Biosensor Measurement of the Binding of Insulin-like Growth Factors I and II and Their Analogues to the Insulin-like Growth Factor-binding Protein-3*

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Most insulin-like growth factor (IGF) molecules in the circulation are found in a 150-kDa complex containing IGF-binding protein-3 (IGFBP-3) and an acid-labile subunit, which does not itself bind IGF. Affinities ($K_d$ values) between 0.03 and 0.5 nM have been reported for IGF-I/IGFBP-3 binding, but no kinetic data are available. In this study we measured the high affinity binding of unlabeled IGFs and IGF analogues to recombinant unglycosylated IGFBP-3, using a BIAcore™ instrument (Pharmacia Biosensor AB). IGF-I binding showed fast association and slow non-first-order dissociation kinetics, and an equilibrium $K_d$ of 0.23 nM. IGF-II had similar kinetics with slightly higher affinity. Analogues with mutations in the first 3 amino acids of the B-region (des(1-3) IGF-I and long IGF-I) showed 25 and 50 times lower affinity than IGF-I. Replacement of residues 28–37 by Gly-Gly-Gly-Gly or deletion of residues 29–41 in the C-region had little effect on the kinetic parameters, contrasting with the markedly impaired binding of these analogues to the IGF-I receptor. Swapping of the disulfide bridges in IGF-I and the C-region mutants decreased the affinity dramatically for IGFBP-3, primarily by decreasing the association rate. Insulin had approximately 1000 times lower affinity than IGF-I.

Insulin-like growth factor-I and -II (IGF-I and IGF-II)† are small proteins that stimulate a variety of growth-promoting and metabolic effects via an interaction with the IGF-I receptor (1). The peptides consist of four regions: A- and B-regions, which are homologous to the A and B chains of insulin; a C-region, which is analogous to but unrelated to the C peptide of insulin that connects the A- and B-regions; and a short carboxyl-terminal D-region, with no counterpart in insulin (2, 3).

IGF-I and -II form complexes with six different IGF-binding proteins (IGFBPs) in the circulation and in the extracellular environment (4). In the circulation the majority of IGFs are bound in a 140-kDa complex consisting of IGFBP-3 of 40–50 kDa, an approximately 85-kDa so-called acid-labile subunit, and IGF-I or IGF-II (5–10). The IGFs bind to IGFBP-3 with high affinity, and the acid-labile subunit is then capable of binding to the formed complex with a somewhat lower affinity (11). The major biological function of IGFBP-3 is believed to be to extend the circulating half-lives of IGFs, since IGFs bound to the 140-kDa complex are cleared from the circulation much slower than free IGFs (12).

Wild type IGFBP-3 is heavily glycosylated, which results in an apparent molecular mass of 40–50 kDa on denaturing SDS-polyacrylamide gel electrophoresis, while the non-glycosylated molecule has a molecular mass of approximately 29 kDa. The non-glycosylated protein’s binding characteristics are reported to be identical to those of the wild type molecule (13).

Attempts have been made to identify the regions of IGF-I involved in the high affinity binding to IGFBP-3 by constructing insulin/IGF-I hybrids and by using site-directed mutagenesis of the IGF-I gene. From the studies involving insulin/IGF-I hybrids, it was established that the B-region but not the D-region of IGF-I is important for binding to IGFBP-3 (14–16). From the studies using site-directed mutagenesis, it was found that the C-region is of less importance than the B-region for high affinity binding to IGFBP-3 (17), and that residues 3 and 4, the region between residues 8 and 18, and residues 49–51 appear to be most important for IGFBP-3 binding (11). In this study we have investigated the detailed association and dissociation binding kinetics between IGFBP-3 and IGF-I, IGF-II, insulin, and seven synthetic IGF-I analogues with mutations in the B- or C-regions and with normal and swapped disulfide bridges. In order to generate detailed kinetics, we have employed the BIAcore™ instrument developed by Pharmacia, and analyzed the data by using computer fitting programs developed at the Hagedorn Research Institute.

The BIAcore™ technology makes it possible to visualize macromolecular interactions directly and in “real time” (18). The BIAcore is a biosensor-based instrument that uses surface plasmon resonance as the detection principle. One molecule of the interaction to be studied is immobilized covalently to a sensor chip, and the other interactant is then passed over the chip in solution. The detection system measures and displays on a computer screen a signal proportional to the mass of protein bound to the surface. In this way, the association phase can be directly visualized as the ligand-containing solution flows over the surface, and the subsequent dissociation is similarly displayed after the flow switches to buffer containing no ligand (18).

The determination of the binding kinetics of IGF-I analogues toward IGFBP-3 is important for the development of IGF-I analogues with reduced affinity for IGFBP-3 and normal affinity for the IGF-I receptor, which could be of clinical interest.

MATERIALS AND METHODS

Equipment and Reagents—The BIAcore™ instrument, sensor chip CMS (certified), surfactant P20, and the amine coupling kit containing N-hydroxysuccinimide (NHS), N-ethyl-N’-(3-diethylaminopropyl)carbo-
diimide (EDC), and ethanolamine hydrochloride were acquired from Pharmacia Biosensor AB (Uppsala, Sweden). The cloned non-glycosylated insulin-like growth factor-biding protein-3 (IGFBP-3) was produced in Escherichia coli by Ceftrix Pharmaceuticals, Inc. (Santa Clara, CA). IGF-I, IGF-II, long IGF-I, and des(1-3)-IGF-I were from GroPep Pty. Ltd. (Adelaia, Australia). Mini-IGF-I, 4-Gly-IGF-I, IGF-I swap, mini-IGF-I swap, and 4-Gly-IGF-I swap were produced in Saccharomyces cerevisiae at Birkbeck College, London. All other chemicals used were of analytical grade, and distilled water was used for buffers.

Immobilization of IGFBP-3 on Sensor Chip—Equal volumes of 0.1 M NHS and 0.1 M EDC were mixed by the BIAcore system's robotics, and 8 µl of the mixture was injected over the surface of the sensor chip to activate the carboxymethylated dextran. 10 µl of IGFBP-3 solution (11 µg/ml in 10 mM sodium acetate, pH 4.5) was then injected over the activated surface, followed by 35 µl of 1 M ethanolamine to deactivate remaining active carboxyl groups. Immediately after the immobilization 35 µl of 100 mM HCl was injected in order to remove remaining noncovalently bound IGFBP-3. This was followed by a 3-h wash with HBS buffer (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.05% P20, pH 7.4), to ensure a stable baseline. During the wash, the baseline variation was typically less than 3%h. Immediately before the injection of ligand, the surface was once again exposed to 100 mM HCl to ensure equal conditions for all the injected ligands. The immobilization procedure was carried out at 25 °C and at a constant flow rate of 5 µl/min HBS buffer. Approximately 650 resonance units (RU) of IGFBP-3 were coupled (0.65 ng/mm²) (18). An example of an immobilization is seen in Fig. 1. A more thorough description of the immobilization procedure can be found elsewhere (19).

Kinetic Assays on the BIAcore—All experiments were carried out at 25 °C with a constant flow rate of 5 µl/min HBS buffer. 32 µl of purified ligand (e.g., IGF-I) diluted to 50 µM in HBS buffer was injected over the immobilized IGFBP-3 (association phase), followed by a 20-min period where pure HBS buffer was passed over the surface (dissociation phase). Since handling of the buffer during injection is enough to cause a small change in refractive index, there is an upward shift in baseline at the beginning and a downward shift at the end of the association phase (see Fig. 2). The up and down shifts were estimated by close-up inspection of the transition regions on a computer screen, and the total refractive index shift was approximated by a straight line interpolation between these two estimated values. The sensogram was corrected for this effect by subtracting the line from the association phase data. All kinetic assays were followed by an injection of 35 µl of 100 mM HCl in order to dissociate the remaining ligand from the binding protein (regeneration phase). All the steps described were fully automated and were carried out by the BIAcore system's robotics. Association, dissociation, and regeneration phases were followed in real time as a change in signal expressed in resonance units (RU) (4). 1000 RU represents approximately 1 ng/mm² of protein bound to the sensor chip surface. Curves derived from these assays were used to generate kinetic constants. An example of a kinetic assay done with IGF-I is shown in Fig. 2.

Data Analysis—Kinetic constants were generated from the association and dissociation curves from the BIAcore experiments by fitting to a simple two-site interaction model. This model was chosen since the curves (especially the dissociation phase) were biphasic (Fig. 2). The stoichiometric equations for this system are as follows.

In these equations, H is the hormone (ligand) concentration, $P_1$ and $P_2$ refer to the concentration of free binding sites 1 and 2, respectively, on IGF-BP-3, and $B_1$ and $B_2$ refer to the concentration of ligand bound to $P_1$ and $P_2$. $k_{a1}$ and $k_{a2}$ (i = 1, 2) are the respective kinetic association and dissociation rate constants. During a BIAcore experiment, the ligand concentration is assumed to be constant in the association phase because of the continuous flow and zero in the dissociation phase. These assumptions result in the following solutions, for association:

$$B = \frac{k_{a1}H}{k_{a1}H + k_{d1}} (1 - e^{-k_{d1}t + k_{a1}t}) + \frac{k_{a2}H}{k_{a2}H + k_{d2}} (1 - e^{-k_{d2}t + k_{a2}t})$$

and for dissociation:

$$B = B_1 e^{-k_{d1}t} + B_2 e^{-k_{d2}t}$$

Here $B_{10}$ and $B_{20}$ are the total concentrations of binding sites 1 and 2, respectively, and $B_{10}$ and $B_{20}$ are the amount of ligand bound to sites 1 and 2.
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RESULTS

To study the binding of IGF-I/analogue to IGFBP-3, the binding protein was immobilized to the BIAcore sensor chip as described above. Kinetic binding assays were carried out for IGF-I, IGF-II, human insulin, and seven IGF-I analogues with the following code names and mutations: des(1–3) IGF-I (deletion of amino acids 1–3), long IGF-I (possessing a 13-amino acid extension at the NH₂ terminus, and amino acid at position 3 changed from Glu to Arg), 4-Gly IGF-I (IGF-I with residues 28–37 replaced by a 4-glycine bridge), mini-IGF-I (deletion of the C-region residues 28–41), and 4-Gly IGF-I swap, 4-Gly IGF-I swap, and mini-IGF-I swap, where swap indicates that the disulfide bridge normally connecting residue 52 to 47 now connects residue 52 to 48, and the disulfide bridge normally connecting residue 6 to 48 now connects residue 6 to 47. The analogues can be divided into four groups according to their mutations; the wild type hormones IGF-I, IGF-II and insulin, the C-region mutants 4-Gly IGF-I and mini-IGF-I, the B-region mutants des(1–3) IGF-I and long IGF-I, and finally the analogues with swapped disulfide bridges IGF-I swap, 4-Gly IGF-I swap, and mini-IGF-I swap. Fig. 3 shows examples of association and dissociation curves for the analogues grouped according to their mutations. Sensorgrams are shown prior to correction for retractive index shift, and the figures show the curve for IGF-I for comparison. In each experiment, the assays were carried out at a ligand concentration of 50 nM on a surface with the same amount of immobilized IGFBP-3. Fig. 3a shows that IGF-I and IGF-II have very similar curves with fast association and slow dissociation, whereas insulin has a very low binding affinity for the binding protein. In Fig. 3b, curves for the two analogues with the C-region mutations are shown. Both analogues have almost identical kinetics, which resemble the kinetics of IGF-I, although they do display a slower association and a faster dissociation rate.

In Fig. 3c are shown kinetic curves for the two analogues with B-region mutations. These mutations reduce the analogue’s affinity for IGFBP-3 when compared to wild type IGF-I, by decreasing the association rate and increasing the dissociation rate. This finding is in agreement with previously published work carried out with crude acid-stable serum-binding proteins (21) and also with work carried out with a 4-kDa binding protein secreted from bovine kidney cells (22). Fig. 3d shows binding curves for the three IGF-analogues with swapped disulfide bridges. It is clear from this picture that swapping of the disulfide bridges in IGF-I greatly reduces its affinity for IGFBP-3.

Data points obtained from the above-mentioned curves were used to calculate kinetic constants. This was done by fitting the binding model described earlier to the data points after correction for the bulk refractive index shift. Fig. 4 shows an example of a curve fit done on corrected data from IGF-I, IGF-II, and insulin experiments. The association, dissociation, and equilibrium dissociation constants calculated from these data are presented in Table I. The analogues in the table have been grouped according to their mutations. IGF-I and IGF-II have the fastest association rate and the slowest dissociation rate of all the analogues, resulting in the highest affinities, with IGF-II having about twice the affinity of IGF-I for IGFBP-3. The calculated affinities of the other analogues are in good agreement with the degree of binding observed in the kinetic binding curves in Fig. 3.

DISCUSSION

In this study non-glycosylated IGFBP-3 was found to have comparable affinity for IGF-I and IGF-II with a slight preference for IGF-II, which is in agreement with what has been reported previously (23). We found the equilibrium dissociation constants for IGF-I and IGF-II to be 0.23 nM and 0.12 nM, respectively, which is approximately 5 times higher than reported by Martin et al. and Sommer et al. (23, 24). The discrepancy might be explained by differences in the assays used.
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| Peptide          | $k_+ \times 10^4 \pm \text{S.E.}$ | $k_2 \times 10^{12} \pm \text{S.E.}$ | $K_d \pm \text{S.E.}$ |
|------------------|----------------------------------|----------------------------------|-------------------|
| IGF-I            | 3.5 $\pm$ 0.37                    | 0.78 $\pm$ 0.11                   | 0.23 $\pm$ 0.04   |
| IGF-II           | 5.2 $\pm$ 0.74                    | 0.62 $\pm$ 0.005                  | 0.12 $\pm$ 0.02   |
| Insulin          | 0.04 $\pm$ 0.008                  | 9.5 $\pm$ 2.1                     | 251 $\pm$ 91      |
| 4-Gly IGF-I      | 1.8 $\pm$ 0.11                    | 1.4 $\pm$ 0.15                    | 0.8 $\pm$ 0.096   |
| Mini-I GF-I      | 1.4 $\pm$ 0.2                    | 2.3 $\pm$ 0.2                    | 1.5 $\pm$ 0.24    |
| Des (1-3) IGF-I  | 1.6 $\pm$ 0.21                   | 0.9 $\pm$ 0.66                    | 5.6 $\pm$ 0.85    |
| Long IGF-I       | 0.53 $\pm$ 0.06                  | 6.3 $\pm$ 0.003                   | 11.9 $\pm$ 1.3    |
| 4-Gly Swap       | 0.32 $\pm$ 0.19                  | 2.1 $\pm$ 0.44                    | 6.6 $\pm$ 1.4     |
| Mini-Swap        | 0.36 $\pm$ 0.14                  | 2.3 $\pm$ 0.29                    | 6.4 $\pm$ 2.6     |
| IGF-I Swap       | 0.11 $\pm$ 0.05                  | 1.7 $\pm$ 1.0                     | 15.5 $\pm$ 11.8   |

It is clear from the low affinities of IGF-I swap, 4-Gly IGF-I swap and mini-IGF-I swap that swapping of the disulfide bridges of IGF-I greatly impairs the hormone's ability to bind to IGFBP-3. This is primarily due to a decreased association rate. The swapped IGF-I analogues also have extremely low IGF-I receptor affinity, which indicates that swapping of the disulfide bridges causes a major conformational change of the entire hormone as found by Miller et al. (28). These findings are important as IGF-I swap is secreted from recombinant organisms (Saccharomyces cerevisiae and E. coli) and arises from the refolding of denatured IGF-I in quantities similar to those of the normally folded product (29–31).

In this study we found IGFBP-3 to have a high and a low affinity binding site for the IGFs, where the high affinity binding site is responsible for approximately 90% of the binding. However, it is uncertain whether the low affinity binding site has any biological relevance, since in these experiments IGFBP-3 is immobilized to the sensor chip via amine groups in a random way, and some IGFBP-3 molecules might be bound to the sensor chip close to the ligand binding site, thus impairing their affinity for the ligand.

We have found that the BIACore assay used in this study has several advantages over the classic 125I-based tracer assays. Most importantly, the BIACore system is label-free and shows the binding of IGF-I analogues to IGFBP-3 in real time. The BIACore system is also time-saving since the system's robotic unit allows for large series of samples to be assayed automatically. Since the other five IGF-I-binding proteins are structurally related to IGF-I (3), we believe that it should also be possible to use the assay described here for the study of interactions between these proteins and IGF-I analogues.

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