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Comparative conformational analysis of human somatotropin and biosynthetic methionyl human somatotropin by spectroscopic titrations

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Summary

Recombinant DNA techniques have been used to prepare human growth hormone (Met-HGH). It differs from the hormone obtained from human pituitaries (HGH) in that it has an additional amino acid (methionine) in the N-terminal position. The proteins from the two sources were subjected to spectroscopic studies, in order to detect any conformational differences resulting from the extra methionine residue. Regardless of origin, they can be separated into a main component (b-component) and a component with higher anodic mobility (c-component) by polyacrylamide gel electrophoresis. In each case the b-component can be transformed into the c-component by heat treatment at 50°C with a reaction rate constant of about 1×10^{-6} s^{-1}. The components have been studied with spectrophotometric, tryptophan fluorescence and spectropolarimetric methods by alkaline titrations (up to about pH 12). No differences could be detected between the HGH- and Met-HGH-forms. During the spectrophotometric titration all the tyrosines were titrated in all components with the same average pK_a. The tryptophan fluorescence was quenched with increasing pH in the same way in all the components. The decreasing fluorescence was attributed to the ionization of a tyrosine residue with pK_a 10.2. Also in CD titrations no differences between HGH and Met-HGH were detected. However, the sensitivity of the technique applied was shown to be high, since a small difference in ellipticity between the respective b- and c-components was found indicating a small change in the environment of the tryptophan.

Abbreviations: CD, circular dichroism; HGH, human growth hormone; Met-HGH, methionyl human growth hormone; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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Introduction

Human somatotropin (human growth hormone, HGH) is used in the treatment of certain forms of dwarfism and is isolated from human pituitaries obtained post-mortem. It has a molecular weight of 22,000 (Bewley and Li, 1975). A plasmid containing the partially synthetic cDNA for HGH has been constructed by recombinant DNA techniques. The plasmid was introduced into *E. coli* K12 and was found to produce a biosynthetic methionyl human somatotropin (Goeddel et al., 1979). The biosynthetic product (Met-HGH) was biologically active and showed the same biological potency as HGH in weight gain and tibia growth tests in hypophysectomized rats (Fryklund et al., 1982)

Several physico-chemical tests, e.g. electrophoresis and gel permeation chromatography, have indicated that HGH and Met-HGH have identical properties. For instance, they both give two bands by polyacrylamide gel electrophoresis (PAGE) (Fryklund et al., 1982). The major ones are the intact proteins, while for HGH the minor one is proposed to represent a desamidated form (Lewis et al., 1970). The bands have been named as b- and c-components, respectively, by Skyler et al. (1977) and this nomenclature has been used throughout this paper. The HGH b- and c-components are directly related to each other in such a way that the c-component can be obtained from the b-component by heat treatment in alkaline buffer (Lewis et al., 1970).

Sensitive physicochemical techniques have already been applied to study the conformation of different growth hormones and to detect subtle conformational differences (Aloj and Edelhoch, 1972). Circular dichroism (CD) studies in the near ultraviolet region indicated non-identical environments of the aromatics and disulfides, arising from dissimilarities in amino acid sequence and local conformation (Holladay et al., 1974; Holladay and Puett, 1977). Reduction of the two disulfide bridges in HGH and alkaline titration of the protein also profoundly altered the CD-spectra (Aloj and Edelhoch, 1972; Bewley and Li 1970; Kawauchi et al., 1976). These changes were reversible by reoxidation and reverse titration, respectively. The tryptophan fluorescence was strongly quenched during alkaline titration due to energy transfer to ionized tyrosines or due to increasing hydroxyl ion concentration or both (Aloj and Edelhoch, 1972; Kawauchi et al., 1976). In contrast to the CD reverse titration, the reverse fluorescence titration exhibited some hysteresis (Kawauchi et al., 1976) and did not return to exactly the original quantum yield.

One of the main concerns in using biosynthetic methionylated proteins in man is the potential immunogenicity imposed on these products by the additional methionine residue and the possible side-effects caused by the immunological response. Studies on these aspects cannot for obvious reasons be performed on laboratory animals. Consequently, information has to be obtained from carefully planned clinical trials. Such trials should be preceded by physicochemical studies to detect if any conformational difference exists between the native and the biosynthetic methionylated forms, which may affect the immunogenic properties. X-Ray crystallography can be used for such purposes, if suitable crystals of the protein are available. Comparative spectrophotometric, spectropolarimetric and fluorescence

titrations may, however, also give relevant information especially under conditions which possibly will induce conformational transitions, e.g. higher pH. With HGH and Met-HGH some immunochemical studies have been performed. While no conclusive information on the immunogenicity has yet been presented, some in vitro studies with monoclonal antibodies in immunoassays (Jonsdottir et al., 1981; Retegui, 1984) and with rabbit antibodies (Fryklund et al., 1982) have shown that HGH and Met-HGH have the same reactivity, indicating that the same antigenic determinants are retained in Met-HGH. However, antibodies raised in rabbits against the N-terminal pentadecapeptide of HGH and the methionylated peptide have different reactivities against HGH and Met-HGH, as well as a monoclonal antibody raised against HGH. (Personal communication from Dr. Linda Fryklund, Stockholm). It is thus obvious that Met-HGH has a conformation which is immunometrically different from that of HGH. However, regarding the antibody inducing capacity of Met-HGH in man, no conclusive information is yet available.

In the present work, the b- and c-forms of both HGH and Met-HGH have been compared by tryptophan fluorescence, circular dichroism and spectrophotometric titrations up to pH about 11–12 to evaluate the possibilities to detect any changed transitions in the respective titration curves, which can be related to the different N-terminal structures.

Materials and Methods

Samples

Human pituitary growth hormone, somatotropin, HGH (Crescormon, batch 82972, KabiVitrum AB) and biosynthetic methionyl human growth hormone, Met-HGH (Somatonorm, batch 80826, KabiVitrum AB) were used for the preparation of the respective b- and c-components by ion exchange chromatography. In some cases the hormones were incubated at +50°C for 50–60 h to yield a larger proportion of the c-component. The incubations were performed at 1 IU/ml in phosphate-buffered solutions (5 mM sodium phosphate, 0.5 M glycine, pH 7.3). Aliquots were removed for determination of rate constants for the transformation from the b- to the c-form.

The preparation of the pure components was performed on a K16/20 column (Pharmacia Fine Chemicals) on DEAE-TrisAcryl M (LKB) which had been equilibrated with 10 mM sodium phosphate buffer pH 8.0 at a flow rate of 50 ml/h. Elution was performed using a linear gradient from 0 to 0.2 M NaCl (2 × 150 ml) in the buffer. The absorbance at 280 nm was monitored with a Uvicord S (LKB). The column was regenerated with 1 M NaCl.

The isolated hormone components were either kept frozen until use or lyophilized. The buffer composition was changed before the spectroscopic studies to 1 mM sodium phosphate, 0.15 M NaCl, pH 6.8 by gel filtration on Sephadex G-25 (PD-10 column, Pharmacia Fine Chemicals).

Gel filtration

Analytical gel filtration was made on a TSK G 2000SW column (Varian) 0.7 × 30 cm in 0.05 M sodium phosphate buffer pH 7.5, containing 1% 2-propanol, at a flow

rate of 0.3 ml/min. The pump was a Constametric II from Laboratory Data Control, the injector (G6K) and fixed wavelength detector (model 440 at 280 nm) were both from Waters. The data from the chromatograms were treated with a 3388 integrator from Hewlett-Packard.

Electrophoresis

The purity of the isolated components was determined by PAGE in gels with T = 10% and C = 3% (Hjertén, 1962). Gel slabs (8 × 8 × 0.3 cm) were cast from preweighed acrylamide/bisacrylamide (Bio-Rad).

The samples were diluted to a protein concentration of 0.5 mg/ml determined by absorption at 277 nm using $A^{1\%}_{1cm} = 8.9$ as found in this work. To 300 μl aliquots of the samples a few crystals of sucrose and 5 μl of a 1% solution of bromophenol blue in water were added. Of each sample solution, 30 μl was added to the gel. The gels had been equilibrated before sample application by electrophoresis in Tris-borate buffer pH 8.4 (0.09 M Tris, 0.08 M boric acid, 2.5 mM Na EDTA) for 0.5 h at 40 mA/gel (constant). All electrophoresis runs were performed in a GE 2/4 equipment (Pharmacia Fine Chemicals) and with a power supply, type 2103, LKB.

The electrophoresis was stopped when the tracking dye reached the bottom of the gel. Staining and destaining were performed by the use of a Gel Destainer II equipment (Pharmacia Fine Chemicals). After electrophoresis the gels were stained in Coomassie Blue R-250 (0.1% in methanol–acetic acid–water 5 : 1 : 5) at 24 V for 15 min. Destaining was done at the same voltage but in a mixture of methanol–acetic acid–water (2 : 3 : 35) until a clear background was obtained. Adsorbent bag for anionic dye (Pharmacia Fine Chemicals) was used in the destaining step.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was run in the same type of gels as in PAGE, with the addition of 0.2% SDS to the Tris-borate buffer. Samples were pre-treated with 10% SDS at 60°C for 1 h. Disulfide bridges were reduced in the presence of 1% 2-mercaptoethanol. After SDS-PAGE the gels were fixed for 15 min at 24 V in 2-propanol–acetic acid–water (5 : 2 : 13) before staining.

The destained polyacrylamide gels were scanned in a Zeiss KM-3 densitometer at 600 nm. The slit was 1.0 × 0.1 mm and the scanning speed was 20 mm/min. The absorbancy zones were integrated on a Hewlett-Packard 3390A integrator.

Amino acid analysis

Known amounts of samples were subjected to amino acid analysis on a Durrum D-500 automatic amino acid analyzer after hydrolysis in 6 M HCl at 110°C for 24 h.

Spectrophotometric titration

Titration of the b- and c-components were performed in a Zeiss DMR 10 spectrophotometer. The difference spectra were scanned between 260 and 330 nm with a slitwidth set for a 0.8-nm bandpass. The concentration of the sample was 0.1–0.4 mg/ml and the starting pH was raised by the addition of 0.1 or 1 M NaOH and measured by a Radiometer PHM-62 pH-meter. The volume changes during the titrations were negligible. The absorption difference for peptide-linked tyrosine was estimated by titration of a solution of N-acetyl-tyrosine-amide (Sigma).

Fluorescence measurements

Fluorescence studies were performed on an Aminco-Bowman Corrected Spectra SPF in standard mode. The excitation light was from a high-pressure xenon lamp. All fluorescence measurements were made in 10×10 mm quartz cells, thermostated to 20°C. The entrance and exit slits were 1.0 and 2.0 mm, respectively. Emission spectra were recorded at a speed of 50 nm/min. Excitation wavelength was 290 nm in order to monitor the change in the intrinsic tryptophan fluorescence emission.

The sample solutions contained 0.03–0.06 mg hormone/ml in phosphate-buffered saline (0.15 M NaCl, 1 mM sodium phosphate), pH 6.8. Titrations were performed with 0.1 M NaOH and pH was read in the cuvette before and after fluorescence measurement. The difference never exceeded 0.05 pH units. A standard pH-meter (Radiometer PHM-62) was used, equipped with a micro-combination electrode Type MI-410 (Microelectrodes, New Hampshire). Reverse titrations were performed with 1 M HCl. The volume changes during the titrations were negligible.

Circular dichroism spectra

CD-spectra were recorded with a spectropolarimeter, JASCO J-41A, Japan Spectroscopic Co. (Tokyo). Measurements were made with 10 or 20 mm rectangular cells from 350 nm to 250 nm with 1 nm spectral band width. The pathlength was chosen to give highest possible ellipticities at photomultiplier voltage not exceeding 700 V. Scanning speeds were not faster than 5 nm/min. Protein solutions were filtered through 0.45 μm membrane filter (Millipore).

During spectropolarimetric titrations, the pH of each protein solution (1.2–2.0 mg/ml) was raised in steps of about 0.3 pH units (above pH 8.5) by addition of 1 M NaOH. The pH was read before and after the CD spectra had been recorded (Radiometer, standard pH-meter, type PHM-62). The difference never exceeded 0.15 pH units. At least two titrations were made with each component. Reverse titrations were performed by stepwise addition of 1 M HCl.

The ellipticity, θ, is expressed as molar ellipticity, $\langle\theta\rangle$ (degree $\times$ cm^2 $\times$ dmol^{-1}), using the equation $\langle\theta\rangle = (\theta \times \mathrm{M})/(1 \times \mathrm{c} \times 10)$, where M is the molecular weight, 1 is the optical path length in cm and c is the concentration in grams per ml. A molecular weight of 22,098 was used for HGH and 22,247 for Met-HGH.

Results

Prior to the isolation of the b- and c-components, preparations of HGH and Met-HGH contained about 15% and 5%, respectively, of the c-component as revealed by PAGE. Incubation of these preparations at +50°C caused an increase to about 50% of c after 60 h. Usually an even faster component (the d-form) can be seen on PAGE, but always in amounts less than 5%. The rate of conversion from b to c was calculated for both HGH and Met-HGH. The rate constant for HGH-b conversion to HGH-c was found to be $(1.2 \pm 0.1) \times 10^{-6}$ s^{-1}. The corresponding value for Met-HGH-b under the same conditions was found to be nearly the same $(1.1 \pm 0.1) \times 10^{-6}$ s^{-1}. By ion exchange chromatography at least 95% pure compo-

nents were obtained, as shown by PAGE (Fig. 1). Freezing, thawing, lyophilization or titration of the isolated components did not significantly change the electrophoretic pattern in PAGE. In SDS-PAGE each purified component migrated as a single band with the same mobility, indicating the same molecular weight. In analytical gel filtration more than 95% of each component was eluted as monomer.

The amino acid composition was determined after hydrolysis. Apart from the extra methionyl residue in Met-HGH compared to HGH the respective c-components do not differ in composition from the b-components.

The absorption coefficient ($A^{1\%}_{277nm}$) was calculated using the absorption at 277 nm after correction for light scattering (Beaven and Holiday, 1952) and the concentration obtained from amino acid analysis. The mean value of $A^{1\%}_{277nm} = 8.9$ was used for determination of the protein concentrations in this study.

In the spectrophotometric titrations all components showed a peak in the difference spectrum at 292 nm and an isosbestic point at 277 nm. The molar difference absorption coefficient at 292 nm for ionized N-acetyl-tyrosine-amide was in the present study found to be 2292 $M^{-1} \times cm^{-1}$. In all cases 8 tyrosines could be titrated and an apparent pK_a-value of around 10.9 was found for all components. No difference could be detected between the titration curves.

Fig. 1.

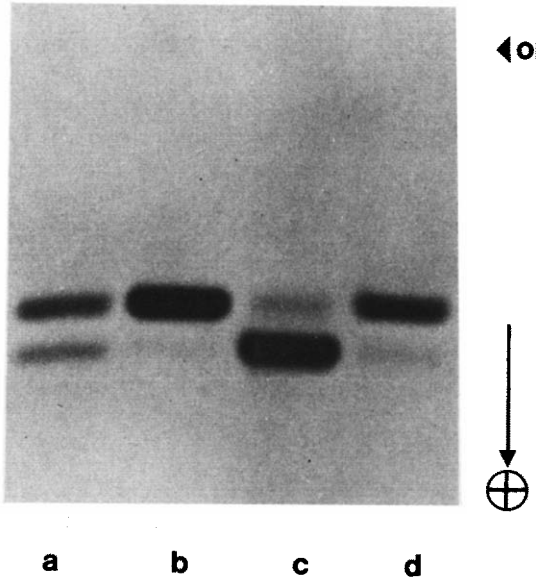

Fig. 1. Polyacrylamide gel electrophoresis of (a) Met-HGH, heat-treated for 40 h at 50°C; (b) isolated Met-HGH b; (c) isolated Met-HGH c; and (d) Met-HGH before heat-treatment. Anode is at the bottom of the figure.

Fig. 2.

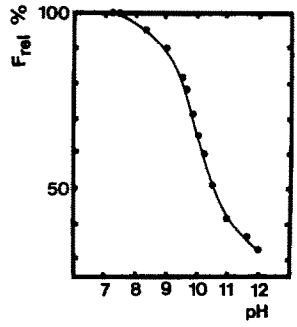

Fig. 2. The quenching of the tryptophan fluorescence emission at 340 nm of Met-HGH-b as a function of pH. Excitation wavelength was 290 nm.

The tryptophan fluorescence was studied in the pH range 7–12. For all components the fluorescence decreased with increasing pH. The emission maximum was obtained in all cases around 340 nm. During the spectral titrations no significant shift in the position of the emission peak could be observed. In Fig. 2 the fluorescence is plotted versus pH. The apparent pK_a-values were determined from the midpoints of the titration curves and were found to be 10.2 for all components. No difference in behaviour was observed between the components when titrated in the alkaline region. In reverse titration, to neutral pH, about 80% of the fluorescence was regained for all four components.

The CD-spectra of all the components studied showed the same general characteristics as presented by Aloj and Edelhoch (1972) and Kawauchi et al. (1976). At neutral pH-values a strong positive maximum originating from the tryptophan transition was situated at 292 nm (Strickland et al., 1969). As a consequence of the ionization of tyrosine residues at higher pH-values a negative maximum was successively formed at 298 nm (Kawauchi et al., 1976), which decreased the maximum at 290–292 nm. At the same time the ionization of the tyrosines also produced a positive change in the ellipticity below the isodichroic point at about 287 nm. The fine structure in the CD-spectra at 260 and 267 nm originating from the phenylalanine side chains (Horwitz et al., 1969) was, however, easily discernible all the time indicating the stability of the protein conformation within the pH-region

Fig. 3.

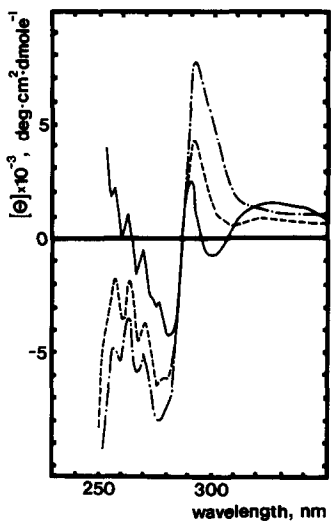

Fig. 3. The CD spectra of Met-HGH-b (1.3 mg/ml) in the region of side-chain absorption, obtained during alkaline titration of the protein. (– · –) pH 6.9; (— — —) pH 9.7; (———) pH 10.2.

Fig. 4.

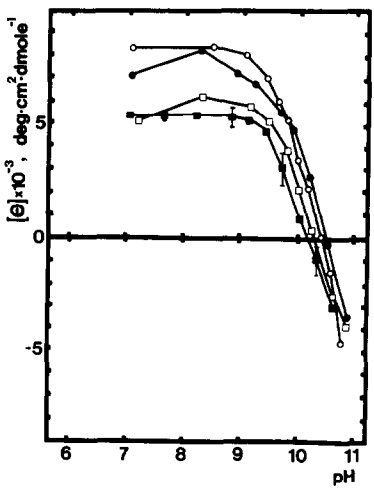

Fig. 4. The molar ellipticity of Met-HGH b (○———○), HGH b (●———●), Met-HGH c (□———□) and HGH c (■———■) at the maximum at 290–292 nm as a function of pH. To investigate the reproducibility of the curves 3 different titrations were performed with HGH-c. The curve shown is the average of all titrations obtained with the standard deviation marked at some representative pH-values.

studied. The spectra of the different hormone components and the changes described at increasing pH were qualitatively very similar in all cases (Fig. 3).

The CD-spectra obtained with the different components were similar, but some quantitative differences could be detected. The ellipticities at the peak around 290–292 nm of the different spectra have been plotted against pH (Fig. 4). The ellipticities depend on the conformation around the tryptophan residue, the tyrosine residues and the degree of ionization of the tyrosines. As is evident, some subtle differences can be detected. The two b-forms have higher ellipticities than the corresponding c-forms. These differences can be regarded as real considering the large number of spectra forming the curves. Unfortunately, the final plateau to be reached after complete titration of the tyrosines cannot be estimated, since the phenolate CD-band (at 298 nm) eventually will hide the tryptophan peak (at 290–292 nm) around pH 11. It is therefore not possible to definitely conclude that the differences seen in the crossing-over points (at ellipticities = 0, Fig. 4) for the b- and c-forms, respectively, can be regarded as further evidence for different conformations of the b- and c-forms.

The c-form is normally present only in small amounts in Met-HGH preparations, and had to be prepared from the b-form by heat treatment at 50°C. To study whether the heat treatment affects the conformation irreversibly, the c-form was isolated from HGH after heat treatment of the b-component, and compared with a c-component not subjected to heat treatment. No significant difference in CD spectra was observed.

Discussion

Biosynthetically produced proteins may contain an N-terminal methionine, which might impose conformational alterations in the native structure. Such conformational changes—if present—will be difficult to detect especially in a comparatively large polypeptide. As X-ray crystallography data are not available other applicable techniques will be ultraviolet spectroscopy, fluorescence and spectropolarimetric methods. Even with these techniques it may be difficult to detect a possible effect on the protein conformation, at neutral pH, from the extra methionyl residue. At this pH proteins are expected to assume stable conformations. No differences between Met-HGH and HGH have thus been established at pH 7–8 by CD or fluorescence measurements. In addition, the rate constants for the transformation of the b-forms of HGH and Met-HGH, at +50°C, to the respective c-forms are the same, and of the same order a those found previously at somewhat different conditions (Lewis et al., 1970). By this treatment asparagine-152 is desamidated according to Lewis et al. (1981). The finding indicates that the environment around this residue is the same for HGH and Met-HGH.

Obviously, the reason for the stability of a protein at neutral pH is the relatively few changes induced in the charge distribution of the protein, around this pH. Only histidine, which is a relatively rare amino acid in proteins, is titrated around pH 7. At pH 3–4, aspartic and glutamic acid side-chains are titrated, but to reach this pH range the isoelectric point is passed resulting in serious complications such as

precipitation. In this work, no results from studies in this pH region have been presented, since we have encountered such difficulties and since a preliminary investigation did not reveal any differences between the HGH- and Met-HGH-forms.

In the alkaline pH region, tyrosine and some lysine side-chains are titrated. In the former case a highly hydrophilic negative phenolate group is formed and in the latter case a positive charge is lost. If the titration does not induce a rapid denaturation, spectroscopic titration is of great potential interest for studying subtle differences in the stability of local conformations around chromophoric groups, e.g. in phenylalanine, tryptophan and tyrosine side-chains. However, the changes of the spectroscopic properties, when tyrosine residues are ionized may complicate the interpretation of the spectra as seen in the present case.

In some cases the transition pH has been established for proteins having discrete neutral and basic forms. Wilting et al. (1980) have, for instance, studied the N → B-transition of human serum albumin and have been able to show the influence of calcium- and chloride-ions on the transition pH by circular dichroism titrations. In the present case, the purpose was to determine—if possible—a transition pH of two conformational forms of HGH, and to study the influence of the extra methionine residue on the conformational changes.

No marked plateau corresponding to an alkaline conformation has been established for any of the protein forms with the methods used. Consequently, any mid-point pH representing a discrete conformational transition has not been identified. However, the ionization of the tyrosines (and the neutralization of the positively charged lysines) certainly are related to the specific environment around the titratable groups. The *changes* of the studied parameters (absorption, fluorescence and ellipticity) with pH will therefore also reflect the three-dimensional structures of the different proteins. The more specific the method is, the easier it is to detect differences. On the other hand, the chance to detect differences will obviously decrease with the number of chromophores studied.

The results obtained from the spectrophotometric titrations were qualitatively the same as those earlier presented by Bewley et al. (1969). Thus, both the b- and c-forms of HGH and Met-HGH showed maxima in the difference spectra at 292 nm and isosbestic points at 277 nm. Furthermore, the apparent pK_a of the tyrosines was about 10.9 in both studies.

All four forms of somatotropin studied showed the same behaviour with respect to fluorescence during titration in the alkaline range. The single tryptophan was excited at 290 nm and the peak of the emission was at 340 nm in the whole pH range studied. This result indicates that the tryptophan residue is buried within the molecule (Burstein et al., 1973) and no change in the environment of tryptophan occurs at higher pH. The fluorescence signal decreased with increasing pH. This effect can be explained by the titration of a tyrosine residue, since the ionized tyrosine residue quenches the tryptophan fluorescence. In the titration curve (Fig. 2) a pK_a of about 10.2 is obtained which is close to the expected value of about 10.0 (Edelhoch and Chen, 1980) for a freely rotating tyrosine residue in a hydrophilic environment. Furthermore, in reverse titration to neutral pH, hysteresis was found as previously reported (Kawauchi et al., 1976).

The CD titration technique is the most discriminating method applied in this work and some differences have been seen between b- and c-forms, which can form a basis for an analysis. The titration curve has been based on the molar ellipticity of the peak at 290–292 nm, which at lower pH-values (< 9) originates from the tryptophan band. Above pH 9, the tyrosines will be titrated and the more acidic ones will increasingly influence the value of the ellipticity. The curves will thus show the titration of some tyrosines *and* the possible change of the conformation around the tryptophan. As could be seen from Fig. 4, the titrations were carried out with reasonably high precision. The differences between the two b-forms and between the two c-forms were small and of the same magnitude as the experimental error involved. However, differences were detected between the curves representing the b- and c-forms of the respective hormones. Thus, the tryptophan ellipticity below pH 9 was smaller for the c-forms and the titration curves were not so steep as those obtained with the b-forms.

In conclusion, no conformational differences have been detected between HGH and Met-HGH by spectrophotometric, tryptophan fluorescence or spectropolarimetric titrations. Obviously, this does not necessarily imply that no differences exist. In this context it is important to stress, however, that the CD titration did detect differences between the b- and c-forms. Lewis et al., (1981) found that the location of the structural difference between these two electrophoretically and chromatographically distinguishable forms of HGH was in the residue 152, being Asn in the b-form and Asp in the c-form. If our preparations differ in the same way, we can conclude that our CD-method has been sensitive enough to show the conformational consequences of a hydrolyzed amido group. However, no consequences have been detected from the additional N-terminal methionine residue. It seems therefore reasonable to conclude that there are only very small—if any—conformational differences between Met-HGH and HGH, especially when considering all the other physicochemical and biological parameters (Goeddel et al., 1979; Lewis et al., 1970) examined.

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