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# Inactive conformation of an insulin despite its wild-type sequence*

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**Abstract**

The peptide group between residues B24 and B25 of insulin was replaced by an ester bond. This modification only in the backbone was meant to eliminate a structurally important H-bond between the amide proton of B25 and the carbonyl oxygen of A19, and consequently to enhance detachment of the C-terminal B-chain from the body of the molecule, exposing the underlying A-chain. According to a model derived from the effects of side-chain substitutions, main-chain shortening, and crosslinking, this conformational change is prerequisite for receptor binding.

Contrary to the expectation that increased flexibility would increase receptor binding and activity, depsi-insulin ([B24–B25 CO-O]insulin) has turned out to be only 3–4% potent. In search of an explanation for this observation, the solution structure of depsi-insulin was determined by two-dimensional $^1$H-NMR spectroscopy. It was found that the loss of the B25–A19 H-bond does not entail detachment of the C-terminal B-chain. On the contrary, it is overcompensated by a gain in hydrophobic interaction achieved by insertion of the Phe B25 side chain into the molecule's core. This is possible because of increased rotational freedom in the backbone owing to the ester bond. Distortion of the B20–B23 turn and an altered direction of the distal B-chain are consequences that also affect self-association. The exceptional position of the B25 side chain is thus the key feature of the depsi-insulin structure. Being buried in the interior, it is not available for guiding the interaction with the receptor, a crucial role attributed to it by the model. This seems to be the main reason why the structure of depsi-insulin represents an inactive conformation.

**Keywords:** conformation; insulin; NMR-spectroscopy; structure

Among the numerous insulin mutants and derivatives investigated in structure/function studies during the last 25 years, the mini proinsulins crosslinked between the amino groups of Gly A1 and Lys B29 by bifunctional reagents have been particularly revealing (Lindsay, 1971; Brandenburg, 1972, 1973a, 1973b; Wollmer et al., 1974; Dodson et al., 1980; Cutfield et al., 1981; Srinivasa & Carpenter, 1983; Nakagawa & Tager, 1989; Wollmer et al., 1989; Brems et al., 1991). They fold spontaneously in high yields, with the correct disulfide bonds being formed (Brandenburg & Wollmer, 1973; Wollmer et al., 1974; Markussen et al., 1985). Bridges of widely different lengths are accommodated easily, and the native three-dimensional structure of the insulin moiety is perfectly preserved (Cutfield et al., 1981). The biopotency as a function of their length, however, passes through a maximum of only 13% at 15 atoms (between Cα A1 and Cα B29) in the bridge (Freychet et al., 1974; Gliemann & Gammeltoft, 1974; Jones et al., 1976; Kobayashi et al., 1989; Nakagawa & Tager, 1989; Krüger et al., 1994). The decrease observed with bridges longer than that, to about 2% in the extreme case of proinsulin, is probably due to their steric interference with the docking process. The diaminosuberoyl bridge (14 atoms), which ideally matches the distance between the groups connected, leads to a submaximal activity of only 7%. In the case of an extremely short bridge, as in single-chain des-(B30)insulin, where the A1 and B29 backbone termini are linked by a peptide bond (Markussen et al., 1985), the structure still is unperturbed (Derewenda et al., 1991), but the receptor binding capacity is almost totally lost (<0.1%) (Markussen et al., 1985). With bridges of adequate or shorter length (≤14 atoms), the accessibility of the surface area known to be essential for receptor binding is also largely maintained (Cutfield et al., 1981). In these cases, the only plausible explanation remaining is that the bridge interferes with the dynamics of the molecule and impairs the functional properties by restraining the conformational flexibility prerequisite for induced fit (Markussen et al., 1985; Wollmer et al., 1989; Krüger et al., 1994). From the observation that shortening of the C-terminal B-chain by five residues (Gattner, 1975) is compatible with full activity provided the charge of the new C-terminus is neutralized as in des-(B26–B30)insulin-B25-amide (DPI-amide) (Cosmatos et al., 1979; Fischer et al., 1985), it was concluded that,

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prior to or upon receptor binding, the C-terminal B-chain is detached from the body of the molecule (Mirmira & Tager, 1989; Derewenda et al., 1991; Hua et al., 1991, 1992, 1993; Murray-Rust et al., 1992; Nakagawa & Tager, 1992), whereby Ile A2 and Val A3 become more accessible (Hua et al., 1991, 1992). It is obvious that a movement of this kind is restricted or even prevented by a short A1–B29 link.

In the unlinked native molecule, a prominent hydrogen bond exists between the amide group of Phe B25 and the carbonyl oxygen of Tyr A19 (Baker et al., 1988) that helps to keep the C-terminal B-chain attached. Therefore, we believed that abolition of that hydrogen bond would facilitate detachment and, consequently, increase the affinity for the receptor. For that purpose, the B24–B25 peptide bond was replaced by an ester bond (Fig. 1; Wollmer et al., 1994), which cannot act as a donor in hydrogen bond formation (Wollmer et al., 1994). To our knowledge, this was the first replacement of a main-chain atom reported for the hormone. Chemically, the difference between the resulting depsi-insulin and native insulin could not be more minute: the side chains are untouched and the length of the ester bond is the same as that of the peptide bond within narrow limits. Only the rotational energy barrier for the former is substantially lower.

Contrary to expectation, however, the receptor binding capacity and biopotency of depsi-insulin, rather than being increased, were found to be decreased to 1.7 and 3.5%, respectively (Wollmer et al., 1994). The same applies to depsi-DPI-amide (Wollmer et al., 1994). In search of an explanation for this behavior, the solution structure of depsi-insulin was determined by NMR spectroscopy, which is described in this communication.

## Results

### *Sequential assignment*

Most of the proton frequencies observed for depsi-insulin (Table 1) and depsi-DPI-amide are virtually identical and closely similar to those of human wild-type (wt) insulin (Hua & Weiss, 1991), [Gly B24]insulin (Hua et al., 1991, 1992), [Asp B9]insulin (Kristensen et al., 1991), and [His B16]insulin (Ludvigsen et al., 1994) studied under nearly the same conditions. Therefore, sequence-specific assignment was feasible on a comparative basis. In unclear cases, frequencies were assigned as described by Wüthrich (1986).

Residue Phe B25, which lacks the amide proton, was identified as follows: First the TOCSY (total correlation spectroscopy) frequencies of Pro B28 were searched for, because there is only one proline residue in insulin. These frequencies served as a starting point for identifying the amide proton resonance of residue $i - 1$ (Thr B27) in the NOESY spectra. The same procedure was applied to identify Tyr B26. Once the amide proton resonance of Tyr B26 was known, the $H_\alpha$ frequency of Phe B25 was identified easily through its downfield shift due to the deshielding effect of the neighboring oxygen atom of the ester group. This finding was confirmed subsequently in the TOCSY spectrum, where only one cross peak to the $H_{\beta 1,2}$ atoms of Phe B25 is present. Frequency assignment to residues B26–B30 of depsi-insulin was also possible by comparison with depsi-DPI-amide.

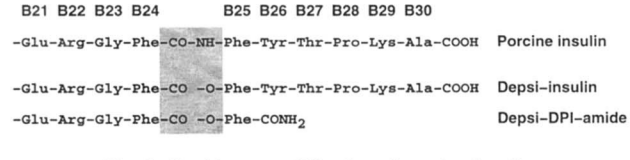


**Fig. 1.** Backbone modification of porcine insulin.

### *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 A13–A20, joined by an extended stretch, A9–A12, and a central helix, B9–B19, followed by a $\beta$-turn, B20–B23 in the B-chain, which is extended at each end, B1–B8 and B24–B30.

By NMR, $\alpha$-helices can be identified through the observation of characteristic $d_{\alpha N}(i + 3)$ and $d_{\alpha\beta}(i + 3)$ sequential connectivities in the NOESY spectra. The analysis of the sequential NOE patterns for the B-chain (Fig. 2) clearly shows the existence of a helical region between residues B9–B19. Moreover, the regularities of nested $(i, i + 3)$ connectivities associated with the strong $d_{NN}$ NOEs (see Fig. 3) and the amide exchange experiment suggest that this $\alpha$-helix is well defined. Another possibility to identify secondary structure elements is given by the chemical shift indices (CSIs) of the $C_\alpha$ protons. A minimum of four consecutive negative CSI values is indicative of a helical arrangement that can continue until a positive value appears (Wishart et al., 1992). In the case of the B-helix, 10 consecutive negative values are found, from B9 to B18 (Fig. 4).

On the same basis, a helix 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, suggesting 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) and, therefore, their assignment is not unambiguous. The CSI suggests a helix between residues A2 and A5 and helical characteristics, $i, i + 3$ NOEs, are found between Val A3 and Cys A6 (Fig. 2). Furthermore, $i, i + 4$ NOE cross peaks are present between Val A3 and Cys A7, as well as Glu A4 and Thr A8, indicating a helix between residues A2 and 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 Gly B20–Gly 23 form a type I $\beta$-turn. In the NMR structures (Hua & Weiss, 1991 and Hua & Weiss, 1990, respectively), contacts characteristic of this turn have been identified between protons on the following atoms: NArg B22/$C_\alpha$Gly B20; $C_\beta$Arg B22/$C_\alpha$Cys B19; NGlu B21/NArg B22; NArg B22/NGly B23; $C_\alpha$Glu B21/NGly B23. The turn is in contact with the C-terminal A-chain as indicated by long-range NOEs between protons of $C_\beta$Arg B22 and NAsn A21. Of this list of contacts, only NArg B22/$C_\alpha$Gly B20 and those between the amide protons of Glu B21, Arg B22, and Gly B23 are also present in depsi-insulin and depsi-DPI-amide. Hence, the criteria of a type-I $\beta$-turn (Wüthrich, 1986) are no longer met. The conformation is determined by sequential amide proton NOEs and consecutive $(i, i + 1)$ $C_\alpha$/N proton NOEs from Cys B19 to Gly B23.

### *Structure calculations*

Because of the highly similar NOESY spectra of depsi-insulin and depsi-DPI-amide, structure calculations were restricted to the former.

**Table 1.** *$^1H$ chemical shifts of depsi-insulin at 25 °C (20%/10%/70% v/v/v $CD_3COOD/D_2O/H_2O$)*[a]

| Residue | NH | $C^{\alpha}H$ | $C^{\beta}H$ | Others | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| A1 Gly | | 3.83, 3.97 | | | | | | | |
| A2 Ile | 8.49 | 3.97 | 1.33 | $C^{\gamma}H_2$ | 1.22, 1.15 | $C^{\gamma}H_3$ | 0.74 | $C^{\delta}H_3$ | 0.60 |
| A3 Val | 8.09 | 3.63 | 1.90 | $C^{\gamma}H_3$ | 0.90, 0.85 | | | | |
| A4 Glu | 8.08 | 4.18 | 2.08 | $C^{\gamma}H_2$ | 2.48 | | | | |
| A5 Gln | 8.24 | 4.05 | 2.07 | $C^{\gamma}H_2$ | 2.38, 2.47 | | | | |
| A6 Cys | 8.26 | 4.84 | 2.84, 3.35 | | | | | | |
| A7 Cys | 8.24 | 4.82 | 3,30, 3.76 | | | | | | |
| A8 Thr | 8.24 | 4.03 | 4.37 | $C^{\gamma}H_3$ | 1.22 | | | | |
| A9 Ser | 7.40 | 4.76 | 3.87, 4.01 | | | | | | |
| A10 Ile | 7.80 | 4.33 | 1.53 | $C^{\gamma}H_2$ | 1.04, 0.39 | $C^{\gamma}H_3$ | 0.62 | $C^{\delta}H_3$ | 0.48 |
| A11 Cys | 9.71 | 4.86 | 3.10, 2.85 | | | | | | |
| A12 Ser | 8.75 | 4.57 | 4.28, 3.99 | | | | | | |
| A13 Leu | 8.60 | 3.82 | 1.31 | $C^{\gamma}H$ | 1.39 | $C^{\delta}H_3$ | 0.77, 0.71 | | |
| A14 Tyr | 7.46 | 4.13 | 2.88, 2.96 | C2,6H | 7.021 | C3,5H 6.81 | | | |
| A15 Gln | 7.53 | 3.95 | 2.01, 2.38 | $C^{\gamma}H_3$ | 2.37 | | | | |
| A16 Leu | 8.11 | 4.16 | 1.72, 1.89 | $C^{\gamma}H$ | 1.72 | $C^{\delta}H_3$ | 0.83, 0.74 | | |
| A17 Gln | 8.06 | 4.15 | 2.01, 2.08 | $C^{\gamma}H_2$ | 2.31, 2.55 | | | | |
| A18 Asn | 7.39 | 4.45 | 2.51, 2.58 | $N^{\delta}H_2$ | 7.13, 6.49 | | | | |
| A19 Tyr | 7.89 | 4.47 | 3.33, 3.02 | C2,6H | 7.32 | C3,5H 6.72 | | | |
| A20 Cys | 7.43 | 4.84 | 3.25, 2.83 | | | | | | |
| A21 Asn | 8.24 | 4.68 | 2.80, 2.86 | $N^{\delta}H_2$ | 7.42 | | | | |
| B1 Phe | | 4.23 | 3.13 | C2,6H | 7.19 | C3,5H 7.31 | C4H | 7.26 | |
| B2 Val | 8.11 | 4.10 | 1.89 | $C^{\delta}H_3$ | 0.83 | | | | |
| B3 Asn | 8.47 | 4.71 | 2.69 | $N^{\delta}H_2$ | 7.5, 6.89 | | | | |
| B4 Gln | 8.41 | 4.48 | 2.08, 1.89 | $C^{\delta}H_2$ | 2.13, 2.24 | | | | |
| B5 His | 8.63 | 4.42 | 3.52, 3.23 | C2H | 8.63 | C4H | 7.36 | | |
| B6 Leu | 8.98 | 4.48 | 1.77, 0.87 | $C^{\gamma}H$ | 1.58 | $C^{\delta}H_3$ | 0.87, 0.72 | | |
| B7 Cys | 8.33 | 4.77 | 3.19, 2.93 | | | | | | |
| B8 Gly | 9.27 | 3.98, 3.83 | | | | | | | |
| B9 Ser | 9.09 | 4.10 | 3.88 | | | | | | |
| B10 His | 7.99 | 4.51 | 3.57, 3.30 | C2H | 8.54 | C4H | 7.35 | | |
| B11 Leu | 7.06 | 4.00 | 1.89, 1.23 | $C^{\gamma}H$ | 1.33 | $C^{\delta}H_3$ | 0.93 | | |
| B12 Val | 7.14 | 3.33 | 2.04 | $C^{\gamma}H_3$ | 0.95, 0.79 | | | | |
| B13 Glu | 7.92 | 4.09 | 2.15 | $C^{\gamma}H_2$ | 2.53 | | | | |
| B14 Ala | 7.72 | 4.08 | 1.47 | | | | | | |
| B15 Leu | 8.02 | 3.85 | 1.47, 1.28 | $C^{\gamma}H$ | 1.47 | $C^{\delta}H_3$ | 0.66, 0.48 | | |
| B16 Tyr | 8.12 | 4.24 | 3.13 | C2,6H | 7.11 | C3,5H 6.73 | | | |
| B17 Leu | 7.77 | 4.08 | 1.91, 1.64 | $C^{\gamma}H$ | 1.82 | $C^{\delta}H_3$ | 0.91 | | |
| B18 Val | 8.52 | 3.84 | 2.07 | $C^{\gamma}H_3$ | 1.00, 0.86 | | | | |
| B19 Cys | 8.72 | 4.77 | 3.22, 2.93 | | | | | | |
| B20 Gly | 7.64 | 3.92 | | | | | | | |
| B21 Glu | 8.42 | 4.10 | 2.14, 2.06 | $C^{\gamma}H_2$ | 2.50 | | | | |
| B22 Arg | 7.96 | 4.19 | 1.94 | $C^{\gamma}H_2$ | 1.73 | $C^{\delta}H2$ | 3.22 | $N^{\epsilon}H$ | 7.07 |
| B23 Gly | 7.60 | 3.97, 3.85 | | | | | | | |
| B24 Phe | 8.04 | 4.82 | 3.17, 3.1 | C2,6H | 6.81 | C3,5H 7.02 | | | |
| B25 Phe | | 5.31 | 3.15 | C2,6H | 7.16 | C3,5H 7.20 | C4H 7.14 | | |
| B26 Tyr | 7.52 | 4.57 | 2.80, 2.74 | C2,6H | 6.85 | C3,5H 6.70 | | | |
| B27 Thr | 7.81 | 4.47 | 4.01 | $C^{\gamma}H_3$ | 1.14 | | | | |
| B28 Pro | | 4.31 | 2.25, 1.89 | $C^{\gamma}H$ | 1.35 | $C^{\delta}H_2$ | 3.59, 3.55 | | |
| B29 Lys | 8.12 | 4.23 | 1.79, 1.72 | $C^{\gamma}H_2$ | 1.42 | $C^{\delta}H_2$ | 1.65 | $C^{\epsilon}H_2$ | 2.96 |
| B30 Ala | 7.79 | 4.34 | 1.14 | | | | | | |

[a]The chemical shifts are given relative to the residual $CH_3$ resonance of acetic acid, presumed to be 2.03 ppm.

Following the procedure described in Materials and methods, 16 structures were generated restraining 347 distances (176 intraresidual, 78 sequential, 45 medium-range, and 48 long-range NOEs). The RMS deviations (RMSDs) after fitting to the $C_{\alpha}$ atoms and to all atoms are 1.8 Å and 2.9 Å, respectively. A $C_{\alpha}$ atom representation of the ensemble and the averaged structure are shown in Figure 5. The RMSDs of the $C_{\alpha}$ atoms of depsi and wt insulin are plotted along the amino acid sequence in Figure 6. This plot indicates a rather well-defined structure in both cases, except for the distal C-terminal B-chain.

*Tertiary structure*

Several NOEs define the relative arrangement of the secondary structure elements within a single and between the two polypeptide chains. These contacts are in general very similar to those observed

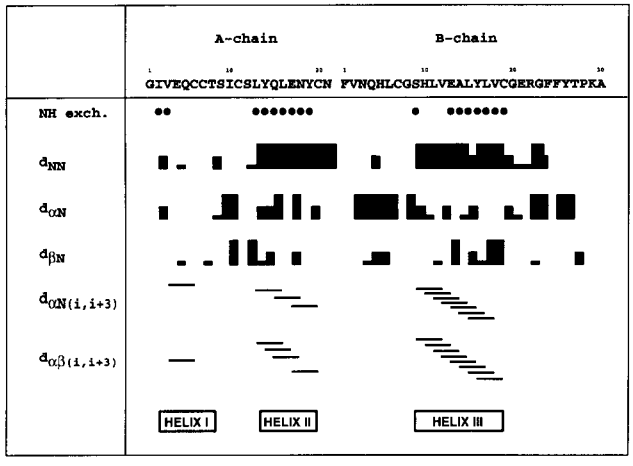


**Fig. 2.** Schematic representation of the observed backbone NOEs, classified into strong, medium, and weak as indicated by the height of bars. Slowly exchanging amide protons are indicated by filled circles. Deduced elements of secondary structure are indicated by boxes in the bottom line.

in the solution structure of human wt insulin (Hua & Weiss, 1990, 1991), except for the C-terminal region of the B-chain, which will be described below.

The orientation of the N- and C-terminal helices in the A-chain is determined by long-range NOEs between the side chains of Ile A2 and Tyr A19 and the A6–A11 cystine bridge. The structural relation of the A- and the B-chain is determined mainly by NOEs between protons of the following pairs of side chains: Asn B3/Ile A10, His B5/Ile A10, Leu B11/Val A3, Leu B11/Cys A6, Leu B15/Tyr A19, and Val B18/Glu A17.

### *Conformation of the aromatic residues Phe B24 and Phe B25*

The aromatic region of a 2D-NOESY spectrum of depsi-insulin is depicted in Figure 7. The ring of Phe B24 experiences contacts with Leu B15, Cys B19, and Gly B20. In human wt insulin, those with Gly B20 are lacking, but further ones with Val B12 and Tyr B16 are observed instead (Hua & Weiss, 1991).

For Phe B25, no NOEs beyond the sequential ones are observed in human wt insulin (Hua & Weiss, 1991), which indicates that the side chain is solvent exposed and highly mobile. The Phe B25 ring of depsi-insulin, however, is in close proximity to the $\delta$-methyl group of Ile A2, a $\delta$-methyl group of Val B12, and a $\gamma$-methyl group of Leu B15. This means that it is inserted into a hydrophobic pocket made up of the aforementioned side chains. The same applies to depsi-DPI-amide. A 0.08 ppm frequency shift for the $C_\alpha$ proton and the loss of one of the NOEs between a ring proton and a $\gamma$-methyl proton of Leu B15 indicates a slightly different orien-

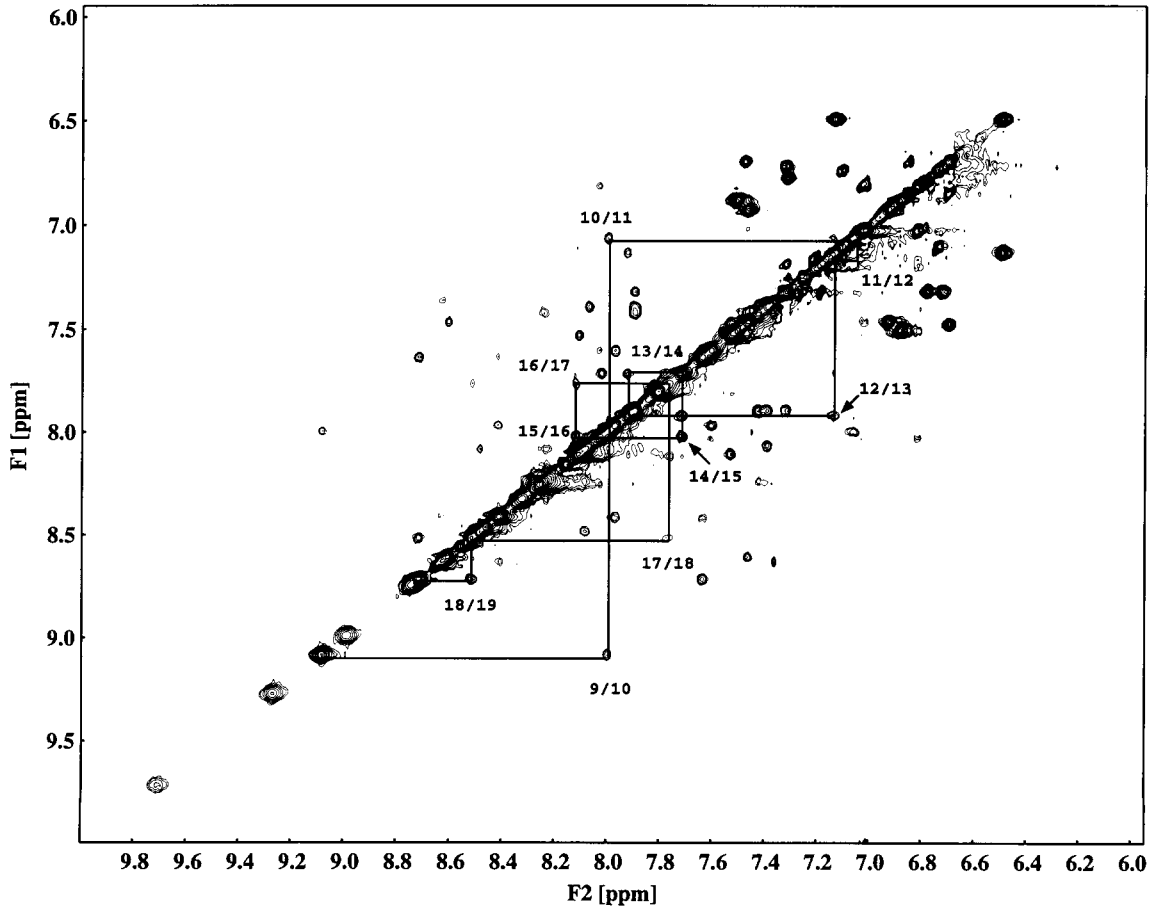


**Fig. 3.** Region of the 2D NOESY spectrum (200 ms mixing time) showing the amide–amide cross peaks with the connectivity lines for the B-helix included.

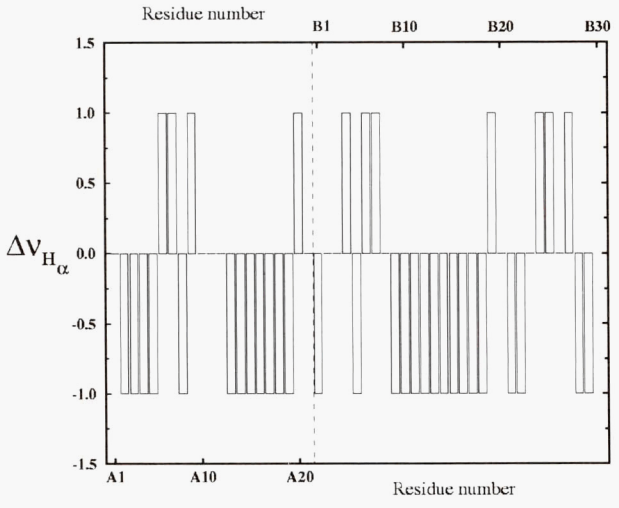


**Fig. 4.** $^1H_\alpha$ CSI (Wishart et al., 1992) calculated for depsi-insulin. Three helical regions at residues A2–A5, A13–A19, and B9–B18 are apparent.

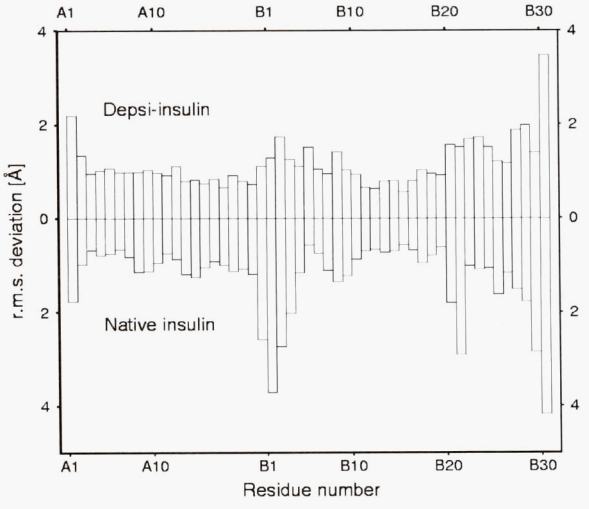


**Fig. 6.** RMS distances between corresponding $C_\alpha$ atoms in the ensemble of 16 NMR structures of depsi-insulin (upper half) and of structures of wt insulin (lower half) (Hua & Weiss, 1990, 1991) after optimum fit.

tation. They are accompanied by 0.03 and 0.10 ppm shifts for the amide proton frequencies of Phe B24 and Gly B23, respectively.

## Discussion

Strangely, the complex of insulin with its receptor has resisted efforts at crystallization and, hence, structural analysis. While efforts to identify sites on the receptor that interact with the hormone have been started, the description of the receptor-binding region of insulin has not yet come to an end. The dynamics of the induced fit, however, are largely unknown. For the receptor interaction of the classical binding region (Pullen et al., 1976), more specifically the C-terminal B-chain, dynamic aspects have been addressed in a model suggested by Tager and coworkers (Nakagawa & Tager, 1986) in 1986 and refined during several years following (Nakagawa & Tager, 1987, 1989, 1992; Mirmira & Tager, 1989; Mirmira et al., 1991).

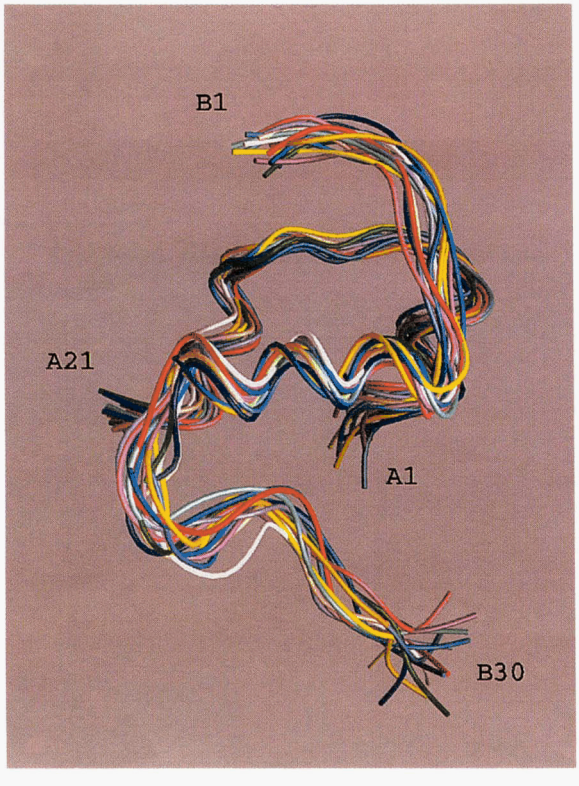


**Fig. 5.** Superposition of 16 depsi-insulin structures generated by distance geometry calculations onto their $C_\alpha$ atoms. The backbones are shown in spaghetti representation. Helices seen are at residues A2–A8, A13–A19, and B9–B19, respectively.

### *Accommodation of the distal C-terminal B-chain*

The side chains of residues B24, B25, and B26 are assumed to contact the receptor in ways that lead to local conformational

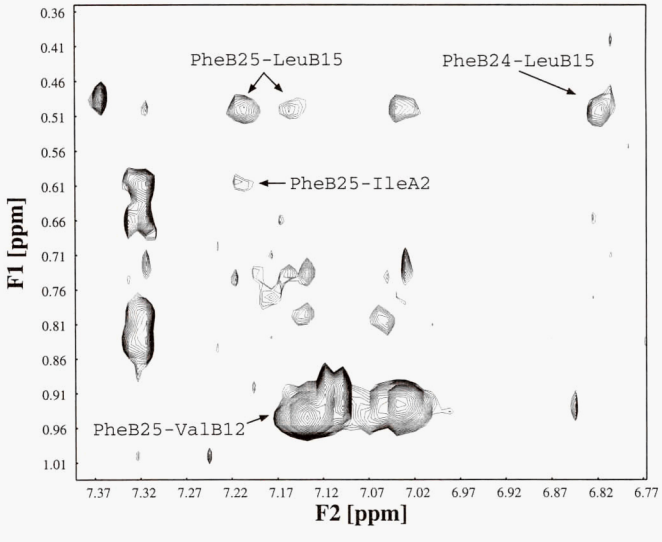


**Fig. 7.** Section of the 2D NOESY spectrum (200 ms) showing contacts between protons of aromatic and aliphatic side chains that are important for the establishment of the tertiary structure of depsi-insulin.

adjustments necessary for high-affinity interactions. The wild-type aromatic triplet Phe-Phe-Tyr, however, is not the optimum sequence in this respect. Each of these residues can be replaced in a way that the receptor binding affinity of wt insulin is exceeded by factors between 1.5 and 11: Phe B24 → D-Ala, 1.5-fold (Mirmira & Tager, 1989); Phe B25 → His, 3-fold (Casaretto et al., 1987); Tyr B26 → sarcosine, 11-fold (Leyer et al., 1995). The higher affinities of the B25 and B26 mutants, however, are observed only if the mutated residues are in the C-terminal position with their carboxylate function neutralized, for instance by amidation. If the B-chain is continued to its full length, the receptor affinity is reduced to much below normal by substitutions at residues 24–26, with few exceptions. This means that the state of the highest affinity possible causes problems with the accommodation of the distal C-terminal B-chain. Although not contributing to the free energy of binding, it cannot just protrude into free solvent, but seems to "dwell" in a comfortable, but limited, space provided by the receptor topology. Apparently, the high-affinity fit achieved by certain B25 or B26 side chains entails conformational restrictions for the distal chain that do not allow it to occupy the site that it occupies in the complex with the wild type.

### *Hydrogen bonding*

The model of Nakagawa and Tager (1986), however, underlines the importance not only of the B24–B26 side chains, but also of the hydrogen bond potential of the main chain between B23 and B26. Whereas the carbonyl and amide hydrogen groups of both B24 and B26 form the hydrogen bond system that stabilizes the intermonomer antiparallel $\beta$-sheet of the dimer, those of B25 are not involved. The B23 carbonyl and B25 amide hydrogen groups establish intramolecular hydrogen bonds with B21 and A19, respectively (Baker et al., 1988). The carbonyls of B24 and B25 have also been considered as candidates for hydrogen bonds with the receptor, because their reduction to $\Psi$ ($CH_2$-NH) decreases the relative binding affinity to 0.1 and 0.4%, respectively (Nakagawa et al., 1993). Whereas in depsi-insulin the B24 carbonyl is present, a hydrogen bond with the receptor has been discussed in this case for the amide proton of B25 (Wollmer et al., 1994).

### *Conformational mobility*

Introduction of the ester bond (or of the methylene-imino bond; Nakagawa et al., 1993), however, not only abolishes hydrogen bond potential, as intended, but also causes an increase in rotational freedom. Before the structure was solved, we speculated that the inactivity of depsi-insulin might be due to excessive mobility of the C-terminal B-chain as an undesired side-effect. Excessive mobility might interfere with accommodation of the distal chain to the space at the receptor surface considered above. This possibility, however, seems to be ruled out by the finding that the binding affinity and biopotency of depsi-DPI-amide are as low (1.7 and 3.5%, respectively) as those of full-length depsi-insulin.

Very high conformational mobility, for instance, results from replacement of Phe B24 by Gly (Hua et al., 1991, 1992). $^{13}C$-isotope-edited 2D-NMR studies have shown that even the whole B-chain C-terminal of the central helix, i.e., residues B20–B30, is partially unfolded (Hua et al., 1991, 1992). Nevertheless, it can be immobilized and adjusted to form the crucial contacts in the receptor complex, because [Gly B24]insulin exhibits near-native bioactivity (78%) (Mirmira & Tager, 1989). In the case of depsi-insulin, however, even though all wt side chains are present, the normative influence of the receptor's recognition surface is insufficient to induce the active conformation.

The resistance to adopt the native conformation is also reflected in the self-association of depsi-insulin. Quaternary structure formation of wt insulin with increasing concentration and zinc ions, respectively, is accompanied by characteristic changes of the CD spectrum in the near and far UV (Wood et al., 1975). Concentration- and zinc-dependent CD spectral changes are also observed with depsi-insulin, but they are different and much less pronounced (Wollmer et al., 1994). Corresponding effects are not encountered with depsi-DPI-amide (Wollmer et al., 1994), nor are they expected, because they are also lacking in the case of DPI and DPI-amide (Fischer et al., 1985), which tend to remain monomeric. Taking all considerations together, there must exist reasons other than excessive mobility in the present case, which are deducible from the NMR structure.

### *Inactive conformation*

In Figure 8, the NMR structure of depsi-insulin is superposed onto that of human wt insulin (Hua & Weiss, 1991) and onto the X-ray structures of wt insulin molecules 1 and 2 (Baker et al., 1988). The key feature of the depsi-insulin structure is the location of the Phe B25 side chain.

The increased rotational freedom owing to the ester bond, which was speculated to make the C-terminal B-chain excessively mobile, instead allows the phenylalanine ring to be buried in the molecule's core. The loss of the B25–B19 hydrogen bond is thus overcompensated by an increase in hydrophobic interaction. This key feature seems also to be the reason for the further deviations of depsi-insulin from the wt structures: Tyr B26 is more solvent

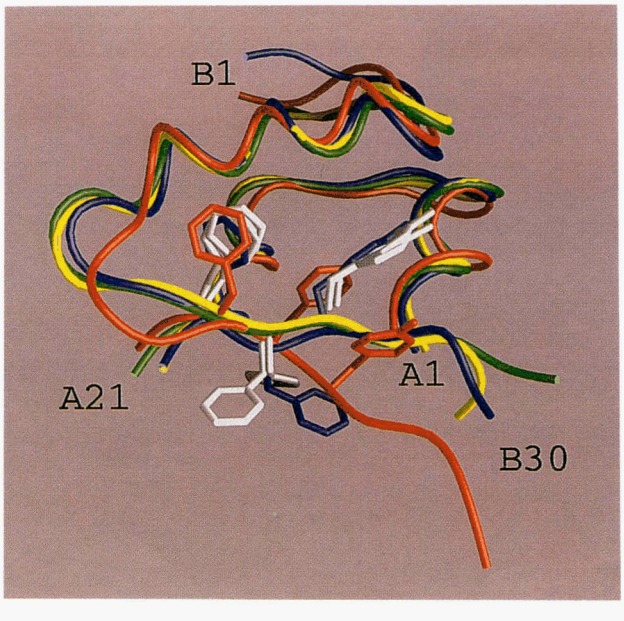


**Fig. 8.** Superposition of the NMR backbone structures of porcine depsi-insulin (red) and wt insulin (blue) (Hua & Weiss, 1990, 1991) and of the X-ray backbone structures of insulin molecule 1 (green) and 2 (yellow) (Baker et al., 1988). Side chains of the aromatic triplet Phe B24–Phe B25–Tyr B26 are included explicitly (left to right). Those of the X-ray structures are in white.

exposed, whereas the position of the Phe B24 ring is virtually unchanged. Important consequences are distortion of the B20–B23 turn and an altered direction of the distal C-terminal B-chain. As a matter of fact, the structure of depsi-insulin as it presents itself in Figures 5 and 8, for instance, is incompatible with normal dimerization: the distortion of the B20–B23 turn also affects the orientation of the B24 amide and carbonyl group and, hence, weakens their intermonomer hydrogen bonding. The direction of the distal B-chain leads to reciprocal bad contacts with the respective partner monomer, a steric hindrance that can be released as demonstrable by model building. The active conformation, however, cannot be restored. Two major requirements of Tager's model (Nakagawa & Tager, 1986), detachment of the C-terminal B-chain and guidance of receptor binding by the B25 side chain, are not met. Being buried in the molecule's core, Phe B25 is not available for that role and also causes the C-terminal B-chain not to be detached sufficiently. Direct involvement of Phe B25 in receptor binding has been demonstrated by Kurose et al. (1994), who obtained 70% crosslinking of photo-reactive [*p*-azido-Phe B25]DPI-amide to the $\alpha$-subunit. In accordance with this observation and with the model, the immersion of Phe B25 into the molecule's core allowed by the B24–B25 ester bond thus seems to be the main reason why the structure of depsi-insulin described here (and that of depsi-DPI-amide) is an inactive conformation.

## Materials and methods

### *NMR spectroscopy*

The preparation of depsi-insulin and depsi-DPI-amide was described previously (Wollmer et al., 1994). The lyophilized materials were dissolved in 70%/20%/10% $H_2O$/AcOD/$D_2O$ (v/v/v). The concentrations were 1.5 mM. Sets of two-dimensional homonuclear $^1$H-NMR TOCSY, DQF-COSY, and NOESY spectra were recorded at 500 MHz and 25 °C on a Varian Unity500 spectrometer. 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 rf 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. Prior to Fourier transformation, a 60° shifted sine bell window function was applied in both dimensions and the spectra were zero filled in $\omega_1$ so that 1,024 × 1,024 data points were obtained. Finally, the spectra were baseline corrected with a polynomial function.

All data processing was performed on a SGI Indigo using the programs SNARF and GIFA written by F.H.J. van Hoesel (Groningen, The Netherlands) and M.A. Delsuc (Delsuc, 1988), respectively. All subsequent procedures, such as spectral assignment, cross peak integration, and distance determination, were performed using the NMRVIEW program (Johnson & Blevins, 1994). Chemical shifts were measured in parts per million as observed relative to the residual $CH_3$ resonance of acetic acid, presumed to be 2.03 ppm.

### *Structure calculations*

Three-hundred forty-seven NOEs were derived from the 2D-NOESY spectra of depsi-insulin and converted into their corresponding interatomic distances. Distance restraints were calculated from NOE intensities measured in a series of NOESY experiments with increasing mixing times. The measured distances, increased by at least 0.1 Å, were taken as the 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 matrix, and then 16 3D-coordinate frames were calculated using Crippen's embed algorithm (Crippen & Havel, 1978). 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 calculation (DDD) (Kaptein et al., 1988; Scheek et al., 1989). The first 1,000 time steps of the DDD calculation were performed for an elevated temperature (1,000 K), followed by 1,000 step annealing, during which the kinetic energy was taken away slowly 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.

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