

Comparison of a structural and functional binding domain in human growth hormone



Abstract

Biacore's SPR technology was used in a comprehensive investigation into the importance of 30 side-chains buried at the interface between human growth hormone (hGH) and the extracellular binding domain of its receptor, hGHbp [1]. Attachment of hGHbp to the sensor chip surface was specifically designed to eliminate receptor dimerization. The effect of replacing each buried residue in hGH with alanine on the kinetics and affinity of binding showed that only 7 of the side chains account for a major part of the affinity. The predominant role of these side chains, which are clustered near the centre of the structural binding domain, is to decrease the dissociation rate of the complex. Replacement of certain charged residues affected the association rate constant, suggesting that electrostatic interactions are involved in guiding the hormone-receptor binding.

Introduction

The complex between human growth hormone (hGH) and the extracellular domain of its receptor (hGHbp) is one of the best characterized hormone-receptor complexes from a structural point of view [2]. One molecule of hGH binds sequentially to two molecules of hGHbp, involving separate sites on the hormone molecule [3,4] and resulting in dimerization of the receptor. The structural binding domain involved in the first binding site has been identified and has been shown to be comprised of 30 contact residues [2]. Biacore's SPR technology measures the progress of macromolecular interactions on a sensor chip surface in real time [5,6], allowing evaluation of the kinetics as well as the affinity of interactions. This Application Note describes the use of Biacore's SPR technology to determine the effect of replacing each of the 30 contact residues in the hGH site 1 structural binding domain with alanine. Attachment of hGHbp to the sensor chip surface was specifically designed to allow site 1 interaction but prevent receptor dimerization, eliminating complications which might arise from site 2 interactions.

The results indicate that only one quarter of the residues account for the major part of the binding energy. Thus the functional binding domain is considerably smaller than the structural binding domain. These results are potentially valuable in the design of hormone analogues for therapeutic purposes, identifying critical residues for the binding interaction and implying that it might be possible to design smaller hormone mimics.

Materials and methods

Figure 1
Sensorgrams showing binding of wild-type hGH and (G120R)hGH to immobilized S237C hGHbp.

Schematic illustration of the binding reaction, showing how binding of one hGH molecule leads to dimerization of hGHbp. The mutant (G120R)hGH is unable to induce dimerization.

Figure 2
Sensorgrams showing binding of wild-type hGH and (G120R)hGH to immobilized (S201C)hGHbp.

Schematic illustration of the binding reaction. The mutant (S201C)hGHbp is unable to form dimers when immobilized through cysteine at position 201.

Sensor Chip CM5, 2-(2-pyridinyldithio)-ethaneamine (PDEA) and amine coupling kit were obtained from Biacore AB, Sweden. Alanine mutations of residues buried at site 1 in hGH were available from previous work [6] or constructed by site-directed mutagenesis [7].

Mutant hGHbp was immobilized on Sensor Chip CM5 by thiol coupling with PDEA at a single cysteine residue, introduced at one of two specific sites (S201C or S237C). The thiol variants of hGHbp were expressed in *E. coli* and purified as described elsewhere [7]. Immobilization was performed with 50 $\mu\text{g/ml}$ hGHbp in 50 mM Na-acetate pH 4.5, to give approximately 1000 RU (1 ng/mm²) immobilized protein.

Association rates were determined from the binding phase of sensorgrams obtained by injecting a range of concentrations of each hGH variant. Five serial dilutions in 150 mM NaCl, 10 mM Na-PO₄ pH 7.4, 0.02% Tween 20 were used, starting at

200 or 1000 nM hGH depending on the affinity for the hGHbp. The flow rate was maintained at 20 $\mu\text{l/min}$ to minimize mass transport effects [9]. The sensor chip surface was regenerated after each injection with a 20 s pulse of 4.5 mM MgCl₂. Control experiments showed that this was sufficient to remove all bound hormone and that sensor chips could be regenerated up to 50 times with no significant change in binding kinetics.

Dissociation rates were measured by saturating the sensor chip with 5 μM hGH, and then observing dissociation of material from the surface during buffer flow at 20 $\mu\text{l/min}$. Rate constants were derived from the dissociation kinetics during the first 10 minutes of buffer flow to minimize potential rebinding effects. Rate constants were evaluated from the sensorgrams using software provided by Biacore AB [10].

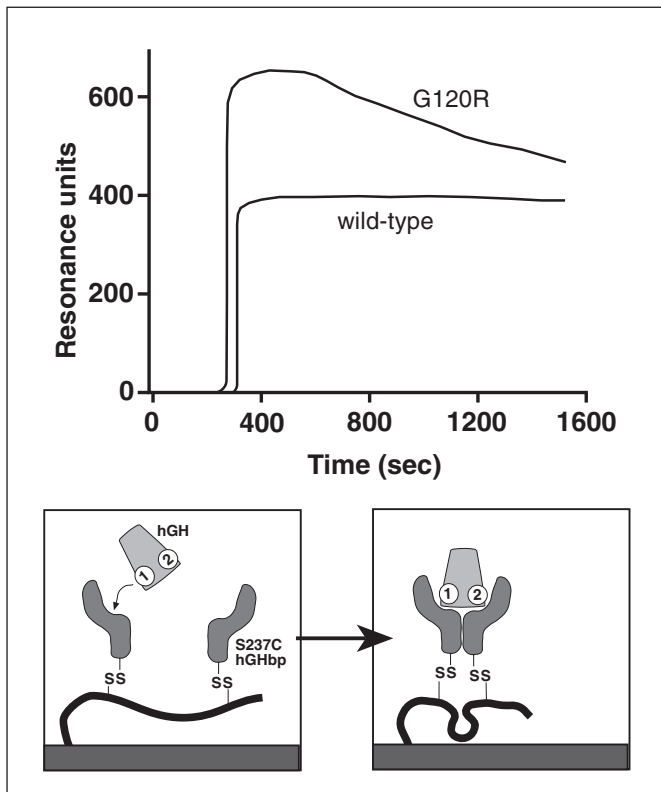


Figure 1

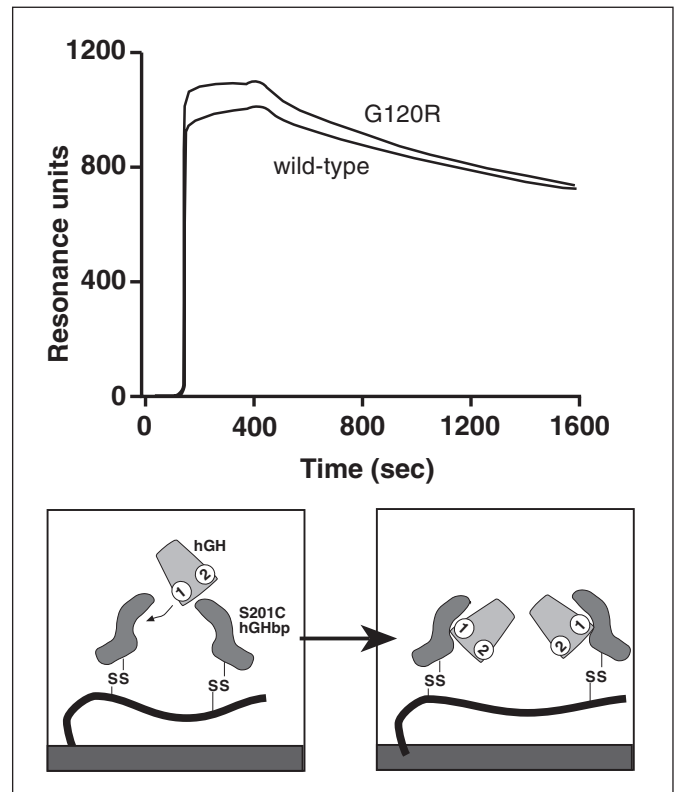


Figure 2

Results

Hormone-receptor interactions on the sensor surface

When hGHbp was attached to the sensor chip surface through a cysteine residue at position 237, wild-type hGH binds rapidly and reached a stable level corresponding to 0.4 molecules of hGH per hGHbp. Dissociation of hGH after the injection pulse was hardly detectable. Mutant (G120R)hGH, which lacks the ability to dimerize receptor, bound with a stoichiometry of 0.7 and dissociated appreciably after the pulse (Figure 1). The association rates of wild type and mutant hGH were comparable. The difference in off rates between wild type and mutant hGH reflects the cooperative effect of the 2:1 binding. These results were taken to indicate that the wild-type hGH induced dimerization of the receptor on the sensor chip surface.

According to the crystallographic structure of the hGH(hGHbp) complex, the two receptor molecules have a contact site at serine-201. When this serine was replaced by cysteine and the S201C variant attached to the sensor chip through this single SH-group, hGH bound to the receptor with a

stoichiometry of 0.84 and (G120R)hGH with a stoichiometry of 0.94 (Figure 2). The kinetics of (G120R)hGH binding to the two receptor variants were closely similar (not shown). Thus by proper positioning of the thiol coupling site on hGHbp, it was possible to orient the receptor on the sensor chip surface to select only binding to site 1 on the hormone. The single-site variant (S201C)hGHbp was used in the analyses described below.

The average standard deviations within triplicate determinations on the same sensor chip was 7% for association constants and 4% for dissociation constants, which compares very favourably with results from RIA determinations [7,11,12].

Analysis of the functional binding domain

For each of the 30 buried residues in the structural receptor-binding domain, a variant hGH was constructed with alanine replacing the residue. All 30 residues have larger side chains than alanine, so direct steric hindrance effects of the substitutions were not expected. The kinetics of binding of each variant to immobilized (S201C)hGHbp are summarized in Figure 3.

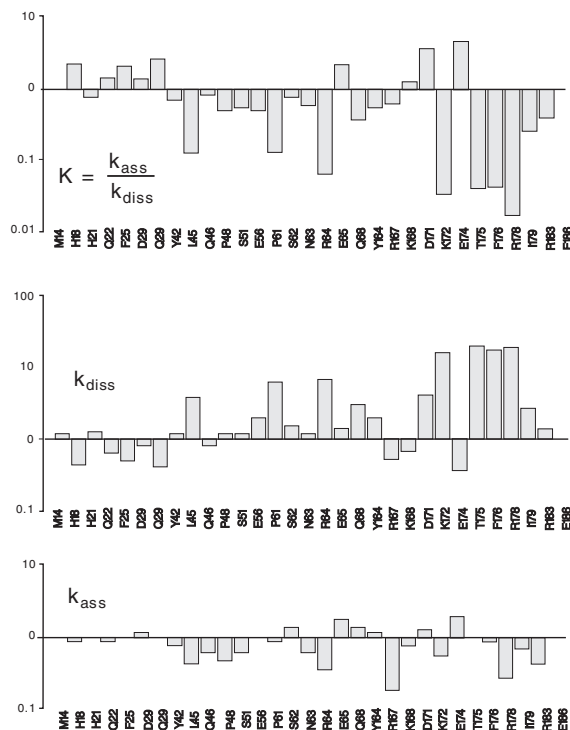


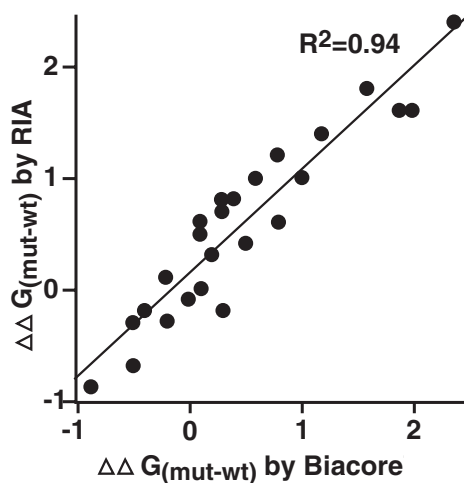
Figure 3. Changes in kinetic constants for binding of wild-type hGH to (S201C)hGHbp as a result of alanine substitution at buried residues in the receptor-binding domain of hGH. Each panel shows the factor change in the respective constant relative to wild-type hormone. Measured constants for the wild-type were k_{ass} $3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, k_{diss} $2.7 \times 10^{-4} \text{ s}^{-1}$, K_D $1.1 \times 10^9 \text{ M}^{-1}$.

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Figure 4. Correlation between the change in free binding energy resulting from alanine substitutions in hGH, calculated from kinetic constants measured with Biacore (horizontal axis) and RIA (vertical axis).

Figure 4 shows tight correlation between changes in binding energies estimated with Biacore's SPR technology and previous measurements with RIA [7,11,12], indicating that the sensor chip surface matrix does not introduce systematic binding artifacts. Of the 30 buried side chains, only 7 alanine substitutions reduced the affinity more than 5-fold, while 5 significantly increased the affinity of the interaction in the opposite direction. Substitution at 11 of the 30 positions affects the affinity by less than a factor of 2. The functionally important residues cluster in a small region near the centre of the structural binding domain (not illustrated). In terms of rate constants, off-rate effects are generally much larger than on-rate effects. The greatest effect on on-rates is produced by substitution of charged side chains: replacement of arginine at positions 64, 167 and 178 decreases the association rate constants while replacement of glutamic acid at positions 65 and 174 increases the association rate constants. These results indicate that electrostatic interaction with negatively charged side chains on the receptor may be important for guiding the hormone to the receptor binding site. However, these are not general electrostatic effects since substitution of other charged residues (e.g. D26, E56, K168, K172, E186) has little or no effect on the on rate.



Conclusions

Establishment of a clearly defined experimental situation for the Biacore® studies was aided by the specific immobilization chemistry on the sensor chip. By immobilizing the receptor through a single cysteine residue introduced at a chosen position, the properties of the surface-bound interaction could be closely controlled. This is a major advantage in comparison with other less-specific surface immobilization methods such as general amine coupling reactions (often used in Biacore systems) or antibody-mediated adsorption (for conventional immunological techniques).

It may reasonably be inferred that the structural receptor-binding domain identified by x-ray studies on hGH-hGHbp complexes includes residues important for the binding function. The Biacore studies described here complement the structural information in an important way, and identify a subset of the structural binding domain residues as a “functional binding domain”. The detailed information provided by real-time kinetic studies allows a precise description of the involvement of residues in the functional binding domain in terms of separate effects on association and dissociation rates.

This work demonstrates the value of Biacore's SPR technology in analysing functional aspects of macro-molecular interaction. The results are of considerable potential value in designing smaller and site-directed growth hormone analogues.

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