Recent studies utilizing alanine scanning mutagenesis have identified a major ligand binding domain of the secreted recombinant insulin receptor composed of two subdomains, one between amino acids 1 and 120 and the other between amino acids 704 and 716. In order to obtain a more detailed characterization of these subdomains, we examined the binding of an insulin superanalog, des(B25–30)-[His-A8, Asp-B10, Tyr-B25] α-carboxamide[insulin, to alanine mutants of the ligand binding determinants of these subdomains. cDNAs encoding mutant secreted recombinant receptors were transiently expressed in 293 EBNA cells, and the binding properties for this analog of the expressed receptors were evaluated. In general des(B25–30)-[His-A8, Asp-B10, Tyr-B25] α-carboxamide[insulin binding correlated with insulin binding, suggesting that both peptides bound to the receptor in a similar manner. Alanine mutations of eight amino acids (Asn15, Phe64, Phe705, Glu706, Tyr708, Leu709, Asn711, and Phe714) of the receptor produced the most profound decreases in affinity for des(B25–30)-[His-A8, Asp-B10, Tyr-B25] α-carboxamide[insulin, suggesting that interactions with these amino acids contributed the major part of the free energy of the ligand-receptor interaction. Mutation of Arg14 and His710 to Ala produced receptors with undetectable insulin binding but an affinity for des(B25–30)-[His-A8, Asp-B10, Tyr-B25] α-carboxamide[insulin only 8–23-fold less than for native receptor. Further analog studies were performed to elucidate this paradox. The receptor binding potencies of His-A8 and Asp-B10 insulins for these receptor mutants appeared to parallel their relative potencies for native affinity receptor binding. COOH terminus of the B-chain, which is essential for high affinity receptor binding, has been the subject of intensive study. The structure of insulin, as well as that of many analogs, has been solved at high resolution (for review see Ref. 10). However, it is only recently that a coherent model of its interaction with its receptor has been developed. A current consensus is that isoleucine A2, valine A3, valine B12, phenylalanines B24 and B25, tyrosine A19, asparagine A21, and the partially buried residues leucines A16 and B15 are the major determinants of the receptor binding site with threonine A8, serine B9, histidine B10, glutamate B13, and tyrosine B16 making minor contributions.\(^1\) The role of phenylalanines B24 and B25 in receptor binding have been subject to particular scrutiny (11–13), because mutations in these positions have been associated with diabetes (14). Systematic studies of insulin analogs modified in these positions (11–13) have lead to the suggestion that these residues are responsible for initial interaction with the receptor and that this interaction produces a conformational change in the COOH terminus of the B-chain, which is essential for high affinity receptor binding.

Considerably less is known about the ligand binding sites of the insulin receptor. The results of affinity labeling experiments (15–18) and studies with chimeric insulin receptors (19–21) point to the involvement of at least three separate regions of the α subunit in the process of insulin binding; amino acids 1–120 (15, 16, 19, 20), 450–524 (17, 20, 21), and 714–718 (18). More recently utilizing alanine scanning mutagenesis, we have provided evidence for a major ligand binding site composed of two subdomains widely separated in the primary sequence. The amino-terminal subdomain is located between amino acids 1 and 120 (22) and is composed of 14 amino acids arranged in four discontinuous peptide segments. The carbonyl-terminal β subunit, which is both extra- and intracellular, linked by disulfide bonds. Insulin binding leads to the activation of the intrinsic tyrosine kinase activity of the receptor β subunit cytoplasmic domain and phosphorylation of intracellular substrates of the receptor, including IRS-1 (2), IRS-2 (3), and SHC (4, 5), leading to the intracellular events responsible for signal transduction (6). Structural and mutational studies indicate that the receptor kinase is activated by transphosphorylation of the receptor’s cytoplasmic domain (7, 8), but the molecular basis of the regulation of this process by ligand binding still remains obscure. It is thought that by analogy to the ligand-induced dimerization of the monomeric receptor tyrosine kinases, insulin binding to the extracellular domain of the receptor either produces an approximation of or a reorientation of the cytoplasmic domains permissive for the transphosphorylation event (for review see Ref. 9).

The initial event in this pathway, the binding of insulin to its receptor, has been the subject of intensive study. The structure of insulin, as well as that of many analogs, has been solved at high resolution (for review see Ref. 10). However, it is only recently that a coherent model of its interaction with its receptor has been developed. A current consensus is that isoleucine A2, valine A3, valine B12, phenylalanines B24 and B25, tyrosine A19, asparagine A21, and the partially buried residues leucines A16 and B15 are the major determinants of the receptor binding site with threonine A8, serine B9, histidine B10, glutamate B13, and tyrosine B16 making minor contributions.\(^1\)

\(^1\) G. Dodson, personal communication.
subdomain, amino acids 704–716, consists of 12 amino acids (23) organized in three discontinuous segments. Unfortunately, although these studies were adequate for localization of this ligand binding domain, its detailed functional characterization was not possible due to technical limitations of the assay employed. Thus in the present study we have further characterized this ligand binding site by evaluating the binding of the des-(B25–30)[His-A8, Asp-B10, Tyr-B25 α-carboxamidem]insulin (X92)\(^2\) (24), an insulin superanalog, to alanine mutants of the amino acids composing this binding site. During this study, two alanine mutations were identified in which the affinity of insulin was disproportionately decreased compared with that of X92. Subsequent studies with other insulin analogs showed that this compromise in affinity for insulin was dependent on the presence of the amino acids of the COOH terminus of the B-chain of the insulin molecule.

**MATERIALS AND METHODS**

**General**—Mutant insulin receptor cDNAs were generated and expressed in transient transfection experiments as described previously (22). The mutants are designated by the amino acids mutated to alanine using the single letter code followed by the number(s) indicating their position in the sequence of the insulin receptor, thus T704A is a mutant in which Thr704 of the receptor has been mutated to alanine. It should be noted that subsequent studies of the mutant V715A, which we previously reported to have a very low affinity for insulin (23), have shown that it has normal affinity for both insulin and X92 (data not shown). This mutant was therefore not utilized in this study. Human insulin and insulin analogs were synthesized as described previously (24). Carrier free \(^{125}\)I (IMS 30) was from Amersham Corp. Protease inhibitors were from Boehringer Mannheim. 293 EBNA cells were purchased from Invitrogen (San Diego, CA). Medium for tissue culture was from Mediatech (Herndon, VA), and serum was from Sigma. Anti-insulin receptor monoclonal antibody 18–44 (25) was generously provided by Dr. M. Soos and K. Siddle (University of Cambridge, Cambridge, UK). Affinity purified goat anti-mouse IgG was from Pierce.

**Radioiodination of Insulin and Analog**—For ligand binding studies insulin and X92 were iodinated with \(\text{I}^{125}\)I by the lactoperoxidase method. Monoiodo-Tyr-A14 isomers were separated from the iodination reactions by reverse phase high pressure liquid chromatography using a C18 column (26).

**Insulin and Analog Binding Assays**—Soluble insulin receptor binding assays were performed using a micropotter antibody plate assay (27, 28). Microtiter plates (Immulon 4, Dynal Inc., Lake Success, NY) were incubated with affinity purified goat anti-mouse IgG (50 

**RESULTS**

**Wild type and mutant insulin receptor cDNAs were transiently expressed in 293 EBNA cells. Equilibrium binding studies were performed on conditioned medium with labeled and unlabeled X92. As described previously for insulin binding to this form of recombinant wild type receptor (22, 23), binding of X92 displayed simple kinetics with a linear Scatchard plot (data not shown). Analysis with the LIGAND program indicated a single population of binding sites with a dissociation constant of 3.3 \(\pm\) 0.2 \(\mu\)M (mean \(\pm\) S.E., \(n = 6\)).**

**TABLE I**

| Mutant     | \(K_d\) wild type | \(K_d\) mutant/\(K_d\) wild type |
|------------|-------------------|---------------------------------|
| NH\(_2\) terminal subdomain |                   |                                 |
| D12A       | 6.2               | 0.9                             |
| H13A       | 19.0              | 8.7                             |
| R14A       | —                 | 7.6                             |
| N15A       | 134.0             | 32.8                            |
| Q38A       | 3.2               | 1.6                             |
| L36A       | 1.6               | 2.5                             |
| M38A       | 3.6               | 1.3                             |
| F39A       | 10.0              | 9.9                             |
| E44A       | 2.9               | 1.4                             |
| P64A       | —                 | 367.0                           |
| Y67A       | 3.2               | 1.6                             |
| E99A       | 4.8               | 7.0                             |
| H710A      | 9.4               | 3.0                             |
| Y91A       | 3.6               | 0.38                            |
| COOH-terminal subdomain |                   |                                 |
| T704A      | 167.0             |                                 |
| F705A      | —                 | 1652.0                          |
| E706A      | —                 | 748.1                           |
| Y708A      | 218.0             | 218.2                           |
| L709A      | 70.1              | 159.9                           |
| H710A      | 23.2              |                                 |
| N711A      | 190.3             |                                 |
| F714A      | 182.0             | 72.1                            |
| V715A      | 8.1               | 3.6                             |

The results of the analyses of alanine mutants of the insulin receptor are shown in Table I; results are expressed as a ratio of the \(K_d\) of the alanine mutant to the \(K_d\) of the wild type receptor, and the corresponding values for insulin are shown for comparison. In general, for mutants in which quantitation for both ligands was technically feasible, the decrease in affini-

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\(^{2}\) The abbreviations used are: X92, des-(B25–30)–[His-A8, Asp-B10, Tyr-B25 α-carboxamidem]insulin; X8, His-A8 insulin; X10, Asp-B10 insulin; X137, des-(B25–30)–[Tyr-B25 α-carboxamidem]insulin; [\(^{125}\)I-Tyr-A14]insulin, insulin radioiodinated at tyrosine 14 of the A-polypeptide chain; [\(^{125}\)I-Tyr-A14]X92, des-(B25–30)–[His-A8, Asp-B10, Tyr-B25 α-carboxamidem]insulin radioiodinated at tyrosine 14 of the A-polypeptide chain.
Binding of analogs to secreted recombinant human insulin receptor and the mutant receptors R14A and H710A were determined using the microtiter plate antibody capture assay described under "Materials and Methods." The IC_{50} values for the displacement of [125I-Tyr-A14]X92 by unlabeled X92, insulin and X8, X10, and X137 were determined from the competition curves fitted to a four parameter logistic plot. Each result is the mean of two independent determinations.

| Mutant     | Insulin | X8 | X10 | X137 | X92 |
|------------|---------|----|-----|------|-----|
| Wild type  | 1.7 × 10^{-9} | 2.0 × 10^{-10} | 4.2 × 10^{-10} | 2.2 × 10^{-10} | 8.4 × 10^{-12} |
| R14A       | 1.1 × 10^{-8}  | 1.1 × 10^{-7}  | 2.0 × 10^{-7}  | 7.9 × 10^{-9}  | 1.1 × 10^{-10} |
| H710A      | 1.6 × 10^{-8}  | 2.3 × 10^{-7}  | 3.8 × 10^{-7}  | 1.4 × 10^{-8}  | 2.7 × 10^{-10} |

**TABLE II**

Analog binding properties for receptor mutants

The differences in the binding free energy for X92 between alanine-substituted mutants and wild type secreted recombinant insulin receptor (ΔΔG) were calculated from the data in Table I using the equation ΔΔG = RTln(K_r mutαnt/K_r wild type), where R is the gas constant and T is the absolute temperature.

**DISCUSSION**

In the present study we have used insulin analog binding to perform a detailed characterization of a major ligand binding site of the insulin receptor. In initial studies we examined the binding of the insulin superanalog X92 to alanine mutants of the receptor with compromised affinity for insulin. This analog has an affinity for the receptor that is 180–200-fold that of insulin and is produced by a triple modification of the native insulin molecule. Substitution of threonine at position B8 by histidine, as is found in avian insulins, increases the affinity of insulin for the insulin receptor 8–10-fold (28); substitution of histidine at position B10 by aspartate produces a 4–5-fold increase in affinity, as originally described for a human mutant insulin (29); and deletion of the COOH-terminal pentapeptide combined with the replacement of phenylalanine B25 by tyrosine a-carboxamide also produces a 8–10-fold increase in affinity (24). With the exception of the removal of the COOH-terminal pentapeptide of the insulin B-chain, the precise structural mechanisms by which these modifications affect affinity are unclear. However, it is probable that the overall structure of this molecule and its conformation in the high affinity receptor complex are therefore likely to be very similar to that of insulin, and it is thus a valid tool for characterizing insulin-receptor interactions. This is confirmed by the overall

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2 The differences in the binding free energy for X92 between alanine-substituted mutants and wild type secreted recombinant insulin receptor (ΔΔG) were calculated from the data in Table I using the equation ΔΔG = RTln(K_r mutαnt/K_r wild type), where R is the gas constant and T is the absolute temperature.

3 The differences in the binding free energy for X92 between alanine-substituted mutants and wild type secreted recombinant insulin receptor (ΔΔG) were calculated from the data in Table I using the equation ΔΔG = RTln(K_r mutαnt/K_r wild type), where R is the gas constant and T is the absolute temperature.
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similarity in the changes in affinity for the two peptides produced by the mutations, with the exceptions Asp^{12}, Arg^{14}, Asn^{15}, Tyr^{91}, Thr^{704}, and His^{710} to alanine (Table I). The discrepancies between the effects of these mutations on the affinities for insulin and X92 are presumably a reflection of the differences in the molecular mechanisms underlying the interactions of the two peptides with the insulin receptor; this is discussed further below for the Arg^{14} and His^{710} to alanine mutations.

In a review of the additivity of the effects of mutations on proteins, Wells concluded that for the majority of protein-protein interactions, the free energy changes due to multiple mutations exhibited simple additivity (30). However, it was noted that large deviations from simple additivity could occur when the mutations produced large structural perturbations or when the sites of mutation interacted with one another. In the present study, the sum of the calculated ΔG values (33.8 kcal/mol) for all the mutations we have evaluated (Fig. 1) is double that of the free energy of ligand binding (14.9 kcal/mol) calculated from the dissociation constant of X92 for the native receptor. It is unlikely that this is due to methodological errors arising from the precision of the assay (see Table II). This deviation from additivity has therefore probably arisen from major structural perturbations or changes in intramolecular interactions caused by the mutations. As we have discussed in previous studies of these receptor mutants (21, 22), it is unlikely that there is any major structural perturbation of the mutant proteins, and thus it is probable that some of these mutations compromise insulin and X92 binding by alteration of intramolecular interactions within the ligand binding site of the receptor.

When the effects of the individual mutations on the free energy of the interaction of X92 with the insulin receptor are considered individually, it is apparent that mutations in the COOH-terminal subdomain produce the largest effects. Alanine substitution of phenylalanine 705 and glutamate 706 had the greatest impact (>3 kcal/mol; Fig. 1), and with the exception of Val^{715} and His^{710} (see below for more detailed discussion) all other substitutions in this subdomain (Tyr^{708}, Leu^{709}, Asn^{711}, and Phe^{714}) also produced profound decreases in free energy of ligand binding (2–3 kcal/mol); the perturbation of binding produced by mutation of Thr^{704} to Ala appears to be an indirect effect (see Ref. 23 for detailed discussion). In the NH_{2}-terminal subdomain only two substitutions, Asn^{15} and Phe^{64} produced comparable changes in free energy. Alanine mutation of all other amino acids critical for high affinity insulin binding within this subdomain only produced free energy changes ranging from ~0.3 to 1.3 kcal/mol (Fig. 1). These findings are in marked contrast to the findings of Clackson and Wells for growth hormone-receptor interactions (31). This interaction has a dissociation constant (0.3 × 10^{-11} M) similar to that of the insulin-receptor interaction. Mutational analysis of the ligand binding site of the receptor indicates that the free energy of the interaction is dominated by the contributions of two tryptophan residues that contribute ~4 kcal/mol each to the energy of ligand binding. With the exception of one residue that contributes approximately 3.2 kcal/mol, the contribution of each of the remaining 29 residues of the ligand binding site, when considered individually is less than or equal to 2 kcal/mol. Thus, the molecular mechanisms underlying the ligand-receptor interactions may be somewhat different in the two systems. In the growth hormone system the free energy of binding is generated from the major contributions of two amino acids, whereas in the insulin system it seems to be generated from more modest contributions of a larger number of amino acids. This conclusion should, however, be interpreted with caution because some of the insulin receptor mutations that we have studied are probably disrupting intramolecular interactions within the binding site rather than direct ligand receptor interactions, as noted in the discussion in the preceding paragraph.

The striking discrepancies observed between the affinities of R14A and H710A mutants for insulin and X92 are worthy of further discussion. Studies with the individually substituted analogs suggest that this is predominantly due to the removal of the COOH-terminal pentapeptide of the insulin B-chain. This mirrors findings with certain insulin analogs. Systematic studies of substitutions for phenylalanine B25 of the insulin molecule indicate that the presence β aromatic ring in this position is essential for high affinity receptor insulin binding. This appears only to apply to the full-length molecule because removal of the COOH-terminal pentapeptide restores the receptor binding potency of such substituted analogs (12, 13). These studies have lead to the suggestion that an initial interaction with the receptor via B25 phenylalanine leads to a conformational change in the main chain of the COOH terminus of the insulin molecule that is essential for the completion of the interaction with the receptor (12, 13). This is supported by the finding that insulin analogs that are cross-linked between glycine A1 and lysine B29, in which movement of the COOH terminus of the B-chain is constrained, exhibit varying decreases in biological activity, depending on the length and flexibility of the cross-link (32, 33), despite the lack of major perturbation of their structure. Solution NMR studies of the structure of Gly-B24 insulin provide further insights into possible structural mechanisms underlying these findings (34). In this analog the COOH terminus of the B-chain is unfolded and disordered, exposing residues in the core of the molecule, in particular valine A3, which has been shown to be essential for receptor binding (35). It is therefore possible that interaction of phenylalanine B25 with the receptor produces a similar change in conformation of the insulin molecule, allowing completion of interaction with the receptor.

Thus, in light of the finding that the interaction of the full-length insulin molecule with the receptor mutants R14A and H710A resembles that of insulin analogs substituted in the B25 position with wild type insulin receptor, it is tempting to suggest that the mutated residues, Arg^{14} and His^{710}, interact with B25 phenylalanine of the insulin molecule to produce the conformational changes in the main chain of the COOH terminus of the B-chain necessary for high affinity insulin binding. Further studies of these mutants with insulin analogs are in progress to test this hypothesis. It is, however, unlikely that the effects of these mutations on the affinity of the receptor for insulin are confined to disruption of interactions with the β aromatic ring of Phe-B25 of the insulin molecule because they produce a 500-1000-fold decrease in affinity (Table II), whereas substitution of Phe-B25 by alanine only results in a 100-fold decrease in affinity (12). Further, the relative receptor binding potencies of X137 for the mutants R14A and H710A are 36- and 64-fold less than that of this analog for native receptor, respectively (Table II). High resolution structural analysis of the hormone-receptor complex will be essential to fully elucidate the molecular mechanisms underlying the perturbation of the ligand-receptor interaction produced by these mutations.

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