Biological Activity of a Fluorescein Human Growth Hormone Derivative Prepared by Specific Covalent Labeling of Lysine-70†

Edna Sakal,‡ Arieh Gertler,*,‡ and Yoram Shechter§,‖

Department of Biochemistry, The Faculty of Agriculture, The Hebrew University of Jerusalem, and Department of Hormone Research, The Weizmann Institute of Science, Rehovot 76100, Israel

Received April 11, 1991; Revised Manuscript Received June 17, 1991

ABSTRACT: Modification of human growth hormone (hGH) with a low equimolar concentration of fluorescein isothiocyanate (FITC) yielded a derivative containing 1 mol of fluorescein/mol of protein. The site of modification was identified as lysine-70. Lysine-70 of hGH is about 3-fold more reactive than a "normal" lysine in a protein, having pseudo-first-order kinetics K_{obs} = 110 ± 7 M^{-1} min^{-1} at pH 10.5. The pK_a of the lysine was estimated to be 10.7, within the normal range of normal ε-lysine moieties in proteins. This higher chemical reactivity seems to favor selective labeling of this moiety at low FITC concentrations. To obtain monomodified derivatives, hGH was derivatized with 0.6 equiv of FITC, and the modified derivatives were separated from unreacted hormone by means of HPLC using a Mono Q column. Its biological activity, determined by Nb_2 bioassay, decreased to 40%, and its affinity toward lactogen receptors in Nb_2 cells and toward somatogen receptors in bovine liver decreased respectively to 30% and 20%. The present study indicates that out of the seven amino groups of human growth hormone, the ε-amino group of lysine-70 is excessively reactive toward FITC. Second, this particular amino group contributes to receptor binding and receptor activation. Lysine-70 is located in the loop between the first and second helix and close to the carboxy-terminal end of the first helix. This contribution is most likely the result of the formation of an electrostatic interaction between the hormone and the receptor. Fluorescein$_{Lys\text{-}70}$-hGH may therefore be utilized in further chemophysical studies, since the derivative largely preserves its binding and biological potency and the fluorescein moiety is located within the hormonal binding domain of GH to its biological receptor.

Growth hormone is an essential pituitary hormone which regulates growth and development of peripheral tissues (Li & Evans, 1944; Li & Liu, 1964; Isaksson et al., 1985). The hormone is composed of 1 polypeptide chain of known sequence consisting of 191 amino acids and 2 intramolecular disulfide bonds (Nicoll et al., 1986). A partial model for the polypeptide's three-dimensional structure is available (Abdel-Meguid et al., 1987). Recently, the amino acid sequence of the growth hormone receptor was also elucidated (Leung et al., 1987).

Several approaches are currently available for studying structure–function relationships of proteins. One includes selective chemical modifications of side-chain moieties followed by identifying the site(s) of modification or the biological properties of the modified protein. Reagents which are selective toward a certain type of amino acid side chain are available. There is a clear preference toward modification of "surface" (exposed) moieties as opposed to those residues buried within the hydrophobic core of the protein (Hammes & Scheraga, 1966). In general, no such preference is expected among hydrophobic surface side-chain moieties. Certain residues exhibit unusually high reactivity due to steric reasons and favorable microenvironment. In enzymes, such reactivity is often associated with the active site and is unique to residues participating in binding or catalysis. Examples for unusually high reactivities are found in the seryl moieties of serine proteases and within the cysteinyl residue of papain (Kitz & Wilson, 1962; Alexander et al., 1963; Sluyterman, 1968).

In the present study, we demonstrate that a particular lysine of human growth hormone (hGH)[1] (out of seven lysines and one α-amino group) is more reactive toward alkylation with FITC. Consequently, this moiety is modified exclusively at low concentrations of FITC. Proteins in general can incur a considerable degree of lysine modifications before being substantially inactivated. Here the alkylation is focused on a biologically essential domain. The site of modification and some of its basic features are identified here.

Experimental Procedures

Materials

Recombinant human growth hormone was the generous gift of Dr. Avraham Nimrod of Biotechnology, Israel. α-Lactalbumin, fluorescein isothiocyanate, dithiothreitol, and iodoacetamide were purchased from Sigma (St. Louis, MO). TPCK-treated trypsin was obtained from Worthington. Cyanogen bromide and urea were obtained from British Drug Houses. All other reagents used in this study were of analytical grade. The QHRS/5 Mono Q column was purchased from

† This research was supported by Grant 2761 from the National Council for Research and Development, The Ministry of Science and Technology, Israel, and by the Kay Foundation of the Hebrew University of Jerusalem.

* Correspondence should be addressed to this author at The Faculty of Agriculture, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel.

‡ The Hebrew University of Jerusalem.

§ The Weizmann Institute of Science.

‖ Incumbent of the Charles Hollenberg Chair for Diabetes and Metabolic Research.

[1] Abbreviations: hGH, human growth hormone; FITC, fluorescein isothiocyanate; $F_{Lys\text{-}70}$-hGH, hGH derivative in which the ε-amino group of lysine-70 was specifically modified with FITC; TLC, thin-layer chromatography; αLA, α-lactalbumin; RCAM-αLA, α-LA derivative in which the four disulfide bonds were fully reduced and carbamoylmethylated; bGH, bovine growth hormone; HPLC, high-pressure liquid chromatography.

0006-2960/91/0430-8899$02.50/0

Pharmacia (Uppsala, Sweden).

Methods

Reaction of hGH with Fluorescein Isothiocyanate. hGH was dissolved in 3.0 mL of 0.1 M Na_2CO_3 (pH 10.5) to make a solution of 22 mg/mL (1 mM). Fluorescein isothiocyanate at various concentrations was then added to make the required molar ratio of the reagent and the hormone. The reaction proceeded for 3 h, and the mixture was then loaded on a G-50 column equilibrated and run with 0.1 M Na_2CO_3. The fractions corresponding to the protein peak were pooled and dialyzed for 2 days against several changes of distilled H_2O and lyophilized. The extent of the modification was determined by dissolving the derivative (0.2 mg/mL) in 0.1 M $NaHCO_3$ (pH 8.5) and measuring its absorbance at 495 nm according to Fothergill (1969). The concentration of the protein was determined according to Bradford (1976) or by acid hydrolysis of an aliquot of the derivative and determination of its amino acid composition.

Kinetic Constants. Pseudo-first-order kinetic constants were evaluated by reacting hGH (1 mM) with 0.2 mM FITC and 0.1 M Na_2CO_3, pH 10.5 and 25 °C. At intervals, aliquots of 50 μL were withdrawn and transferred to test tubes containing glycyl ethyl ester in excess (final concentrations, 0.3 M). The aliquots were then dialyzed extensively, and the extent of fluorescein incorporated into the protein was determined spectrophotometrically. Pseudo-first-order rate constants (K_{obs}) were calculated by using the equation $K_{obs} = \ln 2/(\text{concentration})t_{1/2}$ where concentration is in molar and $t_{1/2}$ in minutes. It is assumed in these calculations that all ε-amino groups of the studied proteins are potentially available for the reaction. The pK_a value of the modified lysine was estimated by reacting GH with FITC at three pH values (pH 8.5, 9.5, 10.5) and using the equation $-\log K_{obs} = \text{pH}(0.71\text{p}K_a)$ (Stark, 1965).

Purification of the Fluorescently Labeled Peptide from FITC–hGH. (*a*) *CNBr Cleavage.* The derivative (30 mg, containing 0.73 ± 0.01 mol of fluorescein/mol of hGH) was dissolved in 20 mL of 70% formic acid. Solid cyanogen bromide (40 mg) was added, dissolved, and allowed to react with the protein for 24 h at room temperature. Solvent and excess CNBr were then evaporated, and the residue was twice dissolved in H_2O and lyophilized.

(*b*) *First Gel Filtration.* The mixture (a) was dissolved in 1.0 mL of 50% acetic acid, loaded on a preequilibrated Sephadex G-50 column (100 × 2 cm), and run with the same solvent. The labeled peak which emerged near the "void volume" (peaked at 54 mL) was pooled and lyophilized.

(*c*) *Reduction and Carbamoylmethylation of the Labeled Fragment.* The lyophilized peak was dissolved in 0.6 mL of 0.3 M Tris-HCl, pH 7.9, and 8 M urea. Dithiothreitol was then added to make a final concentration of 1 mM. The reduction proceeded for 20 min after which iodoacetamide (final concentration 20 mM) was added. The reaction proceeded for an additional 20 min, after which glacial acetic acid (0.6 mL) was added.

(*d*) *Second Gel Filtration.* The reduced and carbamoylmethylated yellow peak was reloaded on the same Sephadex G-50 column (100 × 2 cm) equilibrated and run with 50% acetic acid. The single yellow peak (eluted now in the included volume, peaked at 73 mL) was pooled and lyophilized.

(*e*) *Trypsin Degradation.* The yellow CNBr-cleaved fragment was suspended in H_2O to a final concentration of ~10 mg/mL. The suspension was adjusted to pH 7.5, and cleaved with TPCK-treated trypsin (about 2% w/w) for 24 h at 37 °C.

Table I: Reaction of hGH with FITC

mol of FITC added/mol of hGH[a]	mol of FITC covalently incorporated into protein	efficiency index (%)
0.1	0.06 ± 0.05	60
0.2	0.13 ± 0.01	67
0.4	0.34 ± 0.01	85
0.7	0.67 ± 0.04	95
1.1	1.10 ± 0.10	100
1.5	1.40 ± 0.15	93

[a] Reaction was carried out with 1 mM hGH in 0.1 M Na_2CO_3, pH 10.5 at 25 °C, and increasing concentrations of FITC for 3 h.

(*f*) *Separation of the Labeled Peptide by Thin-Layer Chromatography.* The peptide mixtures were run on a silica-coated thin-layer plate developed with butanol/acetic acid/H_2O, 4:1:1 (w/w). The band corresponding to the yellow peptide was scratched and eluted with 3.0 mL of 50% acetic acid. Following concentration under vacuum, it was reapplied to a TLC plate that was developed with butanol/pyridine/acetic acid/H_2O (4:1:0.2:1 w/w). The yellow peptide was eluted with 50% acetic acid. The concentration of the peptide was determined by its absorbance at 445 nm using the molar extinction coefficient ϵ_{445nm} = 12000. Amino acid analyses were performed after acid hydrolysis with 6 M HCl for 22 h using a Dionex BioLC amino acid analyzer.

HPLC Chromatography. HPLC anion-exchange chromatography of fluoresceinated hGH was performed in a Merck-Hitachi apparatus equipped with a D-2000 integrator and an L-6200 controller on a QHR5/5 column (Pharmacia). Twenty-five milligrams of FITC–hGH dissolved in 0.2 mL of 0.4% $NaHCO_3$ was applied to the column previously equilibrated with buffer A (0.02 M Tris-HCl, pH 7.0). The column was washed for 5 min with buffer A, and then a linear gradient of 0–100% of 25 mL of buffer B (1 M NaCl in buffer A) was employed for 30 min. The effluent was monitored by the UV absorbance at 280 nm. The flow rate was 30 mL/h, and 0.5-mL fractions were collected.

Biological Assays for hGH. (*a*) *Biological activity* of hGH was determined by using the Nb_2 rat lymphoma cell bioassay (Tanaka et al., 1980) as modified by Gertler et al. (1985).

(*b*) *Binding Determination.* The displacement of ^{125}I-hGH from Nb_2 cell homogenates (lactogen receptors) was assayed as described earlier (Elberg et al., 1990). The displacement of ^{125}I-bGH from bovine liver microsomal fraction (somatogen receptors) was assayed as described earlier (Gertler et al., 1984). IC_{50} was calculated as the amount of native or modified GH causing 50% displacement of the ^{125}I-GH, by plotting the specific binding as a function of GH concentration.

Results

Reaction of hGH with FITC. The reaction between hGH (1 mM) and FITC (0.1–1.0 mM) in 0.1 M Na_2CO_3 (pH 10.5) occurred with high labeling efficiency (Table I); namely, about 1 mol of FITC/mol of hGH was incorporated into the protein when 1 equiv of FITC was added. Therefore, the extent of the reaction could be controlled with relative ease by the proper addition of the reagent to protein (Table I).

Kinetic Studies. The rate of reaction of GH with FITC was evaluated by using an excess of GH over the reagent at pH 10.5 and 25 °C. This was compared to that of a model protein (native and RCAM-α-lactalbumin) under identical experimental conditions. Pseudo-first-order rate constants were 110 ± 10, 41 ± 3, and 45 ± 3 M^{-1} min^{-1} for growth hormone, α-lactalbumin, and RCAM-αLA, respectively (values derived from Figure 1 and summarized in Table II). The pK_a value of the modified amino group in GH was estimated to be 10.7

Table II: Pseudo-First-Order Kinetic Constants for the Reaction of FITC with hGH and Other Model Proteins[a]

protein	k_{obs} (M^{-1} min^{-1})	pK_a value at 25 °C
α-lactalbumin	41 ± 3[b]	ND[d]
RCAM-α-lactalbumin	45 ± 3[b]	ND
human growth hormone	100 ± 7[b]	10.7 ± 0.1[c]

[a] Reactions were carried out at pH 10.5 and 25 °C with 0.2 mM FITC and proteins to make a final concentration of amino groups = 10 mM. [b] Values were derived from Figure 1. [c] Calculated according to Stark (1965) (see also Experimental Procedures). [d] Not determined.

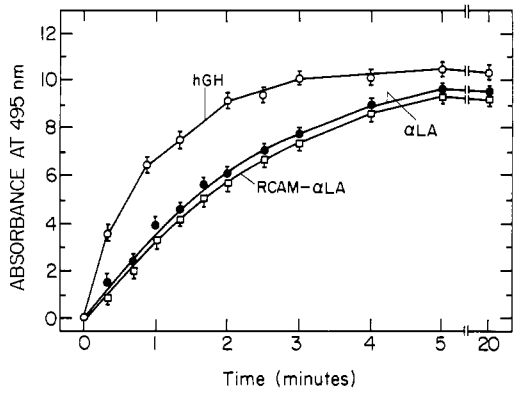

FIGURE 1: Rate of the reaction of hGH with FITC. Comparison to model proteins. The reaction performed at pH 10.5 and 25 °C contained either 1.4 mM hGH (○) or 0.83 mM α-lactalbumin (●) to make a final concentration of ε-amino groups = 10 mM and 0.2 mM FITC. At intervals, aliquots were withdrawn to determine the extent of the modification (Experimental Procedures). Calculated as units OD_{495} incorporated per 1 μmol of protein. Each point in the curve is the mean ± SE of three separate experiments.

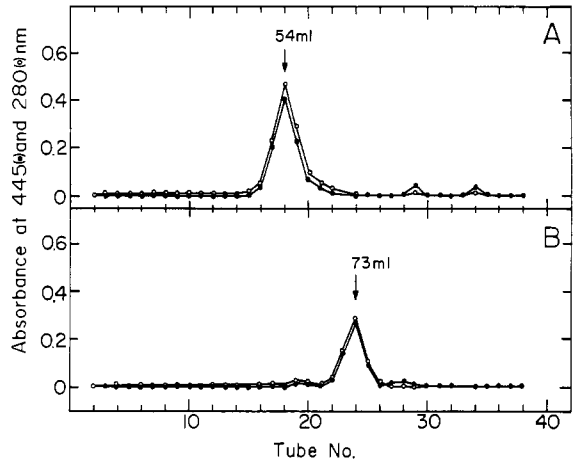

FIGURE 2: Separation of the FITC-labeled CNBr fragments by two-dimensional gel filtration. FITC-hGH (0.73 mol/mol) was fragmented with cyanogen bromide and applied to a Sephadex G-50 column, preequilibrated, and run with 50% acetic acid. Fractions of 3.0 mL were collected. The absorbance at 280 and 445 nm was recorded (A). Fractions 17–20 [in (A)] were pooled, evaporated, reduced, carbamoylmethylated, and reapplied to the same Sephadex G-50 column. (B) Fractions 23–25 were then pooled for further processing (Experimental Procedures).

(Experimental Procedures and Table II). This value is within the normal pK_a values expected for ε-amino groups of proteins (Elmore, 1968). Hence, excess reactivity of the amino moiety in question toward FITC is not due to an abnormally lower pK_a value.

Identification of the Site of the Modification. To identify the site of the modification, the hormone was labeled with 0.75 equiv of FITC to obtain a derivative containing 0.73 ± 0.01 mol of fluorescein/mol of protein. This derivative was fragmented with cyanogen bromide. The yellow fragment was purified to homogeneity by "two-dimensional" gel filtration as follows: The CNBr fragments were first fractionated on a Sephadex G-50 column (100 × 2 cm) prior to reduction and carbamoylmethylation of the disulfide bonds. Under these conditions, the yellow peak emerged in the excluded volume and could be purified from lower molecular weight fragments (Figure 2A). The labeled peak was then pooled, lyophilized, reduced, carbamoylmethylated, and reapplied to the same G-50 column where it was eluted at 73 mL (Figure 2B), pooled, and lyophilized. Amino acid analysis revealed that it was eluted at 73 mL (Figure 2B) and was then pooled and lyophilized. Amino acid analysis revealed it to be fragment 15–125 of GH (Table III).

Table III: Amino Acid Composition of the Purified Yellow Cyanogen Bromide Fragment[a]

amino acid	calculated for CNBr fragment 15–125	found
aspartic acid	9	9.8
threonine	5	5.7
serine	12	10.8
glutamic acid	21	21
proline	5	4.4
glycine	2	2.5
alanine	5	4.8
valine	4	3.6
methionine		
isoleucine	5	5.1
leucine	18	17.6
tyrosine	5	4.95
phenylalanine	6	6.8
lysine	4	4.8
histidine	2	1.6
arginine	5	5.2
fluoresceinyllysine[b]	1	1

[a] Amino acid analysis was performed following hydrolysis with 6 M HCl for 22 h. [b] Estimated by the absorbance at 445 nm prior to acid hydrolysis.

Table IV: Amino Acid Composition of the Purified Fluorescein-Labeled Tryptic Peptide[a]

amino acid	calculated for peptide 66–78	found
aspartic acid	1	1.3
threonine	1	1.0
serine	1	1.0
glutamic acid	5	5.6
proline		0.07
glycine		0.1
alanine		0.1
valine		
methionine		
isoleucine		
leucine	3	2.8
tyrosine		
phenylalanine		0.1
lysine	0.5–1[c]	0.9
histidine		
arginine	1	0.8
fluoresceinyllysine[b]	1	1.0

[a] Determined after hydrolysis with 6 M HCl for 22 h. [b] ε-Fluoresceinyllysine is hydrolyzed substantially back to lysine on acid hydrolysis [i.e., see Shechter et al. (1978)]. [c] Determined spectroscopically (at 445 nm) prior to acid hydrolysis.

Separation by Thin-Layer Chromatography. The yellow CNBr fragment was trypsinized and the peptide mixture applied to a TLC plate that was first developed with butanol/acetic acid/H_2O (4:1:1). The yellow band (R_f = 0.4) was eluted, concentrated, and reapplied to a TLC plate now developed with butanol/acetic acid/pyridine/H_2O (4:1:1:0.2). The peptide (R_f = 0.2) was reextracted. Amino acid analysis revealed it to be peptide 65–77 of growth hormone (Table IV),

Table V: Extent of Modification and Biological Activities of FITC–hGH Fractions Separated by the Mono Q Column

tube[a] no.	protein[b] (nmol/tube)	absorbance at 495 nm	mol of FITC–hGH/mol of protein[c]	biological activity		
				Nb_2[d] growth	binding to membranes[e] liver	Nb_2
hGH				100	10	5
19	65.5	0		104	12	7
20	16.0	0		ND[f]	ND	ND
21	40.3	2.122	0.92	42	42	16
22	17.0	0.863	0.90	45	ND	ND
23	14.3	1.080	1.32	37	58	21
24	6.8	0.431	1.30	33	ND	ND
25	3.6	0.275	1.32	25	ND	ND

[a] Tube number corresponds to Figure 3. [b] As determined by Bradford (1976) (see Experimental Procedures). [c] Calculated according to Fothergill (1969). [d] Residual activity. Quantified by Nb_2 bioassay as described under Experimental Procedures. Unmodified hGH = 100%. [e] IC_{50}, nanograms per tube. [f] Not determined.

indicating that lysine-70 is the modified residue.

Separation of the Modified Hormone on HPLC and Detection of the Biological Activity of the Separated Derivatives. In order to obtain a derivative of hGH fully and selectively modified on Lys-70, 0.75 μmol of hGH was treated with 0.45 μmol of FITC as described earlier. After extensive dialysis, an aliquot (0.2 mL containing 1.7 mg) was separated on a Mono Q column (Figure 3A), and 0.5-mL fractions were collected. Three main peaks appeared: tube 19 that corresponds to the unmodified hormone (Figure 3A) and tubes 21 and 23–24 that contain the modified derivatives. The protein concentration of each fraction was determined according to Bradford (1976) and confirmed by amino acid analysis after hydrolysis (not shown). The extent of FITC incorporation was determined by measuring the absorption at 495 nm and calculated according to Fothergill (1969). As shown in Table V, the peak at tube 19 contained the unmodified hGH, and its biological activity as determined with the Nb_2 lymphoma cells bioassay was almost equal to that of the untreated hormone. Incorporation of 1 mol of FITC/mol of hGH decreased the biological activity of the modified analogue to 42–45%. Modification of additional lysine or lysines (tubes 23–24) reduced the activity to 33–37%.

Binding Studies. Studies were performed to elucidate whether the decreased bioactivity resulted from a decreased affinity toward the hGH receptor. Experiments using Nb_2 cell homogenates as the receptor source were performed (Table V). Since the Nb_2 cells contain lactogen receptor, binding experiments using bovine liver membranes as a source of somatogen receptors and ^{125}I-bGH were performed in parallel to measure the effect of the modification on the binding to somatogen receptors. As shown in Table V, the decrease in the binding capacity toward Nb_2 lactogen receptor was almost parallel to the loss of bioactivity. The binding capacity toward the somatogen liver receptor was reduced to a higher degree.

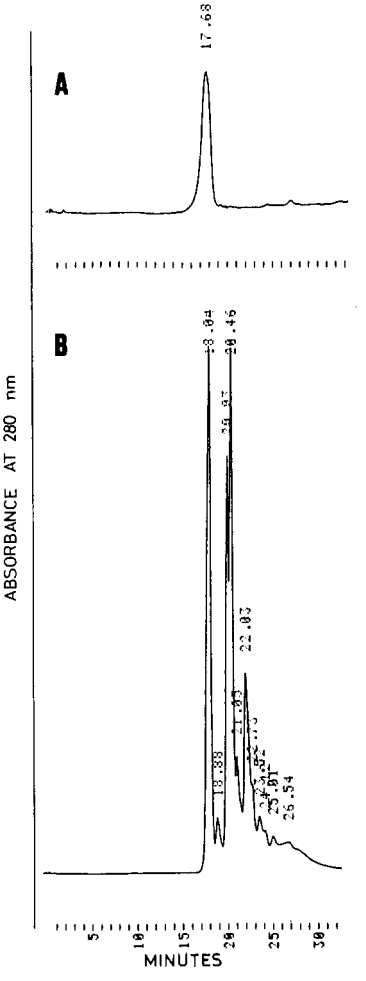

FIGURE 3: HPLC elution pattern of unmodified hGH (A) and FITC–hGH (B). 1.75 mg of hGH or FITC–hGH was applied on a Mono Q column, and 0.5-mL fractions were collected. Chromatography conditions are described under Experimental Procedures.

Discussion

The ε-amino moieties of proteins are positively charged and hydrophilic and, as such, are generally orientated to the aqueous surface of globular proteins and exposed equally to the action of water-soluble modifying reagents. Preferential alkylation may occur with α-amino groups which have a considerably (about 2 log units) lower pK_a value than ε-amino groups of proteins.

In this study, we initially observed that low concentrations of FITC (less than 1:1 molar ratio) added to hGH decrease the hormone's biological potency. Further studies revealed that the main modification (<90%) occurred at lysine-70. The structural analysis was performed prior to the separation on a Mono Q column; it is quite reasonable to assume that the second peak after HPLC separation (Figure 3), in which nearly 1 mol of FITC was incorporated per mole of hGH, is a homogeneously modified hGH molecule.

According to the three-dimensional structure of porcine GH (Abdel-Meguid et al., 1987) and assuming that the structure of hGH is similar, lysine-70 is located in the loop (residues 54–74) between the first and second α-helix. Lys-70 appears to be very close to the COOH-terminal portion of helix 4, which was suggested (Cunningham et al., 1989; Cunningham & Wells, 1989) to form an essential part of the binding site.

Selective modification toward a specific moiety may result from a favorable microenvironment within the protein molecule

Table VI: Amino Acid Sequence of the C-Terminal Part of the Nonidentical Region Connecting the First and the Second α-Helix in Growth Hormones from Various Species

species	sequence	reference
human	T^{60}PSNREETQQK^{70}SNLELL	Nicoll et al. (1986)
whale	T^{60}PANKDEAQQR^{70}SDVELL	Nicoll et al. (1986)
horse	A^{60}PTGKDEAQQR^{70}SDMELL	Nicoll et al. (1986)
cow	A^{60}PTGKNEAQQK^{70}SDLELL	Nicoll et al. (1986)
sheep	A^{60}PTGKNEAQQK^{70}SDLELL	Nicoll et al. (1986)
pig	A^{60}PTGKDEAQQR^{70}SDVELL	Nicoll et al. (1986)
rat	A^{60}PTGKEEAQQR^{70}TDMELL	Nicoll et al. (1986)
mouse	A^{60}PTGKEEAQQR^{70}TDMELL	Nicoll et al. (1986)
chicken	A^{60}PTGKDDAQQK^{70}SDMELL	Nicoll et al. (1986)
duck	A^{60}PTGKDDAQQK^{70}SDMELL	Chao et al. (1989)
bullfrog	A^{60}PTGKDNTHQK^{70}SDIDLL	Chao et al. (1989)
carp	A^{60}PTGKDET-QK^{70}SSMLKL	Chao et al. (1989)
salmon	S^{60}PVDKHET-QK^{70}SSVLKL	Chao et al. (1989)
tuna	S^{60}PIDKHET-QR^{70}SSVLKL	Chao et al. (1989)
yellow tail	S^{60}PIDKHET-QR^{70}SSVLKL	Chao et al. (1989)
bonito	S^{60}PIDKHET-QR^{70}SSVLRK	Noso et al. (1991)

and/or a lower pK_a value of the residue in question. The pK_a value of the lysine seems not to differ from that of normal ε-amino groups in proteins (pK_a = 10.7, Table II). Kinetic analyses revealed the amino group of lysine-70 to be about 3-fold more reactive than a normal lysine in a model protein (Table II, Figure 1). This 3-fold overreactivity suggests that lysine-70 is particularly exposed to the outer surface and is located within a microenvironment favoring the alkylation with FITC as well. The modification partly abolishes the biological activity as assayed in the Nb_2 bioassay. Binding analyses revealed a similar decrease in the affinity for lactogen receptors from Nb_2 cells while the affinity toward somatogen receptors from bovine liver was much more reduced. This finding may indicate that the replacement of the positively charged residue by a bulky hydrophobic group may be less critical for the interaction with the lactogen receptor, consistent with the fact that position 70 in all prolactins is occupied by a neutral amino acid having either a small hydrophilic (serine, threonine) or an aliphatic chain (isoleucine, valine, methionine).

A similar 2.4-fold decrease in the affinity toward the soluble portion of the cloned liver hGH receptor was also observed by Cunningham and Wells (1989) in the hGH K70A recombinant analogue despite the fact that the Lys side chain was replaced by a small methyl group rather than by the bulky fluorescein moiety. These results prompt us to suggest that the positive charge of the ε-amino group may play a role in the binding site of the hormone. The 4-fold decrease in the IC_{50} value (Table V), which most likely represents a similar decrease in the association constant, indicates an ~0.55 kcal/mol decrease in the free energy, which is consistent with changing of the electrostatic interactions or bonds by hydrophobic interaction. This conclusion is also substantiated by the fact that, in all GHs, this portion is occupied either by Lys or Arg (Table VI).

The IC_{50} value for bovine placental lactogen that competes for binding to the liver microsomal fraction with ^{125}I-bGH is ~5-fold lower than that of bGH (Krivi et al., 1989), consistent with the fact that position 70 in bovine placental lactogen is occupied by threonine (Schuler et al., 1988). More recently, it was also documented that mutations at the loop region such as P59A or P61A resulted in an up to 2.5-fold decrease in the somatogen receptor mediated biological activity. Removal of the 62–67 portion, which most likely induced a more drastic change in the microenvironment of the loop structure, caused a 600-fold decrease in the biological activity (Uchida et al., 1990). Yet, in all prolactins that also bind to the Nb_2 lactogen receptor with the same affinity (Tanaka et al., 1980; Ashkenazi et al., 1987), position 70 is occupied by several neutral amino acids (Nicoll et al., 1986). Thus, it is reasonable to conclude that the lack of the positively charged side chain at this position in prolactins is compensated for by other interactions.

From a practical point of view, F_{Lys-70}-GH preserved much of its biological potency; the precise location of the fluorescent probe is known, and the derivative can therefore be utilized in future studies for determining parameters such as lateral mobility of the receptor and rate of receptor-mediated ligand internalization as well as the fate and distribution of the internalized hormone. Such experiments are currently carried out in our laboratory.

It seems logical to assume that the higher reactivity of lysine-70 toward FITC and the decrease in the hormonal activity upon its modification are mutually related. The common denominator may be the biologically essential three-dimensional structure (i.e., a pocket or a cleft) within the protein molecule that also can accumulate FITC favorably. As we mentioned before, higher chemical reactivities were noted among amino acid moieties constituting enzymatic domains (Means & Feeney, 1971). To the best of our knowledge, this phenomenon was not observed among peptide and protein hormones, thus making growth hormone an example in this respect.

Acknowledgments

We thank Dr. Y. Nimrod of Biotechnology (Israel) for the recombinant hGH and Ms. Marsha Rockitter and Rona Levin for typing the manuscript.

References

Abdel-Meguid, S. S., Shieh, H. S., Smith, W. W., Dayringer, H. E., Violand, B. N., & Bentle, L. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6434–6437.

Alexander, R., Wilson, B. I., & Kitz, R. (1963) *J. Biol. Chem. 238*, 741–744.

Ashkenazi, A., Cohen, R., & Gertler, A. (1987) *FEBS Lett. 210*, 51–55.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Chao, S. C., Pan, F. M., & Chang, W. C. (1989) *Biochim. Biophys. Acta 1007*, 233–236.

Cunningham, B. C., & Wells, J. A. (1989) *Science 244*, 1081–1084.

Cunningham, B. C., Jhurani, P., Ng, P., & Wells, J. A. (1989) *Science 243*, 1330–1336.

Cunningham, B. C., Henner, D. J., & Wells, J. A. (1990) *Science 247*, 1461–1465.

Elberg, G., Kelly, P. A., Djiane, J., Binder, L., & Gertler, A. (1990) *J. Biol. Chem. 265*, 14770–14776.

Elmore, D. T. (1968) in *Peptide and Proteins* (Ebsworth, E. A., Padley, P. J., & Schofield, K., Eds.) pp 42–72, Cambridge University Press, Cambridge, U.K.

Fothergill, J. E. (1969) in *Fluorescent Protein Tracing* (Nairn, R. C., Ed.) pp 35–54, Livingstone Ltd., Edinburgh.

Gertler, A., Ashkenazi, A., & Madar, Z. (1984) *Mol. Cell. Endocrinol. 34*, 51–57.

Gertler, A., Walker, A., & Friesen, H. G. (1985) *Endocrinology 116*, 1636–1644.

Hammes, G. G., & Scheraga, H. A. (1966) *Biochemistry 5*, 3690–3693.

Isaksson, O. G. P., Eden, S., & Jansson, J. O. (1985) *Annu. Rev. Physiol. 47*, 483–499.

Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245–3249.

Krivi, G. G., Hauser, S. D., Stafford, J. M., Collier, R. J., & Byatt, J. C. (1989) Program of the 71st Annual Meeting

of the Endocrine Society, Seattle, WA (Abstr. 1523).
Leung, D. W., Spencer, S. A., Cachianes, G., Hammonds, R. G., Collins, C., Henzel, W. J., Barnard, R., Waters, M. J., & Wood, W. I. (1987) *Nature 330*, 537-543.
Li, C. H., & Evans, H. M. (1944) *Science 99*, 183-184.
Li, C. H., & Liu, W. R. (1964) *Experientia 20*, 169-178.
Means, G. E., & Feeney, R. E. (1971) in *Chemical Modifications of Proteins*, Holden-Day Inc., San Francisco, CA.
Nicoll, C. S., Mayer, G. L., & Russell, S. H. (1986) *Endocr. Rev. 7*, 169-203.
Noso, T., Yasuda, A., Kawazone, I., Takehara, H., Takahashi, A., Sakai, K., & Kawauchi, H. (1988) *Int. J. Pept. Protein Res. 32*, 579-589.
Schuler, L. A., Shimomura, K., Kessler, M. A., Zieler, C. G., & Bremel, R. D. (1988) *Biochemistry 27*, 8443-8448.
Shechter, Y., Schlessinger, J., Jacobs, S., Chang, K.-J., & Cuatrecasas, P. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2135-2139.
Sluyterman, L. A. E. (1968) *Biochim. Biophys. Acta 151*, 178-187.
Stark, G. R. (1965) *Biochemistry 4*, 1030-1036.
Tanaka, T., Shiu, R. P. C., Gout, P. W., Beer, C. T., Noble, R. L., & Friesen, H. G. (1980) *J. Clin. Endocrinol. Metab. 51*, 1058-1063.
Uchida, E., Shimokawa, S., Nishida, Y., Takasu, H., Ikehara, M., Uesugi, S., Tomita, K., Tanaka, A., Morikawa, M., Hayakawa, T., & Nishikawa, S. (1990) *Biochem. Biophys. Res. Commun. 172*, 357-363.

Synthesis, Isolation, and Characterization of Endogenous β-Galactoside-Binding Lectins in Human Leukocytes[†,‡]

Howard J. Allen,[*,§] Sally Gottstine,[§,∥] Ashu Sharma,[§] Richard A. DiCioccio,[⊥] Richard T. Swank,[#] and Hua Li[#,○]

Departments of Surgical Oncology, Gynecologic Oncology, and Cell and Molecular Biology, Roswell Park Cancer Institute, Buffalo, New York 14263, and Biology Department, Canisius College, Buffalo, New York 14208

Received April 25, 1991; Revised Manuscript Received June 28, 1991

ABSTRACT: Galaptin, a β-galactoside-binding lectin, was isolated from human buffy coat cells (peripheral leukocytes) and spleen by affinity chromatography. The molecular weight (32K) of the native buffy coat galaptin was similar to that for splenic galaptin. Their subunit molecular weight (14.5K), p*I* (4.60–4.85), and amino acid composition were identical. Both galaptins showed the presence of a single polypeptide when subjected to reversed-phase HPLC. Monospecific rabbit polyclonal antiserum raised against the 14.5-kDa subunit of splenic galaptin reacted with a 14.5-kDa polypeptide present in buffy coat cells, Epstein–Barr virus-immortalized B lymphoblastoid cells, and HL-60 promyelocytic leukemia cells. However, galaptin was not synthesized in vitro by buffy coat cells. Rather, a monomeric β-galactoside-binding protein of M_r 15.5–16.5K that is immunologically distinct from galaptin was synthesized. This galactoside-binding protein was separable from galaptin by polyacrylamide gel electrophoresis and by anion-exchange chromatography. In contrast, immunoprecipitation experiments confirmed that galaptin was synthesized by the B lymphoblastoid cells. cDNA corresponding to the B lymphoblastoid cell mRNA encoding galaptin was amplified by the polymerase chain reaction. The amplified product was partially sequenced, and 299 nucleotides were identified. The derived amino acids corresponded to residues 6–65, 84–114, and 118–126 found to be present in human splenic galaptin. Immunohistochemical analyses revealed that galaptin was distributed throughout the cytoplasm of B lymphoblastoid cells rather than being localized to the cell surface. The results presented here demonstrate that galaptin is present in a variety of leukocytes including buffy coat cells. Although buffy coat cells may accumulate galaptin, they do not synthesize it in vitro. The 15.5–16.5-kDa β-galactoside-binding lectin that is synthesized does not appear to accumulate in the buffy coat cells, and it may be a secretory protein [Allen, H. J., et al. (1986) *Immunol. Invest.* 15, 123–138].

The S-type lectins (Drickamer, 1989) are soluble, generally cation-independent, thiol-dependent proteins and are found in a wide variety of tissues and cells (Barondes et al., 1988). Studies of the physicochemistry of the S-type lectins have revealed a variety of expressed proteins with *N*-acetyllactosamine-binding specificity. The major S-type lectin that is frequently isolated from mammalian sources consists of a 30-kDa dimer composed of identical subunits (Allen et al., 1987a,b). In some cases, the monomer may be the primary form isolated (Merkle et al., 1989). For convenience, we have chosen to refer to this particular S-type lectin as galaptin. This is a more narrow definition than that originally stated (Harrison & Chesterton, 1980).

The amino acid sequence of galaptin of human origin has been directly determined (Hirabayashi & Kasai, 1988; Sharma et al., 1990). The gene coding for human galaptin has been cloned from cDNA from different sources, and it has been

[†] This work was supported by NIH Grants CA42584 (H.J.A.), DK32161 (R.A.D.), and GM33559 (R.T.S.) and by NIH Institutional BRSG Grant 2S07RR05648-22.
[‡] The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J05338.
* Address correspondence to this author.
[§] Department of Surgical Oncology, Roswell Park Cancer Institute.
[∥] Biology Department, Canisius College.
[⊥] Department of Gynecologic Oncology, Roswell Park Cancer Institute.
[#] Department of Cell and Molecular Biology, Roswell Park Cancer Institute.
[○] Present address: Frederick Cancer Research Facility, Frederick, MD 21701.

0006-2960/91/0430-8904$02.50/0