Immunochemical characterization of charge isomers of bacteria-derived human growth hormone with monoclonal antibodies

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Monoclonal antibodies were used to study the immunochemical nature of charge isomers of bacterially produced methionyl human growth hormone. After isoelectric focusing of the hormone the 12 monoclonal antibodies reacted similarly in immunoblotting experiments and none of them could discriminate between the two isolated charge isomers in ELISA. This indicates that the generation of charge isomers of met-hGH does not result in loss of the determinants recognized by the monoclonal antibodies and that the conformation of the two main charge isomers is identical within these determinants.

Human growth hormone *Charge isomer* *Monoclonal antibody* *Immunoblotting* *ELISA*

1. INTRODUCTION

Pituitary human growth hormone, hGH, is a single-chain polypeptide of 191 amino acids cross-linked by two disulphide bridges [1]. Bacterially synthesized hGH is identical to the pituitary hGH except for an extra NH_2-terminal methionine and thus a met-hGH [2].

Preparations of pituitary hGH show charge heterogeneity when analysed by gel electrophoresis and isoelectric focusing [3,4]. It has been shown [5] that the main charge isomerism is related to desamidations. Also bacterially produced met-hGH preparations have been shown to contain charge isomers [6], which have been chemically characterized [7]. If the generation of charge isomers creates variation in the biological activity of the hormone [5] this might be reflected by alterations in the antigenic determinants. Therefore, we investigated the immunochemical nature of the charge isomers using 12 monoclonal antibodies against pituitary hGH, which all react indistinguishably with the met-hGH [8]. Cross-reactivity with hCS indicates that the Mabs react with at least 3 different determinants on the hGH molecule [8]. Recent experiments indicate that they recognize at least 5 independent antigenic determinants (to be published).

The reactivity of the 12 Mabs with the charge isomers of met-hGH was studied by the immunoblotting technique [9] after isoelectric focusing of the hormone. Further, the main charge isomer (b) representing the intact hormone, and a more acidic isomer (c) were isolated by ion-exchange chromatography and compared for reactivity with the different Mabs in a competitive ELISA.

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunoadsorbent assay; GαMIg-ALP, alkaline phosphatase conjugated goat anti-mouse immunoglobulin; hGH, human growth hormone; IEF, isoelectric focusing; Mab, monoclonal antibody; met-hGH, bacterially produced methionyl hGH; PBS, phosphate-buffered saline; r.t.; room temperature

2. MATERIALS AND METHODS

2.1. *Hormone source*

Bacterially produced met-hGH was Somatonorm® from KabiVitrum AB (Stockholm).

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2.2. *Isoelectric focusing*

Ten μg samples of met-hGH were applied on a 0.5 mm thin layer of 1% (w/v) agarose gel (Isogel™, LKB, Stockholm) with 3% Ampholine carrier ampholytes, pH 4.0–6.5 (LKB), and run at a constant power of 4.0 W/110 mm length of gel for 1 h. pH measurements were performed with a surface pH electrode (Dr W. Ingold AG, Zürich, Switzerland).

2.3. *Immunoblotting*

The method was a modification of that devised in [9]. The focused protein was transferred from the agarose gel to the nitrocellulose (Trans-Blot™, BioRad Laboratories, Richmond, CA) by diffusion. The remaining protein binding sites were saturated with 3% BSA (Sigma, St. Louis, MO) in PBS at r.t. overnight. The nitrocellulose was then cut and each strip was incubated at r.t. for 2 h with slow agitation in culture supernatant from one of the hybridomas diluted 1/5 in PBS-0.05% Tween. The strips were then washed 3–4 times in PBS-Tween and incubated with agitation at r.t. for 2 h in GαMIg-ALP (Sigma) diluted 1/1000. After washing, the strips were developed by incubation in 1 mM α-naphthyl phosphate (Sigma) and 2 mM Fast blue salt B (Merck, Darmstadt) in 50 mM Tris–HCl buffer, 10 mM $MgCl_2$ (pH 8.3) for 5–15 min.

2.4. *Purification of charge isomers*

The preparation of pure components was performed on a K16/20 column (Pharmacia Fine Chemicals, Uppsala) 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 isolated charge isomers were kept frozen until use.

2.5. *Competitive microELISA*

Competitive microELISA was performed as in [8]. A limited amount of Mab was incubated with

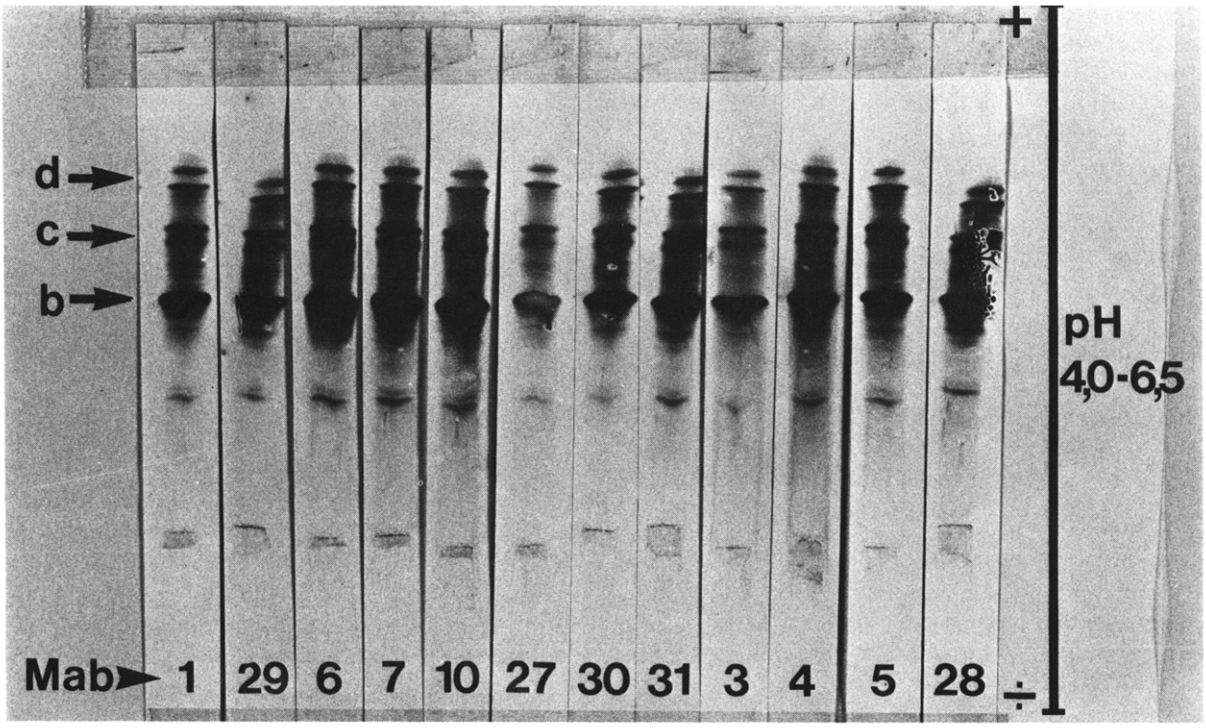

Fig. 1. Immunoblotting of met-hGH IEF pattern with the Mabs indicated at the bottom of each lane. Charge isomers b, c and d are indicated by arrows.

serial 5-fold dilutions of the competing antigen in hGH-coated microELISA plates (Dynatech M-129B, Dynatech, Switzerland). Bound Mab was determined with GαMIg-ALP (Sigma) and the plates were developed by adding *p*-nitrophenyl phosphate (Sigma), and reading the absorbance at 405 nm in a Multiskan automated spectrophotometer (Flow Laboratories, Irvine, Scotland). The results are expressed as % Mab bound at a given antigen concentration, 100% being Mab alone.

3. RESULTS

As previously reported met-hGH preparations obtained by recombinant DNA technique show charge heterogeneity [6], comparable to that of pituitary hGH preparations [7]. When the IEF pattern of met-hGH was transferred to a nitrocellulose acetate sheet and subjected to immunoblotting by the 12 Mabs (fig.1), they all showed a very similar reactivity pattern. The main isomer b and two more acidic isomers c and d are recognized by all the Mabs, and in addition some minor components. From the immunoblotting experiments we conclude that all the antigenic determinants recognized by the 12 Mabs are present on isomers b, c and d. The reactivity of individual charge isomers with the different Mabs, however, cannot be quantitatively determined by this method. Therefore the major b-component and the minor c-component were isolated by ion-exchange chromatography on DEAE-Trisacryl using a salt gradient. The elution pattern is shown in fig. 2. The charge isomers b and c are separated by about 0.1 pH unit in IEF [7]. The band c-components are >95% pure, as shown by PAGE [10].

The purified charge isomers b and c were compared with each other and with the intact met-hGH preparation for reactivity with the 12 Mabs in a competitive ELISA (fig.3). For all the Mabs the in-

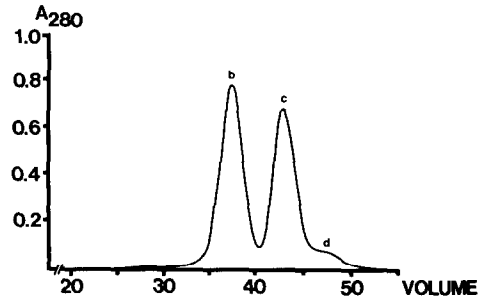

Fig. 2. Ion-exchange chromatography on DEAE-Trisacryl M of heat-treated met-hGH. The fractions containing the main hGH component (b) and the charge isomer (c) were collected and pooled separately for further analysis.

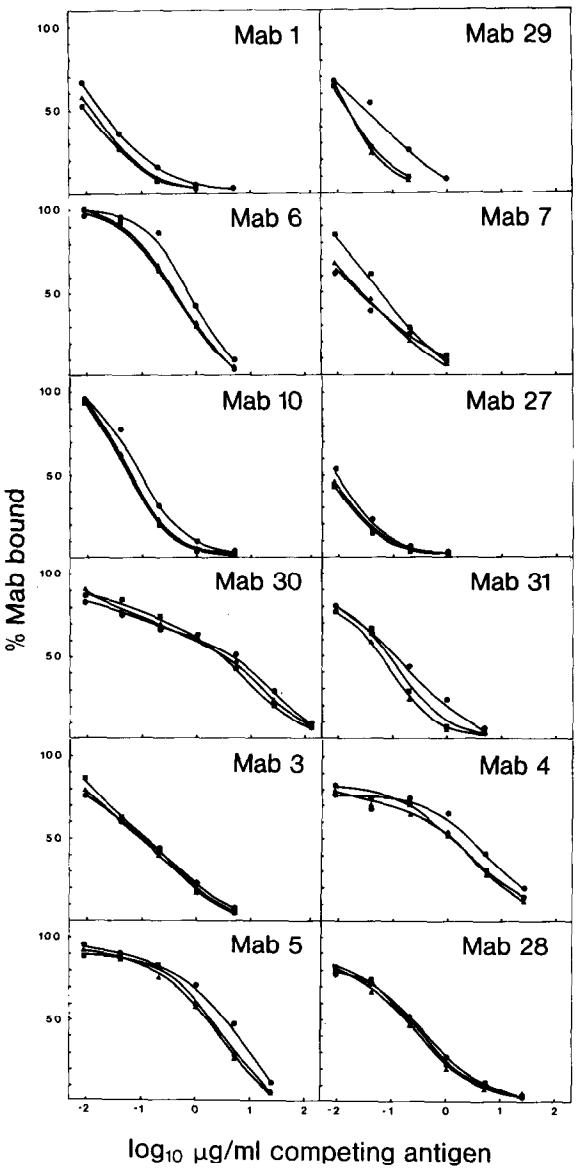

Fig. 3. Competitive ELISA: abscissa; $\log_{10}$ concentration of competing antigen (µg/ml). (●) met-hGH, (■) charge isomer b, (▲) charge isomer c. Ordinate; % binding of Mab. 100% is A_{405} of Mab alone.

hibitory curves of the charge isomers b and c are virtually overlapping and almost identical to those of the intact met-hGH preparation. This indicates that the charge isomers b and c do not differ in the antigenic determinants recognized by the 12 Mabs.

4. DISCUSSION

The charge heterogeneity of pituitary hGH has been extensively studied [3,4]. In [5] the charge isomerism was shown to be related to desamidations and it was proposed that the desamido-hGH is a physiologically relevant isohormone [5]. Recently charge heterogeneity was described for bacterially produced met-hGH [6]. Upon chemical characterization the charge isomers of met-hGH behave similarly to those of pituitary hGH, indicating that isomer c is a desamidated form of isomer b [7]. Decreased immunoreactivity of desamidated pituitary hGH in the NIH-hGH RIA has been reported [11]. Recently Mabs to hGH have been successfully applied for characterization of size variants [12] and proteolytically modified forms of hGH [13,14]. However, immunochemical characterization of hGH charge isomers at the level of individual antigenic determinants has not been previously described. The similar reactivity pattern of our 12 different Mabs in the immunoblotting experiments (fig.1) indicates that the main charge isomers of met-hGH are not generated with loss of any of the antigenic determinants recognized by these Mabs. Although all the antigenic determinants recognized by our Mabs show strong conformational dependency [8] none of the Mabs discriminate between the purified b and c charge isomers of met-hGH in the ELISA (fig.3). Therefore our results indicate conformational identity within the antigenic determinants of met-hGH charge isomers b and c as recognized by these Mabs.

Desamido-hGH has been suggested to be a component of the functioning pituitary gland [5]. Although pituitary and met-hGH have been shown to desamidate only slowly in vitro [10] it is reasonable to believe that charge isomers of the bacteria-derived met-hGH are generated during purification or storage. To answer the question of the physiological relevance of charge isomerism of hGH, chemical and immunochemical studies must be combined with studies of the biologial activity of the purified met-hGH charge isomers.

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