the sequential connectivity of the 4 helices and simplify the structure of hGH. The three dimensional structure of hGH is not yet known, but is assumed to be similar to that of pGH based on amino acid sequence homology (11). hGH is assumed to be essentially composed of 4 helices, but with an unusual connectivity relative to other 4-helix bundle proteins (Fig. 1 left) such as cytochrome b-562 and myohemerythrin (11). In this design, the connectivity was changed to directly connect the antiparallel helices, resulting the deletion of two long surface loops (Fig. 1 right). This rearrangement decreases the molecular weight from 23 kDa of original hGH to 14 kDa.

The residues which were considered to be involved in the helix formation were indicated previously (11). These were used with only minor changes, and were arranged in hGH-hv so as to join the helices in the order of 2-3-1-4 (Fig. 2). As a result of surface loop deletion, hydrophobic residues Leu81, Leu162

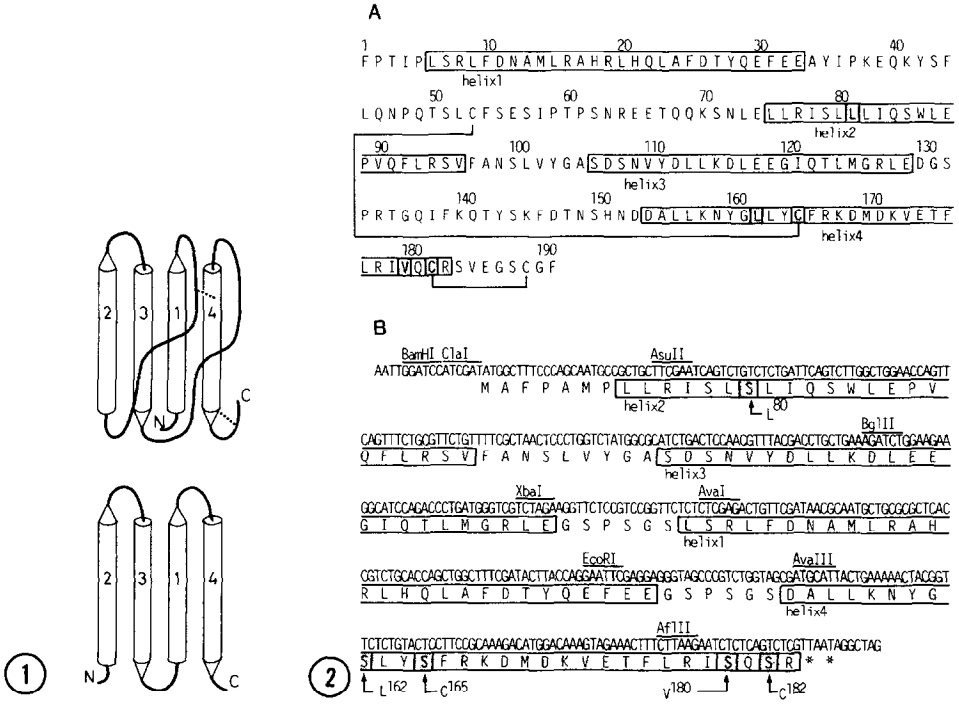
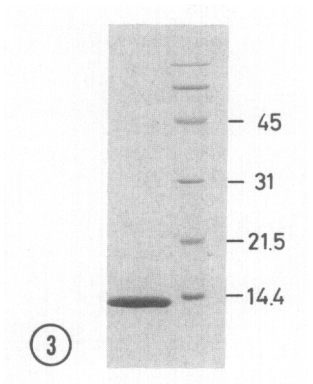


Figure 1. Schematic drawings of expected secondary structural connectivity in hGH (top) and hGH-hv (bottom). Helices are numbered from the NH_2-terminal of hGH (11). Two dotted lines show disulfide bonds.

Figure 2. The amino acid sequences of (A) hGH (15) and (B) hGH-hv with the gene sequence. Amino acid residues considered to be involved in helix formation (11) are boxed, and the mutated residues before and after mutation are indicated in shaded boxes. Two disulfide bonds in hGH are also indicated with lines connecting cysteine residues. In the gene sequence of hGH-hv, unique restriction enzyme sites are indicated above the DNA sequence. Asterisks show stop codons.



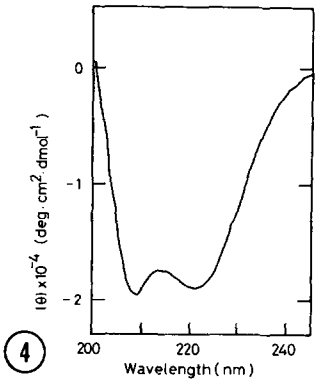


Figure 3. (left) SDS-PAGE of purified hGH-hv. Six μ g of purified hGH-hv was electrophoresed on 15% SDS-PAGE according to the method of Laemmli (16), and stained by Coomassie Brilliant Blue R-250. Right lane shows molecular weight markers.

Figure 4. (right) CD spectrum of hGH-hv at a concentration of 10μ M in 25 mM Tris-HCl (pH8.0) containing 1% OG.

and Val180 were speculated to be exposed to the solvent from the structural model (11) and helical wheel projection. Accordingly, they were changed to serine in hGH-hv. Two cysteines, Cys165 and Cys182, were also changed to serine, since their counterparts, with which they formed disulfide bonds in hGH (12), were deleted in hGH-hv. Newly generated turns connecting the helices 3 and 1, and 1 and 4 were designed to be Gly-Ser-Pro-Ser-Gly-Ser referring to the generalized models of loop connecting α-helices (13). The NH_2-terminal residues preceding helix 1 in pGH were also attached in front of helix 2 in hGH-hv (Fig. 2). The secondary structure prediction (14) showed that for helix formation of helix 2 in hGH-hv this NH_2-terminal sequence is preferable rather than that from hGH. The amino acid sequence of this designed variant of hGH, designated as hGH-hv, is shown in Fig. 2.

Expression and Purification of hGH-hv

A DNA fragment encoding the new protein hGH-hv was designed as in Fig. 2. The entire gene was placed under the control of trp promoter and expressed in

Table I. Molecular weight of hGH-hv determined by gel permeation chromatography

Relevant condition*1	Apparent molecular weight*2 (dalton)
OG 1%	37000
OG 0.5%, CHAPS 0.5%	28000
CHAPS 1%	27000, 13000

*1 Samples were dissolved in 25 mM Tris-HCl (pH8.0) containing the ingredients.
*2 The apparent molecular weights were determined as described in Materials and Methods.

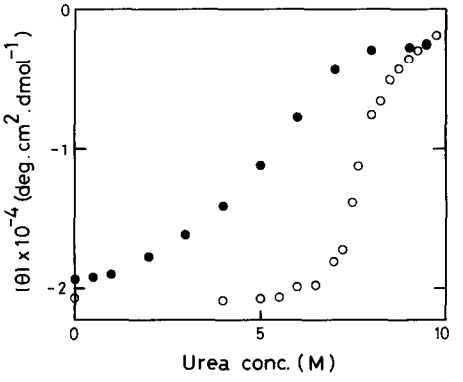


Figure 5. Urea-induced changes of the ellipticity at 220nm of hGH-hv (•) and hGH (o). Each solution contains hGH-hv or hGH, 1% OG, 25 mM Tris-HCl(pH8.0) and appropriate amount of urea. 0.5% β-mercaptoethanol is also added to the hGH solutions.

E.coli. After expression the protein was found in an insoluble form. It was solubilized by 7M urea and purified to apparent homogeneity by the combination of DEAE and gel filtration chromatographies in the presence of 7M urea. Purified hGH-hv had a molecular weight of 14 kDa calculated from SDS-PAGE (Fig. 3), which was consistent with the expected value. The NH_2-terminal amino acid sequence analysis revealed a mixture of the proteins with (>85%) and without (<15%) the initiation methionine. The both proteins had the expected amino acid sequences over 15 residues. In this study, the separation of two proteins was not carried out. The amino acid composition was also consistent with the expected sequence.

Refolding of urea-denatured hGH-hv was accomplished by the aid of 1% OG. Other detergents such as deoxycholic acid or CHAPS also assisted refolding of hGH-hv, but for subsequent spectroscopic studies they had the disadvantage of rather high absorbance in the far UV region. These detergents may assist refolding by masking hydrophobic residues unexpectedly exposed to the solvent or by eliminating the aggregation of partially folded forms. The assistance of non-denaturing detergents in refolding was also reported in the case of rhodanese, where the active enzyme was recovered (17). hGH-hv was recovered from the supernatant fraction after centrifugation at 100,000 x g. It was uncertain whether hGH-hv would retain any of the original hGH biological activity even if the protein were to fold into a definite tertiary structure. Therefore, the structural state of protein from the supernatant was not assayed through function, but was regarded as being folded at this stage. Without the detergents, the recovery of hGH-hv from the soluble fraction decreased drastically.

Secondary structure formation

The CD spectrum of hGH-hv, shown in Fig. 4, has two distinctive negative minima at 208 and 222 nm, which is typical of a highly helical protein (18).

The protein was estimated to contain 73% of α-helix and no β-sheet by the method of Provencher and Grockner (19) which is consistent with the designed structure.

Tertiary strucutre formation

To confirm that hGH-hv folded into a compact globular protein, the molecular weight was estimated (Table I). The apparent molecular weight of 37 kDa under the non-denaturing condition demonstrates two possibilities; hGH-hv forms a trimer or hGH-hv is partly denatured. The addition of the zwitterionic detergent CHAPS dissociated 37 kDa species to 27 kDa and 13 kDa. The 13 kDa species arising by the CHAPS addition indicates that hGH-hv folds into a compact globular form, but is oligomerized by OG. Such an aggregation induced by OG has also been found in the case of hGH solutions (data not shown). Moreover, the fluorescence maximum of the unique Trp residue in hGH-hv shifted from 338 nm in non-denaturing condition to 348 nm upon denaturation by urea. Since such a red-shift is a result of the exposure of Trp residue buried inside a protein to surrounding water upon denaturation (20), the result obtained is also a good indication of a tertiary structure formation of hGH-hv.

These results show that the designed hGH-hv folds into a compact helical form, but additional investigations should be necessary to confirm the spatial arrangement of the 4 helices. In hGH-hv, the conservation of the secondary structural interaction: hydrophobic interaction and packing of the side chains, which are known to affect the stability of already folded molecule (21), may be sufficient to direct the assembly of secondary structural elements into a folded conformation.

Structural stability

The stability of hGH-hv and natural hGH were compared (Fig. 5). The urea dependency and the mid point of the denaturation curves were totally different from each other. hGH-hv obtained by this work is fairly destabilized relative to the natural hGH, even the protein folds into a definite tertiary structure.

ACKNOWLEDGMENTS

The authors thank Dr. T.J.P.Hubbard for critical reading of the manuscript. The authors thank Misses C.Seko and M.Hayashi for technical assistance. Amino acid composition was determined by Toray Research Center, Inc.

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