Use of calcium dependence as a means to study the interaction between growth hormones and their binding proteins in rabbit liver

Ross BARNARD and Michael J. WATERS

Department of Physiology and Pharmacology, University of Queensland, St. Lucia, Queensland 4067, Australia

The affinity of 22000- M_r human growth hormone (22 K-hGH) for GH binding proteins in rabbit liver is increased approx. 19-fold by 25 mM- Ca^{2+} . In contrast, ovine growth hormone (oGH) binding is Ca^{2+} -independent up to 10 mM, and decreased by greater Ca^{2+} concentrations. The 20000- M_r hGH variant (20K-hGH), lacking residues 32–46, exhibits intermediate behaviour. Without Ca^{2+} there is a residual 40 % of maximum specific binding to liver microsomes, and this increases to 65 % with liver cytosolic GH binding proteins. In contrast with 22K-hGH, Scatchard analysis of 20K-hGH binding to liver microsomes produces curvilinear plots in the presence of 25 mM- Ca^{2+} . From these results and inhibition studies with monoclonal antibodies to the GH binding proteins, it is concluded that deletion of the region 32–46 from 22K-hGH has eliminated one component of high-affinity Ca^{2+} -potentiable binding. The Ca^{2+} -mediated increase in K_a for the 22K-hGH-binding protein interaction is consistent with convergence of unit negative charges on the hormone and binding protein towards an intercalated Ca^{2+} ion. A positive charge in the critical region of nonprimate GHs would render their interactions Ca^{2+} -independent and of lower K_a compared with 22K-hGH. A likely candidate for the negatively charged interactive residue is glutamate-33, since it is unique to human GH and is replaced by a positively charged arginine in non-primate GHs. Its absence in 20K-hGH could explain the altered calcium-dependence of 20K-hGH binding to what is probably the type 2 binding protein [Barnard & Waters (1986) *Biochem. J.* 237, 885–892]. The Ca^{2+} -dependence of 20K-hGH binding to a subset of GH binding proteins provides both a verification and a mechanistic basis for the proposal [Hughes, Tokuhito, Simpson & Friesen (1983) *Endocrinology* (Baltimore) 113, 1904–1906] that 20K-hGH binds with high affinity to only a subset of binding proteins in rabbit liver membranes.

INTRODUCTION

We previously demonstrated the calcium dependence of human growth hormone binding to GH binding proteins in rabbit serum and cytosol [1]. In contrast, ovine GH binding does not require Ca^{2+} [1]. Ca^{2+} -dependent hGH binding is displaceable by oGH, so both ligands share the same sites, but with different cation-dependence properties. Fix *et al.* [3] reported earlier that the binding of rat and bovine GH to rabbit liver membranes is independent of Ca^{2+} concentrations, whereas hGH binding was cation-dependent.

These results prompted us to postulate that neutralization of opposed negative charges by Ca^{2+} is necessary to facilitate hGH, but not oGH (or bovine or rat GH), binding to the rabbit liver binding proteins. In order to obtain support for our proposed model for the role of Ca^{2+} in the hormone-binding protein interaction, we have measured the free energy changes which characterize the 22K-hGH-binding protein interaction both in the presence and absence of Ca^{2+} and compared these with the free-energy change for oGH binding.

The 20000- M_r variant of hGH lacks residues 32–46 of 22K-hGH [4]. To investigate the possible location of residues in hGH critical for generation of Ca^{2+} -dependence, we have studied the Ca^{2+} -dependence of 20K-

hGH binding to rabbit liver microsomes and cytosolic GH binding proteins and compared this with the 22K-hGH and non-primate GH binding interaction.

MATERIALS AND METHODS

Materials

Recombinant-DNA-derived human growth hormone was a gift from Genentech Inc. (San Francisco, CA, U.S.A.). The pituitary-derived 20000- M_r variant of hGH was a generous gift from Dr. G. E. Chapman and was purified in his laboratory (Department of Biochemistry, University of Auckland, Auckland, New Zealand). 20K-hGH from this source has been independently shown to electrophorese identically with recombinant-DNA-derived 20K-hGH [5]. Ovine growth hormone (oGH I-1 or oGH-13) was provided by the National Hormone and Pituitary Program (Baltimore, MD, U.S.A.), or was purified in this laboratory by the method of Ellis [6] to equipotency with oGH I-3. Human and ovine growth hormones were iodinated by the lactoperoxidase method of Thorell & Johansson [7] as described previously [8]. 20K-hGH was iodinated by the Iodogen method of Salacinski *et al.* [9] to obtain a satisfactory specific radioactivity. Essentially the same Ca^{2+} -dependence behaviour

Abbreviations used: hGH, oGH, human and ovine growth hormones; 22K-hGH, 20K-hGH, the 22000- M_r and 20000- M_r variants of hGH; oPRL, ovine prolactin.

was observed with 20K-hGH iodinated at lower (50 $\mu\text{Ci}/\mu\text{g}$) or higher (> 120 $\mu\text{Ci}/\mu\text{g}$) specific activity. Iodinated hormones were fractionated on a Sephadex G-100 column. Specific radioactivities were in the range 50–150 $\mu\text{Ci}/\mu\text{g}$.

Preparation of liver microsomes and cytosol

The liver microsomes used in the Ca^{2+} -dependence studies were prepared by the method described previously [10] from frozen non-pregnant female or male rabbits. Microsomes were resuspended in the cation-free storage buffer of Haro *et al.* [11] and kept at -20°C . The cytosol preparation used in the present and previous studies [1,12,13] was a filtered 100000 g supernatant of the liver microsomal pellet.

Calcium-dependence assays

Liver microsomes and cytosol were first titrated in a standard radioreceptor assay [8] to determine the dilution yielding optimum specific binding of the various radiolabelled hormones. To determine Ca^{2+} -dependence of hormone binding to liver microsomes, 100 μl portions of microsomes were diluted in 25 mM-Tris/HCl/1 % bovine serum albumin, pH 7.5 (TB buffer), and incubated with 100 μl of radiolabelled hormone (approx. 100000 c.p.m.), 100 μl of TB buffer and 300 μl of TB buffer plus either calcium at twice final assay concentration or EDTA at 10 mM (for zero- Ca^{2+} tubes). Specific binding of ^{125}I -labelled hormone was determined in all assays by addition of unlabelled oGH-13 to 0.4 or 1.0 $\mu\text{g}/\text{ml}$ in a parallel series of tubes. Incubation proceeded overnight at 4°C , with shaking at 80 rev./min. To determine Ca^{2+} -dependence of hormone binding to cytosol, the method was modified by the inclusion of a non-(hormone binding site)-directed monoclonal antibody (MAb 1 [12,14]) to enable poly(ethylene glycol) precipitation of ligand-binding protein complexes [1,12,13]. The cytosolic GH binding proteins are poly(ethylene glycol) soluble unless complexed with a monoclonal antibody. Assays using cytosol were stopped by adding 1 ml of cold 0.2 % (w/v) bovine γ -globulins and 1 ml of 30 % (w/v) poly(ethylene glycol) 6000. It was also necessary to

terminate the assays with liver microsomes with poly(ethylene glycol) to ensure compact and complete pellet formation in the absence of cations. Because of this, non-specific binding of hormones was somewhat increased. In some assays (Figs. 5 and 6) a monoclonal antibody (263) which inhibits binding to a subset of GH binding proteins [1,12,13] was incorporated into the incubation mixture at a 1:6000 final dilution.

Determination of affinity constants

Affinity of cytosolic GH binding proteins for the different growth hormones was estimated by Scatchard analysis [15] of ^{125}I -labelled hormone binding to cytosol in the presence of increasing concentrations of unlabelled hormone. MAb 1 was included in the 0.6 ml incubation volume at 1:6000 (v/v) final dilution to enable poly(ethylene glycol) precipitation of binding protein-hormone complexes. TB assay buffer was used with 25 mM- Ca^{2+} or without Ca^{2+} plus 10 mM-EDTA. Incubation was overnight at 4°C plus 3 h at 25°C . The data analysis and curve fitting were performed using the EBDA [16] and LIGAND [17] programs adapted by McPherson [18] for the IBM personal computer. The software was purchased from Elsevier Biosoft (Cambridge, U.K.). Scatchard plots shown in the Results section were traced from plots generated by the LIGAND program. For comparison of hGH and oGH data, correction was made for bindability of labelled hormones, as determined in a radioreceptor assay [8] with liver microsomes.

RESULTS

Effect of Ca^{2+} on 22K-hGH binding to liver microsomes and cytosol

Specific binding of 22K-hGH to rabbit liver microsomes or cytosol is enhanced 4–5-fold in the presence of Ca^{2+} (Figs. 1a and 1b). Note that there is no increase in the proportion of Ca^{2+} -independent binding in the cytosol compared with liver microsomes.

Fig. 1. Ca^{2+} dependence of 22K-hGH binding to (a) liver microsomes and (b) cytosol

Liver microsomes were added at (approx. 0.1 mg of protein/ml) 1:100 in TB buffer (plus Ca^{2+} or without Ca^{2+} plus EDTA). Specific binding was determined with excess unlabelled oGH added at 2 $\mu\text{g}/\text{ml}$. Maximum specific binding of ^{125}I -hGH was 11.2 %; non-specific binding was 10.5 %. (b) Liver cytosol was added at 1:10 dilution in TB buffer (plus Ca^{2+} or without Ca^{2+} plus EDTA). Specific binding was determined with excess unlabelled oGH added at 5 $\mu\text{g}/\text{ml}$. Maximum specific binding of ^{125}I -hGH was 15.5 %; non-specific binding was 9 %. Specific activity of ^{125}I -hGH was 104 $\mu\text{Ci}/\mu\text{g}$.

Fig. 2. Scatchard analysis of 22K-hGH binding in the presence (●) and absence (○) of 25 mM- Ca^{2+}

●, Female liver cytosol was added at 1:40 dilution in TB buffer. MAb 1 was incorporated in the incubation mixture at 1:6000 final dilution. Specific activity of ^{125}I -hGH was $56 \mu\text{Ci}/\mu\text{g}$. Specific binding was 9% of added trace; non-specific binding was 10% of added trace. ○, The same female cytosol was added at 1:8 dilution in TB buffer with 10 mM-EDTA. MAB 1 was incorporated at a final dilution of 1:6000. Specific binding was 13% of added trace; non-specific binding was 19% of added trace. The Figures, including lines of best fit, were traced from the plot generated by the LIGAND program (see the Materials and methods section). The lower curve (○) has been normalized and plotted on the same axis as the upper curve (●). K_a with $\text{Ca}^{2+} = 17 \times 10^9 \text{ M}^{-1}$; K_a minus Ca^{2+} , $1.2 \times 10^9 \text{ M}^{-1}$ for the experiments shown.

Effect of Ca^{2+} on the affinity of the hGH-binding protein interaction

Scatchard analyses in the presence of 25 mM- Ca^{2+} or in the absence of Ca^{2+} (plus 10 mM-EDTA) gave linear Scatchard plots in both cases (Fig. 2). From three determinations on female rabbit cytosol the affinity constant for hGH binding in the presence of Ca^{2+} was enhanced an average 19-fold [19 ± 1.4 (S.E.M.), $n = 3$] over the value in the absence of calcium ($1.29 \pm 0.06 \times 10^9 \text{ M}^{-1}$ without calcium, $2.46 \pm 0.13 \times 10^{10} \text{ M}^{-1}$ with calcium).

Effect of Ca^{2+} on ovine and human GH binding to liver microsomes

Fig. 3 shows that concentrations of Ca^{2+} greater than 10 mM significantly reduce the binding of oGH to rabbit liver microsomes. Note that essentially the same result is obtained with oGH iodinated to low or high specific activity, indicating that the iodination process has not perturbed the Ca^{2+} -dependence characteristics of the interaction. For this reason, Scatchard analyses of oGH binding were subsequently performed with the inclusion of 10 mM-EDTA in calcium-free buffer. Fig. 4 shows a typical Scatchard plot for oGH binding to sites precipitable from female liver cytosol by MAB 1. Three such Scatchard analyses of female rabbit cytosol in the absence of Ca^{2+} resulted in an affinity estimate for oGH

Fig. 3. Inverse Ca^{2+} -dependence of oGH binding to rabbit liver microsomes

Female liver microsomes were added at 1:4 dilution (approx. 2 mg of protein/ml) in TB buffer (plus calcium or without calcium plus EDTA). ●, oGH I-1; ○, oGH purified by the method of Ellis [6]. Maximum specific binding of oGH I-1 and 'Ellis' oGH [6] was 16% and 13.6% respectively. Specific activities were $121 \text{ Ci}/\mu\text{g}$ and $30 \text{ Ci}/\mu\text{g}$ oGH I-1 and 'Ellis' oGH [6] respectively.

Fig. 4. Scatchard analysis of oGH binding in the absence of Ca^{2+}

Female liver cytosol was added at 1:5 dilution in TB buffer with 10 mM-EDTA. MAB 1 was incorporated at a final dilution of 1:6000. Specific activity of ^{125}I -oGH was $50 \mu\text{Ci}/\mu\text{g}$, and the iodinated hormone was 60% specifically bindable. Specific binding of total added ^{125}I -oGH was 11%; non-specific binding was 12% of added trace. Average K_a for three such determinations was $(1.58 \pm 0.12) \times 10^9 \text{ M}^{-1}$.

of $1.58 \pm 0.12 \times 10^9 \text{ M}^{-1}$ (S.E.M., $n = 3$). Since the affinity estimate for hGH binding to the same female cytosol with 25 mM- Ca^{2+} added was $15 \pm 1 \times 10^9 \text{ M}^{-1}$ (S.E.M., $n = 4$; e.g. Fig. 2), the affinity of hGH for the cytosolic GH binding proteins is approx. 9-fold greater than the affinity of oGH.

Effect of Ca^{2+} on 20K-hGH binding to liver microsomes and cytosol

To investigate the effect of the deletion of residues 32–46 of 22K-hGH we studied the calcium-dependence of the 20000- M_r variant.

Fig. 5. Effect of calcium on 20K-hGH binding to liver microsomes

Liver microsomes (the same preparation as used in Fig. 1a) were added at a 1:8 dilution (approximately 1 mg of protein/ml) in TB buffer. ○, MAb 263 added at 1:1000 dilution; ●, without MAB 263. Specific binding was determined with excess unlabelled oGH added at 2 µg/ml. Maximum specific binding was 8.5 %; non-specific binding was 15 % of added trace. Specific activity of ¹²⁵I-20K-hGH was 50 µCi/µg. Points represent means of triplicate determinations with S.E.M. indicated. Corresponding points differ significantly as indicated (**P* < 0.01); 44 % of maximum specific binding was observed in the absence of calcium in this experiment.

With microsomes prepared from non-pregnant rabbits, approx. 40 % (39 ± 4 %) (S.E.M., *n* = 3) of maximum specific binding of 20K-hGH is observed without calcium (Fig. 5). With liver cytosol, the proportion of Ca²⁺-independent binding is increased to 65 ± 2 % (S.E.M., *n* = 3) (Figs. 6 and 8). This could be attributed to the increased proportion of type 2 binding protein in cytosol [12,13] in which case 20K-hGH binding to the type 2 binding protein would be Ca²⁺-independent. With 22K-hGH there is no increase in the proportion of Ca²⁺-independent binding to cytosol compared with liver microsomes (Figs. 1a and 1b), consistent with a Ca²⁺ requirement for 22K-hGH binding to all binding proteins.

Three other lines of evidence are consistent with the existence of distinct subsets of Ca²⁺-dependent and Ca²⁺-independent binding sites for 20K-hGH in rabbit liver microsomes and in liver cytosol.

1. Inhibition of 20K-hGH binding by MAb 263. MAb 263 is one of a panel of anti-(GH receptor/binding protein) monoclonal antibodies which inhibit 22K-hGH binding to the type 3 membrane subtype of GH binding proteins in rabbit liver [1,12,13]. Since this subtype is absent from cytosol [12], the increased proportion of Ca²⁺-independent binding of 20K-hGH in cytosol compared with microsomes could be explained by the absence of calcium-dependent 20K-hGH binding to type 3 (Fig. 6). This suggestion is supported by the data of Fig. 5,

Fig. 6. Effect of calcium on 20K-hGH binding to liver cytosol

Liver cytosol was added at 1:8 dilution in TB buffer. MAB 1 was incorporated into the incubation mixture at 1:6000 final dilution. Specific binding to sites precipitable by MAB 1 was determined with excess unlabelled oGH added at 5 µg/ml. Specific activity of ¹²⁵I-20K-hGH was 120 µCi/µg. ○, MAB 263 added at 1:1000 dilution; ●, without MAB 263. Points represent means of triplicate determinations with S.E.M. indicated. Points are not significantly different; 65 % of maximum specific binding (5 % of added trace) was observed in the absence of calcium, in this experiment (see Fig. 8 for another). Non-specific binding was 20 % of added trace. Trace was 60 % bindable in the presence of bivalent cations (20 mM).

showing that 20K-hGH binding to liver microsomes has a greater proportion of Ca²⁺-dependent binding and approximately half of this Ca²⁺-dependent binding is inhibited by MAB 263 (i.e. type 3).

2. Scatchard analysis of 20K-hGH binding to rabbit liver microsomes. If 20K-hGH binding to a subset of sites in the rabbit liver differs in terms of Ca²⁺-dependence, one might expect curvilinear Scatchard plots for 20K-hGH binding in the presence of Ca²⁺. Scatchard analysis of 20K-hGH binding to liver microsomes with 25 mM-Ca²⁺ consistently produced curvilinear plots (Fig. 7). In the absence of calcium, the high-affinity component was not detectable and Scatchard plots were linear with an affinity similar to that of the low-affinity component seen in the presence of Ca²⁺ (Fig. 7). This contrasts with 22K-hGH binding, for which Scatchard plots are linear either with or without Ca²⁺ (Fig. 2; [12]).

3. Competition by ovine prolactin for 20K-hGH binding sites in cytosol. Specific binding of 20K-hGH (or other GHs) to cytosolic GH binding proteins is detectable by poly(ethylene glycol) precipitation in the presence of an anti-(GH receptor) monoclonal antibody which does not interfere with ligand binding (MAB 1 [1,14]). Ovine prolactin (oPRL) at 0.5 µg/ml does not reduce the Ca²⁺-dependent increment in 20K-hGH binding, but competes for the Ca²⁺-independent component only (Fig. 8). In the absence of MAB 1, no prolactin-displaceable 20K-hGH binding is measurable; therefore the observed competition between 20K-hGH and oPRL is not due to interference by PRL receptors. Thus it appears that oPRL shows specificity overlap for the Ca²⁺-independent 20K-hGH binding site.

Fig. 7. Scatchard analysis of 20K-hGH binding to liver microsomes in the presence of 25 mM- Ca^{2+} (●) or without Ca^{2+} (○)

Microsomes from a non-pregnant female rabbit were added at a 1:40 dilution (approx. 0.2 mg of protein/ml) in TB buffer. Specific binding was 12% in the presence of 25 mM- Ca^{2+} and 3% in the absence of Ca^{2+} ; non-specific binding was 32% and 20% in the presence and absence of Ca^{2+} respectively. Trace was 70% bindable. Specific activity of ^{125}I -20K-hGH was 120 $\mu\text{Ci}/\mu\text{g}$. The affinity of interaction in the absence of Ca^{2+} was approx. $1.7 \times 10^8 \text{ M}^{-1}$. In the presence of Ca^{2+} a two-site model gave a significantly better fit than a single-site model ($P < 0.05$). A two-site fit with the site capacity ratio constrained to 45:55 was not significantly different from an unconstrained fit and was better than a single-site fit ($P < 0.05$). K_a estimates for high- and low-affinity components were $1.6 \times 10^9 \text{ M}^{-1}$ and $1.7 \times 10^8 \text{ M}^{-1}$ respectively for the curve shown. For three such experiments the mean K_a values were $(2.0 \pm 0.2) \times 10^9 \text{ M}^{-1}$ (S.E.M.) $(2.5 \pm 0.3) \times 10^8 \text{ M}^{-1}$ (S.E.M.).

DISCUSSION

In view of the major importance of charge interactions in determining the geometric specificity of protein-protein interactions [19] and in the short-range stabilization of enzyme-ligand complexes [20], we wish to propose the following model to account for these observations.

Our results show that the absence of the sequence 32-46 from 22K-hGH diminishes the extent of Ca^{2+} -dependent high-affinity interaction which occurs between 22K-hGH and liver GH binding proteins. The interaction between ovine GH and the same GH binding-proteins in the liver is not Ca^{2+} -dependent or -potentiated. We propose that the role of Ca^{2+} in 22K-hGH binding is to neutralize a repulsive interaction between the binding protein and the hormone. The requirement for Ca^{2+} would then imply the existence of strategically located negative charges on both the binding protein and 22K-hGH. The existence of such opposed negative charges is supported by the magnitude of the free energy change mediated by Ca^{2+} ions. The 19-fold increase in affinity is equivalent to a 7.3 kJ/mol decrease in free energy at 25 °C. Using the effective dielectric constant of 60

Fig. 8. Competition by oPRL for 20K-hGH binding sites precipitable from liver cytosol by MAb 1

Liver cytosol from a non-pregnant female rabbit was added at a 1:4 dilution in TB buffer. MAb 1 was incorporated in the incubation mixture at a final dilution of 1:6000. Specific binding to sites precipitable by MAb 1 was determined by displacement with excess unlabelled oGH (10 $\mu\text{g}/\text{ml}$ added concentration): ●, without added oPRL-16; ○, with oPRL-16 added at 0.5 $\mu\text{g}/\text{ml}$. In the absence of MAb 1, no PRL or oGH displaceable binding of 20K-hGH was detectable. * Indicates that corresponding points do not differ significantly ($P > 0.1$), with the difference between the 0 mM- Ca^{2+} points (●, ○, at 0 mM) subtracted. Points represent means of triplicate determinations with S.E.M. indicated. Maximum specific binding was 7.7%; non-specific binding was 20% of added trace. Specific activity of ^{125}I -20K-hGH was 120 $\mu\text{Ci}/\mu\text{g}$. Trace was 70% bindable in the presence of bivalent cations (10 mM).

measured by Wells *et al.* [21] for the inter-charge microenvironment of the subtilisin-substrate complex, such a free energy change could be generated by convergence of unit negative charges to within 0.6 nm on opposite sides of an intercalated Ca^{2+} ion.

If a critical negative charge on hGH were replaced by a positive charge at the corresponding interacting position in ovine GH, the simple electrostatic model would be sufficient to explain the greater affinity of hGH for its binding proteins relative to oGH, since the latter would involve only unit charge interaction, whereas the hGH interaction is enhanced by the double charge of Ca^{2+} interacting with both negative charges. If structural constraints on the convergence of opposite charges are similar in the hGH- Ca^{2+} -binding protein and oGH-binding protein complex, an inter-charge distance of less than 0.8 nm in both complexes would be consistent with the 9-fold difference in affinity constants between hGH and oGH binding seen in this study (Figs. 2 and 4).

This electrostatic model is also consistent with the observed inverse Ca^{2+} -dependence of oGH binding (Fig. 3). Thus, high calcium concentrations would hinder the interaction between a critical negative charge on the binding protein and the positively charged residue on oGH.

The electrostatic model is consistent with the ability of the singly charged cation Na^{+} to facilitate hGH binding, albeit less effectively than Ca^{2+} [1].

A possible candidate for the critical interactive negative charge in hGH is glutamate-33, since this is the only unit

negative for unit positive substitution in the entire hGH sequence relative to the aligned oGH sequence [22] and it is absent from 20K-hGH. All subprimate GHs characterized to date have a positively charged arginine at the corresponding position [22]. This residue is adjacent to the highly conserved and probably external Tyr³⁵-Ile³⁶-Pro³⁷ sequence [22].

The model proposed here explains Ca²⁺-dependence phenomena in terms of the local involvement of cations in the binding protein-hormone complex. Other, more complex, allosteric models have been proposed in which Ca²⁺ is postulated to induce gross changes in the rigidity or size of the hGH molecule, which facilitate hormone binding [23]. Such gross changes would be likely to condition the formation of several secondary interactions with the binding protein. However, since individual secondary interactions yield free energy changes of 8–20 kJ/mol [24] the resultant shift in affinity constant would be expected to be considerably greater than the 19-fold observed here. It is also difficult to envisage how a general tightening of the molecule by Ca²⁺ would produce on one hand a positive Ca²⁺-dependence (hGH) and on the other a negative dependence (oGH) with no change for most of the 20K-hGH binding.

The explanation of the Ca²⁺-dependence characteristics of 20K-hGH binding appears to be more complex than that for hGH or oGH. The observation that ovine PRL at 0.5 µg/ml competes only for the Ca²⁺-independent component of 20 K-hGH binding (Fig. 8) supports the concept of Ca²⁺-dependent and Ca²⁺-independent subsets of 20K-hGH binding sites. The existence of discrete subsets of high-affinity Ca²⁺-dependent and low-affinity Ca²⁺-independent binding sites for 20K-hGH is also evidenced by the curvilinear Scatchard plots which are linearized by removing Ca²⁺ (Fig. 7). The increased proportion of Ca²⁺-independent binding of 20K-hGH to cytosolic binding proteins relative to membrane-associated binding proteins (Figs. 5 and 6) is also supportive of this idea. The proportions of Ca²⁺-dependent and -independent binding are such that a correspondence of types 1 and 3 with Ca²⁺-potentiated binding and type 2 with Ca²⁺-independent binding is possible (see model in [12]).

Hughes *et al.* [2] have proposed that 20K-hGH is bound with high affinity by only a subset of GH binding sites in the rabbit liver involved in the growth-stimulating action of GH. Our findings support his interpretation of competitive binding studies, and for the first time provide a mechanistic basis for it.

The mechanism by which the deletion of residues 32–46 perturbs the calcium-dependence behaviour of hGH remains to be established definitively. It seems likely that the critical negatively charged residue is contained within the deletion sequence 32–46. If this is the case, since the binding of 20K-hGH to some sites remains Ca²⁺-potentiable, a different domain of the hGH molecule would have to be involved in interactions with the remaining Ca²⁺-potentiable sites. The concept of more than one active centre in hGH and primate GHs already has some support [25–27]. Alternatively, if an allosteric mechanism is involved, the component of binding which remains Ca²⁺-dependent must be less

susceptible to the possible allosteric perturbations induced by deletion of residues 32–46.

Supported by an NHMRC grant to M.J.W.

REFERENCES

- Barnard, R. & Waters, M. J. (1986) *Biochem. J.* **237**, 885–892
- Hughes, J. P., Tokuhira, E., Simpson, J. S. A. & Friesen, H. G. (1983) *Endocrinology* (Baltimore) **113**, 1904–1906
- Fix, J. A., Leppert, P. & Moore, W. V. (1981) *Horm. Metab. Res.* **13**, 508–515
- Lewis, U. J., Bonewald, L. F. & Lewis, L. J. (1980) *Biochem. Biophys. Res. Commun.* **92**, 511–516
- Tinsley, F. C., Grinnan, E. L., Baker, S. H., Powell, J. G., Bemis, K. G. & Shaar, C. J. (1986) *Biochem. Biophys. Res. Commun.* **138**, 342–348
- Ellis, S. (1961) *Endocrinology* (Baltimore) **69**, 554–570
- Thorell, J. I. & Johansson, B. G. (1971) *Biochim. Biophys. Acta* **251**, 363–369
- Waters, M. J. & Friesen, H. G. (1979) *J. Biol. Chem.* **254**, 6815–6825
- Salacinski, P. R. P., McLean, C., Sykes, J. E. C., Clement-Jones, V. V. & Lowry, P. J. (1981) *Anal. Biochem.* **117**, 136–146
- Tsushima, T. & Friesen, H. G. (1973) *J. Clin. Endocrinol. Metab.* **37**, 334–337
- Haro, L. S., Collier, R. J. & Talamantes, F. J. (1984) *Mol. Cell. Endocrinol.* **38**, 109–116
- Barnard, R., Bundesen, P. G., Rylatt, D. B. & Waters, M. J. (1985) *Biochem. J.* **231**, 459–468
- Barnard, R. & Waters, M. J. (1986) *J. Receptor Res.* **6**, 209–225
- Barnard, R., Bundesen, P. G., Rylatt, D. B. & Waters, M. J. (1984) *Endocrinology* (Baltimore) **115**, 1805–1813
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
- McPherson, G. A. (1983) *Comput. Programs Biomed.* **17**, 107–114
- Munson, P. J. & Rodbard, D. (1980) *Anal. Biochem.* **107**, 220–239
- McPherson, G. A. (1985) *J. Pharmacol. Methods* **14**, 213–228
- Hopp, T. P. (1986) *J. Immunol. Methods* **88**, 1–18
- Getzoff, E. D., Tainer, J. A., Weiner, P. K., Kollman, P. A., Richardson, J. S. & Richardson, D. C. (1983) *Nature* (London) **306**, 287–290
- Wells, J. A., Powers, D. B., Bott, R. R., Graycar, T. P. & Estell, D. A. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1219–1223
- Nicoll, C. S., Mayer, G. L. & Russell, S. M. (1986) *Endocrine Rev.* **7**, 169–203
- Moore, W. V., Wong, K. P. & Rawitch, A. B. (1978) *Program Annu. Meet. Endocr. Soc. U.S.A.* 60th, Miami, abstr. 603
- Watson, J. D. (1976) *Molecular Biology of the Gene*, 3rd edn., p. 100, W. A. Benjamin, Menlo Park
- Wallis, M. (1978) in *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins: A Survey of Recent Developments*, vol. 5 (Weinstein, B., ed.), pp. 213–320, Marcel Dekker, New York
- Phares, C. K. & Salem, M. A. M. (1986) *Program Annu. Meet. Endocr. Soc. U.S.A.* 68th, Anaheim, abstr. 749.
- Thomas, H., Green, I. C., Wallis, M. & Aston, R. (1987) *Biochem. J.* **243**, 365–372