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Disposition of the Phenylalanine B25 Side Chain during Insulin–Receptor and Insulin–Insulin Interactions†

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ABSTRACT: By the semisynthesis of both full-length insulin analogues and their des-pentapeptide-(B26–B30)-α-carboxamide counterparts, we have examined the importance of the electronic character and bulk of the position B25 side chain both in directing insulin interaction with its receptor on isolated canine hepatocytes and in determining the ability of insulin to self-associate in solution. Analogues include those in which Phe^{B25} was replaced by cyclohexyl-Ala; Tyr; *p*-nitro-, *p*-fluoro-, *p*-iodo-, or *p*-amino-Phe; or *p*-amino-Phe in which the aromatic amino function had been acylated by the acetyl, hexanoyl, decanoyl, or 1-adamantanoyl group. Our findings identify that (a) the β-aromatic side chain at position B25 is indeed critical for high-affinity ligand–receptor interactions, (b) neither electron withdrawal from nor electron donation to the β-aromatic ring perturbs ligand–receptor interactions in major ways, (c) considerable lattitude is allowed the placement of linear or polycyclic apolar mass at the para position in *p*-amino-Phe^{B25}-substituted analogues with respect both to receptor binding affinity and to biological activity in vivo, and (d) para apolar mass at position B25 is readily accommodated during the self-association of insulin monomers, as assessed by analytical tyrosine radioiodination and spectroscopic analysis of analogue complexes with Co^{2+} and Co^{3+}. These findings are discussed in terms of a model for insulin–receptor interactions at the cell membrane in which the position B25 side chain defines the edge of intermolecular contact.

The COOH-terminal region of the insulin B-chain and invariant residue Phe^{B25} are known to be important in the high-affinity interactions of insulin with its plasma membrane receptor (Nakagawa & Tager, 1986, 1987; Mirmira & Tager, 1989; Inouye et al., 1981; Wollmer et al., 1981), as well as in the formation of the β-sheet structure between insulin monomers in each of the three identical dimers of the 2-Zn insulin hexamer (Blundell et al., 1972; Baker et al., 1988). Although we have proposed that the position B25 side chain functions in part to induce favorable main-chain adjustments in the COOH-terminal region of the insulin B-chain (rather than to confer direct binding energy), and although deletion of residues B26–B30 from the carboxyl terminus of the B-chain ameliorates to one degree or another the negative effect that attends replacement of Phe^{B25} by various amino acids (Nakagawa & Tager, 1986, 1987), the importance of Phe^{B25} in insulin–receptor interactions has yet to be completely resolved. That is, side-chain structure at position B25 can influence significantly the affinity of receptor for ligand, even in truncated analogues, and the effect of replacement of Phe^{B25} in these shortened analogues can be either beneficial or detrimental. As important examples, des-pentapeptide-(B26–B30)-[His^{B25}-α-carboxamide]insulin and des-pentapeptide-(B26–B30)-[Tyr^{B25}-α-carboxamide]insulin exhibit nearly 300% of the receptor binding potency of insulin (Nakagawa & Tager, 1986; Casaretto et al., 1987), whereas des-pentapeptide-(B26–B30)-[Gly^{B25}-α-carboxamide]insulin and des-pentapeptide-(B26–B30)-[Leu^{B25}-α-carboxamide]insulin exhibit only about 20% of normal binding potency (Nakagawa & Tager, 1986; Mirmira & Tager, 1989).

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The structural disposition of the Phe^{B25} side chain, as judged by crystallographic analysis and NMR, is the most variable of any residue in insulin and is seen by many approaches to occupy multiple different conformations [see Derewenda et al. (1990) for a review]. First, in the 2-Zn and 4-Zn crystallographic structures of insulin (Blundell et al., 1972; Baker et al., 1988; Bentley et al., 1976), the side chain of Phe^{B25} in molecule II of the insulin dimer is directed across the dimer interface toward molecule I, whereas that of Phe^{B25} in molecule I is displaced backward toward the surface of the same molecule. Second, molecule I like and molecule II like forms are observed in both the T- and R-states of the insulin hexamer, states in which residues B1–B7 occur in extended or α-helical conformation, respectively (Derewenda et al., 1989, 1990; Bentley et al., 1976). Third, in the 2-Zn crystallographic structure, disorder in the electron density map suggests that the side chain of Phe^{B25} in molecule II sometimes occupies an orientation similar to that of the same residue in molecule I (Baker et al., 1988). Fourth, evidence from NMR studies indicates that the side chain of Phe^{B25} in the insulin monomer seems to occupy a molecule II like orientation seen in the 2-Zn and 4-Zn crystallographic structures (Weiss et al., 1989). Fifth, in the crystallographic structure of des-pentapeptide-(B26–B30)-insulin (an insulin analogue in which the COOH-terminal residues B26–B30 have been removed and which still retains substantial receptor binding potency; Nakagawa & Tager, 1986; Fischer et al., 1985; Rieman et al., 1983), the side chain of Phe^{B25} is turned completely away from the body of the molecule, occupying a position very different from that of the same residue in the other insulin structures described (Liang et al., 1985).

In view of the structural and biological evidence accumulated to date, important questions remain concerning (a) the structural disposition of the Phe^{B25} side chain both when it is in solution and when it is receptor bound, (b) the character of the receptor region contacting residue B25 in full-length and des-pentapeptide-(B26–B30)-insulins, and (c) the potential role that side-chain structure at position B25 may play in determining the potencies of various insulin analogues. To approach a further understanding of the importance of insulin residue B25, we constructed full-length and truncated semisynthetic insulin analogues with replacements and modifications at position B25 selected to vary both the electronic character of the aromatic ring and the ultimate disposition of the side chain. Our data identify that electron-withdrawing or electron-donating substituents on the position B25 aromatic side chain actually cause only small perturbations in receptor binding potency and that very significant hydrocarbon bulk is surprisingly well tolerated at the para position. On the basis of our findings, we propose a model in which Phe^{B25} undertakes a conformation that points away from the ligand–receptor interface when the hormone is bound to its hepatic receptor.

Materials and Methods

Peptide Synthesis. All insulin analogues were prepared by trypsin-catalyzed peptide bond formation (Inouye et al., 1981) between the α-carboxyl group of Arg^{B22} of bis(*t*-butyloxycarbonyl(Boc)[1]-des-octapeptide-(B23–B30)-insulin and the α-amino group of various synthetic octa- and tripeptides. Octapeptides containing residues B23–B30 were synthesized with Ala replacing Lys^{B29} by use of solid-phase methods and an Applied Biosystems Model 430A peptide synthesizer. Peptides were cleaved from the resin and deprotected with anhydrous HF. All reagents for peptide synthesis were purchased from Applied Biosystems (Foster City, CA). Octapeptides were purified by reverse-phase HPLC essentially as described before (Nakagawa & Tager, 1987). The N^{α}-Boc derivatives of cyclohexyl-Ala, *p*-nitro-Phe, *p*-fluoro-Phe, and *p*-iodo-Phe were purchased from Bachem (Torrance, CA). Tripeptides were synthesized as their α-carboxamides in solution with use of N^{α}-Boc amino acids purchased from Bachem. *p*-Chloro-Phe and *p*-bromo-Phe (both purchased from Bachem) were converted to their N^{α}-Boc derivatives by standard methods (Moroder et al., 1976). N^{α}-Boc amino acids were converted to their active *N*-hydroxysuccinimidyl esters and were used either in the formation of their corresponding amino acid α-carboxamides or directly in solution-phase synthesis (Bodanszky & Bodanszky, 1984).

The N^{α}-Boc derivatives of the octa- and tripeptides bearing *p*-nitro-Phe at position B25 were catalytically hydrogenated according to methods reported in the literature (Schwyzer & Caviezel, 1971) to convert the aromatic *p*-nitro group to the aromatic *p*-amino group. Subsequent to the reduction, 10 μmol of the octa- or tripeptide, dissolved in 5 mL of dimethylformamide, was treated with 100 μmol of acetic anhydride, hexanoic anhydride, decanoic anhydride, or 1-adamantanecarbonyl chloride (all purchased from Aldrich Chemical Co., Milwaukee, WI) for 20 min at 22 °C. The reactions were terminated by the addition of 10 mL of water, and the solutions were freeze-dried. The peptides bearing acylated *p*-amino groups were treated with trifluoroacetic acid to remove the α-amino-protecting group and were subsequently applied to Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA), which were washed and eluted as described before (Mirmira et al., 1991). Trypsin-catalyzed peptide semisynthesis proceeded in the manner detailed previously (Nakagawa & Tager, 1986). The syntheses of [Gly^{B25}]insulin and [Tyr^{B25}]insulin have been described before (Mirmira & Tager, 1989; Mirmira et al., 1991). Semisynthetic products were found to be >90% pure as assessed by HPLC. All insulin analogues were found to have the expected amino acid compositions after hydrolysis in 6 N HCl at 110 °C for 24 h.

Receptor Binding Studies. Receptor binding experiments based on the use of isolated canine hepatocytes were performed as previously described (Nakagawa & Tager, 1986). HPLC-purified [[^{125}I]iodo-Tyr^{A14}]insulin for receptor binding experiments was a gift from Lilly Research Laboratories (Indianapolis, IN). Binding of radiolabeled insulin and unlabeled insulin or analogue occurred during incubations for 30 min at 30 °C.

Analytical Radioiodination of Insulin Analogues. Radioiodination of metal ion free insulin analogues for the assessment of analogue structure and ability to self-associate and subsequent analysis of the distribution of ^{125}I over the four tyrosine residues in the insulin analogues proceeded in the manner detailed previously (Mirmira et al., 1991). Treatment of the iodinated analogues with tosylphenylalanine chloromethyl ketone treated trypsin followed by reverse-phase HPLC allowed the detection of the radiolabeled octapeptide containing iodo-Tyr^{B26} and the three radiolabeled isomers of des-octapeptide-(B23–B30)-insulin, containing (in order of elution) iodo-Tyr^{A19}, iodo-Tyr^{B16}, or iodo-Tyr^{A14} (Mirmira et al., 1991).

In Vivo Biological Studies. The biological potency of insulin and [*p*-hexanoyl-Pap^{B25},Ala^{B29}]insulin were determined by the rabbit blood glucose assay (Pingel et al., 1985). Twelve male New Zealand White strain rabbits, weighing 2.5–3.0 kg, were fasted 15–18 h prior to use. Each of six rabbits received a

[1] Abbreviations: Boc, *t*-butyloxycarbonyl; HPLC, high-performance liquid chromatography; Pap, *p*-amino-L-phenylalanine.

subcutaneous injection of 0.75 nmol/kg of insulin or insulin analogue prepared in 0.5 mL of 150 mM NaCl. Arterial blood samples (0.5 mL, anticoagulated with ethylenediaminetetraacetic acid) were taken 5 min before injection and 0.5, 1, 2, 3, 4, 5, and 6 h after injection. Plasma glucose levels were measured with the use of the glucose assay kit No. 510 obtained from Sigma Chemcial Co. (St. Louis, MO).

Spectroscopic Studies. Absorption data, based on the formation of complexes between insulin and Co^{2+} or Co^{3+} (Roy et al., 1989; Thomas & Wollmer, 1989; Kruger et al., 1990), were obtained by use of a Beckman DU-40 UV–visible spectrophotometer. Spectroscopic measurements were carried out at 22 °C in 0.050 M Tris brought to pH 8.0 with HCl at peptide concentrations of 0.17 mM (as determined by quantitative HPLC analysis) and metal ion concentrations of 0.060 mM. Details of the addition of other components (including phenol and H_2O_2) are provided in the figure legend for the spectroscopic experiments. Insulin and insulin analogues were prepared Zn-free by passage through a Bio-Gel P-4 column equilibrated with 3 M acetic acid and were subsequently freeze-dried before use.

Results

Receptor Binding Studies. Our approach to the preparation of insulin analogues considered (a) the synthesis of multiple tripeptides and octapeptides corresponding to residues B23–B25 and B23–B30 of the insulin B-chain, respectively, each bearing amino acid substitutions at position B25, and (b) the potential for the chemical modification of the *p*-amino function in peptides bearing Pap at position B25. Synthesis of octapeptides was simplified by constructing peptides with Ala at position B29 (in place of Lys, a residue known to be of little importance in insulin–receptor interactions; Nakagawa & Tager, 1986; Blundell et al., 1972; Fischer et al. 1985), subjecting the peptides to side-chain modification (as applicable), and proceeding with trypsin-catalyzed semisynthesis, all as detailed under Materials and Methods. Figure 1a and Table I document both the validity of our synthetic approach and the importance of side-chain character at position B25. Whereas [Ala^{B29}]insulin (an analogue bearing the natural residue Phe at position B25) exhibits close to full receptor binding potency (75% of that of insulin), [cyclohexyl-Ala^{B25},Ala^{B29}]insulin retains only 0.7% of the potency of insulin. On the one hand, these data parallel previous findings documenting the low binding potency of insulin analogues bearing amino acid replacements at position B25 (Nakagawa & Tager, 1986, 1987; Mirmira & Tager, 1989; Inouye et al., 1981; Wollmer et al., 1981; Kobayashi et al., 1982). On the other, they emphasize the specific importance of the β-aromatic ring of Phe relative to the β-aliphatic ring of cyclohexyl-Ala at position B25 for the high-affinity interaction of insulin with its receptor.

Since the structure of the β-aromatic side chain at position B25 is critical to receptor binding potency in full-length insulin analogues [Figure 1a; see also Nakagawa and Tager (1986)], and since the character of the position B25 side chain can affect the affinity of receptor even in des-pentapeptide-(B26-B30)-insulin analogues (Nakagawa & Tager, 1986, 1987; Mirmira & Tager, 1989; Casaretto et al., 1987), we synthesized full-length and truncated insulins bearing modifications of the β-aromatic ring at position B25 that differ specifically in electronic character and size. Table I identifies all analogues within this series (peptides 4–15) as possessing significant binding potency (≥25% relative to insulin) and only small differences between the full-length and truncated counterparts of insulins bearing particular position B25 sub-

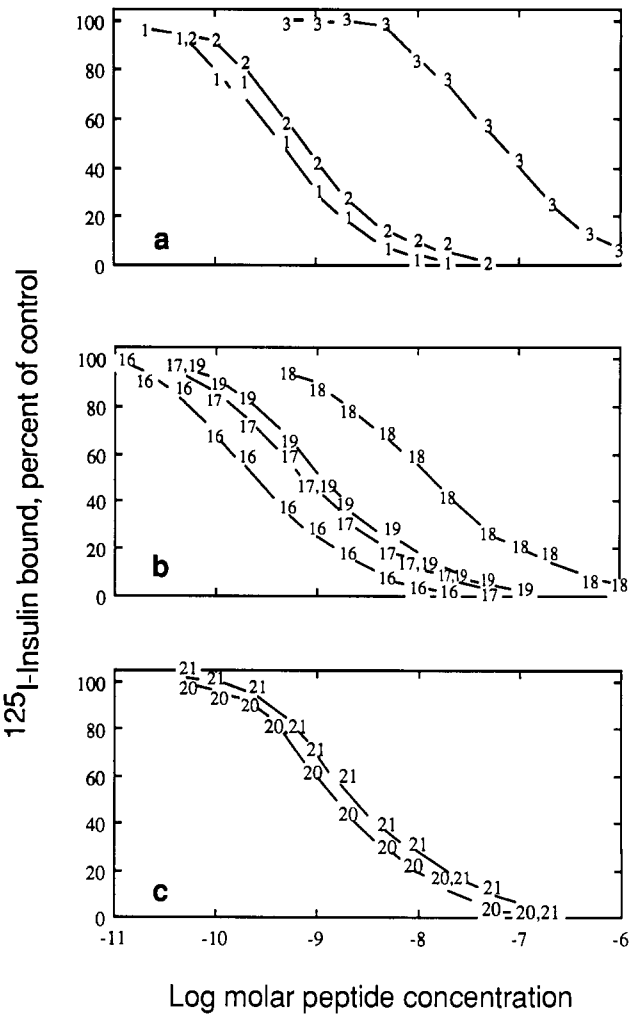

FIGURE 1: Inhibition of ^{125}I-labeled insulin binding to isolated canine hepatocytes by insulin and by insulin analogues with replacements at position B25. The amount of ^{125}I-insulin bound to cells as a percentage of control binding (that occurring in the absence of added competitor) is plotted versus competitor concentration on a logarithmic scale in all panels. Details of cell incubation conditions and of analogue preparation are provided under Materials and Methods. The identifying numbers provided in Table I (rather than symbols) are used to indicate individual data points. Panel a: 1, insulin; 2, [Ala^{B29}]insulin; 3, [cyclohexyl-Ala^{B25},Ala^{B29}]insulin. Panel b: 16, des-pentapeptide-(B26–B30)-[*p*-acetyl-Pap^{B25}-α-carboxamide]insulin; 17, des-pentapeptide-(B26–B30)-[*p*-hexanoyl-Pap^{B25}-α-carboxamide]-insulin; 18, des-pentapeptide-(B26–B30)-[*p*-decanoyl-Pap^{B25}-α-carboxamide]insulin; 19, des-pentapeptide-(B26–B30)-[*p*-1-adamantanoyl-Pap^{B25}-α-carboxamide]insulin. Panel c: 20, [*p*-hexanoyl-Pap^{B25},Ala^{B29}]insulin; 21, [*p*-1-adamantanoyl-Pap^{B25},Ala^{B29}]-insulin. See text and Table I for further details.

stitutions. Thus, (a) replacement of Phe^{B25} by Tyr or Pap (residues containing groups that both are strongly electron-donating to the aromatic ring and have the potential for serving as hydrogen-bond donors or acceptors) yields full-length analogues with 103 and 66% of the receptor binding potencies of insulin, respectively, and des-pentapeptide-(B26–B30) derivatives that exhibit receptor binding potencies about 2-fold greater than the potencies of their full-length counterparts (Nakagawa & Tager, 1986; Casaretto et al., 1987), (b) replacement of Phe^{B25} by *p*-nitro-Phe or *p*-fluoro-Phe (residues containing groups that are strongly electron withdrawing from the aromatic nucleus) yields full-length analogues and des-pentapeptide-(B26–B30) derivatives with very similar potencies in the range 70–90% relative to insulin, and (c) the replacement of Phe^{B25} by *p*-iodo-Phe yields a full-length analogue with 78% of the potency of insulin and a des-pentapeptide derivative

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

identifying no.	peptide	relative potency
1	insulin	100
2	[Ala^{B29}]insulin	76 ± 4
3	[cyclohexyl-Ala^{B25},Ala^{B29}]insulin	0.85 ± 0.20
4	des-pentapeptide-(B26–B30)-[Tyr^{B25}-α-carboxamide]insulin	185 ± 17
5	des-pentapeptide-(B26–B30)-[Pap^{B25}-α-carboxamide]insulin	124 ± 4
6	des-pentapeptide-(B26–B30)-[p-nitro-Phe^{B25}-α-carboxamide]insulin	78 ± 2
7	des-pentapeptide-(B26–B30)-[p-fluoro-Phe^{B25}-α-carboxamide]insulin	83 ± 2
8	des-pentapeptide-(B26–B30)-[p-chloro-Phe^{B25}-α-carboxamide]insulin	54 ± 8
9	des-pentapeptide-(B26–B30)-[p-bromo-Phe^{B25}-α-carboxamide]insulin	38 ± 9
10	des-pentapeptide-(B26–B30)-[p-iodo-Phe^{B25}-α-carboxamide]insulin	27 ± 4
11	[Tyr^{B25}]insulin	103 ± 11
12	[Pap^{B25},Ala^{B29}]insulin	66 ± 7
13	[p-nitro-Phe^{B25},Ala^{B29}]insulin	67 ± 4
14	[p-fluoro-Phe^{B25},Ala^{B29}]insulin	87 ± 5
15	[p-iodo-Phe^{B25},Ala^{B29}]insulin	78 ± 10
16	des-pentapeptide-(B25–B30)-[p-acetyl-Pap^{B25}-α-carboxamide]insulin	120 ± 10
17	des-pentapeptide-(B26–B30)-[p-hexanoyl-Pap^{B25}-α-carboxamide]insulin	43 ± 4
18	des-pentapeptide-(B26–B30)-[p-decanoyl-Pap^{B25}-α-carboxamide]insulin	2.4 ± 0.5
19	des-pentapeptide-(B26–B30)-[p-1-adamantanoyl-Pap^{B25}-α-carboxamide]insulin	38 ± 2
20	[p-hexanoyl-Pap^{B25},Ala^{B29}]insulin	33 ± 6
21	[p-1-adamantanoyl-Pap^{B25},Ala^{B29}]insulin	26 ± 2

[a] The semisynthetic insulin analogues and their respective receptor binding potencies (relative to the potency of porcine insulin) are identified above. Details of the synthetic and semisynthetic methods used are provided under Materials and Methods. Cell preparation and incubation conditions have been described before (Nakagawa & Tager, 1986). Relative receptor binding potency is defined for these purpose 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 to [[^{125}I]iodo-Tyr^{A14}]insulin to receptor)) × 100. All curves describing the concentration dependence for the inhibition of radiolabeled insulin binding to receptor were parallel and complete. Each value represents the mean ± SD of three separate determinations. The value for [cyclohexyl-Ala^{B25},Ala^{B29}]insulin represents the mean of two determinations. The concentration of insulin causing half-maximal inhibition of radiolabeled insulin binding was 0.45 ± 0.11 nM (N = 13). Since ≤10% of the approximately 15 fmol of radiolabeled insulin became cell-associated in the experiments reported (even in the absence of competitor), the data were not significantly affected by variations in ligand concentration due to receptor binding. The relative binding potencies reported in the table can therefore be considered to reflect relative binding affinities.

with an unexpected 3-fold decrease in receptor binding potency.

Overall, substitution of the side chain of Phe^{B25} with F, Cl, Br, or I (in the order both of decreasing electronegatively and of increasing atomic size) yields truncated analogues with 83, 54, 38, and 27% of the receptor binding potency of insulin, respectively. Given the much smaller effects exerted by p-nitro and p-amino substituents in related molecules (see above), it would appear that in these analogues the sizes of the substituents on the β-aromatic ring of the position B25 side chain are more important than their relative electronegatives in determining the potencies of analogue interactions with the insulin receptor.

The high binding potency of the full-length and des-pentapeptide-(B26–B30) analogues [p-iodo-Phe^{B25},Ala^{B29}]-insulin and [p-nitro-Phe^{B25},Ala^{B29}]insulin and the significant potency of the corresponding analogues of [2-naphthyl-Ala^{B25}]insulin [cf. Nakagawa and Tager (1986)] suggest that the receptor region contacting residue Phe^{B25} displays tolerance for bulk, as long as that bulk is carried by a β-aromatic ring. To understand better the nature of the receptor region that

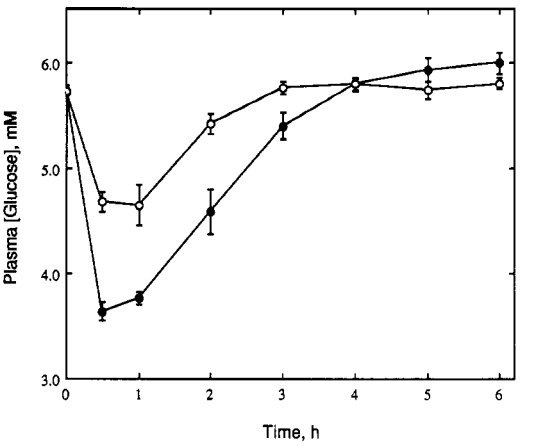

FIGURE 2: Effects of insulin and [p-hexanoyl-Pap^{B25},Ala^{B29}]insulin on blood glucose levels in vivo. The peptides were administered to fasting rabbits by subcutaneous injection at a dose of 0.75 nmol/kg of body weight as described under Materials and Methods. Plasma glucose levels (ordinate) were determined after the periods identified on the abscissa. ●, insulin; ○, [p-hexanoyl-Pap^{B25},Ala^{B29}]insulin. N = 6 in both cases; error bars identify values for SEM. See text for further details.

potentially contacts insulin residue B25, we examined the effects of acylation of the p-amino function of Pap^{B25} on the receptor binding potency of full-length and truncated insulin analogues. Figure 1b and Table I demonstrate that (a) the introduction of the acetyl, hexanoyl, and decanoyl groups at this site in the corresponding des-pentapeptide-(B26–B30)-insulins leads to a progressive decline in the affinity of the insulin receptor for ligand (with the derivatized analogues exhibiting 120, 43, and 2% of the potency of insulin, respectively), and (b) the introduction of the 1-adamantanoyl group at position B24 in des-pentapeptide-(B26–B30)-[Pap^{B25}-α-carboxamide]insulin results in an analogue with as much as 38% of the potency of insulin, notwithstanding that the 1-adamantanoyl group possesses a relative molecular mass even greater than that of the decanoyl group. It thus appears that both linear and polycyclic groups are readily accommodated at the para position of the β-aromatic ring at position B25 in truncated analogues and that significant three-dimensional bulk is easily tolerated during ligand–receptor interactions. Figure 1c and Table I document the ease with which the mass of the hexanoyl and 1-adamantanoyl groups is also accommodated in full-length analogues (for which the hexanoyl and 1-adamantanoyl derivatives exhibit 33 and 26% of the receptor binding potency of insulin, respectively).

Although the acylation of insulin at position B25 (through its introduction of hydrophobic mass) might be expected to alter considerably the physical properties of the molecule, we found no evidence either for the binding of the ^{125}I-labeled hexanoyl or decanoyl derivatives of des-pentapeptide-(B26–B30)-[Pap^{B25}-α-carboxamide]insulin to the albumin used in cell incubation buffer or for the nonspecific cellular binding of the analogues by isolated canine hepatocytes (data not shown). As important, Figure 2 demonstrates that in the subcutaneous rabbit blood glucose assay performed in vivo, [p-hexanoyl-Pap^{B25},Ala^{B29}]insulin exhibits on a molar basis about 45% of the blood glucose lowering potential of insulin, a value little different from the relative receptor binding potency of the analogue with use of the isolated canine hepatocyte system. Notwithstanding that analogues of full-length insulin and des-pentapeptide-(B26–B30)-insulin may differ in their requirements for a β-aromatic side chain at position B25 [cf. Nakagawa and Tager (1986, 1987), Mirmira and Tager (1989), and Casaretto et al. (1987)], our results imply that

Table II: Distribution of ^{125}I among the Four Tyrosines after Radioiodination of Insulin Analogues[a]

peptide	concn (μM)	degree of iodination relative to residue A14			
		Tyr^{B26}	Tyr^{B16}	Tyr^{A19}	Tyr^{A14}
[Ala^{B29}]insulin	1	1.19 (43)	0.28 (10)	0.33 (12)	1 (36)
	100	0.21 (13)	0.23 (14)	0.16 (10)	1 (62)
des-octapeptide-(B23–B30)-insulin + insulin octapeptide-(B23–B30)	1	12.6 (83)	0.65 (4.3)	0.91 (6)	1 (6.6)
[Gly^{B25}]insulin	1	1.76 (51)	0.41 (12)	0.28 (8)	1 (29)
	100	0.33 (16)	0.51 (25)	0.20 (10)	1 (49)
[cyclohexyl-Ala^{B25},Ala^{B29}]insulin	1	0.63 (29)	0.26 (12)	0.30 (14)	1 (46)
	100	0.30 (17)	0.26 (15)	0.19 (11)	1 (57)
[*p*-hexanoyl-Pap^{B25},Ala^{B29}]insulin	1	0.29 (17)	0.28 (16)	0.16 (9)	1 (58)
	100	0.18 (12)	0.29 (19)	0.046 (3)	1 (66)

[a] The distribution of ^{125}I over residues Tyr^{A14}, Tyr^{A19}, Tyr^{B16}, Tyr^{B26} for insulin analogues at 1 and 100 μM concentrations are identified above. For each tyrosine residue in a given peptide, relative ^{125}I incorporation is defined as (radioactivity observed in the fragment isolated by HPLC bearing the relevant radioiodinated tyrosine residue)/(radioactivity observed in the fragment isolated by HPLC bearing [^{125}]iodo-Tyr^{A14}). The percent of total radioactivity appearing in each of the fragments isolated by HPLC is given in parentheses. Variations in the percent of total radioactivity associated with Tyr^{A14}, a residue that is equivalently solvent-exposed in both monomers and dimers (Blundell et al., 1972; Baker et al., 1988), arise from the differential association of ^{125}I with tyrosine residues A19, B16, and B26 as the result of relative shielding or deshielding. Each values represents the mean of duplicate determinations.

the site and mode of interaction of the β-aromatic side chain (when it is present) is fundamentally conserved for full-length and truncated insulin analogues.

Studies on Self-Association. As noted in the introduction, the disposition of insulin residue B25 can differ markedly in the insulin monomer, dimer, and hexamer. To explore the consequences of replacing Phe^{B25} by residues with varying bulk and structure, we examined the accessibilities of the four spatially separated tyrosines of insulin analogues by analytical radioiodination using a method developed previously to assess both insulin solution structure and insulin self-association (Mirmira et al., 1991). Table II identifies (a) the high degree of iodination of Tyr^{B26} in [Ala^{B29}]insulin at a 1 μM concentration, (b) the protection of Tyr^{B26} (a residue that occurs at the monomer–monomer interface in the insulin dimer) when the analogue concentration is raised to 100 μM, and (c) the lesser, but significant, protection of Tyr^{A19} that simultaneously occurs when the analogue concentration is raised from 1 to 100 μM. Table II also shows the results of analytical radioiodination studies performed on an equimolar mixture of des-octapeptide-(B23–B30)-insulin and the octapeptide corresponding to residues B23–B30 of insulin at 1 μM concentration. Whereas the reactivities of tyrosine residues B16 and A19 in des-octapeptide-(B23–B30)-insulin are increased approximately 3-fold relative to the reactivity of Tyr^{A14} (in comparison to the same residues in [Ala^{B29}]insulin at 1 μM peptide), the degree of reactivity of the tyrosine residue in the free octapeptide is more than 10-fold greater than that of Tyr^{B26} in [Ala^{B29}]insulin at the same concentration. These findings suggest that packing of the COOH-terminal B-chain domain (residues B23–B30) against the core of the insulin molecule (even in dilute solution) results in the significant shielding of residues Tyr^{B16} and Tyr^{A19} and in the very major shielding of Tyr^{B26}.

The results of analytical radioiodination experiments applied to [Gly^{B25}]insulin (an analogue exhibiting 1.5% of the binding potency of insulin; Mirmira & Tager, 1989) and to derivatives of [Ala^{B29}]insulin in which Phe^{B25} was replaced by cyclohexyl-Ala or *p*-hexanoyl-Pap are presented in Table II. Notwithstanding differences that apply to the extent of iodination of Tyr^{B26} in these analogues at 1 μM concentrations (differences that most likely arise from altered flexibility of the extended COOH-terminal B-chain domain in the analogues), all of the analogues exhibit protection of both Tyr^{B26} and Tyr^{A19} when peptide concentrations are raised to 100 μM. It should be noted that equivalent protection is not applicable to [Gly^{B24}]insulin or to other analogues that do not dimerize in solution [cf. Mirmira et al. (1991)]. These results suggest that insulin is capable of dimer formation when the Phe^{B25} side chain is replaced either by a hydrogen atom or by very bulky substituents and imply that contacts involving Phe^{B25} play little role in insulin's ability to self-associate.

Further experiments were designed to probe by spectroscopic measurements the ability of analogues to form the metal ion hexamers typical of insulin in solution (Roy et al., 1989; Thomas & Wollmer, 1989). Two systems were studied. The first examined the ability of insulin to complex with Co^{2+} and to undergo a phenol-dependent transition from the T- to the R-state (a transition that changes the Co^{2+} ligand field coordination in the insulin$_6$(Co^{2+})$_2$ complex from octahedral to tetrahedral geometry, results in an intense absorbance between 500 and 650 nm, and is accompanied by a structural adjustment in which the extended conformation of residues B1–B8 becomes α-helical; Derewenda et al., 1989; Roy et al., 1989; Thomas & Wollmer, 1989). Figure 3a demonstrates the ready ability of [Gly^{B25}]insulin and [*p*-hexanoyl-Pap^{B25},Ala^{B29}]insulin to form the respective metal ion coordinated hexameric structures identified above. Importantly, insulin, [cyclohexyl-Ala^{B25},Ala^{B29}]insulin, and [*p*-1-adamantanoyl-Pap^{B25},Ala^{B29}]insulin yielded spectra identical with those shown for the two analogues described above (data not shown), and [Gly^{B24}]insulin (a nondimerizing analogue; Mirmira & Tager, 1989; Mirmira et al., 1991) failed to yield an absorbance change upon the addition of Co^{2+} and phenol (Figure 3a); the last analogue is presumably incapable of forming the hexameric structures typical of insulin and of the insulin analogues studied here.

The second system addressed the rates of oxidation of Co^{2+} by H_2O_2 in preformed insulin$_6$(Co^{2+})$_2$ complexes (to yield Co^{3+} complexes that retain the octahedral geometry of the metal ion but that result in a 5-fold increase in absorbance at about 490 nm; Thomas & Wollmer, 1989; Storm & Dunn, 1989). On the one hand, Figure 3b shows that the rate of oxidation of Co^{2+} in preformed complexes of the analogue in which Phe^{B25} is replaced by *p*-1-adamantanoyl-Pap is indistinguishable from that in preformed complexes with insulin, whereas comparable rates are about 2-fold greater for complexes in which Phe^{B25} is replaced by cyclohexyl-Ala or *p*-hexanoyl-Pap and are about 4-fold greater for complexes in which Phe^{B25} is replaced by Gly. On the other hand, Figure 3c identifies the similar spectroscopic character of the peptide–metal ion complexes formed with Co^{3+} in the analogues studied. Taken together, these results identify a somewhat looser packing of the Co^{2+} metal ion complexes with the analogues relative to

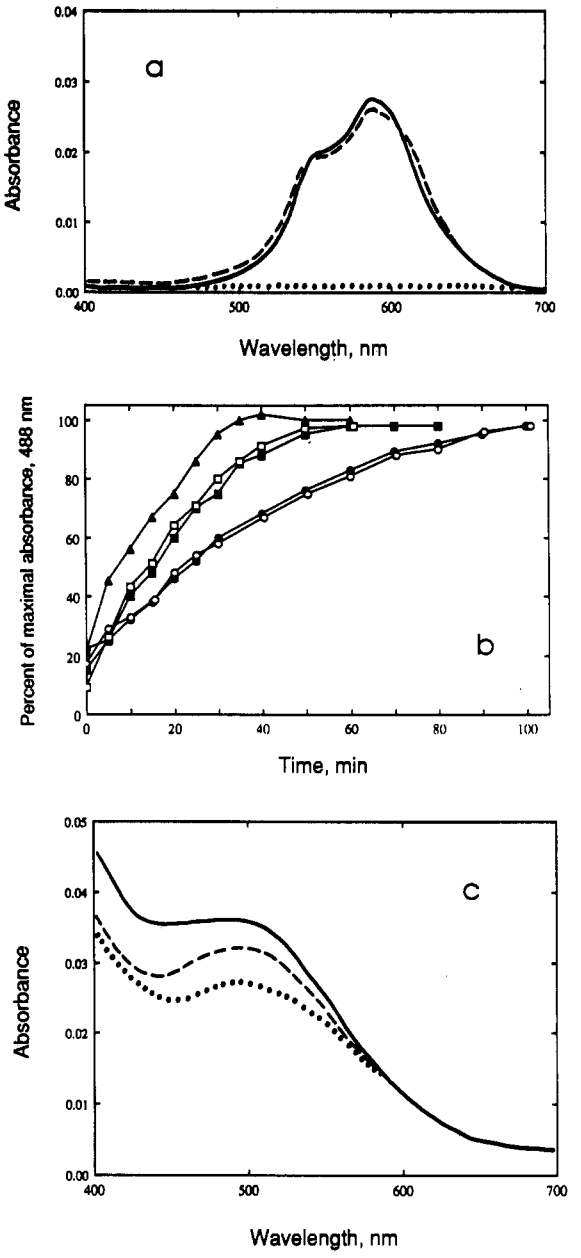

FIGURE 3: Spectroscopic analysis of complexes formed between insulin or insulin analogues and Co^{2+} or Co^{3+}. The rationale for these studies involving the formation of insulin$_6(Co^{2+})_2$ (T_6) insulin complexes, related $T_6 \rightarrow R_6$ conformational transitions induced in these complexes by the addition of phenol, and the oxidation of cobalt in insulin$_6(Co^{2+})_2$ complexes by H_2O_2 is presented in the text. Panel a: visible spectra of complexes formed between insulin analogues (0.17 mM) and $CoCl_2$ (0.060 mM) in 0.05 M Tris (pH 8) in the presence of 50 mM phenol; (—) [*p*-hexanoyl-Pap^{B25},Ala^{B29}]insulin; (---) [Gly^{B25}]insulin, (···) [Gly^{B24}]insulin. It should be noted that the spectrum obtained for insulin is no different from that shown for [*p*-hexanoyl-Pap^{B25},Ala^{B25}]insulin and has been omitted only to improve visual clarity. Panel b: time course for the enhancement of absorbance observed at 488 nm as the result of the addition of 44 mM H_2O_2 to solutions of insulin or insulin analogues (0.17 mM) incubated beforehand with $CoCl_2$ (0.060 mM) in 0.05 M Tris (pH 8); (●) insulin, (○) [*p*-1-adamantanoyl-Pap^{B25},Ala^{B29}]insulin, (■) [*p*-hexanoyl-Pap^{B25},Ala^{B29}]insulin, (□) [cyclohexyl-Ala^{B25},Ala^{B29}]insulin, (▲) [Gly^{B25}]insulin. In each case, the data have been normalized to the percent of maximal absorbance observed at 488 nm (i.e., after 100 min of incubation at 22 °C). Panel c: visible spectra of complexes formed between insulin or insulin analogues (0.17 mM) and $CoCl_2$ (0.060 mM) in 0.05 M Tris (pH 8) 100 min after the addition of 44 mM H_2O_2 (to oxidize Co^{2+} to Co^{3+}); (—) [Gly^{B25}]insulin, (---) insulin, (···) [*p*-hexanoyl-Pap^{B25},Ala^{B29}]insulin. See Materials and Methods and text for further details.

insulin (leading to the more ready oxidation of Co^{2+} to Co^{3+} by H_2O_2), notwithstanding that these analogues all demonstrate the strong ability to form dimers and hexamers in solution.

Discussion

Our results on the binding potencies and structures of insulin analogues bearing selected amino acid substitutions at position B25 provide further insights into evaluation of the structural and functional role of the COOH-terminal B-chain domain of insulin during ligand–receptor interactions. Studies on the binding potency of [cyclohexyl-Ala^{B25},Ala^{B29}]insulin and analogues bearing modifications of the β-aromatic side chain at position B25 identify that (a) a β-aromatic ring at position B25 is clearly critical for high-affinity ligand–receptor interactions [see also Nakagawa and Tager (1986)], (b) substituents on the β-aromatic ring at position B25 designed to withdraw or to donate electron density to the ring or potentially to participate in hydrogen bonding do not perturb the affinity of receptor for ligand in major ways, and (c) residues B26–B30 in full-length insulin analogues tend to mitigate the small effects of specific β-aromatic amino acid replacements at position B25 that are observed in their truncated counterparts. (Compare the analogue pairs [Tyr^{B25}]insulin and des-pentapeptide-(B26–B30)-[Tyr^{B25}-α-carboxamide]insulin, [*p*-iodo-Phe^{B25},Ala^{B29}]insulin and des-pentapeptide-(B26–B30)-[*p*-iodo-Phe^{B25}-α-carboxamide]insulin, and [*p*-fluoro-Phe^{B25},Ala^{B29}]insulin and des-pentapeptide-(B26–B30)-[*p*-fluoro-Phe^{25}-α-carboxamide]insulin, respectively, Table I.) Taken together, these findings suggest that the roles of the position B25 side chain and the sequence B26–B30 in insulin–receptor interactions are often intertwined and that the placement of mass within the region (rather than the details of side-chain functionality and potential energy-conferring interactions) govern overall insulin's fit with its receptor. Whereas the high affinity of the insulin receptor for des-pentapeptide-(B26–B30)-[Tyr^{B25}-α-carboxamide]insulin (180% of that of insulin) is unexpected, the beneficial effect of replacing Phe^{B25} by Tyr in the truncated analogue apparently does not identify a framework on which to construct insulin analogues with still higher potencies for receptor interactions.

The substantial receptor binding potencies of full-length and truncated Pap^{B25}-substituted analogues bearing linear (hexanoyl) or polycyclic (1-adamantanoyl) apolar mass at the para position (analogues retaining >25% of the receptor binding potency of insulin, Figure 1 and Table I) further suggest that the site and mode of interaction of residue B25 with receptor is fundamentally the same in both full-length and truncated analogues of insulin and that the insulin receptor can accommodate significant bulk in the direction taken by para substituents on the β-aromatic ring at insulin position B25. Our studies assessing the importance of the structure of the position B25 side chain during insulin self-association can provide clues as to the potential conformation of residue Phe^{B25} during ligand–receptor interactions. Thus, (a) analytical radioiodination shows that the ability of [Gly^{B25}]insulin and [*p*-hexanoyl-Pap^{B25},Ala^{B29}]insulin to self-associate in solution (as measured by the relative shielding of residues Tyr^{B26} and Tyr^{A19} at 100 μM analogue compared with 1 μM analogue) is retained despite major alterations in the nature of the side chain at position B25, and (b) spectroscopic analysis of the same analogues (as well as of the 1-adamantanoyl derivative) in the presence of Co^{2+} and phenol shows that these insulin derivatives can readily undergo the T → R structural transformation that is typical of insulin hexamers in solution.

Taken together, results presented above identify that metal ion independent self-association and metal ion dependent R_6 hexamer formation can occur even in the absence of a side chain at position B25 and that even bulky groups at position B25 are readily accommodated. Notwithstanding that side-chain structure at position B25 can apparently influence the three-dimensional packing of insulin analogues about the coordinated metal ion in the T_6 hexameric state (as assessed by high rates of oxidation of Co^{2+} to Co^{3+} in peptide$_6$(Co^{2+})$_2$ complexes of [Gly^{B25}]insulin and [*p*-hexanoyl-Pap^{B25},Ala^{B29}]insulin relative to those of insulin), none of the structural perturbations at position B25 examined actually seem to preclude the self-association of analogues in the ways typical of insulin itself.

Structures a–e of Figure 4 depict the conformations of the Phe^{B25} side chain as they occur at the monomer–monomer interface of the insulin dimer (within the metal ion hexamers of various crystallographically determined structures) and as they might occur at the interface between insulin and receptor. Figure 4a shows the molecule I and molecule II conformations of Phe^{B25} seen in the crystallographic structure of the 2-Zn insulin hexamer (Derewenda et al., 1990). In molecule I, Phe^{B25} is folded back over the surface of the insulin molecule and is in van der Waals contact with Tyr^{A19}, whereas in molecule II Phe^{B25} is directed across the monomer–monomer interface toward molecule I (Blundell et al., 1972; Baker et al., 1988; Derewenda et al., 1989, 1990; Bentley et al., 1976). Figure 4b depicts a model in which both B25 phenylalanines would occupy molecule I like orientations and in which para-directed mass at position B25 would be readily accommodated. Additional data point to the potential formation of the two molecule I like conformations of Phe^{B25} depicted in Figure 4b. First, evidence from the crystallographic analysis of the 2-Zn insulin hexamer has identified that Phe^{B25} of molecule II can sometimes occupy a molecule I like conformation (Casaretto et al., 1987). Second, both molecules of insulin within the cubic crystal exhibit molecule I like conformations of Phe^{B25} (Derewenda et al., 1990; Dodson, 1978). Third, energy minimization studies indicate that, in the absence of crystal lattice forces, the molecule I like orientation of Phe^{B25} is the energetically more favorable (Wodak et al., 1984). Fourth, analytical radioiodination of [*p*-hexanoyl-Pap^{B25},Ala^{B29}]insulin at 1 and 100 μM identifies significant shielding of residue Tyr^{A19} compared to the control ([Ala^{B29}]insulin) at the same concentrations, a result consistent with a molecule I like orientation of the β-aromatic ring at position B25 in which the hexanoyl group at the para position of Pap^{B25} protects Tyr^{A19} from iodination (Table II). It should be noted, however, that NMR studies have identified the Phe^{B25} side chain in the native insulin monomer to undertake a flexible, externally rotated conformation more similar to that observed in crystallographic molecule II (Weiss et al., 1989).

Parts c–e of Figure 4 present alternative conformations of Phe^{B25} that might apply when insulin is bound to its receptor. Parts c and d depict residue Phe^{B25} as assuming an outwardly directed, molecule II like orientation in which the side chain might make contact with receptor (from above or below, respectively) by means of a tangential extension of the receptor surface beyond the COOH-terminal β-sheet. Figure 4e presents Phe^{B25} in an alternatively directed, molecule I like conformation (in which the Phe^{B25} side chain folds back over the monomer surface) by means of a tangential retraction (or limitation) of the receptor surface toward the COOH-terminal β-sheet. All of the structures of parts c–e are drawn to reflect observations demonstrating that (a) high-affinity receptor

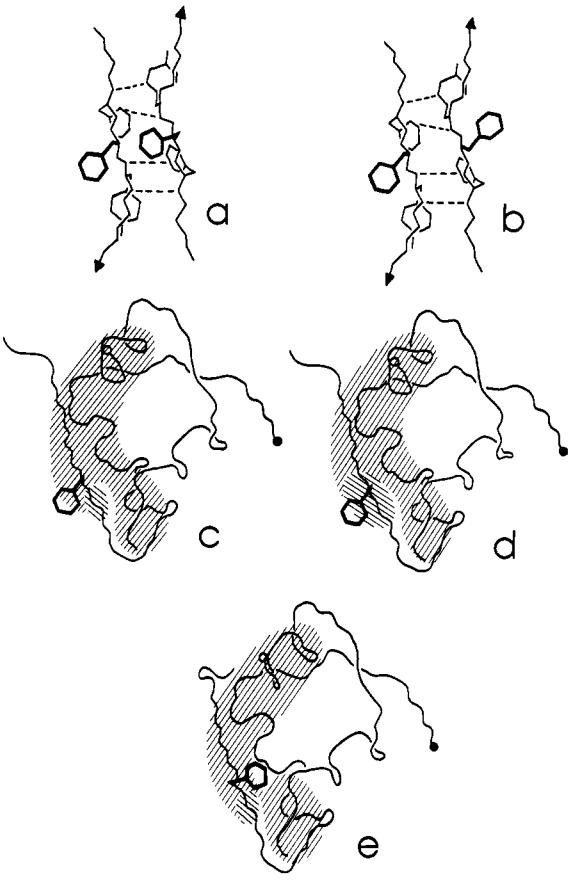

FIGURE 4: Diagrammatic structures of insulin–insulin and insulin–receptor interactions relevant to the conformation of the residue B25 side chain. Panel a: a view down the 2-fold axis of the COOH-terminal B-chain domains of interacting molecules I (left) and II (right) at the monomer–monomer interface in the 2-Zn crystallographic insulin hexamer [taken from Blundell et al. (1972)]. Panel b: a modeled view related to the one shown in panel a in which both molecules of insulin at the monomer–monomer interface exhibit a molecule I like conformation of Phe^{B25}. The side chains of Phe^{B24}, Phe^{B25} (heavy lines), and Tyr^{B26} are shown explicitly; dashed lines indicate hydrogen bonds between the main chains of the two monomers within the dimer. Panels c–d show structures of insulin as they might occur in combination with the insulin receptor; the view is taken from Derewenda et al. (1990). The amino terminus of the B-chain is indicated by a solid dot, and that of the A chain is indicated by an open circle; the side chain of Phe^{B25} is shown by a heavy line. The receptor surface that might contact the insulin monomer is indicated by hatching (left to right) for surfaces above the molecule and right to left for surfaces below the molecule (including those that might contact Phe^{B24} [cf. Mirmira and Tager (1989) and Fischer et al. (1985)]. The hatched areas take into consideration the importance of the COOH-terminal domains of the insulin A- and B-chains, the NH_2-terminal domain of the insulin A-chain, and the orientation of the Phe^{B25} side chain but are not meant to define the receptor surface precisely. Panel c: a view in which the side chain of Phe^{B25} takes a molecule II like conformation and is contacted by receptor from above. Panel d: a view in which the side chain of Phe^{B25} takes a molecule II like conformation and is contacted by receptor from below. Panel e: a view in which the side chain of Phe^{B25} takes a molecule I like conformation and is contacted by receptor from above. See text for further details and interpretations.

interactions define the need for a β-aromatic ring at insulin position B25 [this paper and Nakagawa and Tager (1986)], (b) significant binding potency is retained when the para position of Pap at position B25 is acylated by substituents of significant bulk (this paper), (c) only small variations in receptor binding potency occur in full-length insulin analogues or des-pentapeptide-(B26–B30)-[Phe^{B25}-α-carboxamide]insulin analogues in which the side chain of Phe^{B25} is replaced with β-aromatic substituents of varying structures [this paper and

Nakagawa and Tager (1986)] and (d) a pair of *o*-iodine atoms is well tolerated in the interactions of [3,5-diiodo-Tyr^{B25},Phe^{B26}]insulin with the hepatocyte insulin receptor (Mirmira et al., 1991). Most important, the structures of parts c–e of Figure 4 all identify the Phe^{B25} side chain as crossing the ligand–receptor boundary, rather than as occupying a pocket formed by the receptor itself. Interestingly, the orientation of the Phe^{B25} side chain proposed from a molecular graphic simulation of the insulin–insulin receptor complex (DeMeyts et al., 1990) is the same as that shown in Figure 4e. Although we cannot yet know whether receptor-bound insulin retains a molecule I like or molecule II like structure, we conclude overall that insulin, when it is in a high-affinity state of interaction with its receptor, undertakes a conformation in which Phe^{B25} defines the limit of the solvent-exposed surface between the receptor and the hormone.

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