Characterization of Ligand Binding of a Soluble Human Insulin-like Growth Factor I Receptor Variant Suggests a Ligand-induced Conformational Change*

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Details of the signal transduction mechanisms of the tyrosine kinase family of growth factor receptors remain elusive. In this work, we describe an extensive study of kinetic and thermodynamic aspects of growth factor binding to a soluble extracellular human insulin-like growth factor-I receptor (sIGF-IR) variant. The extracellular receptor domains were produced fused to an IgG-binding protein domain (Z) in transfected human 293 cells as a correctly processed secreted α-β'-Z dimer. The receptor was purified using IgG affinity chromatography, rendering a pure and homogenous protein in yields from 1 to 5 mg/liter of conditioned cell media. Biosensor technology (BIACore) was applied to measure the insulin-like growth factor-I receptor (IGF-I), des(1–3)-IGF-I, insulin-like growth factor-II, and insulin ligand binding rate constants to the immobilized IGF-I-R-Z. The association equilibrium constant, \( K_a \), for the IGF-I interaction is determined to 2.8 × 10^8 M\(^{-1}\) (25 °C). Microcalorimetric titrations on IGF-I/IGF-I-Z were performed at three different temperatures (15, 25, and 37 °C) and in two different buffer systems at 25 °C. From these measurements, equilibrium constants for the 1:1 (IGF-I:((α-β'-Z)_2)) receptor complex in solution are deduced to 0.96 × 10^8 M\(^{-1}\) (25 °C). The determined heat capacity change for the process is large and negative, −0.51 kcal (K mol\(^{-1}\)). Further, the entropy change (ΔS) at 25 °C is large and negative. Near- and far-UV circular dichroism measurements display significant changes over the entire wavelength range upon binding of IGF-I to IGF-I-R-Z. These data are all consistent with a significant change in structure of the system upon IGF-I binding.

Cellular receptor structure and function relationships have become the focus of an increasing amount of research, relevant for biological understanding as well as for possible pharmaceutical applications. The insulin-like growth factor-I receptor (IGF-I\(^{R1}\)) is a transmembrane glycoprotein and belongs to the receptor tyrosine kinase family that includes, among others, the insulin (insR), epidermal growth factor, and platelet-derived growth factor receptors. IGF-I is a major regulator of both cellular growth and metabolism (3). The components of the IGF hormone signaling system are tightly regulated, both in tissue-specific and developmentally specific manners. The IGF molecular system contains three known peptide ligands (IGF-I, IGF-II, and insulin), three types of cellular receptors (IGF-I\(^R1\), insR\(^R2\), and IGF-II/mannose 6-phosphate), and six known subtypes of circulating IGF-binding proteins (IGFBPs) (IGFBP-1 to IGFBP-6). The molecular components of the IGF signaling system have been the subject of several recent review articles (3, 4). The mechanism by which insulin and IGF molecules interact with their respective receptor to mediate signaling is still not understood in great detail at the molecular level. The ligand-induced receptor activation has been suggested to involve a conformational switch in the quaternary structure upon ligand binding, with movements of the extracellular α parts and a congregation of the cytoplasmic tyrosine kinase regions to enable activation (5, 6). However, the complexity of IGF-I signaling remains rather elusive almost 10 years after the molecular cloning of the human IGF-I receptor gene (7).

IGF-I\(^R1\) is active as a preformed heterotetrameric receptor containing two extracellular α-domains with ligand binding sites and two transmembrane-spanning β-domains, also harboring the cytoplasmic tyrosine kinase activity (Fig. 1A). The chains are disulfide-linked in a β-α-α-β arrangement with possibly three disulfide linkages between the α chains and one disulfide bond between each α-β chain pair (8). The gene is transcribed and translated as a single α-β polypeptide chain and posttranslationally processed and assembled into a mature receptor. It has been shown for the homologous insulin receptor that receptor maturation is achieved through proteolytic processing, oligosaccharide attachment, and disulfide-linked homodimerization during the transport from endoplasmic reticulum to Golgi and further to the cell surface (9).

In this paper, the ligand binding properties of a purified extracellular portion of the IGF-I\(^R1\) have been characterized using BIACore biosensor analysis. From the BIACore data, molecular association and dissociation rates as well as binding equilibrium constants were calculated. Binding properties of the soluble IGF-I\(^R1\) receptor variant were analyzed for the ligands IGF-I, des(1–3)-IGF-I, IGF-II, and insulin, respectively. Near- and far-UV circular dichroism measurements were performed to establish an estimation of possible secondary structure changes upon ligand association.

Microcalorimetric titration experiments of IGF-I to the heterotetrameric IGF-I\(^R1\)-Z were performed at three different temperatures, from which enthalpies, \( ΔH \), Gibbs free energies, \( ΔG \), entropies, \( ΔS \), and heat capacity, \( ΔC_p \), of the binding process could be calculated. In this work, we have used the model proposed by Freire et al. (10–13) to analyze microcalorimetric titration data of the binding of IGF-I to the heterotetrameric IGF-I\(^R1\)-Z.
Fig. 1. A, schematic representation of the full-length IGF-I receptor; B, the produced extracellular receptor domains fused to the Z affinity handle. The α-chain contains two homologous domains, L1 and L2, separated by a cysteine-rich region. The β-chain contains extracellular fibronectin type III type repeats (Fn3), a single transmembrane region, and the intracellular tyrosine kinase domain. Black horizontal bars indicate potential α-α cysteine disulfide bonds. The topology and cysteine arrangement is based on the work by Ward et al. (8). The transmembrane and intracellular domains of the produced soluble receptor are replaced by a single IgG binding affinity handle, Z. The position of the inserted thrombin cleavage site between the receptor and Z is indicated.

EXPERIMENTAL PROCEDURES

Ligand Proteins—Native IGF-I, des(1–33)IGF-I, and IGF-II were produced in Escherichia coli and purified as described previously (14, 15). Recombinant human insulin was purchased from Sigma.

Production Vector Construction—A vector was constructed for the production of IGF-Ih, including an affinity handle for purification and analysis purposes. Human IGF-I receptor cdNA was cloned using linked reverse transcriptase-polymerase chain reaction of placental total RNA (Clontech) into a vector containing cytomegalovirus promoter and enhancer regions, a single Z domain gene, a stop codon followed by SV40 poly(A)-signal and 3′-untranslated regions. A sequence coding for a thrombin-sensitive cleavage linker, SGLVPRGSGS, was inserted between the receptor β-3′ and Z-5′ coding regions at amino acid position 933 according to the numbering of Ullrich et al. (7). The outline of the expression cassette of the resulting plasmid, pKGE978, is shown in Fig. 2.

Transfection and Expression—Human primary kidney 293 cells were transfected for transient expression using the calcium phosphate method (16). Cells were incubated for 24 h in Dulbecco’s modified Eagle’s medium/McCoy’s 5A 1:1 (Life Technologies, Inc.) plus 5% bovine calf serum (HyClone), upon which the medium was replaced by serum-free medium and cells were incubated for another 24 h. Stable transfected cell lines were established from 293 human kidney cells transfected with pKGE978 and co-transfected with plasmid pKGE800 carrying a neomycin resistance gene. Selection was performed using G418, Geneticin, (Sigma) at 1 mg/ml.

Receptor Purification—Cell media harvested after 48–72 h of growth were centrifuged to remove cell debris and further diluted with 1/10 volume of water and 1/10 volume of 100 mM Tris·HCl, 50 mM NaCl, 0.05% Tween 20, pH 7.4 (20, 21) to buffer the media to a pH suitable for the chromatography step. Diluted and buffered medium was filtered through a 0.22-μm filter to remove insoluble material prior to chromatography. Affinity chromatography purification was performed using an IgG-Sepharose FF column (Pharmacia Biotech, Sweden), previously equilibrated with 1 × Tris·HCl. Unspecifically bound proteins and impurities were removed with 10 column volumes of 1 × Tris·HCl and 2 volumes of 5 mM ammonium acetate, pH 5.0. Bound fusion protein was subsequently eluted by 0.2 M acetic acid, pH 2.8. Fractions were immediately neutralized through collection in tubes containing 1/10 fraction volume of 1 M Tris·HCl, pH 8.0. The buffer of the eluted material was exchanged to 10 mM HEPES, pH 7.4, through several rounds of dialysis and concentration using a Filtron ultrafiltration unit with a 30-kDa molecular mass cut-off (Filtron). Purified and concentrated receptor was stored in aliquots at −80 °C.

Biochemical Characterization—Quantitative amino acid analysis was performed by acid hydrolysis of the peptide chain in 6 M HCl at 155 °C for 45 min, followed by analysis using an ion exchange column and ninhydrin derivative detection. The analysis was performed on a Beckman 6300 amino acid analyzer, equipped with a System Gold data handling system (Beckman).

The N-terminal protein sequence was determined using a HP G1005A protein sequencing system with on-line phenylthiohydantoin analysis, version 2.2 chemistry (Hewlett-Packard).

The oligosaccharide content of the receptor was determined by analysis of hexose content as described by Kenne and Strömberg (17). Briefly, hexose content was determined by acid hydrolysis and alditol acetate derivative of released sugars. Sugar alditol acetates were separated by gas chromatography-mass spectrometry on a fused silica capillary column (25 m × 0.2 mm) at 250 °C. An HP 5970B mass selective detector (Hewlett-Packard) was used for monitoring eluted peaks. Samples were compared with calibration curves constructed using defined sugar standards. N-Acetylenuraminic acid (sialic acid) content was analyzed by cleavage of the glycosylating carbohydrate chains with hydrogen chloride in methanol, transferring the sialic acid to its methyl ester methyl glycoside and further separation by gas chromatography.2 Protein homogeneity was evaluated by SDS-polyacrylamide gel electrophoresis (18) using NOVEX precast 4–20% gradient polyacrylamide gels (Novel Experimental Technology) stained with Coomassie Blue staining. The Z affinity tail was detected using semidry transfer to nitrocellulose membranes and peroxidase anti-peroxidase staining as detailed previously (19). Apparent molecular mass of both free and complexed receptor was determined using laser light-scattering analysis, performed with a Protein Solutions DynaPro 801 TC (Protein Solutions). The receptor was analyzed at a concentration of 1 mg/ml in 10 mM phosphate buffer, pH 7.4, at seven different temperatures in the range 4–25 °C.

Partial Reduction and Alkylation of the Receptor—Partial reduction of receptor heterotetramers from (αβ′-Z)2 to αβ-Z was performed essentially according to the protocol of Sweet et al. (20, 21). A 5-ml volume of 1 M Tris, pH 10.5, was added to 30 μg of receptor in 50 μl of 50 mM Heps buffer, pH 7.6, giving a final pH of 8.5. The mixture was incubated at room temperature for 25 min before the addition of reduced dithiothreitol to a final concentration of 2 mM. Following a 15-min incubation, free thiols of the partially reduced receptor were alkylated by adding iodoacetic acid to a final concentration of 100 mM. The alkylation reaction was allowed to proceed for 60 min at room temperature in the dark. The sample was then desalted on a PD-10 Sephadex G50 column (Pharmacia Biotech, Inc.), preequilibrated with 50 mM ammonium acetate, pH 6.8, and lyophilized. The partial reduction was analyzed using SDS-polyacrylamide gel electrophoresis on a 4–20% precast NOVEX gradient gel stained using Coomassie Brilliant Blue.

Ligand Binding Assay—Analytical ligand binding assays were performed using phosphate, pH 7.5, 150 mM NaCl, 0.05% Tween 20 as assay buffer and 125I-labeled IGF-I (Amersham, UK). Pre-IgG-coated Scintistrip microwell plates (Wallac, Finland) were incubated with the
Fig. 2. A, outline of the expression vector pKGE978. The receptor nucleotide sequence starts at position 13 and proceeds to position 2842 (amino acids 30 to 902). Transcription is initiated from the xcmv promoter region (p/E CMV), shown as a black arrow, followed by the human IGF-I receptor cDNA sequence and the SV40 3'-untranslated sequence. Restriction enzyme cleavage sites are shown above the vector outline. B, the translated protein product is shown with signal sequence, amino acids 30 to 91, as a white box; the α-domain, amino acids 1–740, is shown as a light gray box; and the β extracellular domain, β’, amino acids 741–932, is shown as a black box. The IGF-I receptor is shown with Z as a hatched box. RKRR indicates the intradomain cleavage sequence. The inserted thrombin cleavage site between β and Z domains is indicated.

receptor preparation. Plates were washed three times with PBS-T and three times with assay buffer. One series of dilutions was incubated with 125I-IGF-I (20,000–30,000 cpm/0.1 ml). A second series was incubated with 125I-IGF-I and unlabeled recombinant IGF-I (1 μg) for control of unspecific binding. After incubation at 4 °C for 3 h at room temperature for 1 h, plates were rapidly washed with assay buffer and allowed to dry. For competitive ligand binding assays, plates were coated with a defined receptor concentration and washed. Receptors were incubated with 100 μl of 125I-IGF-I in all wells. Second, serial dilutions were made starting with 50 μl of 125I-IGF-I solution containing 3 μg unlabeled IGF-I. After incubation, wells were treated as described above. Bound radioactivity was measured using a γ counter (Wallac).

Circular Dichroism—Far-UV circular dichroism spectra were recorded using a Jasco J 720 spectropolarimeter. Spectra were recorded from 250–184 nm at a step resolution of 0.1 nm using a scanning speed of 10 nm/min. Each shown spectrum is the average of three accumulations. Protein was dissolved in 15 mM potassium phosphate buffer, pH 7.0, to a concentration of 0.2 mg/ml. Cuvette path length was 0.1 cm. Actual protein concentration was determined using quantitative absorbance at 280 nm.

Thermodynamic Analysis—The binding of a ligand to a protein and protein folding are thermodynamically analogous processes. Except for hydrogen bonding, electrostatic interactions, or other specific interactions, the most important contribution to the thermodynamic properties of the change in hydrophobic hydration. Changes in hydration can be a result of changes in the binding surfaces of both protein and ligand as well as changes in hydration due to conformational changes of the protein. The heat capacity change, ΔCp (∆Cp = dH/dT), is the thermodynamic property that is most affected by changes in hydrophobic hydration. When transferring a nonpolar surface from a nonpolar environment to an aqueous environment, e.g., dissociation of protein–ligand complexes or unfolding of proteins, the ΔCp is large and positive. A striking feature of transferring a small hydrocarbon compound from a nonpolar environment to an aqueous solution is the high
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degree of group additivity to the heat capacity (29–31). The group additivity rules can be translated into models where the thermodynamic properties are functions of accessible solvent surface area, ∆ASA. Stuter and et al. (32), Livingstone et al. (35), and Freire et al. (11) have similar approaches to divide the contributions from hydration/dehydration of nonpolar and polar-accessible surface areas, ∆ASA_{np} and ∆ASA_{ps}, respectively, by parameterizing ∆H_{p} and ∆C_{p}. It has been shown that the major contributions to ∆C_{p} and ∆H_{p} for protein folding/unfolding or protein-ligand interactions can be rationalized in terms of ∆ASA (11, 32, 33).

From the temperature dependence of the enthalpy, the heat capacity was calculated.

\[
\Delta C_{p}^{b} = d\Delta H / dT \quad \text{(Eq. 1)}
\]

Due to the large heat capacity change, the enthalpy and the entropy are strongly temperature-dependent. Equations 2 and 3 show how the enthalpy, ΔH, and the entropy, ΔS, vary with the temperature, T, relative to a reference temperature, T_{ref}.

\[
\Delta H(T) = \Delta H(T_{ref}) + \Delta C_{p}^{b}(T-T_{ref}) \quad \text{(Eq. 2)}
\]

\[
\Delta S(T) = \Delta S(T_{ref}) + \int \frac{\Delta C_{p}^{a}}{T}dT = \Delta S(T_{ref}) + \Delta C_{p}^{a}(T/T_{ref}) \quad \text{(Eq. 3)}
\]

The proton exchange in the binding process, i.e. proton linkage, was obtained by determining the enthalpy of binding in the two different buffer systems, PBS and HBSS, at 25 °C.

The intrinsic binding enthalpy, ΔH_{int}, and the number of linked protons, Δn, is calculated from Equations 4 and 5.

\[
\Delta H_{\text{int}} = \Delta H_{\text{obs}} + \Delta H_{\text{ion}}\Delta n \quad \text{(Eq. 4)}
\]

\[
\Delta H_{\text{obs}} = \Delta H_{\text{int}} + \Delta H_{\text{link}}\Delta n \quad \text{(Eq. 5)}
\]

where ΔH_{\text{int}} and ΔH_{\text{obs}} are the observed enthalpies of binding in the two different buffers and where ΔH_{\text{link}} and ΔH_{\text{obs}} are the ionization enthalpies for the used buffers.

When analyzing the calorimetric data we have used the method developed by Freire et al. (13) for calculating changes in hydration-accessible nonpolar surface area, ∆ASA_{np}, and accessible polar surface area, ∆ASA_{ps}, upon binding. When corrections have been made for proton linkage, as described by Equations 4 and 5, the enthalpy of binding can be parameterized as follows,

\[
\Delta H(T) = a(T) \cdot \Delta ASA_{np} + b(T) \cdot \Delta ASA_{ps} + \Delta H_{\text{link}} \quad \text{(Eq. 6)}
\]

and the heat capacity can in analogy be rationalized as follows,

\[
\Delta C_{p}^{a} = c(T) \cdot \Delta ASA_{ps} + d(T) \cdot \Delta ASA_{np} \quad \text{(Eq. 7)}
\]

The parameters a(T)/d(T) are fitted parameters calculated by Freire (13); a(25 °C) = −24.2 cal/(mol Å²), b(25 °C) = 40.5 cal/(mol Å²), c(25 °C) = 0.43 cal/(K mol Å²), and d(25 °C) = −0.26 cal/(K mol Å²).

The total change in entropy, ΔS_{tot}, upon binding can be divided into a sum of different contributing effects.

(i) The contribution from the change in hydration upon binding, ΔS_{hydr}, at temperature T is calculated from the heat capacity change using a reference temperature, T_{ref},

\[
\Delta S_{\text{hydr}} = \Delta C_{p}^{a}(T/T_{ref}) \quad \text{(Eq. 8)}
\]

The reference temperature (T_{ref}) is set to 385 K. This temperature is the convergence temperature of the entropy, where the hydrophobic hydration contribution to entropy is zero (34, 35). At T, the entropy of unfolding of proteins converge to the same value. This is also the case for the dissolution of small hydrophobic compounds in aqueous solution (35). The convergence temperature has been interpreted as the temperature at which the hydrophobic contribution to the total entropy has happened.

(ii) There are contributions to the total entropy change due to reductions in rotational/translational degrees of freedom, ΔS_{rot/trans}. Kauzman (36) and Murphy et al. (37) have shown that ΔS_{rot/trans} is well approximated by the cratic entropy, −8 cal K⁻¹ mol⁻¹, for 1:1 binding stoichiometry.

(iii) The entropic contribution due to the change in the number of particles in the system, ΔS_{num part}, is a statistical entropic effect.

When corrections have been made for proton linkage, as described by Equations 4 and 5, the enthalpy of binding can be parameterized as follows,

\[
\Delta H_{\text{int}} = \Delta H_{\text{obs}} + \Delta H_{\text{ion}}\Delta n \quad \text{(Eq. 4)}
\]

\[
\Delta H_{\text{obs}} = \Delta H_{\text{int}} + \Delta H_{\text{link}}\Delta n \quad \text{(Eq. 5)}
\]

where ΔH_{\text{int}} and ΔH_{\text{obs}} are the observed enthalpies of binding in the two different buffers and where ΔH_{\text{link}} and ΔH_{\text{obs}} are the ionization enthalpies for the used buffers.

When analyzing the calorimetric data we have used the method developed by Freire et al. (13) for calculating changes in hydration-accessible nonpolar surface area, ∆ASA_{np}, and accessible polar surface area, ∆ASA_{ps}, upon binding. When corrections have been made for proton linkage, as described by Equations 4 and 5, the enthalpy of binding can be parameterized as follows,

\[
\Delta H(T) = a(T) \cdot \Delta ASA_{np} + b(T) \cdot \Delta ASA_{ps} + \Delta H_{\text{link}} \quad \text{(Eq. 6)}
\]

and the heat capacity can in analogy be rationalized as follows,

\[
\Delta C_{p}^{a} = c(T) \cdot \Delta ASA_{ps} + d(T) \cdot \Delta ASA_{np} \quad \text{(Eq. 7)}
\]

The parameters a(T)/d(T) are fitted parameters calculated by Freire (13); a(25 °C) = −24.2 cal/(mol Å²), b(25 °C) = 40.5 cal/(mol Å²), c(25 °C) = 0.43 cal/(K mol Å²), and d(25 °C) = −0.26 cal/(K mol Å²).

The total change in entropy, ΔS_{tot}, upon binding can be divided into a sum of different contributing effects.

(i) The contribution from the change in hydration upon binding, ΔS_{hydr}, at temperature T is calculated from the heat capacity change using a reference temperature, T_{ref},

\[
\Delta S_{\text{hydr}} = \Delta C_{p}^{a}(T/T_{ref}) \quad \text{(Eq. 8)}
\]

The reference temperature (T_{ref}) is set to 385 K. This temperature is the convergence temperature of the entropy, where the hydrophobic hydration contribution to entropy is zero (34, 35). At T, the entropy of unfolding of proteins converge to the same value. This is also the case for the dissolution of small hydrophobic compounds in aqueous solution (35). The convergence temperature has been interpreted as the temperature at which the hydrophobic contribution to the total entropy has happened.

(ii) There are contributions to the total entropy change due to reductions in rotational/translational degrees of freedom, ΔS_{rot/trans}. Kauzman (36) and Murphy et al. (37) have shown that ΔS_{rot/trans} is well approximated by the cratic entropy, −8 cal K⁻¹ mol⁻¹, for 1:1 binding stoichiometry.

(iii) The entropic contribution due to the change in the number of particles in the system, ΔS_{num part}, is a statistical entropic effect.

In the initial state for a 1:1 stoichiometry there are two particles, while in the final state there is only one particle. R is the gas constant (R = 1.987 cal (mol K)⁻¹). Thus, ΔS_{num part} is for 1:1 binding Rln(½) = −1.1 cal K⁻¹ mol⁻¹.

(iv) In the protein–protein interactions there can be changes in ionization of amino acid residues. This will contribute to the total entropy change as an ionization entropy contribution, ΔS_{ion}. The magnitude to this contribution is dependent on which type of residue that is involved in the change in ionization.

(v) In the binding process involving a protein, it is likely that there will be changes in conformational degrees of freedom. This can be due to conformational changes in the peptide backbone and/or conformational changes of individual residues. The changes in conformational freedom will contribute to the total changes in entropy, ΔS_{conf}.

Adding up the contributions (i–v) we arrive at the following expression for the total change in entropy upon binding.

\[
\Delta S_{\text{tot}} = \Delta S_{\text{hydr}} + \Delta S_{\text{rot/trans}} + \Delta S_{\text{ion}} + \Delta S_{\text{num part}} \quad \text{(Eq. 10)}
\]

RESULTS

The extracellular portion of the receptor was produced as a soluble secreted molecule with an affinity handle fused to the C-terminal end. The use of the Z affinity tail has been successfully implemented in the production of a large variety of proteins in various prokaryotic production systems (38) and is in this report used in an eucaryotic host system.

Cloning/Sequencing—The receptor was cloned and sequenced in the production vector pKGE 978, and the sequence was found to be fully in agreement with the published sequence (7).

Cell Growth—Human primary kidney 293 cells were transfected for transient expression of the IGF-1 receptor. In addition, 293 clones expressing the receptor were selected for establishment of stable cell lines. The selected 293 cell line yielded high and stable expression combined with a minimum of proteolytic degradation.

Protein Characterization—The small scale cell growth used in the initial experiments, up to 50-ml flasks, gave a production yield ranging from 1 to 5 mg of IgG-purified sIGF-I-β/Z/liter of conditioned media. The purity of the receptor was determined using SDS-polyacrylamide gel electrophoresis (Fig. 3A). Detection of the Z affinity handle using peroxidase anti-peroxidase staining (Fig. 3B) reveals a single band in the unreduced sample (lane B2) corresponding in size to full-length soluble receptor. In the reduced sample (lane B1), a band corresponding to the size of the β-Z-chain is stained. No additional bands, and therefore no detectable degradation products, containing the Z tail were found. The determined amino acid composition corre-
sponds to what is expected from theoretical values (data not shown). Five cycles of N-terminal sequencing revealed two different sequences corresponding to a correctly processed $\alpha$-chain N terminus after signal peptide removal and a postcleavage $\beta$-chain N terminus, respectively.

Receptor processing and association status were analyzed by partial reduction analysis. This analysis is based on the fact that the $\alpha-\alpha$ interchain (class I) disulfides are more susceptible to reduction than intrachain (class II) disulfides (20). Limited reduction results in the appearance of $\alpha-\beta'-Z$ in addition to the parent ($\alpha-\beta'-Z_2$) receptor. The gel in Fig. 3A shows the presence of one species in the unreduced sample (lane A2), corresponding approximately to the size of ($\alpha-\beta'-Z_2$) receptor. The totally reduced sample (lane A1) exhibits two bands of approximately 134 and 70 kDa, corresponding to $\alpha$- and $\beta'$-Z chains, respectively. The partially reduced sample (lane A3) shows the presence of two bands corresponding to nonreduced receptor and a band of intermediate molecular mass, 180 kDa, which may correspond to the $\alpha-\beta'$-Z half receptor. The molecular masses determined may deviate from theoretical values due to changes in gel mobility originating from glycosylation and different levels of intrachain reduction in the samples as well as possible charge differences originating from the alkylation agent.

Glycosylation analysis of the receptor reveals a sugar content of 26.1% by mass. The composition of associated sugars is as follows: fucose, 2.2%; mannose, 7.7%; galactose, 4.6%; and glucosamine, 9.6%. The sialic acid component was found to be 2%. Total molecular mass based on the theoretical molecular mass, derived from amino acid composition plus sugar content, is 280 kDa for the ($\alpha-\beta'-Z_2$) receptor. Attempts to determine the molecular mass of the complete receptor assembly using mass spectrometry did not succeed using either laser desorption or electrospray methodology.

Laser light-scattering analysis of both free and ligand-complexed receptor indicates an apparent hydrodynamic radius slightly larger than expected from theoretical calculations. The calculated molecular mass of free receptor at 25 °C was approximately 370 kDa, and the mass of the complex was 410 kDa. The determined value may differ up to 20% from the actual value in this analysis. From the light scattering data the receptor was found to be monodisperse, indicating a homogenous receptor population. The difference in size between free and complexed receptor is much larger than the actual size of the receptor population. The difference in size of free and complexed receptor is approximately to the size of ($\alpha-\beta'-Z_2$) receptor. Attempts to determine the molecular masses of the complete receptor assembly using mass spectrometry did not succeed using either laser desorption or electrospray methodology.

Circular Dichroism—CD spectroscopy was used to detect possible changes in secondary structure content upon ligand binding. The far-UV spectra of free and ligated receptor are shown in Fig. 4A. The spectrum of the free IGF-I ligand is subtracted from the complexed receptor spectrum. Calculations of the secondary structure components using VARS/LC1 variable selection suggest a structure content for the free receptor of 13% $\alpha$-helical, 26% anti-parallel $\beta$-sheet, 9% parallel $\beta$-sheet, 18% turn, and 35% unstructured. Spectra of IGF-I$^{15}$-Z complexed with an excess of ligand (IGF-I) (spectra of the ligand subtracted) reveal small but reproducible differences over the wavelength range suggesting that these differences observed are a direct consequence of binding IGF-I to its receptor. There is a decrease in negative ellipticity for the complex over the entire range. The calculated structural element content of complexed receptor is 14% $\alpha$-helical, 28% antiparallel $\beta$-sheet, 7% parallel $\beta$-sheet, 20% turn, and 31% unstructured. The absolute values of secondary structure may contain added uncertainties due to contribution to negative ellipticity from associated oligosaccharides below 210 nm.

The near-UV spectra of the free receptor, of the ligand-complexed receptor with ligand signal subtracted, and of the sum signal of free receptor and ligand, are shown in Fig. 4B. In contrast to the far-UV spectra, there is an increase in negative ellipticity upon ligand binding in the near-UV spectra. The negative ellipticity at 217 nm is decreased by 9%, and at 286 nm it is increased by 57%. The near-UV spectra is sensitive to aromatic side chain positions and cysteine bridge conformations.

Ligand Binding Assay—The binding displacement of radio-labeled IGF-I to purified IGF-I$^{15}$-Z, using IGF-I as a tracer, is shown in Fig. 5. The half-maximal inhibiting concentration (IC$_{50}$) value calculated from the binding curve is 0.5 nM.

Biosensor Analysis—BIAcore kinetic measurements were performed for a series of known ligands to the IGF-I$^{15}$: IGF-I, IGF-II, des1-3-IGF-I, insulin, and IGF-I$^{10}$-Z. The determined values in the last association constant from all runs was estimated to be less than 12%, calculated as the square root of the sum of the squares of the errors in amino acid analysis, pipetting, and data fitting. Determined kinetic parameters (Table I) indicate that differences in both association rates and dissociation rates underlie the observed differences.
in calculated association equilibrium constants for the tested ligands. The des(1–3)-IGF-I variant has an association rate that is about 1.5 times faster than that of native IGF-I, while the association rate for IGF-II is about 2.8-fold slower than IGF-I. IGF-I and des(1–3)-IGF-I have similar disassociation rates, while that of IGF-II is 2 times faster than that of IGF-I. The insulin association was too slow to be determined, using BIAcore technology at the conditions used. Ligand association was analyzed for insulin at concentrations up to 500 times higher (0.17 mM) than those used for IGF-I. The differences in disassociation rate between the ligands are less pronounced, insulin excluded, than the association rates. The data for insulin disassociation is best fitted to a two-site model in contrast to the other ligands. Out of the two distinct disassociation rates, one is 100 times faster than that of IGF-I, 180 (s$^{-1} \times 10^{-5}$) and the other is 10 times faster, 18 (s$^{-1} \times 10^{-6}$). The calculated association equilibrium constants for IGF-I$^{R-Z}$ are thus about 2-fold higher for des(1–3)-IGF-I than for native IGF-I, while the value for IGF-II is about 5-fold less, accounted for both by slower association and faster release from the receptor.

**Titration Microcalorimetry for IGF-I Binding to IGF-I$^{R-Z}$**

The measured heats of binding were at all temperatures exothermic. The largest heat was obtained at the first injection at each titration series and ranged from 30 to 65 mJ (Fig. 7). At 15 and 25 °C, the measured heat values in the PBS buffer were below the limit of accurate determination of equilibrium constants. However, due to the larger enthalpy at 37 °C in PBS and at 25 °C in HBS, data could be fitted to a 1:1 binding model with acceptable statistical output (Fig. 8). Other thermodynamic binding models, e.g., models including a complex with stoichiometry of 1:2 or 2:1 (IGF-1:IGF-I$^{R-Z}$), were also tested to establish the most probable thermodynamic model for the binding process. These other models could be disregarded based on statistical analysis. At 15 and 25 °C in PBS buffer, the enthalpies of binding were calculated from total integral heats. The results from the titration calorimetric experiments are summarized in Table II. The results from the analysis of the thermodynamic data are listed in Table III. The thermodynamic properties are dominated by the large negative heat capacity. This is typical for protein-protein interactions in which the hydration of nonpolar residues is reduced. Due to the large heat capacity change, the enthalpy and the entropy are strongly temperature-dependent. As outlined under “Experimental Procedures,” the large negative heat capacity change can be explained by dehydration of large hydrophobic surface areas. The large and negative enthalpy also implies a strong temperature dependence of the affinity constant, which indeed can be observed by the difference in affinities at 25 and 37 °C. The number of linked protons in the reaction was determined to be $2.13 \pm 0.01$ at pH 7.4. Important contributions to the entropy change upon binding comes from the solvent, $\Delta S_{sol}$, stemming from the burial of hydrophobic groups in the binding process and due to a decrease in the conformational degree of freedom in the

![Fig. 5. Competition binding curve of $^{125}$I-IGF-I binding to IGF-IR-Z, using IGF-I as a tracer. The IC$_{50}$ value is estimated to approximately 0.5 nM.](image)

![Fig. 6. Biosensor analysis of ligand binding to immobilized IGF-I$^{R-Z}$. The sensorgrams showing the relative response in resonance units after background subtraction versus time in seconds are recorded for the following ligands: IGF-I (A), des(1–3)-IGF-I (B), IGF-II (C), and insulin (D). The concentrations ligands are indicated by numbers in the corresponding graphs.](image)
The titration microcalorimetry-derived thermodynamic parameters, and in particular the large entropy loss upon ligand binding, are consistent with a conformational stabilization induced by ligand binding. Part of this stabilization could be induced by a conformational change. The titration microcalorimetry data presented here suggest that some 4700 Å² get depleted of water when IGF-I binds to IGF-I-RZ. This can be either through the burial of binding surfaces on both the ligand and the receptor or from structural changes leading to a reduction of solvent exposed surface in either of the molecules. Assuming rigid molecules, this would mean that more than half ((4675/2)/4070 = 0.57) of IGF-I gets buried upon binding. This is an unlikely large portion of the molecule, supporting the hypothesis that a conformational change does occur in the receptor. The CD analysis of the IGF-I receptor ectodomain suggests that the secondary structure of the receptor changes upon ligand binding. The far-UV CD spectra of free and IGF-I-ligated IGF-I-R-Z show small changes in negative ellipticity (Fig. 4), and the near-UV spectral changes are somewhat larger, but in both ranges the changes are reproducible and significant. The difference in the spectra between the complex and the sum of the signals of free receptor and free ligand indicates that changes in position of aromatic side chains and, possibly, cysteine bridge dihedral angels occur in the receptor ectodomains upon ligand association. These changes could be due to secondary structural changes, quaternary structural changes, or both (41).

The three-dimensional structure of the insulin receptor cytoplasmic tyrosine kinase domain has been determined using protein crystallography (6). These data suggest that the activation of the tyrosine kinase involves intradomain transphosphorylation made possible by induced close contact of the domains and structural rearrangements in the protein kinase active site region. Thus, it seems possible that signal transduction across the membrane is the result of a ligand-induced quaternary structural change. The nature of this change may, for instance, involve a scissors-like movement of the receptor chains, a twist of the chains, or a combination of these movements. Binding of ligand to both IGF-I and insulin receptors is proposed to involve negative cooperativity (42). The mechanism has been suggested to involve the presence of both high and low affinity binding sites on each subunit. A ligand might bind to a high affinity site on one subunit inducing conformational changes enabling the ligand to bridge the two subunits by also binding to the low affinity site on the second subunit. Our studies on IGF-I-R-Z give a stoichiometry of 1:1 IGF-I:(α-β’-Z)₂ with high affinity, which could include two separate binding sites.
Ligand-induced IGF-I Receptor Conformational Change

Table II
Thermodynamic parameters derived from titration calorimetry measurements of IGF-I binding to IGF-Iβ

| Temperature °C | Buffer | $K$ (M$^{-1}$) | $\Delta H^{\text{obs}}$ (kcal mol$^{-1}$) | $\Delta H^{\text{intr}}$ (kcal mol$^{-1}$) | $\Delta S^{\text{obs}}$ (cal (K mol)$^{-1}$) | $\Delta S^{\text{intr}}$ (cal (K mol)$^{-1}$) | $\Delta G^{\text{obs}}$ (kcal mol$^{-1}$) | $\Delta G^{\text{intr}}$ (kcal mol$^{-1}$) | $\Delta G^{0}$ (kcal mol$^{-1}$) |
|--------------|--------|--------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| 15           | PBS    | 22.8         | 0.57           | 60.4           | 131            | 178            | 9.6            | 57             | 0.51           |
| 25           | PBS    | 30.0         | -1.12          | -88.2          | 0.57           | -27.0          | -0.57          | 191            |
| 25           | HBS    | 9.6          | 43.9           | 27.8           | 31.3           | 10.9           | 70             |
| 37           | PBS    | 0.61         | 34.1           | 27.8           | 31.3           | 10.9           | 70             |

The relative potency of binding to the produced IGF-I receptor for the different ligands is in accordance with previous investigations performed using competitive ligand methods (45). The des(1–3)-IGF-I exhibits a faster association rate than native IGF-I and thus a higher affinity. IGF-II has 5 times lower affinity than IGF-I due to both slower on rates and higher off rates. The observed discrepancies in affinity constant between the BIAcore methodology and the titration microcalorimetry are in accordance with previous studies and may be due to higher degree of conformational freedom for the IGF-I receptor (46) at 25 °C. This reduction in conformational degrees of freedom upon surface immobilization would increase the apparent affinity for the ligand. The receptor binding properties of a number of IGF-I mutants have been tested using both BIAcore technology and classical radiolabeled competition assays. Such studies show that although the absolute value of binding may vary between used methodologies, a series of molecules will have the same relative order of affinities, disregarding the method used. Similar conclusions can be made also for the interaction of human growth hormone mutants to the human growth hormone receptor (44) and for binding of Z mutants and their binding to IgG (45) using displacement and BIAcore methods. In addition, the validity of association equilibrium constants determined by BIAcore kinetic analysis has been addressed in works by Ladbury and Morton et al. (52); in both works the combination of biosensor methodology and isothermal titration calorimetry was used to characterize receptor-ligand interactions. Ladbury and co-workers (51) found that only steady state measurements were reliable in the biosensor setup used. In the work by Morton et al. (52), good agreement was found between kinetic and steady state biosensor measurements as well as titration calorimetry-derived values. We believe the presented kinetic data to be correct based on the following. The association is between a small monomorphic ligand and a large immobilized monodisperse receptor, thus minimizing the possibilities of avidity effects, using self-associating proteins or mass transport limitations, that occur when a large receptor associates with a small immobilized ligand. The plot of $k_{\text{on}}$ against concentration, used to determine the association rate ($k_{\text{off}}$), was linear in the used concentration range. The dissociation rate does not change with concentration in the highest concentrations used, and the addition of free IGF-I-binding IGFBP-1 protein in the dissociation phase does not increase $k_{\text{off}}$ demonstrating that detectable rebinding does not occur. The obtained rate information provides the opportunity to more fully quantify the behavior of the ligands and the effects of introduced mutations in IGF-Iβ ligands. The association rate of insulin binding to IGF-Iβ-Z is too low to be determined using the BIAcore; it is possible, however, to calculate the dissociation rate constant from the data. Interestingly, in contrast to the other ligands, a two-site model gave the best fit for insulin dissociation. The rates of dissociation are approximately 10 and 100 times faster, respectively, for the two insulin off-rates than the corresponding rate for IGF-I. A hypothetical explanation for this difference is that IGF-I binds to the IGF-I receptor and induces a conformational change by simultaneously bridging the high and low affinity sites (42). Insulin, on the other hand, binds to the receptor, one molecule to each α subunit, with significantly lower affinity but is unable to induce conformational changes and is therefore rapidly dissociated from these two sites. Analysis of ligand binding in the presence of different antibodies directed toward the IGF-I receptor (46) shows that signal transduction may be blocked independently of ligand binding. This might be the case if the antibody would bind outside the ligand binding site and block a conformational change, thus inhibiting further signal transduction. In the study by Soos et al. (46) there is a further observation that antibodies potentiating IGF-I ligand binding also increase the affinity of insulin for the IGF-I receptor up to 50-fold. These findings are consistent with the thought that insulin on its own is unable to bridge between the α subunit ligand-binding regions of the IGF-I receptor. The antibody could possibly switch the receptor conformation to an “insulin competent” state by locking the subunits in a conformation allowing formation of an insulin high affinity receptor complex.

| Changes in nonpolar area, $\Delta A_{\text{polar}}$ | -2495 Å² |
| Changes in polar area, $\Delta A_{\text{polar}}$ | -2180 Å² |

| Contributions to change in hydration | Entropy 60.4 kcal/mol | Heat capacity 60.4 kcal/mol |
| Contribution to change in polar hydration | -88.2 kcal/mol | 0.57 kcal/mol |
| Contribution to total change in hydration | -27.0 kcal/mol | -0.57 kcal/mol |
| Contribution due to conformational changes | 131 cal/(K mol) |

| Other entropic contributions | -178 cal/(K mol) |
| Contribution due to proton linkage | -1 cal/(K mol) |

$\Delta n = -3.1$
mation, but the possibility could not be excluded that structural changes may accompany ligand binding and receptor phosphorylation. Schaefer et al. (49) studied the insulin receptor extracellular domains, using a construct analogous to the extracellular IGF-1R presented in this work and found forms of structures similar to those found by Christiansen and collaborators. These studies support the theory of large flexibility of the receptor α domains. The speculation that the conformations found in these reports represent the structures of receptor with or without ligand present is therefore appealing. Further, Schaefer et al. (49) applied CD analysis to the insulin receptor and concluded that changes in secondary structure content occurred when insulin was added.

Detailed characterization of ligand binding of extracellular receptors requires a convenient way to produce sufficient amounts of the mature protein. We have demonstrated the successful use of an affinity handle fusion protein expression system for the production of large amounts of a soluble form of IGF-1R. Purification using the Z-IgG affinity system has high specificity for the production of large amounts of the mature protein. We have demonstrated the release of protons in the binding process and concluded that changes in secondary structure content occurred when insulin was added.

The data presented in this paper support the hypothesis that extracellular IGF-I receptor domains undergo a conformational change upon ligand binding that includes changes in both the amount of accessible surface area and the amount of secondary structure content. The release of protons in the binding process may originate from formation of salt bridges between receptor and ligand. It has been shown by mutational analysis that the charged IGF-I C domain residues are important for high affinity IGF-I receptor binding (50). The proton release could also be part of the conformational change in the receptor structure. These two different possibilities cannot be distinguished without detailed structural or mutational analysis.

In this study, we have shown that the produced soluble IGF-1R-Z binds one IGF-I molecule with high affinity and is capable of structural changes, possibly those involved in the activation of signal transduction. The lack of transmembrane and cytoplasmic regions does not destroy high affinity ligand binding capacity.

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