Maintenance of the B-Chain β-Turn in [Gly^{B24}]Insulin Mutants: A Steady-State Fluorescence Anisotropy Study†

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ABSTRACT: [Gly^{B24}]insulin is a novel insulin analog which maintains nearly full biological activity [Mirmira, R. G., & Tager, H. S. (1989) *J. Biol. Chem. 264*, 6349−6354] even though its structure, as determined by 2D NMR, shows complete loss of the characteristic B-chain β-turn [Hua, Q. X., Shoelson, S. E., Kochoyan, M., & Weiss, M. A. (1991) *Nature 354*, 238−241], which in native insulin allows the extended B-chain C-terminal region to fold against the central B-chain helix. In these studies, steady-state anisotropy measurements and fluorescence quenching analysis of the tryptophan-substituted analogs [Trp^{B25}]insulin and [Gly^{B24},Trp^{B25}]insulin have been used to study the structure of the C-terminal region of the B-chain and have demonstrated that [Gly^{B24}]insulin mutants maintain the normal B-chain conformation to a degree comparable to that of native (Phe^{B24}) insulin at neutral pH. The tryptophan-substituted, B-chain C-terminally truncated analogs [Trp^{B25}-α-carboxamide]despentapeptide(B26−B30)-insulin (DPI) and [Gly^{B24},Trp^{B25}-α-carboxamide]DPI also significantly retain the characteristic insulin B-chain fold in solution with [Gly^{B24},-Trp^{B25}-α-carboxamide]DPI being more tightly folded than its corresponding Phe^{B24}-analog ([Trp^{B25}-α-carboxamide]DPI), as assessed by these methods. The results of anisotropy measurements are consistent with the existence of a correlation between the high-affinity receptor binding of [Gly^{B24}]insulin and the partial maintenance of the B-chain β-turn under physiologic conditions. Thus we conclude that only analogs which possess, or can readily assume, this oriented structure can form high-affinity binding complexes with insulin receptor.

Several studies have addressed the hypothesis that insulin must undergo a conformational change upon binding to its receptor. This notion is supported by studies on insulin analogs having amino acid replacements (Wang et al., 1991; Mirmira & Tager, 1989; Schwartz et al., 1983) or deletions (Fischer et al., 1985; Nakagawa & Tager, 1993), which suggest that individual amino acids do not necessarily provide high-affinity receptor binding but collectively allow a particular structure to be attained (Dodson et al., 1983). An insulin analog, known as a mini-proinsulin (Figure 1B), which possesses a B29 α-carboxy to A1 α-amino peptide linkage has been shown to display barely detectable receptor binding capability (<0.1%) (Markussen et al., 1985). Subsequently, this analog was found to be almost identical in its three-dimensional structure to both the 2-Zn crystallographic and the solution state structures of native insulin (Figure 1A) (Baker et al., 1988; Hua & Weiss, 1991; Derewenda et al., 1991). This adherence to native insulin structure with diminished binding affinity provided further evidence supporting the need for a conformational change upon receptor binding.

Native insulin, as shown in Figure 1A, is a two-chain molecule consisting of an A-chain (21 residues) and B-chain (30 residues) which are disulfide linked, resulting in an insulin monomer (MW ≈ 5800) (Blundell et al., 1972; Baker et al., 1988). The A-chain has two α-helical regions, A1−A8 and A13−A18. The B-chain has an amino-terminal extended chain from positions B1 to B8, an α-helical region from B9 to B19, a (1→4) β-turn from B20 to B23, and a β-strand from B24 to B30 which interacts with the adjacent B-chain α-helix. This conformation is referred to as T-state insulin, as opposed to R-state insulin in which the binding of phenol causes B1−B8 to assume an α-helical conformation extending the preexisting B-chain α-helix (Derewenda et al., 1989; Jacoby et al., 1996). The B-chain β-turn forms the characteristic B-chain fold by causing the C-terminal β-strand to fold back onto the central helix. This structural feature has been found to be maintained in all known crystallographic structures of insulin, including analogs in which five residues of the B-chain C-terminus (B26−B30) have been deleted (despentapeptide-insulin: DPI) (Figure 1C) (Bi et al., 1984). Only one structure has been determined in which the insulin B-chain fold is reported to be absent; this is a derivative having a Phe^{B24} → Gly substitution (see Figure 1D) (Hua et al., 1991, 1992a). In [Gly^{B24}]insulin the B-chain β-strand is displaced away from its corresponding α-helix, exposing the hydrophobic core of the molecule. Despite the gross difference in the structure of [Gly^{B24}]insulin as compared to native insulin (see panels A and D of Figure

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[1] Abbreviations: TPCK, tosylphenylalanine chloromethyl ketone; Boc, *tert*-butyloxycarbonyl; DMF, dimethylformamide; AcOH, acetic acid; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; DOI, desoctapeptide(B23−B30)-insulin; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; $(Boc)_2$-DOI, N^{α}-Gly^{A1},N^{α}-Phe^{B1}-bis(Boc)-desoctapeptide(B23−B30)-insulin; NAWEE, *N*-acetyltryptophan ethyl ester; DPI, despentapeptide(B26−B30)-insulin; Gdn-HCl, guanidine hydrochloride.

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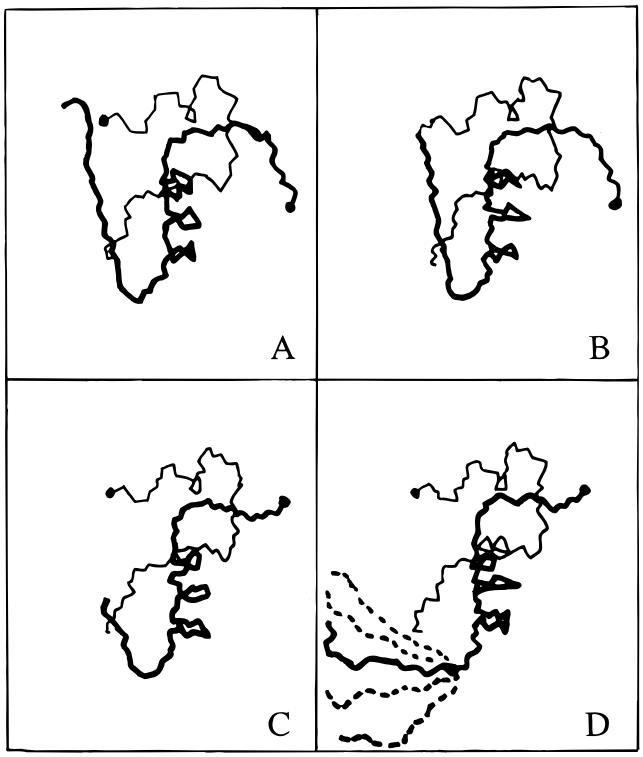

FIGURE 1: Structure of insulin and insulin analogs. Panels A−D represent the structures of (A) 2-Zn insulin (Baker et al., 1988) and the solution state 2D NMR structure of native insulin (Hua & Weiss, 1991), (B) mini-proinsulin (Derewenda et al., 1991), (C) despentapeptide(B26−B30)-insulin (Bi et al., 1984), and (D) [Gly^{B24}]insulin (Hua et al., 1991). In each panel the A-chain is indicated by the thinner line and the B-chain by the thicker line, and the N-terminus of each is designated by a ball. The cross-link from Al to B29 in mini-proinsulin is shown as a thin connecting segment, while the dashed lines in panel D represent the variable positions of the B-chain C-terminus of [Gly^{B24}]insulin.

1), the Phe^{B24} → Gly mutant maintains near-native receptor binding (≈75%) (Mirmira & Tager, 1989). This observation prompted the supposition that insulin might unfold the C-terminal B-chain β-turn upon binding to its receptor, which could explain why this analog retains high-frequency binding (Hua et al., 1991).

In these studies fluorescence anisotropy techniques have been employed to determine the rotational freedom of a single tryptophan substituted at position B25 (Phe^{B25} → Trp) which is suspected to be adjacent to the hydrophobic core in an insulin monomer (Baker et al., 1988; Pittman & Tager, 1995). A plane polarized light source is used to excite a fluorophore (Trp^{B25}) whose emission is measured both parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the excitation plane (Lakowicz, 1983a). The degree of rotational freedom of the fluorescent probe can be determined by the ability of the probe, which is excited in one plane, to rotate out of the excitation plane before emission (depolarization). Fluorescent side chains used as structural probes in proteins display a range of anisotropies from tightly packed side chains, possessing high anisotropy values, to loosely packed fluorescent side chains which are more depolarized and have lower anisotropies (Lakowicz, 1983a; Beechem et al., 1995). Both its sensitivity and specificity, when fluorescent probes are carefully chosen, have made polarization studies a useful technique for investigating the local environment of various side chains in a protein (Bailey et al., 1995; Fa et al., 1995).

Using steady-state fluorescence anisotropy, we have demonstrated that Gly^{B24},Trp^{B25}-analogs essentially maintain the insulin B-chain fold with only minor deviations. This result could explain the relatively well conserved binding affinity of [Gly^{B24}]insulin. On the basis of our results, we hypothesize that the B-chain fold is a prerequisite of high-affinity insulin/insulin receptor interactions and that upon encountering receptor the B-chain β-strand is partially separated from the central B-chain helix as in DPI-NH_2.

MATERIALS AND METHODS

Preparation of Insulin Analogs with Altered B-Chain COOH-Terminal Domains. Each insulin analog 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 octapeptide (Inouye et al., 1981). The octapeptides were synthesized via solid-phase methods previously described (Mirmira et al., 1991). $(Boc)_2$-DOI (1 μmol) and octapeptide (4 μmol) 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 24−72 h (Morihara et al., 1986; Nakagawa & Tager, 1993). 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) (TEAP) and acetonitrile] were 30−50%. The Boc groups were then removed with trifluoroacetic acid (TFA) for all of the analogs except [Gly^{B24},Trp^{B25}]insulin, in which the Boc group was removed after purification. The analogs were then dissolved in 3 M acetic acid and purified via preparative HPLC on a C-4 column by use of a 0.1% aqueous TFA and 0.1% TFA in acetonitrile solvent system. The purified product was desalted on a Bio-Gel P-4 gel filtration column using 3 M acetic acid, collected, and lyophilized. The analogs were then dissolved in 0.1 M Tris brought to pH 7.5 by the addition of hydrochloric acid, and solutions were made to the desired concentrations using extinction coefficients calculated from the values $\epsilon_{Tyr}^{275} = 1340$ and $\epsilon_{Trp}^{275} = 5500$. The value $A_{275}^{mg/mL} = 1.87$ was calculated for [Trp^{B25}]insulin and $A_{275}^{mg/mL} = 1.90$ for [Gly^{B24},Trp^{B25}]insulin. Porcine insulin concentrations were determined using $A_{275}^{mg/mL} = 1.05$ (Frank & Veros, 1968).

Synthesis of Tripeptide α-Carboxamides. H-Gly-Phe-Trp-NH_2 and H-Gly-Gly-Trp-NH_2 were prepared by use of the stepwise active ester method in solution (Bodansky & duVigneaud, 1959). The C-terminal H-Trp-NH_2 (purchased from Sigma) was condensed with Boc-Phe-*N*-hydroxysuccinimide ester (OSu) or Boc-Gly-OSu in DMF (Anderson et al., 1964) followed by removal of the N^{α}-Boc protecting groups by 25% TFA in methylene chloride to yield H-Phe-Trp-NH_2 or H-Gly-Trp-NH_2. The resulting dipeptide α-carboxamides were further acylated with Boc-Gly-OSu and treated with TFA to yield the final tripeptide α-carboxamides. The purity of each compound was determined by thin-layer chromatography on SiO_2 or analytical reverse-phase HPLC on a C-18 column using an acetonitrile/TEAP solvent system as mentioned above.

Preparation of Despentapeptide(B26−B30)-[Trp^{B25}-α-carboxamide(NH_2)]insulin and Despentapeptide(B26−B30)-[Gly^{B24},Trp^{B25}-NH_2]insulin. Each analog was prepared by

trypsin-catalyzed peptide bond formation between the α-carboxy group of Arg^{B22} or $(Boc)_2$DOI (3 μM) and the α-amino group of a tripeptide amide (30 μM) (Nakagawa & Tager, 1986) as described above. Concentrations were determined using $A_{275}^{mg/mL} = 1.809$ and $A_{275}^{mg/mL} = 1.840$ for [Trp^{B25}-NH_2]DPI and [Gly^{B24},Trp^{B25}-NH_2]DPI, respectively.

Receptor Binding Studies. Receptor binding assays of insulin analogs were performed as previously described (Cara et al., 1990) with minor modifications. Human placental membranes were prepared using a previously published method (Marshall et al., 1974). Placental membranes (25 μg of protein/tube) were incubated with [[^{125}I]iodo-Tyr^{A14}]-insulin (≈30 000 cpm) in the presence of selected concentrations of unlabeled hormone for 16 h at 4 °C in a final volume of 0.25 mL of 0.05 M Tris-HCl and 0.25% (w/v) bovine serum albumin at pH 8. Subsequent to incubation, each mixture was diluted with 1 mL of buffer at 0 °C and centrifuged (12000*g*) for 4 min at 4 °C. The supernate was then removed by aspiration, and the membrane pellet was counted for radioactivity. Each determination was performed in duplicate.

Steady-State Fluorescence Anisotropy Studies. Fluorescence spectra were recorded on a PTI fluorometer interfaced with an NEC computer, using a 0.8 mL sample cell (1 cm × 1.2 cm × 4.3 cm) with a 1 cm path length. Fluorescence emission scans were taken from 310 to 450 nm, while exciting at 295 nm for primarily tryptophan fluorescence. Polarizers were set at vertical (V) or horizontal (H) positions as required, and an average of eight scans per spectrum were taken. Each trial was repeated two to five times to determine standard deviations (σ) when necessary. Anisotropies (r) were calculated from the intensity maxima using the quantum yield and instrumental correction factor (G-factor) found by measuring free tryptophan (*N*-acetyltryptophan ethyl ester: NAWEE) in solution ($r \approx 0.02$) and using the equation

$$G = I_{HV}/I_{HH} \quad (1)$$

where I_{HV} is the intensity when excited (first subscript) with a horizontally polarized light source and vertical emission (second subscript) detected, and I_{HH} is the intensity with horizontally polarized excitation and emission detected in the same horizontal plane (Lakowicz, 1983a). $I_{||}$ (parallel intensity) and $I_{\perp}$ (perpendicular intensity) were correlated using the calculated G-factor and the equation:

$$(I_{VV}/I_{VH})/G = I_{||}/I_{\perp} \quad (2)$$

so that the equation

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH}) \quad (3)$$

could be used to calculate the steady-state anisotropies in the typical form of

$$r = (I_{||} - I_{\perp})/(I_{||} + 2I_{\perp}) \quad (4)$$

Nonpolarized Fluorescence Studies. Polarizers were removed, and an average of three runs was used for each of the final spectra; all background effects were subtracted to obtain spectra exclusively representative of changes in the environment of the substituted tryptophan in the insulin analogs. All solutions were in 0.1 M Tris-HCl buffer at pH 7.5 and were at concentrations such that the analogs alone in solution would be monomeric (3.3 μM). Fluorescence quenching studies were performed using acrylamide as a quenching agent. Inner filter effects were found to be minimal. All of the data from the fluorescence quenching studies was modeled using the Stern−Volmer equation (Lakowicz, 1983b):

$$F_0/F = 1 + K_{SV}[Q] \quad (5)$$

where F_0 is the fluorescence intensity in the absence of quencher, F is the fluorescence intensity in the presence of acrylamide quencher, [Q] is the concentration of acrylamide used, and K_{SV} is the dynamic quenching constant.

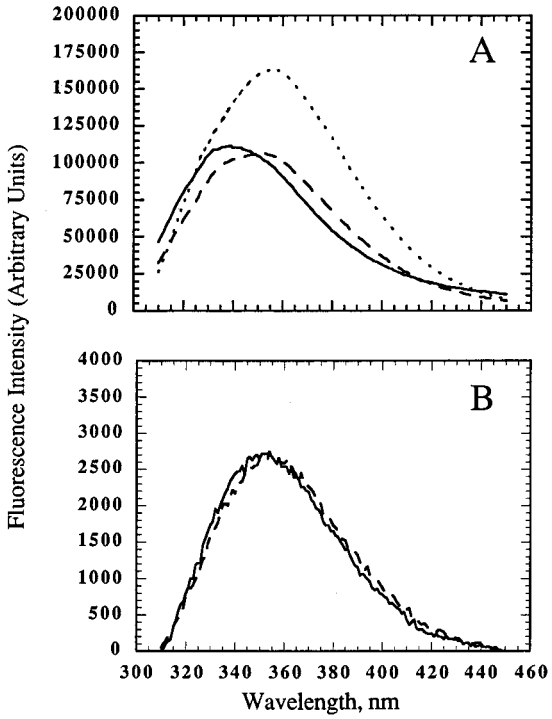

FIGURE 2: Nonpolarized fluorescence analysis of insulin analogs and polarized spectra of *N*-acetyltryptophan ethyl ester (NAWEE). Panel A illustrates the nonpolarized fluorescence emission spectra of 3.3 μM [Trp^{B25}]insulin (—), [Gly^{B24},Trp^{B25}]insulin (− −), and the octapeptide GGWYTPKT (···). Panel B shows the polarized emission spectra of 8 μM NAWEE with the parallel, $I_{||}$ (—), and perpendicular, $I_{\perp}$ (− −) intensities. All samples were excited at 295 nm and are in 0.1 M Tris-HCl (pH 7.5).

RESULTS

Figure 2A illustrates the fluorescence spectra of 3.3 μM [Trp^{B25}]insulin, [Gly^{B24},Trp^{B25}]insulin, and 3.3 μM octapeptide (GGWYTPKT) corresponding to the B-chain C-terminus of the [Gly^{B24},Trp^{B25}]insulin analog, at pH 7.5. These are concentrations at which native insulin, as well as the analogs, has been demonstrated to be primarily monomeric (Goldman & Carpenter, 1974; Pittman & Tager, 1995); i.e., at 3.3 μM native insulin is ≈60% monomeric while the analogs would most likely be >60% monomeric. The emission spectrum of [Gly^{B24},Trp^{B25}]insulin is red shifted compared to that of [Trp^{B25}]insulin; however, the emission maximum of the octapeptide is slightly red shifted compared to that of the Gly^{B24}-analog. This implies that the tryptophan side chain in [Gly^{B24},Trp^{B25}]insulin is not fully solvent exposed. The tryptophan in [Gly^{B24},Trp^{B25}]insulin does, however, appear to be more solvent exposed than in [Trp^{B25}]insulin in which

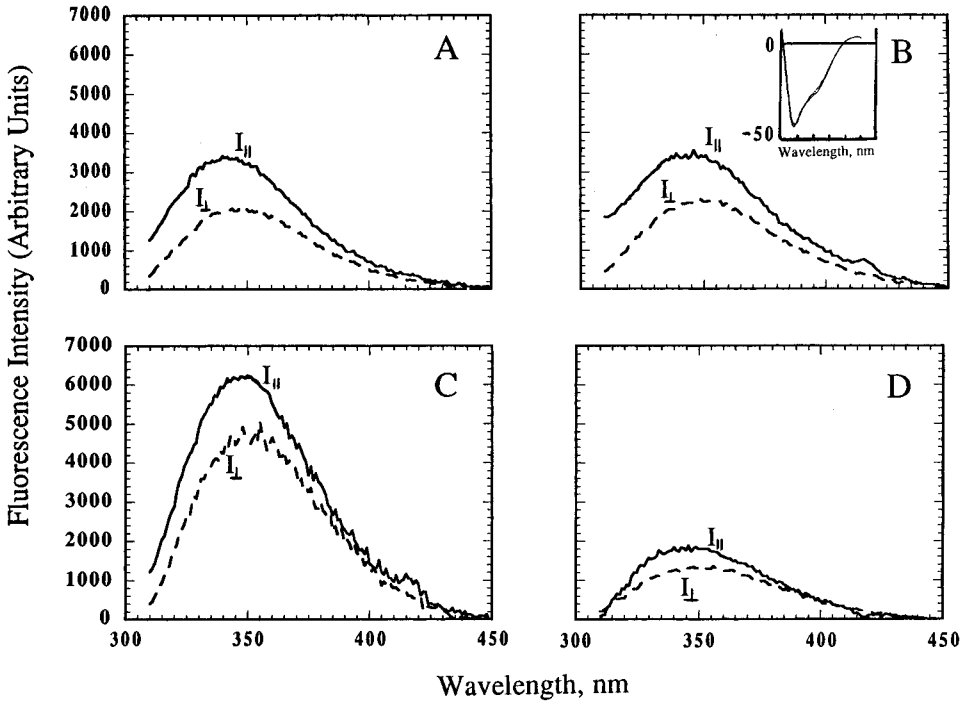

FIGURE 3: Polarized fluorescence emission spectra of [Trp^{B25}]insulin and [Gly^{B24},Trp^{B25}]insulin. Panels A−D illustrate the parallel ($I_{||}$: —) and perpendicular ($I_{\perp}$: − −) emission spectra of 3.3 μM (A) [Trp^{B25}]insulin, (B) [Gly^{B24},Trp^{B25}]insulin, (C) [Gly^{B24},Trp^{B25}]insulin in 6.5 M Gdn-HCl, and (D) [Gly^{B24},Trp^{B25}]insulin in 20% acetic acid. All samples were originally prepared in 0.1 M Tris-HCl, pH 7.5. Excitation was at 295 nm with polarizers positioned as mentioned in Materials and Methods. Anisotropy values (r) are listed in Table 1. The inset in panel B displays the CD spectra (0−50 mdeg; 200−250 nm) of [Lys^{B28},Pro^{B29}]insulin (—) and [Gly^{B24},Lys^{B28},Pro^{B29}) insulin (− - −) in 0.1 M Tris-HCl, pH 7.5 (nonbuffer subtracted).

the fluorescent side chain has previously been demonstrated to be adjacent to the hydrophobic core of an insulin monomer (Pittman & Tager, 1995). This suggests that the insulin B-chain fold is preserved in [Trp^{B25}]insulin and is slightly distorted in the Gly^{B24}-analog.

To further investigate the local environment of the tryptophan side chain in [Trp^{B25}]- and [Gly^{B24},Trp^{B25}]insulin, steady-state anisotropy values were calculated from the fluorescence intensity maxima of parallel ($I_{||}$) and perpendicular ($I_{\perp}$) emission spectra. As previously mentioned, if the tryptophan is free to rotate in solution, then the anisotropy value should be near zero, with $I_{||} = I_{\perp}$, demonstrating depolarization. This is illustrated by *N*-acetyltryptophan ethyl ester (NAWEE; see Figure 2B) which is fully solvent exposed tryptophan analog, can rotate freely, and therefore depolarizes the emission spectra (Lakowicz, 1983a; Bailey et al., 1995) having an anisotropy value $r \approx 0.01$ (see Table 1). The octapeptide also demonstrated a reduced anisotropy indicative of nearly complete depolarization (see Table 1).

Suspecting that the surrounding protein environment would decrease the rotational freedom of the tryptophan side chain in [Trp^{B25}]insulin, polarized emission spectra of this analog were taken and are shown in Figure 3A. It is clearly illustrated that $I_{||}$ and $I_{\perp}$ are not equal, indicating that the tryptophan side chain at position B25 is not freely rotating in this analog. This indicates that the protein scaffolding prohibits such freedom of rotation by effectively packing the fluorescent side chain. Figure 3B illustrates the emission spectra of [Gly^{B24},Trp^{B25}]insulin which also displays a marked difference in $I_{||}$ and $I_{\perp}$, indicating that the tryptophan probe in this Gly^{B24}-analog is also not free to rotate in solution. As shown in Table 1 the anisotropy (r) of [Gly^{B24},-Trp^{B25}]insulin is reduced compared to that of [Trp^{B25}]insulin, but still maintains a significant packing environment for the Trp^{B25} side chain. These results argue that, at neutral pH, the local environment of the tryptophan side chain in [Gly^{B24},Trp^{B25}]insulin is similar to that of [Trp^{B25}]insulin which has previously been demonstrated to maintain the insulin B-chain fold (see Figure 1A) (Pittman & Tager, 1995). The inset of Figure 3B also demonstrates the similarity of the circular dichroic spectra of [Lys^{B28},Pro^{B29}]- and [Gly^{B24},Lys^{B28},Pro^{B29}]insulin, two monomeric analogs with binding affinities similar to those of native insulin and [Gly^{B24}]insulin, respectively (data not shown).

In an attempt to confirm that the B-chain β-turn exists in [Gly^{B24},Trp^{B25}]insulin, fluorescence studies were conducted in 6.5 M guanidine hydrochloride, with the expectation that spectral changes would primarily reflect structural adjustments at the B-chain C-terminus if there was, in fact, some preexisting structure. Figure 3C illustrates that the intensity

Table 1: Fluorescence Anisotropy Values for Insulin Analogs[a]

tryptophan fluorescence source	anisotropy (r)
NAWEE	0.013
octapeptide (GGWYTPKT)	0.044
[Trp^{B25}]insulin	0.178 ± 0.012
[Trp^{B25}]insulin in 6.5 M Gdn-HCl	0.030
[Trp^{B25}]insulin in 20% AcOH	0.161
[Gly^{B24},Trp^{B25}]insulin	0.152 ± 0.014
[Gly^{B24},Trp^{B25}]insulin in 6.5 M Gdn-HCl	0.089
[Gly^{B24},Trp^{B25}]insulin in 20% AcOH	0.111 ± 0.001
[Trp^{B25}-NH$_2$]DPI	0.066 ± 0.005
[Gly^{B24},Trp^{B25}-NH$_2$]DPI	0.074 ± 0.003

[a] The anisotropies are calculated from the parallel ($I_{||}$) and perpendicular ($I_{\perp}$) intensities using the equation: $r = (I_{||} - I_{\perp})/(I_{||} + 2I_{\perp})$ as mentioned in Materials and Methods. The table lists the average of two to five separate experiments. Each analog, and NAWEE (*N*-acetyltryptophan ethyl ester), was measured three to five times with standard deviations (σ) calculated unless anisotropy values were nearly identical, in which case only two trials were performed.

of the emission spectra increases when [Gly^{B24},Trp^{B25}]insulin is in 6.5 M guanidine hydrochloride, and the anisotropy decreases from $r = 0.152$ in Tris-HCl buffer to $r = 0.089$ in guanidine (see Table 1). Both of these spectral changes indicate a change in the local environment of the tryptophan under denaturing conditions. These changes indicate that guanidine releases the tryptophan side chain from its environmental constraints, consistent with our interpretation that, at neutral pH, [Gly^{B24},Trp^{B25}]insulin maintains the B-chain β-turn.

The discrepancy between these results and the previously determined [Gly^{B24}]insulin solution structure (Hua et al., 1991, 1992a) was further investigated by studying the behavior of [Gly^{B24},Trp^{B25}]insulin in 20% acetic acid (Figure 3D), conditions under which the 2D NMR structure was determined for [Gly^{B24}]insulin. The reduced intensity in the presence of the organic acid is due to the quenching effects of hydrogen ions on tryptophan (Lakowicz, 1983b). However, there is also a decrease in the anisotropy of [Gly^{B24},-Trp^{B25}]insulin in 20% acetic acid ($r = 0.111$) which is similar to the anisotropy of the analog in 6.5 M guanidine hydrochloride. It appears that both 6.5 M guanidine and 20% acetic acid release the substituted tryptophan in [Gly^{B24},-Trp^{B25}]insulin from previously existing environmental constraints, reducing the anisotropy value, presumably by denaturing the preexisting folded structure (Figure 1). [Gly^{B24}]Insulin in 20% acetic acid was not studied using CD methods due to the absorbance of acetic acid in the far-UV. Hua et al. (1992a) demonstrated, however, that the molar ellipticity of [Gly^{B24}]insulin is significantly attenuated relative to native insulin at pH 1.9 in aqueous HCl.

To reassess the importance of intramolecular contacts conferred by the B-chain β-strand and their relevance in maintaining the insulin fold in solution, [Trp^{B25}-α-carboxamide]- and [Gly^{B24},Trp^{B25}-α-carboxamide]despentapeptide-(B26−B30)-insulin (DPI) were synthesized and investigated for their fluorescent properties. The crystal structure, as well as the solution state structure, of DPI demonstrates that this analog maintains the insulin B-chain fold in the absence of the additional β-strand contacts which residues B26−B30 confer in native insulin (Bi et al., 1984; Hua & Weiss, 1990, 1991). Previous studies have also demonstrated that the B25 α-carboxamide (NH_2) derivative of DPI (DPI-NH_2) retains higher than native binding affinity (≈140%) for insulin receptor (Nakagawa & Tager, 1993); however, a single Phe^{B24} → Gly mutation in this truncated analog results in a compound with ≤1% binding to insulin receptor (Mirmira & Tager, 1989; Nakagawa & Tager, 1993). This is radically different from the minor reduction in receptor binding caused by the same mutation in full-length [Gly^{B24}]insulin (≈75% binding) and suggests that B24 plays a profound role in the proper orientation of more distal residues. [Trp^{B25}-NH_2]DPI has previously been reported to demonstrate 54% binding affinity for insulin receptor (Casaretto et al., 1987), and in these studies we report binding of 52% for [Trp^{B25}-NH_2]DPI and 0.74% for [Gly^{B24},Trp^{B25}-NH_2]DPI (Table 2). These results indicate that the tryptophan-containing analogs are behaving in a manner similar to that of the non-tryptophan-containing DPI analogs which show a similar trend in receptor binding when a Phe^{B24} → Gly mutation is introduced.

To uncover any structural changes which may result from deletion of the last five amino acids of the insulin B-chain (B26−B30) coincident with the introduction of a Phe^{B24} → Gly mutation, the fluorescence emission spectra of [Trp^{B25}-NH_2]- and [Gly^{B24},Trp^{B25}-NH_2]DPI were investigated (Figure 4A). Interestingly, the nonpolarized emission maximum of [Gly^{B24},Trp^{B25}-NH_2]DPI is slightly blue shifted when compared to that of [Trp^{B25}-NH_2]DPI, suggesting that the tryptophan in [Gly^{B24},Trp^{B25}-NH_2]DPI is in a more hydrophobic environment than in [Trp^{B25}-NH_2]DPI. Thus, [Gly^{B24},-Trp^{B25}-NH_2]DPI appears to maintain the β-turn of the insulin fold with Trp^{B25} folded closer to the hydrophobic interior of this insulin analog. Panels B and C of Figure 4 illustrate the parallel and perpendicular emission spectra of [Trp^{B25}-NH_2]DPI and [Gly^{B24},Trp^{B25}-NH_2]DPI, respectively. Both of these analogs have anisotropy values of $r \approx 0.07$, with [Gly^{B24},Trp^{B25}-NH_2]DPI having a slightly higher anisotropy (see Table 1). Values of this magnitude suggest that Trp^{B25} can rotate more freely in these DPI analogs than in the full-length B-chain insulin analogs. Nonetheless, our data indicate that even the DPI analogs retain a degree of polarization. These results are supported by the previous crystal structure (Bi et al., 1984) and the molten-globule model proposed by Hua et al. (1992b, 1993), in which the insulin B-chain fold is partially maintained in DPI.

Due to the difference in the emission maxima of the truncated analogs, fluorescence quenching studies were conducted to further evaluate the solvent accessibility of the tryptophan probes placed at position B25. Figure 5 illustrates the Stern−Volmer plots of the octapeptide (GGWYTPKT), [Trp^{B25}]- and [Gly^{B24},Trp^{B25}]insulin, as well as [Trp^{B25}-NH_2]-DPI and [Gly^{B24},Trp^{B25}-NH_2]DPI in 0.1 M Tris-HCl (pH 7.5). Comparison of the slope of the Stern−Volmer plot for [Trp^{B25}]insulin with that of [Gly^{B24},Trp^{B25}]insulin (Figure 5A) indicates that the tryptophan in the Gly analog is more solvent accessible, as demonstrated by its larger slope and correspondingly higher dynamic quenching constant (K_{SV}). This is consistent with our previously published results (Pittman & Tager, 1995) and the nonpolarized emission spectra presented earlier in which [Gly^{B24},Trp^{B25}]insulin is red shifted, indicating greater solvent exposure, relative to [Trp^{B25}]insulin (see Figure 2A). Accordingly, the octapeptide is noted to have the most solvent accessible tryptophan and a greater K_{SV} than [Gly^{B24},Trp^{B25}]insulin. Figure 5B illustrates that the C-terminally truncated analog [Trp^{B25}-NH_2]-

Table 2: Relative Receptor Binding Potencies of Insulin and Insulin Analogs[a]

peptide	relative potency (%)
insulin	100
DPI-NH_2	147[b]
[Gly^{B24}]DPI-NH_2	0.98[b]
[Trp^{B25}]insulin	5.7
[Gly^{B24},Trp^{B25}]insulin	0.33[c]
[Trp^{B25}-NH_2]DPI	52
[Gly^{B24},Trp^{B25}-NH_2]DPI	0.74

[a] The analogs used in this study and their relative receptor binding potencies are identified above. Details of semisynthetic methods and of placental membrane 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 analog causing half-maximal inhibition of binding of [[^{125}I]iodo-Tyr^{A14}]insulin in receptor)] × 100. Each value represents the average of two to four trials and displayed considerable consistency. The relative binding potencies reported in this table can be considered under most circumstances to reflect relative binding affinities. [b] Cited from Nakagawa and Tager (1993). [c] Cited from Pittman and Tager (1995).

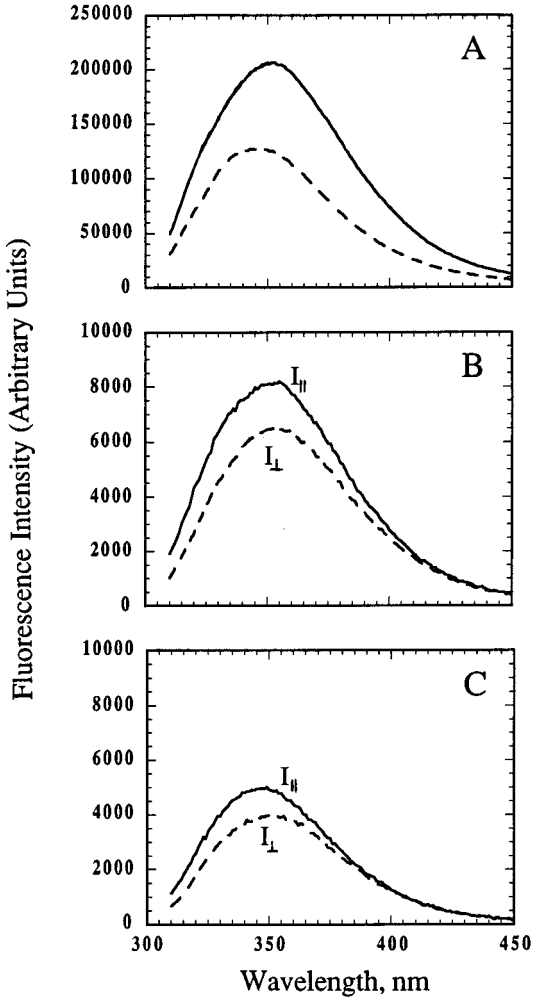

FIGURE 4: Fluorescence analysis of [Trp^{B25}-α-carboxamide]despentapeptide(B26−B30)-insulin and [Gly^{B24},Trp^{B25}-α-carboxamide]-despentapeptide(B26−B30)-insulin. Panel A illustrates the nonpolarized emission spectra of [Trp^{B25}-NH$_2$]DPI (—) and [Gly^{B24},-Trp^{B25}-NH$_2$]DPI (− −). Panels B and C illustrate the polarized emission spectra of [Trp^{B25}-NH$_2$]DPI and [Gly^{B24},Trp^{B25}-NH$_2$]DPI, respectively. $I_{||}$ is represented by a solid line and $I_{\perp}$ is represented by a dashed line. In each figure the protein concentration is 3.3 μM in 0.1 M Tris-HCl, pH 7.5, and excitation is at 295 nm. Anisotropies (r) are listed in Table 1.

DPI has a higher K_{SV} than [Gly^{B24},Trp^{B25}-NH$_2$]DPI (see Table 3), suggesting greater solvent accessibility of the tryptophan in [Trp^{B25}-NH$_2$]DPI. This is also indicated by the red-shifted emission spectra of [Trp^{B25}-NH$_2$]DPI when compared to its truncated Gly^{B24} counterpart (Figure 4A). It should also be noted that, overall, the DPI analogs possess higher dynamic quenching constants (see Table 3), indicating that the tryptophan in both of these analogs is more solvent exposed than the tryptophan in [Gly^{B24},Trp^{B25}]insulin. We interpret this to indicate that the tryptophan in the truncated insulins is less tightly packed than in either full-chain insulin analog.

These results suggest that the insulin B-chain fold is preserved in all of our Trp^{B25} analogs and through packing serves to partially shield the substituted tryptophans from the acrylamide quencher. We find that the tryptophan in [Gly^{B24},Trp^{B25}-NH$_2$]DPI is less solvent exposed than in [Trp^{B25}-NH$_2$]DPI, implying that the extra flexibility conferred by Gly^{B24} in this truncated analog allows the tryptophan side chain to fold in toward the hydrophobic core of the molecule. This is not the case for [Gly^{B24},Trp^{B25}]insulin in which the B-chain C-terminal residues appear to provide a more solvent exposed conformation for this side chain when compared to [Trp^{B25}]insulin. Such results imply that the C-terminal residues of the B-chain may serve in a critically balanced equilibrium to properly orient the B-chain β-strand in insulin at some precise distance from its central α-helical segment. This critical distance is more readily achieved in the absence of the B-chain C-terminus in DPI-NH$_2$, which may be further adjusted when interacting with the insulin receptor. The collapse of this equilibrium in Gly^{B24}-DPI accordingly results in a marked reduction in binding affinity.

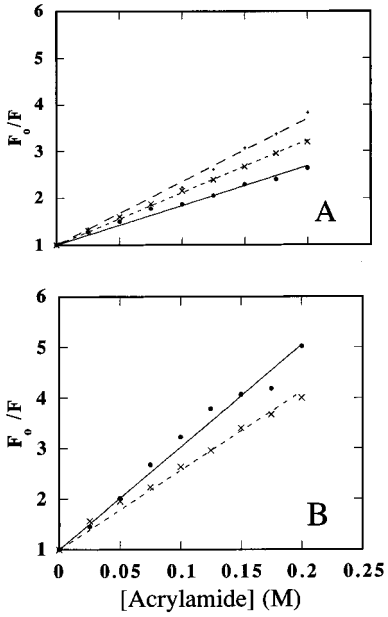

FIGURE 5: Stern−Volmer plots of acrylamide quenching of tryptophan fluorescence. Panel A displays the Stern−Volmer plots of [Trp^{B25}]insulin (—), [Gly^{B24},Trp^{B25}]insulin (- - -), and octapeptide GGWYTPKT (− −); panel B illustrates the plots of [Trp^{B25}-NH$_2$]-DPI (—) and [Gly^{B24},Trp^{B25}]DPI (- - -). All protein solutions are at 3.3 μM in 0.1 M Tris-HCl, pH 7.5, and are plotted as acrylamide concentration, [Q] (0−200 mM), versus F_0/F (degree of fluorescence quenching) modeled via the Stern−Volmer equation $F_0/F = 1 + K_{SV}[Q]$, where F_0 is the tryptophan fluorescence emission in the absence of quenching agent, F is the fluorescence at a given acrylamide concentration, and K_{SV} is the Stern−Volmer dynamic quenching constant. All measurements were taken at the fluorescence emission maximum for the solution being analyzed, which is listed in Table 3 with K_{SV} values; excitation was at 295 nm.

Table 3: Fluorescence Quenching Constants for Stern−Volmer Plots[a]

analogs: tryptophan fluorescence source	wavelength of emission maximum, nm	dynamic quenching constant (K_{SV}), M^{-1}
[Trp^{B25}]insulin	342	8.4 ± 0.2
[Gly^{B24},Trp^{B25}]insulin	350	11.2 ± 0.1
GGWYTPKT (peptide)	354	13.5 ± 0.3
[Trp^{B25}-NH$_2$]DPI	351	19.9 ± 0.4
[Gly^{B24},Trp^{B25}-NH$_2$]DPI	346	14.8 ± 0.3

[a] The dynamic quenching constants (K_{SV}) for each of the insulin analogs studied via acrylamide quenching. The constants were obtained from an average of two to three experiments from graphs in Figure 5, which were modeled by the Stern−Volmer equation: $F_0/F = 1 + K_{SV}[Q]$, where F_0/F is the degree of fluorescence quenching and K_{SV} is the Stern−Volmer dynamic quenching constant. In all cases the buffering solution used is 0.1 M Tris-HCl at pH 7.5. All analogs are at 3.3 μM. All values for the degree of fluorescence quenching, therefore the Stern−Volmer plots in Figure 5, as well as quenching constants, were obtained at the emission maximum of the particular fluorophore.

DISCUSSION

In the studies presented we have used steady-state fluorescence emission techniques to investigate the local environment of tryptophan residues substituted at position B25 (Phe^{B25} → Trp) of human insulin, [Gly^{B24}]insulin, and [B25-α-carboxamide]despentapeptide(B26−B30)-insulin. Position B25 is known to be important in ligand−receptor interactions, as demonstrated by the greatly reduced binding of analogs in which Phe^{B25} has been mutated to other amino acids [Nakagawa & Tager, 1986; cf. Tager (1990) and Mirmira et al. (1991)]. Accordingly, this residue has been shown to cross-link to the insulin receptor α-subunit in its C-terminal region (Kurose et al., 1994). Nonetheless, tryptophan-substituted mutants retain activity and have been proven to be highly useful for studying structural adjustments in insulin analogs (Schiller & Geiger, 1983; Laws et al., 1995; Pittman & Tager, 1995). Their use has been supported by findings that similar structural adjustments occur in native insulin (Derewenda et al., 1989; Hua & Weiss, 1991; Jacoby et al., 1996). Here we provide evidence that [Gly^{B24},Trp^{B25}]-insulin, although having lost the B24 phenylalanyl side chain, appears to significantly maintain the insulin B-chain fold. This is indicated by the inability of the substituted tryptophan to rotate freely, resulting in a marked difference in $I_{\parallel}$ and $I_{\perp}$ for this analog (Figure 3B). We conclude that this restriction of fluorophore mobility is due to the surrounding protein environment and that the tryptophan is not fully solvent exposed, as the model of [Gly^{B24}]insulin would suggest (Figure 1D). Previous studies have demonstrated that Tyr^{B26} of the B-chain C-terminus is only one-tenth as susceptible to radioiodination in [Gly^{B24}]insulin as in the C-terminal octapeptide in solution (Mirmira et al., 1991), indicating that the C-terminal region in [Gly^{B24}]insulin is not freely exposed to solvent as it is in the free octapeptide. Accordingly, we find that the tryptophan in [Gly^{B24},Trp^{B25}]insulin is more solvent exposed than in [Trp^{B25}]insulin, but its emission spectrum is blue shifted relative to that of the corresponding C-terminal octapeptide (GGWYTPKT). We also find that the anisotropy of the tryptophan-substituted octapeptide is greatly reduced when compared to [Gly^{B24},Trp^{B25}]insulin. These results indicate that the tryptophan in [Gly^{B24},Trp^{B25}]-insulin is not fully solvent exposed. All our data suggest that [Gly^{B24},Trp^{B25}]insulin retains the B-chain fold to a degree comparable to that of [Trp^{B25}]insulin, which has previously been demonstrated to form dimeric and hexameric complexes with native insulin (Pittman & Tager, 1995), a correlate of the maintenance of the B-chain fold (Inouye et al., 1981; Mirmira & Tager, 1989).

Figure 3C demonstrates that 6.5 M guanidine causes an increase in fluorescence intensity in [Gly^{B24},Trp^{B25}]insulin and an approximately 8-fold increase in the intensity of NAWEE and the octapeptide studied (data not shown). [Trp^{B25}]insulin did not display an increase in intensity but was found, however, to display a 12 nm red shift (data not shown) along with a reduced anisotropy in the presence of 6.5 M guanidine (see Table 1). This suggests that 6.5 M guanidine is capable of denaturing some previously existing structure in the [Gly^{B24},Trp^{B25}]insulin and [Trp^{B25}]insulin analogs, supporting the existence of some defined structure in the B-chain C-terminus of both analogs. Interestingly, we find that the anisotropy of [Gly^{B24},Trp^{B25}]insulin in 20% acetic acid ($r = 0.111$) is comparable to the anisotropy of this analog in 6.5 M guanidine ($r = 0.089$) (Table 1), indicating that 20% acetic acid has denaturing effects on the analog similar to those of 6.5 M guanidine. This is not the case for [Trp^{B25}]insulin, which essentially maintains comparable anisotropies in the presence ($r = 0.161$) and absence ($r = 0.178$) of acetic acid (see Table 1). If acetic acid were to have a similar denaturing effect on [Gly^{B24}]insulin, it could help randomize the C-terminal β-strand structure at acidic pH, e.g., as illustrated in Figure 1D. Hua et al. (1996) have recently demonstrated that the chemical shifts of a fully active monomeric insulin analog ([Asp^{B10},Lys^{B28},Pro^{B29}]insulin: DKP) display large differences at neutral and acidic pH by 2D NMR analysis, suggesting that the insulin monomer is structurally more defined at neutral pH. Indeed, our data suggest that [Trp^{B25}]insulin and [Gly^{B24},Trp^{B25}]insulin maintain a similarly folded structure at pH 7.5 which, in [Gly^{B24},-Trp^{B25}]insulin, significantly distorts in 20% AcOH.

We have also investigated the ability of [B25-α-carboxamide(NH$_2$)]DPI analogs to retain the insulin B-chain fold in an attempt to elucidate the cause of the markedly reduced receptor binding of these truncated analogs when a Phe^{B24} → Gly mutation (≈140% → <1% binding) is introduced (Nakagawa & Tager, 1993). This is significantly different from the slight reduction in receptor binding caused by the same mutation in native insulin (100%) vs [Gly^{B24}]-insulin (≈75%) (Mirmira & Tager, 1989). Figure 4A illustrates that the emission spectrum of [Gly^{B24},Trp^{B25}-NH$_2$]-DPI is blue shifted relative to [Trp^{B25}-NH$_2$]DPI. This indicates that the tryptophan in [Gly^{B24},Trp^{B25}-NH$_2$]DPI is in a more hydrophobic, solvent-shielded, environment than in the truncated non-Gly^{B24} analog. This indication is supported by quenching data (Figure 5B and Table 3) which demonstrated that the tryptophan in [Trp^{B25}-NH$_2$]DPI is more solvent accessible to acrylamide quenching than in [Gly^{B24},-Trp^{B25}-NH$_2$]DPI. These results indicate that the deletion of residues B26−B30 causes the tryptophan in [Gly^{B24},Trp^{B25}-NH$_2$]DPI to fold inward toward the hydrophobic core of the molecule, forming a more compact structure than [Trp^{B25}-NH$_2$]DPI. We find that this folding effect is quite opposite to that caused by a Phe^{B24} → Gly substitution in the full-length analog [Gly^{B24},Trp^{B25}]insulin, which is partially unfolded relative to [Trp^{B25}]insulin. This is consistent with previous studies that have indicated that the side chain of Phe^{B25} interacts with receptor [cf. Tager (1990) and Kurose et al. (1994)]. The tighter folding of this side chain into the hydrophobic core in the [Gly^{B24},Trp^{B25}-NH$_2$]DPI analog may prevent this presumably important binding interaction. Several investigators have previously proposed that residues B26−B30 may serve to partially unfold the B-chain upon interacting with insulin receptor (Derewenda et al., 1991; Dodson et al., 1983; Markussen et al., 1985); accordingly, our studies support this hypothesis.

The studies presented in this paper imply that the low binding affinity of [Gly^{B24},Trp^{B25}-NH$_2$]DPI is due to the greater flexibility imparted by Gly^{B24} which allows the C-terminal B25 residue to fold inward, yielding a structure that is too tightly packed for appropriate insulin receptor recognition. In contrast, a Phe^{B24} → Gly mutation in the full-length B-chain of native insulin ([Gly^{B24}]insulin) results in additional flexibility at the B-chain C-terminus which may allow partial unfolding of the native β-turn of insulin. On the basis of our results we propose that the insulin receptor may possess the ability to correctly orient the partially

unfolded β-strand of [Gly^{B24}]insulin, allowing near-native receptor binding to occur. This would be energetically favorable and consistent with our findings that [Gly^{B24}]insulin mutants can assume, and seem to partially maintain, the insulin fold at physiologic pH. Shoelson and co-workers have also found by fluorescence energy transfer between a donor/acceptor pair placed at positions A1 and B29 that, upon binding insulin receptor, the intramolecular distance between A1 and B29 does not change as dramatically as is seen in the 2D NMR structure of [Gly^{B24}]insulin (unpublished data). It is also of interest that the A1−B29-linked mini-proinsulin (Figure 1B), an analog that is completely incapable of unfolding the B-chain β-turn, has a greatly reduced binding affinity (<0.1%). We, therefore, can conclude that there is a critical distance from the central B-chain α-helix that the B-chain β-strand must attain during high-affinity ligand−receptor interactions. Attaining this critical distance appears to rely on a greater separation of these B-chain segments than that defined in the classical 2-Zn insulin structure but without loss of the overall insulin fold.

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