The solution structure of a superpotent B-chain-shortened single-replacement insulin analogue

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Abstract

This paper reports on an insulin analogue with 12.5-fold receptor affinity, the highest increase observed for a single replacement, and on its solution structure, determined by NMR spectroscopy.

The analogue is [D-AlaB26]des-(B27–B30)-tetrapeptide-insulin-B26-amide. C-terminal truncation of the B-chain by four (or five) residues is known not to affect the functional properties of insulin, provided the new carboxylate charge is neutralized. As opposed to the dramatic increase in receptor affinity caused by the substitution of D-Ala for the wild-type residue TyrB26 in the truncated molecule, this very substitution reduces it to only 18% of that of the wild-type hormone when the B-chain is present in full length.

The insulin molecule in solution is visualized as an ensemble of conformers interrelated by a dynamic equilibrium. The question is whether the “active” conformation of the hormone, sought after in innumerable structure/function studies, is or is not included in the accessible conformational space, so that it could be adopted also in the absence of the receptor. If there were any chance for the active conformation, or at least a predisposed state to be populated to a detectable extent, this chance should be best in the case of a superpotent analogue. This was the motivation for the determination of the three-dimensional structure of [D-AlaB26]des-(B27–B30)-tetrapeptide-insulin-B26-amide. However, neither the NMR data nor CD spectroscopic comparison of a number of related analogues provided a clue concerning structural features predisposing insulin to high receptor affinity. After the present study it seems more likely than before that insulin will adopt its active conformation only when exposed to the force field of the receptor surface.

Keywords: active conformation; CD spectroscopy; insulin; NMR spectroscopy; receptor binding; structure; superpotency

Insulin delivers its signal by inducing a conformational change in the receptor, which is transduced through the membrane to activate the intracellular tyrosine kinase (Lee & Pilch, 1994). While the structure of the kinase domain has been solved (Hubbard et al., 1994), the only structural information available extracellularly is on the isolated hormone since, strangely, the complex of insulin with its receptor has resisted efforts at crystallization and structural analysis. Recently, however, the crystal structure of the first three

domains of the homologous type-1 insulin-like growth factor (IGF-1) receptor has been solved (Garrett et al., 1998). Unfortunately, this fragment does not bind IGF-1, so that its structure cannot provide detailed information on the interaction.

The complex formation is understood to proceed as an induced fit, which implies that neither partner will have the same conformation in the complex as on its own. For insulin there are many indications of its structural flexibility, which mainly, though not exclusively, concerns either end of the B-chain. Therefore the free hormone in solution is visualized as an ensemble of conformers related by a dynamic equilibrium. The question is whether the “active” conformation, i.e., the conformation it adopts in complex with the receptor, is included in the conformational space accessible in the absence of the latter.

Native insulin by far is not the strongest ligand for its receptor. Analogues have been described with binding affinities up to more than 10 times that of the wild-type (for a selection see Table 1). In most cases the substitutions concern the aromatic residues PheB25 and TyrB26. For the stronger complex to form, the B-chain has to be C-terminally shortened and the new carboxylate charge neutralized, for instance by amidation. The substitutions lending higher

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Abbreviations: BMRB, BioMagResBank; CD, circular dichroism; CSI, chemical shift index; DDD, distance-bound driven dynamics; DOI, Des-(B23–B30)-octapeptide-insulin; DPI, Des-(B26–B30)-pentapeptide-insulin; DQF-COSY, double-quantum-filtered scalar-correlated spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; PDB, Protein Data Bank; RMSD, root-mean-square deviation; RP-HPLC, reverse-phase high-performance liquid chromatography; TOCSY, total correlation spectroscopy; TPPI, time-proportional phase incrementation scheme.

Table 1. Receptor binding affinity and biopotency of selected superpotent insulin analogues^a

Analogue	Receptor-binding affinity (%)	Biopotency (%)	Reference
[AspB10]insulin	534 ± 146	435 ± 144 (L)	Schwartz et al. (1987)
[D-AlaB24]insulin	150 ± 9	n.d.	Mirmira and Tager (1989)
[D-PheB24]insulin	140 ± 9	n.d.	Mirmira and Tager (1989)
[D-TyrB24,PheB26]insulin	163 ± 10	n.d.	Mirmira et al. (1991)
[D-AlaB26]insulin	18.0	n.d.	Siedentop (1997)
[D-ProB8]DDI-NH ₂	143 ± 25	n.d.	Leyer et al. (1995)
DTI-NH ₂	110 ± 3	138 ± 10 (L)	Lenz (1990)
[D-TyrB26-NH ₂]DTI	203 ± 16	315 ± 44 (L)	Lenz (1990)
[AlaB26-NH ₂]DTI	273 ± 36	n.d.	Leyer et al. (1995)
[SerB26-NH ₂]DTI	306 ± 11	225 ± 9 (L)	Sievert (1991)
			Sievert et al. (1993)
[GluB26-NH ₂]DTI	449 ± 8	204 ± 28 (L)	Sievert (1991)
[SarB26-NH ₂]DTI	1,100 ± 240	n.d.	Leyer et al. (1995)
[D-AlaB26-NH ₂]DTI	1,252 ± 173	967 ± 334 (GU)	This paper
[TyrB25-NH ₂]DPI	270	330 (GO)	Nakagawa and Tager (1986)
[TyrB25-NH ₂]DPI	n.d.	228 ± 36 (L)	Casaretto et al. (1987)
[AsnB25-NH ₂]DPI	227	292 ± 62 (L)	Spoden (1988)
			Spoden et al. (1995)
[HisB25-NH ₂]DPI	300	313 ± 52 (L)	Casaretto et al. (1987)
			Spoden (1988)
[AspB10, TyrB25-NH ₂]DPI	1,170 ± 30	1,350 ± 110 (L)	Schwartz et al. (1989)

^aGO, glucose oxidation in adipocytes; GU, glucose uptake in adipocytes; L, lipogenesis in adipocytes; n.d., not determined; Sar, N-methyl glycine.

receptor affinity to the shortened analogues will impair binding to a level much below that of the wild-type, if the B-chain is present in full length, with very few exceptions (see Table 1). This means that formation of the strong complex causes problems with the accommodation of the C-terminal B-chain, which is believed to detach from the body of the insulin molecule (Mirmira & Tager, 1989; Derewenda et al., 1991; Hua et al., 1991, 1992, 1993; Murray-Rust et al., 1992; Nakagawa & Tager, 1992). In the wild-type complex, the distal chain, though not contributing to the free energy of binding, cannot just protrude into free solvent, it rather seems to “dwell” in a comfortable, but limited, space provided by the receptor topology (Kurapkat et al., 1997). Apparently, the high affinity fit of certain B25 or B26 side chains entails conformational restrictions for the distal chain that do not allow it to occupy the site it is normally meant to.

If there were any chance that in the equilibrium ensemble of conformers the active conformation, or at least a state predisposed to adopt it, is populated highly enough to be detectable, this chance should be best in the case of a superpotent analogue. Therefore we determined the NMR structure of [D-AlaB26]des-(B27–B30)-tetrapeptide-insulin-B26-amide ([D-AlaB26-NH₂]DTI) with a receptor-binding capacity of 1,250%, the highest value observed for a single replacement analogue.

Results

Bioassays

[D-AlaB26-NH₂]DTI exhibited a very high affinity for insulin receptors: 1,252 ± 173% ($n = 7$). The biological activity in vitro was

also high: 967 ± 334% ($n = 3$). Both values are referred to wild-type (wt) human insulin = 100%.

Sequential assignment

Most of the proton frequencies observed for [D-AlaB26-NH₂]DTI are closely similar to those of wt human insulin (Hua & Weiss, 1991), [GlyB24]insulin (Hua et al., 1991, 1992), [AspB9]insulin (Kristensen et al., 1991), and [HisB16]insulin (Ludvigsen et al., 1994), studied under almost identical conditions. Therefore sequence specific assignment was feasible on a comparative basis. In unclear cases frequencies were assigned as described by Wüthrich (1986).

To identify the frequencies of residue D-AlaB26, residue PheB24 served as a starting point (Fig. 1, cross peaks 1, 2, 3). Sequential NOE connectivities $d_{\alpha N}$ and $d_{\beta N}$ were found between the amide proton of PheB25, and the H α and H $\beta_{1,2}$ protons of PheB24 (Fig. 1, cross peaks 4, 6, 7), respectively. Subsequently sequential NOE connectivities between the amide proton of D-AlaB26, and the H α and H $\beta_{1,2}$ protons of PheB25, could be identified (Fig. 1, cross peaks 8, 9), and the AMX system of D-AlaB26 (Fig. 1, cross peaks 9, 11) was finally confirmed in the TOCSY spectrum.

Secondary structure analysis

The secondary structure elements known from the X-ray analysis of 2Zn insulin crystals (Baker et al., 1988) are two helices in the A-chain, A2–A8 and A12–A17, joined by an extended stretch, A9–A11, and a central helix, B9–B19, followed by a β -turn, B20–B23 in the B-chain. Both ends of the B-chain, B1–B8 and B24–

Fig. 1. Region of the two-dimensional NOESY spectrum (200 ms mixing time) showing the amide-aliphatic cross peaks. The amide-proton frequencies of residues B24–B26 are indicated by vertical lines. Cross peaks 1,2,3 and 5,6 and 9,11 are intraresidual NOEs of PheB24, PheB25, and D-AlaB26, respectively. The cross peaks 4,7 and 8,10 are sequential NOEs of PheB24/PheB25 and PheB25/D-AlaB26, respectively.

B30, are extended. Within narrow limits the same applies to the solution structure of native insulin and DPI (Hua & Weiss, 1990, 1991), with the extended C-terminal B-chain missing in the latter.

Helical secondary structure elements can be identified by NMR spectroscopy through the presence of characteristic $d_{\alpha N}(i+3)$ and $d_{\alpha\beta}(i+3)$ sequential connectivity patterns in the NOESY spectra. An analysis of these NOE patterns for the B-chain (Fig. 2) clearly indicates an α -helix at residues B9–B19. Moreover, the regularities of the nested $(i, i+3)$ connectivities associated with the strong d_{NN} NOEs and the amide exchange experiment suggest that this α -helix is well defined. Another possibility to identify secondary structure elements is given by the chemical shift index (CSI) of the C_{α} protons. A minimum of four consecutive negative CSI values is indicative of a helical arrangement that is continued until a positive value appears (Wishart et al., 1992). In the case of the B-helix, 10 consecutive negative values are found, from B9–B18 (Fig. 3).

On the same basis, a helical region can be identified in the C-terminal A-chain comprising residues A13–A19 (Fig. 4). Nevertheless, the nesting of the $(i, i+3)$ connectivities is not as complete as in the case of the B-helix, reflecting ambiguous hydrogen

bonding (Fig. 2). The sequential NOE patterns for the helix in the N-terminal A-chain are strongly fragmented (Fig. 2). This is at least partially due to overlapping frequencies of residues A4–A8 (Table 1), so that their assignment is equivocal. The CSI suggests a helix between residues A2 and A5 and helical characteristics, $d_{\alpha N}(i+3)$ NOEs, are found for GlyA1/GluA4, ValA3/CysA6, and GluA4/CysA7, while $d_{\alpha\beta}(i+3)$ connectivities are observable for GlyA1/GluA4 and GluA4/CysA7 (Fig. 2). Furthermore, $(i, i+4)$ NOE cross peaks are present between ValA3 and CysA7, as well as between GluA4 and ThrA8. This observation and the CSI taken together indicate a helix from residue A2–A8.

In the X-ray structures of porcine wt insulin (Baker et al., 1988) as well as of DPI (Bi et al., 1984; Dai et al., 1987), residues GlyB20–GlyB23 form a type I β -turn. In the NMR structures (Hua & Weiss, 1990, 1991), contacts characteristic of this turn have been identified between protons of the following atom pairs: NArgB22/ C_{α} GlyB20, C_{β} ArgB22/ C_{α} CysB19, NGluB21/NArgB22, NArgB22/NGlyB23, and C_{α} GluB21/NGlyB23. The turn is in contact with the C-terminal A-chain as indicated by long-range NOEs between protons of C_{β} ArgB22 and NAsnA21. All these contacts can be identified in the NOESY spectra of [D-AlaB26-NH₂]DTI suggesting a native-like β -turn conformation for its sequence GlyB20–GlyB23.

Residue D-AlaB26

The region of the two-dimensional NOESY spectrum (200 ms mixing time) containing the NOEs of residue D-AlaB26 is shown in Figure 4. The β -protons of the alanine side chain are in contact with the aromatic ring protons of PheB24 (Fig. 4, contacts 5, 6) and PheB25 (Fig. 4, contacts 2, 4, 7). Cross peaks 3 and 8 indicated in Figure 4 are due to contacts between the $H_{\gamma 11}$ -proton of IleA2 (the frequency of which is very similar to that of the β -protons of D-AlaB26), and the aromatic ring protons of TyrA19. No contacts are visible between the side chain of D-AlaB26 and residues of the protein core. Other sequential NOE connectivities are shown in Figure 1.

Structure calculations

Following the procedure described in Materials and methods, 16 structures were generated, restrained by 446 distances (144 intra-residual, 135 sequential, 82 medium-range, and 85 long-range NOEs). A C_{α} representation of the ensemble and the averaged structure are shown in Figure 5. The RMSD after fitting to the C_{α} atoms, and to all atoms of the residues IleA2–AsnA21 and PheB1–CysB19, are 1.45 and 2.64 Å, respectively. The RMSDs of the C_{α} atoms of both [D-AlaB26-NH₂]DTI and wt insulin (Hua & Weiss, 1991) are plotted over the amino acid sequence in Figure 6. The profiles of these plots are remarkably similar, the values in both cases indicating a rather well-defined structure, except at the chain termini.

Tertiary structure

Several NOEs define the relative arrangement of the secondary structure elements within each polypeptide chain, and between the two of them. The orientation of the N- and C-terminal helices in the A-chain is determined by long-range NOEs between the side chains of IleA2 and TyrA19, and by the A6–A11 cystine bridge.

Fig. 2. Schematic representation of the observed backbone NOEs, classified into strong, medium, and weak, as indicated by the height of the bars. Slowly exchanging amide protons are indicated by filled circles. Deduced elements of secondary structure are indicated by boxes in the bottom line.

The relative positions of the A- and the B-chain are mainly determined by NOEs between protons of the following pairs of side chains: AsnB3/IleA10, HisB5/IleA10, LeuB11/ValA3, LeuB11/CysA6, LeuB15/TyrA19, and ValB18/GluA17. In general these

contacts are very similar to those observed in the solution structure of human wt insulin (Hua & Weiss, 1990, 1991).

CD spectroscopy

The CD spectrum of insulin not only reflects the spatial arrangement of chromophores in the secondary and tertiary structure, it also responds to self-association to dimers and hexamers as promoted by increasing concentration and coordination of zinc ions (Wood et al., 1975). To see to which extent the capability of self-association might be preserved in [D-AlaB26-NH₂]DTI, the analogue's CD spectrum was recorded as a function of protein and zinc concentration in 0.25 mM Tris buffer at pH 7.8. The spectrum is virtually insensitive to changes in concentration, whereas zinc ions induce a minute but significant effect in both the near and the far UV (Fig. 7). Essentially the same CD spectra were observed for [L-AlaB26-NH₂]DTI with only the zinc effect being more pronounced (see Fig. 7). The corresponding CD spectra of "wild-type" DTI-NH₂ are depicted in Figure 8. They are clearly concentration dependent and exhibit a still stronger effect of zinc ions, both however much reduced in comparison with full-length wt insulin (Wood et al., 1975). Figure 8 also includes the CD spectrum of [D-TyrB26-NH₂]DTI, which is independent of concentration, that for the zinc form not being accessible because of partial precipitation. The inverted tyrosyl configuration leads to a slight increase and blue shift of the two shortest wavelength bands in the far UV, and the appearance of a positive band around 280 nm. A hint of the latter is common to the near UV spectra of all DTI-NH₂

Fig. 3. The ¹H_α chemical shift index (Wishart et al., 1992) calculated for [D-AlaB26-NH₂]DTI. Three helical regions are apparent at residues A2–A5, A13–A19, and B9–B18.

Fig. 4. Section of the two-dimensional NOESY spectrum (200 ms mixing time) showing contacts between protons of aromatic and aliphatic side chains, which are important for the establishment of the tertiary structure of [D-AlaB26-NH₂]DTI.

analogues depicted here as well as to those of [GlyB26-NH₂]DTI and [SerB26-NH₂]DTI (data not shown).

Discussion

Superactivity to a substantial extent was first described for [AspB10]insulin, a single-replacement analogue with full chain

length (Schwartz et al., 1987). Independently, superpotency was also obtained by substitutions of PheB25 or TyrB26 (see Table 1). The HisB10 → Asp mutation was not anticipated to increase receptor affinity; it was discovered as the reason for a case of familial hyperinsulinemia (Chan et al., 1988). Correspondingly, there is no obvious structural explanation for its effect. The substitutions at positions B25 and B26, on the other hand, were part of system-

Fig. 5. **A:** Superposition of 16 [D-AlaB26-NH₂]DTI structures generated by distance geometry calculations onto their C_α atoms. The backbones are shown in spaghetti representation. The helices seen are at residues A2–A8, A13–A19, and B9–B19, respectively. **B:** Schematic representation of the average backbone structure of [D-AlaB26-NH₂]DTI calculated from the ensemble of the 16 superposed structures shown in Figure 5A. The A-chain is in green; the B-chain is in orange. The side chains of the disulfide bridges (A6/A11, A7/B7, and A20/B19) are shown in yellow.

Fig. 6. RMS distances between corresponding C_{α} atoms in the ensemble of 16 NMR structures of [D-AlaB26-NH₂]DTI (upper half) and of structures of wild-type insulin (lower half; Hua & Weiss, 1991) after optimum fit.

atic exploration of the classical receptor binding region of insulin as defined by Pullen et al. (1976).

Combination of the two modifications in [AspB10, TyrB25-NH₂]DPI resulted in a binding affinity of 1,170% (Schwartz et al.,

1989). This value is matched, unless exceeded, by a des-(B27–B30)-tetrapeptide-insulin-B26-amide with only D-Ala substituted for TyrB26; a residue even of the distal pentapeptide of the B-chain, which can be dispensed with for full activity. Almost any superpotency brought about by a replacement in the C-terminal B-chain depends on the absence of at least residues B29 and B30, and on neutralization of the new terminal carboxylate charge. Formation of the strong complex is incompatible with the distal C-terminal B-chain finding the space left free by the receptor topology, for its nonbinding accommodation in the case of wt insulin. A key role for the interaction with the receptor is attributed to the B25 side chain (Nakagawa & Tager, 1986). The question is whether substitutions at positions B26–B28 are active in the enhancement of receptor affinity (beyond that achievable by replacement of PheB25), by contributing to the binding energy, or permissive only in allowing optimization of the fit by avoiding bad contacts for the distal C-terminal B-chain.

Therefore the behavior of residue D-AlaB26-amide is the focus of interest. Modeling this side chain into the X-ray structure shows that it is directed toward the molecule's interior. Without causing steric problems, this orientation should involve the B26 methyl group in NOEs, which were not observed. Hence, it seems to be highly mobile and consequently adjustable to the receptor surface. In its model-built position, for instance, it would not interfere with dimerization. For NMR spectroscopy, self-association was suppressed by using 20% acetic acid pH 1.9 as solvent. Self-association, however, if it occurs, is normally reflected in the concentration and zinc dependence of the CD spectrum at pH 7.8. The effect mainly

Fig. 7. CD spectra of [D,L-AlaB26-NH₂]DTI (L-Ala; Leyer et al., 1995) in the (A) near and (B) far UV: —, D-Ala, $c = 0.99$ g/L, metal-free; ---, D-Ala, $c = 0.97$ g/L, 0.33 eq. Zn²⁺/monomer; - · -, L-Ala, $c = 1.31$ g/L, metal-free; · · ·, L-Ala, $c = 1.13$ g/L, 0.33 eq. Zn²⁺/monomer.

Fig. 8. CD spectra of [D,L-TyrB26-NH₂]DTI in the (A) near and (B) far UV. For A: —, D-Tyr, 2 g/L > *c* > 0.02 g/L, metal-free; ---, L-Tyr, *c* = 0.22 g/L, metal-free; — · —, L-Tyr, *c* = 2.31 g/L, metal-free; · · · ·, L-Tyr, *c* = 1.99 g/L, 0.33 eq. Zn²⁺/monomer. For B: —, D-Tyr, *c* = 0.90 g/L, metal-free; ---, L-Tyr, *c* = 0.90 g/L, metal-free; — · —, L-Tyr, *c* = 0.90 g/L, 0.33 eq. Zn²⁺/monomer.

originates in the reciprocal interaction of TyrB16 of one monomer with TyrB26 of the other in the dimer.

In the case of [D-AlaB26-NH₂]DTI lacking one of the interaction chromophores, the absence of concentration effects, and a much reduced effect of zinc, must not automatically be interpreted as an indication of impaired self-association. Mobility of the C-terminus, however, may impede its engagement in the fourth hydrogen bond of the intermonomer antiparallel β -sheet between its carbonyl oxygen and the amino proton of PheB24 in the dimer. Dimer formation is enhanced when the equilibrium is shifted further to hexamers upon addition of zinc ions. "Wild-type" DTI amide, however, clearly exhibits a weak concentration dependence showing that dimerization not only involves the hydrogen bonding carbonyl of residue B26, but also its side chain. The effect of zinc, though more pronounced than with [D-AlaB26-NH₂]DTI, produces a near UV spectrum, which is only 60% as intense as that of wt insulin with the maximum at 270 rather than 276 nm. This means that the TyrB26 side chain does not adopt the same position as in the presence of the distal chain, which seems to be largely determined by ProB28, although it could.

Very recently, Ludvigsen et al. (1998) reported the solution structure of [GluB16,GlyB24]des-B30-insulin, the second one they obtained for quasi-physiological conditions, i.e., in water at near-neutral pH. Normally 20% acetic acid is used as co-solvent to overcome association problems. In the new structure, the B-chain termini are more defined, which is also the case with [D-AlaB26-NH₂]DTI in 20% acetic acid (see Fig. 5). The detachment of the C-terminal B-chain, believed to be important for receptor interaction, is restricted to the tail B27–B29, i.e., the residues that the

present analogue is lacking. The structural rearrangements seen in [GluB16,GlyB24]des-B30-insulin are suggested to also occur in native insulin, as they allow to rationalize the receptor binding properties of a series of analogues. However, they are at variance with those of many others, and especially with the key role attributed to the side chain of B25, by the model of Tager (Nakagawa & Tager, 1986). If, as a consequence of the flexibility introduced by GlyB24, PheB25 fits into a pocket normally occupied by PheB24, why should this also occur with PheB24 unsubstituted? Indeed, PheB25 was found to be exposed in the NMR structure of [GluB1,B10,B16,B27]des-B30-insulin, the first one solved at neutral pH (Olsen et al., 1996). Furthermore, how could TyrB25, HisB25, or AsnB25, if turned inward despite their more polar nature, confer superpotency (see Table 1)? The potency of [GluB16,GlyB24]des-B30-insulin is reduced to 15%, though this is attributed to TyrB16 $\rightarrow$ Glu. The side chain of PheB25 was also observed to be buried (even in 20% acetic acid), in the case of depsi-insulin, which is only 3% potent (Kurapkat et al., 1997). Further indication of the direct involvement of B25 stems from quantitative cross-linking, radiolabeling, and activation of the insulin receptor by photoactivatable benzoylphenylalanine incorporated into that position (Shoelson et al., 1993). Seventy percent cross-linking was also observed with photoreactive [p-azidoPheB25-NH₂]DPI (Kurose et al., 1994).

Conclusion

If there were any chance for the "active" conformation, or at least a predisposed state to be populated in solution to a detectable

extent, it should be best with a superpotent analogue. However, in contrast to depsi-insulin ([B24-B25CO-O]insulin) where the solution structure has provided striking evidence why it is so poorly active (Kurapkat et al., 1997), that of [D-AlaB26-NH₂]DTI does not reveal a significant difference from wt insulin that could be considered a feature of the active conformation. All deviations observed are within the limits of thermal mobility or experimental ambiguity. After the present study it seems more likely than before that insulin will adopt its active conformation only when exposed to the force field of the receptor surface.

Materials and methods

Amino acids and reagents were purchased from Merck AG (Darmstadt, Germany). Trypsin and porcine insulin were generous gifts from Dr. R. Obermeier (Hoechst Marion Roussel Deutschland GmbH, Frankfurt, Germany). All chemicals were of analytical grade.

Gel filtrations were performed on Sephadex G-25 or G-50 fine (Pharmacia, Uppsala, Sweden) and reversed-phase chromatography on Eurosil Bioselect 100–20 C18 (Besta, Wilhelmsfeld, Germany).

For gradient RP-HPLC analysis, the liquid chromatograph LC 22 was used (Bruker–Franzen Analytik, Bremen, Germany). A 0.25 M H₃PO₄/Et₃N buffer (pH 2.25) and a linear acetonitrile gradient were used for peptide and protein elution on a nucleosil 100–5 C18 RP-column. To check the purity of the insulin analogue, a gradient of 20–50% acetonitrile over 30 min was applied.

For amino acid analysis (Spackman et al., 1958) on models LC 3000 and LC 5000 (Biotronic, München, Germany), peptides and proteins were hydrolyzed in 6 M HCl in the presence of phenol for 24 h at 110 °C.

Preparation of HOAc·Gly-Phe-Phe-D-Ala-NH₂

The tetrapeptide HOAc·Gly-Phe-Phe-D-Ala-NH₂ was synthesized in solution. Z(Benzyloxycarbonyl)-Gly-Phe-Phe-OH was made exclusively by the N-hydroxysuccinimide ester method starting from the C-terminus, which was protected as a sodium salt in every step of the synthesis (Anderson et al., 1964). Coupling of this tripeptide with D-Ala-NH₂ by the DCC(dicyclohexylcarbodiimide)/HOBT method in DMF (König & Geiger, 1970), removal of the dicyclohexylurea by filtration, precipitation of the highly hydrophobic tetrapeptide with water, filtration of the product, and recrystallization from a large volume of ethanol, yielded the Z-protected peptide. Catalytic hydrogenation produced the amino component for enzymatic synthesis (Wünsch, 1974). Purity was checked by RP-HPLC and amino acid analysis. RP-HPLC purity: 93%. Amino acid analysis: Gly 0.97 (1), Phe 2.00 (2), and Ala 1.01 (1).

Preparation of des-(B23–B30)-octapeptide-insulin

Des-(B23–B30)-octapeptide-insulin (DOI) was prepared by TosPhe (N-tosyl-l-phenylalanyl)CH₂Cl-trypsin digestion of insulin in 0.05 M Tris and 1 mM CaCl₂. A solution of 250 mg (43 μmol) zinc-free porcine insulin in 20 mL of 0.05 M Tris, adjusted to pH 9.5 by addition of solid Tris, was incubated with TosPheCH₂Cl-trypsin at a molar enzyme/substrate ratio of 1/200. After 15 h at room temperature, the reaction mixture was chromatographed on Sephadex G-25f in 10% acetic acid and lyophilized. RP-HPLC

purity: 91%. Amino acid analysis: Asp3.00 (3), Thr0.92 (1), Ser2.44 (3), Glu7.00 (7), Gly3.01 (3), Ala1.01 (1), Val3.69 (4), (Cys)₂ 2.59 (3), Ile1.56 (2), Leu6.00 (6), Tyr2.92 (3), Phe1.01 (1), His2.00 (2), and Arg1.01 (1).

Semisynthesis of [D-AlaB26-NH₂]DTI

The synthesis of the C-terminally shortened insulin analogue was performed by the following general procedure (Moody et al., 1974; De Meyts et al., 1976; Inouye et al., 1979; Sievert, 1991). The amino component (750 μmol) was dissolved in 2 mL DMF. After addition of 100 μL 0.05 M Ca(CH₃COO)₂, 121 mg (25 μmol), DOI was dissolved by shaking, and the mixture was slowly diluted with 2 mL 1,4-butanediol. The resulting solution was incubated with a solution of 20 mg (0.87 μmol) TosPheCH₂Cl-trypsin in 400 μL 0.05 M Ca(CH₃COO)₂. Then the apparent pH value was adjusted to 6.7 by addition of N-methylmorpholine or acetic acid. After a reaction time of 5 h, the enzymatic synthesis was stopped by addition of 4 mL of 30% acetic acid.

Separation of excess amino component and trypsin was achieved by gel filtrations on Sephadex G-25 and G-50 fine in 10% acetic acid. The C-terminally protected shortened insulin was separated from DOI and side products by reversed-phase chromatography. Gradient elution was performed with 500 mL 0.1% trifluoroacetic acid/25% 2-propanol as starting solution, and 500 mL 0.1% trifluoroacetic acid/40% 2-propanol in a linear mode.

The fractionated eluates were collected, concentrated under reduced pressure, and lyophilized. RP-HPLC purity: 97%. Amino acid analysis: Asp3.04 (3), Thr1.00 (1), Ser2.84 (3), Glu7.00 (7), Gly4.00 (3), Ala1.99 (2), Val3.32 (4), (Cys)₂ 2.26 (3), Ile1.34 (2), Leu6.00 (6), Tyr2.98 (3), Phe3.01 (1), His1.98 (2), and Arg1.02 (1).

Preparation of other insulin analogues

Concerning the preparation of further insulin analogues included in this study, refer to the literature cited in Table 1 and Figures 7 and 8.

Bioassays

Receptor binding capacity was determined by the assay of De Meyts, measuring partial displacement of [Tyr(¹²⁵I)A14]insulin from the receptors of cultured human IM-9 lymphocytes by the analogue relative to wt insulin (De Meyts, 1976).

The biopotency of [D-AlaB26-NH₂]DTI was determined by measuring glucose uptake in cultured 3T3-L1 adipocytes. The procedure was described by Frost and Lane (1985).

NMR spectroscopy

Lyophilized [D-AlaB26-NH₂]DTI was dissolved in 70%/20%/10% H₂O/AcOD/D₂O (v/v/v). The concentration was 4 mM. Sets of two-dimensional homonuclear ¹H-NMR TOCSY, DQF-COSY, and NOESY spectra were recorded at 500 MHz and 25 °C on a Varian Unity500 spectrometer. The mixing times for the six NOESY spectra were 50, 100, 150, 200, 250, and 300 ms, respectively.

The spectral width was 6,000 Hz in both dimensions, and the water signal was suppressed by a weak radiofrequency pulse during the relaxation delay. For each spectrum 512 *t*₁ increments were acquired, each with 2,048 complex points using the time-proportional phase incrementation scheme (TPPI). Prior to Fourier transforma-

tion, a 60° shifted sine bell window function was applied in both dimensions and the spectra were zero filled in ω_1 so that $1,024 \times 1,024$ data points were obtained. Finally the spectra were base line corrected with a polynomial function.

All data processing was performed on a SGI Indigo workstation using the program NMRPipe (Delaglio et al., 1995). All subsequent procedures, as spectral assignment, cross-peak integration, and distance determination, were performed by using the NMR-view program (Johnson & Blevins, 1994). Chemical shifts were measured in parts per million as observed relative to the residual CH₃ resonance of acetic acid, presumed to be 2.03 ppm.

For deuterium exchange measurements, the lyophilized protein was dissolved in 80% D₂O/20% CD₃COOD (100 v/v) solution. A series of one-dimensional spectra was recorded with a spectral width of 6,000 Hz. The first spectrum was recorded 15 min, the last one 20 h after dissolving the protein.

The chemical shifts are deposited at the BMRB (accession number 4266), and the coordinates are deposited at the PDB under accession code 1b2v.

Structure calculations

Four hundred-forty-six NOEs were derived from the two-dimensional NOESY spectra of [D-AlaB26-NH₂]DTI and converted into their corresponding interatomic distances. The distance restraints were calculated from NOE intensities measured in a series of NOESY experiments with increasing mixing times. The distances were grouped into classes ($d < 2.8$ Å; 2.8 Å $< d < 3.4$ Å; 3.4 Å $< d < 4.3$ Å; $d > 4.3$ Å) and after pseudo-atom correction were used as upper limits for the distance restraints. The lower limits were always assumed to be the sum of the van der Waals radii of the two atoms involved. These upper and lower restraint limits were collected in a distance matrix, and then 16 three-dimensional coordinate frames were calculated using Crippen's embedded algorithm (Crippen, 1979). An error function, consisting of a distance and a chirality part (Havel et al., 1983), was minimized in three phases. In the first phase, a 300-step conjugate gradient optimization was performed twice. The next phase was a distance-bound driven dynamics (DDD) calculation (Kapteina et al., 1988; Scheek et al., 1989). The first 1,000 time steps of the DDD calculation were performed at an elevated temperature (1,000 K), followed by 1,000-step annealing, during which the kinetic energy was slowly taken away by temperature coupling to an external bath of 1 K (Berendsen et al., 1984). The final phase again was a conjugate gradient optimization, this time of 200 steps.

Circular dichroism spectroscopy

CD measurements were carried out on an AVIV (Lakewood, New Jersey) 62DS spectrometer and a Jasco (Japan Spectroscopic Co., Ltd., Tokyo, Japan) J-600 spectropolarimeter, both calibrated according to Chen and Yang (1977). The spectral bandwidth was 2 nm ($\lambda < 250$ nm) and 1 nm ($\lambda > 250$ nm), respectively, and the temperature 27 °C.

The solvent was 25 mM Tris/HCl buffer throughout. Protein concentrations were determined photometrically (Pye Unicam spectrophotometer PU 8800) using the method of Waxman et al. (1993). The absorption coefficients $A(276 \text{ nm})^{1\text{cm};1\text{g/l}}$ used for the DTI-amides, with D- or L-Tyr, and D- or L-Ala in position B26, were 1.13 and 0.876, respectively.

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