Importance of Main-Chain Flexibility and the Insulin Fold in Insulin–Receptor Interactions†

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ABSTRACT: We have investigated the effects of altering the disposition between the COOH-terminal B chain domain of insulin and the core of the insulin molecule on ligand interactions with the hepatocyte insulin receptor. Analogues include those in which Arg^{B22} of des-octapeptide(B23–B30)-insulin is extended by one to three residues of glycine prior to termination in Phe-NH_2, by one to five residues of glycine prior to termination in Phe-Phe-NH_2, or by an additional residue of glycine prior to termination in more extended sequences derived from insulin or [Gly^{B24}]insulin. Analogues were also examined with respect to their abilities to form hexamers in solution in the presence of Co^{2+}, phenol, and NaSCN. Overall, our studies of ligand–receptor interactions identify that (a) the energetic penalty for the introduction of a single residue of glycine is uniform in all classes of analogues for up to three residues of glycine but diminishes somewhat for analogues with longer insertions and (b) the COOH-terminal residues of the B chain retain their importance for all classes of analogues, no matter the number of glycine residues introduced. Analogues with glycine insertions, but not those with glycine substitutions, readily form thiocyanate-stabilized complexes with Co^{2+} in the presence of phenol. We conclude that the β-turn characteristic of the crystallographic and solution structures of insulin serves mainly as a tether to connect the COOH-terminal B chain domain to the insulin core, that the insulin receptor exhibits considerable capacity to participate in the conformational adjustment of ligand molecules, and that receptor-mediated movements in native insulin lead to important structural adjustments without the need for major changes in the usual insulin fold.

Several recent studies on the structural basis for insulin–receptor interactions and insulin action have emphasized the importance of conformational adjustments in the insulin molecule and deviations in the receptor-bound conformation of the hormone from its crystallographically determined structure. Most important in this respect, (a) the side chain of Phe^{B25} apparently plays a role during insulin–receptor interactions in adjusting the conformation of what would otherwise be a negative structural element in the COOH-terminal domain of the B chain (Nakagawa & Tager, 1986, 1987; Mirmira & Tager, 1991), (b) the side chain of Phe^{B24} has been proposed to guide a change in local dihedral angles in the insulin main chain (Mirmira & Tager, 1989; Mirmira et al., 1991), (c) insulin analogues chemically cross-linked between residues A1 and B29 exhibit diminished (but still significant) receptor binding potency (Brandenburg et al., 1973; Nakagawa & Tager, 1989), (d) a "miniproinsulin" in which Lys^{B29} exists in peptide linkage to Gly^{A1} exhibits immeasurably low ability to bind to the insulin receptor [notwithstanding that its crystallographic structure is very close to that of insulin; see Markussen et al. (1985) and Derewenda et al. (1991)], (e) the solution structure of insulin, determined by NMR methods, is comprised of an ensemble of closely related conformations, all of which maintain the insulin fold (Hua & Weiss, 1991; Hua et al., 1991), and (f) the solution structure of [Gly^{B24}]insulin [an analogue with close to full receptor binding potency; see Mirmira and Tager (1989)] is comprised of a larger ensemble of conformations in which the insulin fold has apparently been lost and the hydrophobic core of the insulin molecule has been exposed to solvent (Hua et al., 1991, 1992). Notably, one insulin analogue that maintains the structure of native insulin (the miniproinsulin) exhibits exceedingly low receptor binding potency, whereas another that can be partially unfolded in solution ([Gly^{B24}]insulin) exhibits nearly normal potency.

The normal fold of the insulin B chain can be described in terms of an NH_2-terminal extended sequence (residues B1–B8), a central α-helix (residues B9–B19), a β-turn (residues B20–B23), and a COOH-terminal β-strand (residues B24–B30) (Blundell et al., 1972; Baker et al., 1988). Structural elements of the crystallographic insulin monomer that participate in stabilizing the β-turn and the overall insulin fold include (a) a hydrogen bond between the peptide oxygen of Gly^{B20} and the peptide NH of Gly^{B23} (with both residues exhibiting dihedral angles typical of the D-amino acids), (b) a hydrogen bond between the peptide oxygen of Gly^{B23} and the peptide NH of Asn^{A21}, (c) a hydrogen bond between the peptide NH of Phe^{B25} and the peptide oxygen of Tyr^{A19}, and (d) charge interactions between the guanidinium group of Arg^{B22} and either the α-carboxy group of Asn^{A21} or the γ-carboxy group of Glu^{A17} (Blundell et al., 1972; Baker et al., 1988). Whereas at least the first two of these structural elements are also present in the solution structure of insulin, all of them would be lost in the solution structure of the high-potency analogue [Gly^{B24}]insulin (Hua et al., 1991). The side chains of Glu^{B21} and Arg^{B22} (residues that are part of the β-turn) are solvent-exposed and somewhat disordered in the crystallographic structure of the hormone (Baker et al., 1988) and can be replaced by other side chains (sometimes including those with D orientation) without major losses in receptor binding potency (Katsoyanis et al., 1975; Schwartz et al., 1983; Wang et al., 1991). Questions remain as to how closely the folded conformation of insulin resembles the structure of the receptor-bound hormone and how changes in conforma-

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tional restriction or flexibility alter the potential of the insulin molecule for interacting with its receptor in an effective way.

To address related issues more completely, we (a) constructed by semisynthetic methods insulin analogues that contain one to five residues of glycine bridging Arg^{B22} of des-octapeptide(B23–B30)-insulin to additional B chain sequences and (b) studied the abilities of these analogues to interact with the insulin receptors on isolated hepatocytes and to form metal ion coordinated hexamers in solution. Results identify a surprising ability of the insulin receptor to participate in the structural adjustment and correct apposition of ligand domains but do not suggest that the insulin molecule need unfold to achieve high-potency interaction with its receptor.

MATERIALS AND METHODS

Materials. Porcine insulin and [[^{125}I]*iodo-Tyr*^{A14}]insulin were obtained from Eli Lilly (Indianapolis, IN). Tosylphenylalanine chloromethyl ketone (TPCK)[1] -treated trypsin was from Worthington (Freehold, NJ). H-Phe-NH_2, H-Gly-NH_2, H-Ala-NH_2, H-Gly-Phe-NH_2, H-Gly-Gly-Gly-Gly-OH, *N-tert*-butyloxycarbonyl-Gly-*N*-hydroxysuccinimide ester (Boc-Gly-OSu), Boc-Ala-OSu, and Boc-Phe-OSu were from Sigma (St. Louis, MO). *N*-9-Fluorenylmethyloxycarbonyl (Fmoc)-Gly-OSu was from Bachem (Torrence, CA).

Preparation of H-Gly-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-(Boc)-Ala-OH. Porcine insulin (120 mg) was converted to N^{α}-Gly^{A1},N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-tris-Boc-insulin by treatment with 2-oximino-2-phenylacetonitrile (45 mg) (Ito et al., 1975) and *N*-methylmorpholine (NMM) (13.2 μL) in dimethyl sulfoxide (2 mL) for 2 h at 25 °C. The resulting mixture was diluted with aqueous acetic acid and was desalted on Bio-Gel P-6, yielding 125 mg of product. The product was incubated with TPCK-treated trypsin (6 mg) for 6 h at 36 °C in 40 mL of 0.1 M NMM–acetate buffer (pH 8) containing 10 mM calcium acetate and 1 mM ethylenediaminetetraacetic acid (EDTA). The resulting mixture was acidified with acetic acid (10 mL), concentrated under reduced pressure, and gel-filtered on Bio-Gel P-4 by use of 3 M acetic acid to yield N^{α}-Gly^{A1},N^{α}-Phe^{B1}-bis-Boc-desoctapeptide(B23–B30)-insulin [$(Boc)_2$-DOI] (97 mg) and N^{ϵ}-Lys^{B29}-Boc-octapeptide(B23–B30) (22 mg). A portion of the octapeptide derivative (12 mg) dissolved in dimethylformamide (DMF) (0.5 mL) was treated with Fmoc-Gly-OSu (20 mg) and NMM (20 μL) for 24 h. Piperidine (0.1 mL) was then added at 0 °C to remove the Fmoc group. After 10 min, the mixture was diluted with ice-cold 3 M acetic acid (3 mL) and the solution was desalted by use of a column of Bio-Gel P-2. The resulting nonapeptide derivative H-Gly-Gly-Phe-Phe-Tyr-Thr-Pro-Lys(Boc)-Ala-OH (10.6 mg) was further purified by reversed-phase high-performance liquid chromatography (HPLC) by use of a C-4 column (1 × 25 cm) and a solvent system consisting of 0.1% aqueous trifluoroacetic acid (TFA) and 0.1% TFA in acetonitrile. A second nonapeptide derivative, H-Gly-Gly-Gly-Phe-Tyr-Thr-Pro-Lys(Boc)-Thr-OH, was prepared according to the procedure described above as applied to the octapeptide derivative H-Gly-Gly-Phe-Tyr-Thr-Pro-Lys(Boc)-Thr-OH, a peptide that was previously synthesized by solid-phase methods for the preparation of [Gly^{B24}]insulin (Mirmira & Tager, 1989).

[1] Abbreviations: TPCK, tosylphenylalanine chloromethyl ketone; Boc, *tert*-butyloxycarbonyl; OSu, *N*-hydroxysuccinimide (ester); Fmoc, *N*-9-fluorenylmethyloxycarbonyl; NMM, *N*-methylmorpholine; EDTA, ethylenediaminetetraacetic acid; $(Boc)_2$-DOI, N^{α}-Gly^{A1},N^{α}-Phe^{B1}-bis-Boc-des-octapeptide(B23–B30)-insulin; DMF, dimethylformamide; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; DOI, des-octapeptide(B23–B30)-insulin.

Synthesis of Peptide α-Carboxamides. Most peptide α-carboxamides were prepared by stepwise solution-phase synthetic methods (Bodanszky & duVigneaud, 1959). Each C-terminal amino acyl-α-carboxamide (H-Phe-NH_2, H-Gly-NH_2, or H-Ala-NH_2) was acylated by use of the appropriate Boc-amino acyl-OSu (Anderson et al., 1964); the N^{α}-Boc group of each peptide was then removed by 25% TFA in CH_2Cl_2 to yield H-Phe-Phe-NH_2, H-Phe-Gly-NH_2, and H-Ala-Ala-NH_2. The procedure was repeated up to four times on the individual peptides to yield H-Gly-Phe-Phe-NH_2, H-Gly-Gly-Phe-Phe-NH_2, H-Gly-Gly-Gly-Phe-Phe-NH_2, H-Gly-Gly-Phe-NH_2, H-Gly-Gly-Gly-Phe-NH_2, H-Gly-Phe-Gly-NH_2, H-Gly-Gly-Phe-Gly-NH_2, H-Gly-Gly-Ala-Ala-NH_2, and H-Gly-Gly-Gly-Ala-Ala-NH_2. The hexapeptide amide H-Gly-Gly-Gly-Gly-Phe-Phe-NH_2 was synthesized by segment condensation between Boc-Gly-Gly-Gly-Gly-OH (derived from H-Gly-Gly-Gly-Gly-OH) and H-Phe-Phe-NH_2 by use of *N*,*N*′-dicyclohexylcarbodiimide in the presence of hydroxybenzotriazole (Konig & Geiger, 1970). The heptapeptide H-Gly-Gly-Gly-Gly-Gly-Phe-Phe-NH_2 was prepared by the addition of one more residue of glycine to the hexapeptide α-carboxamide described above. The purity of each peptide α-carboxamide was determined by thin-layer chromatography on SiO_2 or analytical reversed-phase HPLC on C-18; if necessary, peptides were purified by preparative HPLC by use of a column of C-4 or C-18 and the TFA-based solvent system described above.

Preparation of Insulin Analogues with Altered B Chain COOH-Terminal Domains. Each insulin analogue was prepared by trypsin-catalyzed peptide bond formation between the α-carboxy group of Arg^{B22} of $(Boc)_2$-DOI and the α-amino group of an amino acid amide, a peptide amide, or a longer peptide (Inouye et al., 1981). Typically, $(Boc)_2$-DOI (1 μmol) and peptide (4 μmol for octa- and nonapeptides, 10 μmol for penta-, hexa-, and heptapeptides, and 20 μmol for smaller peptides or amino acid α-carboxamides) were incubated with TPCK-treated trypsin (0.3 mg) in a mixture of DMF/1,4-butanediol/0.2 M Tris acetate (pH 8, containing 10 mM calcium acetate and 1 mM EDTA) in the ratio 35:35:30 v/v/v (100 μL) at 12 °C for 15–24 h (Morihara et al., 1986). The condensation yields [judged by analytical HPLC on a C-18 column in a solvent system of 0.1 M phosphoric acid/0.05 M triethylamine (adjusted to pH 3.0 with sodium hydroxide) and acetonitrile] were 50–60% for the octa- and nonapeptides and 60–85% for the other peptides. Each mixture was diluted with 3 M acetic acid (1–3 mL), filtered through a nylon membrane (0.45 μm), and subjected to purification by preparative HPLC on a column of C-4 by use of the TFA-based solvent system. Finally, Boc-protecting groups were removed by treatment of the peptide derivatives with TFA at 0 °C for 1 h; the peptides were repurified by HPLC if necessary. The purities of the final products (>95%) were confirmed by analytical HPLC.

Additional Methods. Procedures for the isolation of canine hepatocytes and for their use in receptor binding experiments have been described before (Bonnevie-Nielsen et al., 1982; Nakagawa & Tager, 1986). Cells (2 × 10^6/mL) were incubated with [[^{125}I]*iodo-Tyr*^{A14}]insulin plus selected concentrations of insulin or insulin analogues (determined by UV absorbance) for 30 min at 30 °C. The T → R structural transitions of insulin$_6$$(Co^{2+})_2$ complexes (transitions that are induced by phenol and are stabilized by thiocyanate) were examined by methods described previously (Roy et al., 1989; Thomas & Wollmer, 1989; Nakagawa & Tager, 1991). Visible spectra were recorded for peptides (0.17 mM final concen-

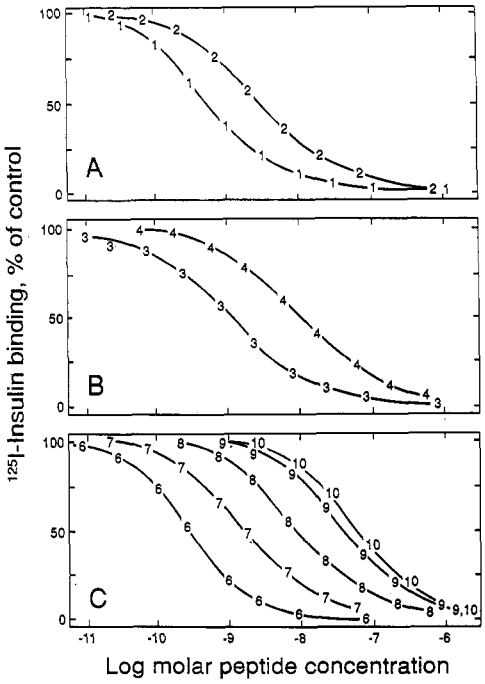

FIGURE 1: Inhibition of binding of ^{125}I-labeled insulin to isolated canine hepatocytes by insulin and by selected insulin analogues. Incubations were performed as described under Materials and Methods. Control binding is defined as the amount of radiolabeled ligand which became cell associated in the absence of competitor; all data have been corrected for so-called nonspecific binding that was detected in the presence of 10 μM insulin. Quantitative information is provided in Table I; identifying numbers in Table I, rather than symbols, are used to indicate the peptide under consideration. (A) 1 and 2, inhibition of binding by insulin and by *endo*-Gly^{B22}-insulin, respectively. (B) 3 and 4, inhibition of binding by [Gly^{B24}]insulin and by *endo*-Gly^{B22}-[Gly^{B24}]insulin, respectively. (C) 6, inhibition of binding by des-pentapeptide(B26–B30)-[Phe^{B25}-α-carboxamide]insulin; 7, 8, 9, and 10, inhibition of binding by analogues in which one, two, three, or four residues of glycine have been inserted between residues Arg^{B22} and Gly^{B23} of peptide 6, respectively. See Table I and text for further details.

tration) dissolved in 0.05 M Tris brought to pH 8.0 by the addition of HCl by use of a Beckman Model DU-40 spectrophotometer. Further details are provided in the legend to Figure 3.

RESULTS

Studies presented here were designed to test the importance of (a) the relative disposition of the COOH-terminal B chain β-strand and the insulin core and (b) the general insulin fold in determining the affinity of insulin–receptor interactions, rather than to examine specifically the roles of individual side chains in determining high-affinity binding. As illustrated in Figure 1A (see Table I), the insertion of a single residue of glycine between Arg^{B22} and Gly^{B23} of insulin (peptide 1) to achieve peptide 2 results in an analogue with 20% of the receptor binding potency of the native hormone. Figure 1B shows results obtained when the same insertion of glycine was applied to [Gly^{B24}]insulin (peptide 3) to achieve peptide 4. The data of Table I identify that the parent analogue retains 67% of the receptor binding potency of insulin, whereas the insertion of glycine leads to a new analogue with about 10% of the potency of the parent. Taken together, these findings suggest that increasing the length of the peptide chain bridging the COOH-terminal β-strand and the insulin core in these full-length molecules has a negative, but not devastating, effect on receptor binding potency.

To simplify considerations with respect to both the insertion of glycine bridges in the insulin molecule and the importance

Table I: Identification and Relative Receptor Binding Potencies of Insulin and Insulin Analogues[a]

identifying no.	peptide[b]	relative potency
1	DOI-GFFYTPKA (insulin)	100
2	DOI-GGFFYTPKA (*endo*-Gly^{B22}-insulin)	19 ± 2 (4)
3	DOI-GGFYTPKT ([Gly^{B24}]insulin)	67 ± 7 (4)
4	DOI-GGGFYTPKT (*endo*-Gly^{B22}-[Gly^{B24}]insulin)	6.3 ± 0.8 (5)
5	DOI-FF-NH_2	0.38 ± 0.03 (4)
6	DOI-GFF-NH_2 (des-pentapeptide-(B26–B30)-[Phe^{B25}-α-carboxamide]insulin)	147 ± 7 (4)
7	DOI-GGFF-NH_2	20 ± 2 (6)
8	DOI-GGGFF-NH_2	2.7 ± 0.6 (5)
9	DOI-GGGGFF-NH_2	1.1 ± 0.1 (5)
10	DOI-GGGGGFF-NH_2	0.78 ± 0.12 (5)
11	DOI-F-NH_2	0.93 ± 0.06 (3)
12	DOI-GF-NH_2 (des-hexapeptide-(B25–B30)-[Phe^{B24}-α-carboxamide]insulin)	4.9 ± 0.1 (3)
13	DOI-GGF-NH_2	0.98 ± 0.16 (3)
14	DOI-GGGF-NH_2	0.28 ± 0.03 (3)
15	DOI-GGFG-NH_2	1.8 ± 0.1 (3)
16	DOI-GGAA-NH_2	0.080 ± 0.012 (3)
17	DOI-GGGAA-NH_2	0.058 ± 0.004 (3)
18	DOI-GFG-NH_2	22 ± 2 (3)
19	DOI-GGG-NH_2	0.20 ± 0.06 (3)

[a] The analogues used in this study and their relative receptor binding potencies are identified above. Details of semisynthetic methods and of cell incubation procedures are provided under Materials and Methods. Relative receptor binding potency is defined as [(concentration of porcine insulin causing half-maximal inhibition of binding of [[^{125}I]*iodo-Tyr*^{A14}]insulin to receptor)/(concentration of analogue causing half-maximal inhibition of binding of [[^{125}I]*iodo-Tyr*^{A14}]insulin to receptor)] × 100. Each value represents the mean ± SD of multiple determinations; the number of separate determinations is shown in parentheses. The concentration of insulin causing half-maximal inhibition of radiolabeled insulin binding was 0.67 ± 0.04 nM (n = 13). Since ≤10% of the 20 pM radiolabeled hormone became cell associated in the experiments reported (even in the absence of competitor), the data were not significantly affected by variations in ligand concentrations caused by receptor binding. The relative binding potencies reported in the table can therefore be considered under most circumstances to reflect relative binding affinities. [b] To enhance ease of presentation and to avoid cumbersome nomenclature, insulin and insulin analogues are described by reference to sequence extensions from the B chain carboxy terminus of des-octapeptide(B23–B30)-insulin (DOI) by use of the one-letter amino acid code. The notation -NH_2 at the right of some sequences indicates that the peptide contains a B chain α-carboxamide group.

of structural arrangements between the COOH-terminal B chain domain and the insulin core, additional studies were directed toward the use of des-pentapeptide(B26–B30)-[Phe^{B25}-α-carboxamide]insulin (peptide 6) as the parent compound. As documented in Table I [cf. Fisher et al. (1985) and Nakagawa and Tager (1986)], peptide 6 [a molecule that retains the β-turn typical of insulin; see Bi et al. (1984) and Hua and Weiss (1990, 1991)] exhibits slightly higher receptor binding potency (147%) than that which applies to insulin. Also, as documented in Table I, deletion of Gly^{B23} from peptide 6 to yield peptide 5 results in an analogue exhibiting only about 0.3% of the receptor binding potency of the parent peptide. On the one hand, deletion of Gly^{B23} (by changing the geometry between the Phe^{B24}-Phe^{B25} dipeptide and the insulin core) undoubtedly prevents the sequence from exerting its positive effects. On the other hand, Figure 1B and Table I show that the stepwise addition of up to four residues of glycine between Arg^{B22} and Gly^{B23} of peptide 6 results in analogues (peptides 7–10) with stepwise decreases in their abilities to interact with the insulin receptor and with overall receptor binding potencies ranging from 0.5 to 14% relative

to peptide 6 (0.8–20% relative to insulin). Importantly, even the analogue bearing the four-residue glycine insertion (peptide 10) retains a relative receptor binding potency approximately 8-fold higher than that of des-octapeptide(B23–B30)-insulin [0.1% potency relative to insulin; see Kikuchi et al. (1980) and Nakagawa and Tager (1986)], a molecule from which the COOH-terminal B chain domain has been deleted.

Table I identifies that relationships very similar to those described above also hold for analogues based on glycine deletion and glycine insertion that are derived through the use of des-hexapeptide(B25–B30)-insulin as the parent compound (peptides 11–14). Accordingly, it seems that stepwise glycine insertions into insulin and insulin analogues (whether or not these analogues can be expected to retain the native insulin fold) exhibit decremental, but somewhat limited, effects on ligand interactions with the insulin receptor.

Further studies were undertaken to determine the extent to which the COOH-terminal B chain sequence Phe-Phe-NH_2 in analogues corresponding to peptides 6–10 (see Table I) actually interacts with the insulin receptor in a defined way (thus to ensure that decrements in relative receptor binding potency due to stepwise glycine insertions arise specifically from extending the distance between the aromatic amino acid pair and the insulin core rather than from increasing main-chain length per se or from secondary alterations in main-chain structure). Three sets of analogues are relevant to this question. Data are presented in Table I. First, within the series comprised of analogues with COOH-terminal B chain sequences corresponding to Gly-Phe-Phe-NH_2, Gly-Gly-Phe-NH_2, Gly-Phe-Gly-NH_2, and Gly-Gly-Gly-NH_2 (peptides 6, 13, 18, and 19, respectively), the separate replacement of Phe^{B24} and Phe^{B25} by glycine results in analogues with 0.7 and 15% potency relative to the parent compound, respectively, whereas the combined replacement results in an analogue with multiplicatively decreased receptor binding potency (0.1% relative to peptide 6). Second, within the series comprised of analogues with COOH-terminal B chain sequences corresponding to Gly-Gly-Phe-Phe-NH_2, Gly-Gly-Gly-Phe-NH_2, and Gly-Gly-Phe-Gly-NH_2 (peptides 7, 14, and 15, respectively), the separate replacement of Phe^{B25} and Phe^{B26} (residues corresponding to Phe^{B24} and Phe^{B25} of insulin or peptide 6) by glycine results in analogues with 0.1 and 9% potency relative to the parent compound, respectively. Third, analogues with COOH-terminal B chain sequences corresponding to Gly-Gly-Ala-Ala-NH_2 and Gly-Gly-Gly-Ala-Ala-NH_2 (peptides 16 and 17, respectively) exhibit only 0.4 and 2% of the respective receptor binding potencies of the corresponding analogues containing the COOH-terminal dipeptide Phe-Phe-NH_2 (peptides 7 and 8, respectively). It is therefore apparent that the dipeptide sequence Phe-Phe-NH_2 is indeed recognized by receptor in the examples presented and that sequence extensions as the result of glycine insertions have their effect in greatest part through increasing the distance between the COOH-terminal dipeptide and the insulin core rather than through altering the character of relevant interactions.

Relationships among the effects of inserting glycine residues between Arg^{B22} and the COOH-terminal B chain domains of insulin and insulin analogues (Table I) become clearer through plotting the logarithms of the relative receptor binding potencies against the relevant number of glycine residues to account for relative distance in terms of the relative free energy of ligand–receptor interactions. Notably, Figure 2 identifies that (a) whether considering insulin (peptide 1), [Gly^{B24}]insulin (peptide 3), des-pentapeptide(B26–B30)-[Phe^{B25}-α-carboxamide]insulin (peptide 6), or des-hexapeptide(B25–B30)-

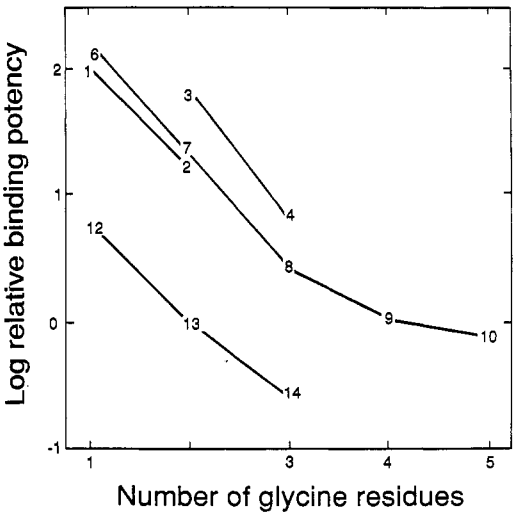

FIGURE 2: Relationships between the receptor binding potencies of insulin and various insulin analogues and the number of residues of glycine occurring between residue Arg^{B22} and the first residue of Phe encountered in the COOH-terminal B chain extension from des-octapeptide(B26–B30)-insulin. The logarithm of relative receptor binding potency is plotted against the relevant number of glycine residues. Identifying numbers in Table I, rather than symbols, are used to indicate the peptide under consideration. 1 and 2, insulin and *endo*-Gly^{B22}-insulin, respectively; 3 and 4, [Gly^{B24}]insulin and *endo*-Gly^{B22}-[Gly^{B24}]insulin, respectively; 6, 7, 8, 9, and 10, des-pentapeptide(B26–B30)-[Phe^{B25}-α-carboxamide]insulin and analogues in which one, two, three, or four residues of glycine have been inserted between residues Arg^{B22} and Gly^{B23} of the parent analogue, respectively; 12, 13, and 14, des-hexapeptide(B25–B30)-[Phe^{B24}-α-carboxamide]insulin and analogues in which one or two residues of glycine have been inserted between residues Arg^{B22} and Gly^{B23} of the parent analogue, respectively. See Table I and text for further details.

[Phe^{B24}-α-carboxamide]insulin (peptide 12) as the parent compound, the insertion of one, two, or three residues of glycine results in a remarkably constant decrement in relative free energy of interaction (one corresponding to 1.1 ± 0.2 kcal/mol per glycine residue for the six relevant peptide pairs), (b) in the series based on peptide 6, the stepwise insertion of additional glycine residues beyond three results in progressively diminished effects, and (c) again for the series based on peptide 6, the value of the plateau that might be reached upon the insertion of even greater numbers of glycine residues exceeds that expected for the interaction of des-octapeptide(B23–B30)-insulin (a peptide with 0.1% relative receptor binding potency) with the insulin receptor. Accordingly, it seems that insertions of glycine between Arg^{B22} and various COOH-terminal B chain sequences have defined and cumulative effects on the relative abilities of the corresponding analogues to interact with the insulin receptor and that these effects in most cases are related simply to the distance between the relevant B chain sequences and the core of the insulin molecule.

Although the determination of the structures of the analogues identified in Table I is beyond the scope of this paper, relevant information can be gleaned by analyzing the ability of related peptides to undergo the T → R structural transition that is typical of insulin (Roy et al., 1989; Thomas & Wollmer, 1989). During this transition, the orientation of insulin hexamers octahedrally liganded with Co^{2+} in solution is altered by the presence of phenol to form tetrahedrally liganded complexes that are stabilized by thiocyanate and that exhibit enhanced electronic absorption in the region 500–650 nm. Figure 3 shows related spectra. Importantly, (a) whereas insulin undergoes the T → R transition in the presence of phenol alone, [Gly^{B24}]insulin exhibits no conformational transition in the presence of phenol and shows only a minor

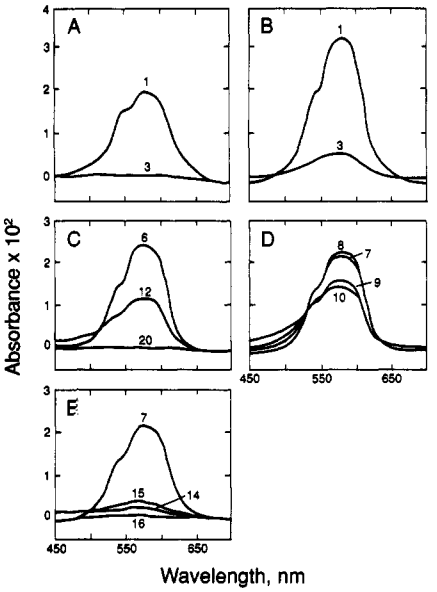

FIGURE 3: Spectroscopic studies of hexamer formation in solution by insulin and selected insulin analogues. Relevant curves are identified by numbers that correspond to the peptides identified in Table I. (A) Spectra obtained as the result of incubating 0.17 mM insulin (peptide 1) and [Gly^{B24}]insulin (peptide 3) with 0.06 mM $CoCl_2$ and 50 mM phenol in 50 mM Tris buffer at pH 8. (B) Spectra obtained as the result of adding 800 mM NaSCN to the insulin solution (peptide 1) and the [Gly^{B24}]insulin solution (peptide 3) identified in panel A. (C) Spectra obtained as the result of incubating 0.17 mM insulin analogues with 0.06 mM $CoCl_2$, 50 mM phenol, and 800 mM NaSCN in Tris buffer at pH 8.0, as presented for panel B: 6, des-pentapeptide(B26–B30)-[Phe^{B25}-α-carboxamide]insulin; 12, des-hexapeptide(B25–B30)-[Phe^{B24}-α-carboxamide] insulin; 20, des-octapeptide(B23–B30)-insulin. (D) Spectra obtained as presented for panel C: 7, 8, 9, and 10, analogues corresponding to the insertion of one, two, three, or four residues of glycine between Arg^{B22} and Gly^{B23} of des-pentapeptide(B26–B30)-[Phe^{B25}-α-carboxamide]insulin (peptide 6). (E) Spectra obtained as presented for panel C: 7, *endo*-Gly^{B22}-des-pentapeptide(B26–B30)-[Phe^{B25}-α-carboxamide]insulin; 14, the analogue of peptide 7 in which Phe^{B25} was replaced by Gly; 15, the analogue of peptide 7 in which Phe^{B26} was replaced by Gly; 16, the analogue of peptide 7 in which Phe^{B25} and Phe^{B26} were replaced by Ala. See Materials and Methods and text for further details.

tendency to undergo the transition even in the presence of phenol plus thiocyanate (Figure 3A,B); (b) although des-octapeptide(B23–B30)-insulin exhibits no tendency to form hexamers in solution and des-hexapeptide(B25–B30)-insulin exhibits only minimal ability to undergo the T → R transition, des-pentapeptide(B26–B30)-insulin when incubated with Co^{2+}, phenol, and NaSCN yields a spectrum little different from that observed for insulin under similar conditions (Figure 3C); (c) the insertion of one to four residues of glycine between Arg^{B22} and Gly^{B23} of des-pentapeptide(B26–B30)-insulin results in analogues which retain the ability to undergo the stabilized T → R transition (Figure 3D); (d) in parallel with effects seen as the result of replacing Phe^{B24} by Gly in insulin (see above), replacement of the equivalent residue (Phe^{B25}) by Gly in peptide 7 to yield peptide 14 results in a major decrease in the ability of the corresponding analogue to undergo the transition in the presence of Co^{2+}, phenol, and NaSCN (Figure 3E); and (e) replacement of the Phe-Phe-NH_2 dipeptide in peptide 7 by Ala-Ala-NH_2 results in a total loss in the ability of the corresponding analogue to undergo the T → R transition (Figure 3E). In contrast to native insulin (see Figure 3A), none of the analogues identified in Figure 3 was observed to undergo the usual T → R transition in the absence of the stabilizing anion (data not shown). These findings demonstrate that insertions of glycine into the β-turn domain of the insulin B chain are compatible with interactions necessary to form ordered analogue complexes and that, in parallel with results derived from receptor binding studies, the character of sequences COOH-terminal to these glycine insertions is critical to determining the ability of the corresponding analogues to form metal ion coordinated complexes capable of undergoing the T → R transition in solution.

DISCUSSION

Results presented here demonstrate the considerable ability of insulin to accommodate glycine insertions into the β-turn domain of the B chain, a region that is well-defined in the crystallographic and NMR structures of the native hormone (Blundell et al., 1972; Baker et al., 1988; Hua et al., 1991), that apparently becomes unfolded in the high-potency analogue [Gly^{B24}]insulin (Hua et al., 1991), and that has been proposed by others to represent a flexible structural element (Wang et al., 1991). Importantly, the β-turn remains intact in the crystallographic and solution structures of des-pentapeptide(B26–B30)-insulin [a high-potency monomeric analogue; see Bi et al. (1984) and Hua and Weiss (1990, 1991)]. While it might seem surprising that des-pentapeptide(B26–B30)-[Phe^{B25}-α-carboxamide]insulin is capable of exhibiting the T → R spectroscopic signature typical of insulin hexamers in the presence of Co^{2+}, phenol, and thiocyanate, the strongly positive heterotropic effects of thiocyanate–metal ion coordination in related insulin hexamers have been documented before (Brader et al., 1991). Analogues of des-tetrapeptide(B27–B30)-insulin containing the α-carboxamide group have also been shown to self-associate in solution (Lenz et al., 1991).

It might seem even more surprising that analogues of des-pentapeptide(B26–B30)-[Phe^{B25}-α-carboxamide]insulin containing insertions of one to four residues of glycine between Arg^{B22} and the terminal Phe-Phe-NH_2 dipeptide also exhibit the thiocyanate-stabilized T → R spectroscopic signature of insulin. Nevertheless, the terminal sequence Gly-Phe-Phe-NH_2 in these analogues could accommodate all of the hydrogen bond interactions typical of the usual insulin β-turn, notwithstanding both that potential charge interactions involving Arg^{B22} and Asn^{A21} or Glu^{A17} would be lost and that the β-turn under most circumstances might be converted into a simple loop connecting the relevant segments of the analogue backbones. The Phe-Phe-NH_2 dipeptide apparently plays an important role in this respect since replacement of either residue of Phe by Gly or replacement of both by Ala yields analogues with severely diminished abilities to combine into organized structures that exhibit the tetrahedral coordination of Co^{2+} characteristic of insulin and other analogues. It remains to be determined whether the inability of the analogues described here to undergo the T → R transition in the absence of thiocyanate arises from their relative resistance to forming stable metal ion coordinated hexamers in the presence of Co^{2+} and phenol alone or from the relative resistance of corresponding hexamers to changes in metal ion coordination (from octahedral to tetrahedral geometry) in the absence of the anion.

Our findings identify that insulin's ability to accommodate glycine insertions in the region of the usual B chain β-turn extends to considerations of the hormone's ability to interact with the insulin receptor. Importantly, all relevant analogues exhibit receptor binding potency greater than that of des-octapeptide(B23–B30)-insulin, and all exhibit receptor binding potency in a range consistent with receptor recognition of both the relevant COOH-terminal B chain domain and the core of the insulin molecule. The uniformity of the effects of

stepwise glycine insertions on the abilities of the relevant ligands to interact with the insulin receptor is notable. Two pertinent questions include why the decline in the free energy of interaction of insulin analogues having glycine insertions between Arg^{B22} and Gly^{B23} is inversely proportional to the number of glycine residues (for small numbers of glycine residues) within all four series of analogues presented in Figure 2 and why the presence of the COOH-terminal B chain dipeptide Phe-Phe-NH_2 is apparently recognized by the insulin receptor even when the dipeptide is separated from residue Arg^{B22} by a bridge of five or more glycine residues.

Analysis of both the enthalpic term and the entropic term of the Gibbs free energy expression is relevant to considering the questions posed above. On the one hand, the uniform slopes of the curves of Figure 2 at low numbers of glycine residues and the increasing slope of one of the curves at higher numbers of glycine residues provide no indication that the insertion of glycine into the structures of the parent peptides at the Arg^{B22}-Gly^{B23} site introduces an important degree of steric interference in analogue–receptor interactions which might otherwise preclude the high-affinity binding of the relevant peptides to the insulin receptor. On the other hand, the same relationships might readily be understood if the major effect on analogue–receptor interactions as the result of glycine insertion arose from considerations of increasing conformational entropy in the increasingly extended members of each analogue series. The change in slope of the curve of Figure 2 that results from addition of the third and fourth residues of glycine to the parent peptide des-pentapeptide(B26–B30)-[Phe^{B25}-α-carboxamide]insulin might well identify a limitation in the degree of conformational freedom that attends the simple insertion of additional residues of glycine (a flexible but still rotationally restricted linkage) to the corresponding analogue molecule. Interestingly, the maximal potential entropic contribution to the decreased free energy of interaction of insulin analogues with receptor as the result of glycine insertion ($\sim$3.6 cal mol^{-1} K^{-1} per residue of glycine) is in the range (proposed originally for substitutions in the reverse direction) for the expected destabilization of proteins by the replacement of Ala by Gly or Pro by Ala [2.4–4.0 cal mol^{-1} K^{-1}; see Nemethy et al. (1966) and Matthews et al. (1987)]. Amino acid insertions might in fact be expected to have greater entropic significance than amino acid replacements.

Figure 4 provides diagrammatic representations of the structures of insulin and three insulin analogues of importance to this study. Several aspects of these structures are worthy of note. First, the structure of insulin both as a Zn^{2+}-containing hexamer in crystals and as the soluble monomer can be characterized by a β-turn connecting the insulin core to the COOH-terminal β-strand (Blundell et al., 1972; Baker et al., 1988; Hua et al., 1991). Second, the structure of the high-potency analogue [Gly^{B24}]insulin (obtained in solution by NMR methods) has been reported to represent a partially unfolded molecule in which the β-turn has been disrupted (Hua et al., 1991, 1992). Nevertheless, the COOH-terminal domain of this analogue seems to retain a degree of order since Tyr^{B26} of [Gly^{B24}]insulin (a residue present in the β-strand) is only about $^1/_{10}$ as susceptible to radioiodination as Tyr^{B26} of the free insulin-derived octapeptide B23–B30 (Mirmira & Tager, 1991). Third, the crystallographic structure of the miniproinsulin described in the introduction (a molecule in which the B chain β-strand could not fold far away from the α-helical insulin core) is essentially isomorphic with the crystallographic structure of insulin (Derewenda et al., 1991). This derivative exhibits immeasureably low potency

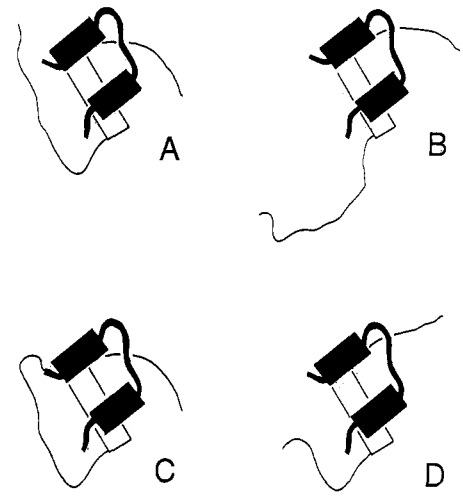

FIGURE 4: Diagrammatic representations of the NMR structure of insulin (A), the NMR structure of [Gly^{B24}]insulin (B), the crystallographic structure of the miniproinsulin in which the α-carboxy group of Lys^{B29} is in peptide linkage to the α-amino group of Gly^{A1} (C), and the crystallographic structure of des-pentapeptide(B26–B30)-insulin (D). The insulin A and B chains are shown in heavy and light lines, respectively. α-Helical regions are depicted as cylinders. The structures are adapted from the works of Blundell et al. (1972), Baker et al. (1988), Derewenda et al. (1991), Hua et al. (1991, 1992), and Bi et al. (1984). The four compounds depicted exhibit 100, 70, <0.01, and 30% of the receptor-binding potencies of native insulin, respectively. See text for further information.

(<0.01% relative to insulin) for binding to the insulin receptor. Fourth, the structure of the cross-linked insulin derivative $N^{\alpha A1}$,$N^{\epsilon B29}$-α,α'-diaminosuberoylinsulin (as determined by crystallographic analysis) is also essentially isomorphic with the crystallographic structure of insulin (Cutfield et al., 1981). Although the receptor binding potency of this conformationally constrained analogue is only 12% that of insulin (Brandenburg et al., 1973), correction for blocking the important Gly^{A1} α-amino group increases the expected potency of the otherwise underivatized but still cross-linked molecule to about 40% of that of insulin [cf. Nakagawa and Tager (1989)]; other insulin analogues cross-linked through N^{ϵ}-Lys^{B29} and N^{α}-Gly^{A1} or N^{ϵ}-D-Lys^{A1} exhibit relative receptor binding potencies in the same range (Nakagawa & Tager, 1989). Fifth, the crystallographic structure of des-pentapeptide(B26–B30)-insulin (a high-potency monomeric analogue) retains the β-turn typical of the insulin fold (Blundell et al., 1972; Baker et al., 1988; Hua et al., 1991).

Taken together, the findings presented above suggest that (a) partially unfolded analogues ([Gly^{B24}]insulin) and conformationally constrained analogues (the chemically cross-linked insulins) can exhibit similar and high potencies for interaction with the receptor, (b) insulin analogues with inherently greater conformational flexibility than that associated with insulin (or with other analogues) exhibit decreased receptor binding potency in proportion to their increased flexibility, and (c) the insulin receptor exhibits surprising ability to participate in the conformational adjustment of various ligands during the binding process. The immeasurably low receptor binding potency of the miniproinsulin may represent a special case resulting from a restriction in potential intramolecular movement beyond that applicable to chemically cross-linked analogues. It seems that the usual β-turn of insulin serves mainly as a tether to link the important COOH-terminal B chain domain to the core of the insulin molecule. Our results show that this tether functions both during ligand–receptor interactions at the hepatocyte plasma membrane and during ligand–ligand interactions in the formation of metal ion

coordinated, anion-stabilized complexes in solution. Overall, it is probable that insulin binds to its receptor in a folded (but not tightly packed) conformation that approximates (but does not mimic) the crystallographic and solution structures of the native hormone. Just as the immunoglobulins seem to bind antigenic peptides by induced fit (Cheetham et al., 1991; Rini et al., 1992) and cyclosporin A seems to bind to cyclophilin with important relaxations of an otherwise compact conformation (Van Duyne et al., 1991; Weber et al., 1991; Fesik et al., 1991), insulin seems to bind to its plasma membrane receptor in a fashion requiring the active participation of both molecules and resulting in concomitant (but possibly rather small) structural alterations in both members of the signaling pair.

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