# Non-equivalent Role of Inter- and Intramolecular Hydrogen Bonds in the Insulin Dimer Interface<sup>\*S</sup>

Received for publication, May 26, 2011, and in revised form, August 3, 2011 Published, JBC Papers in Press, August 31, 2011, DOI 10.1074/jbc.M111.265249

Emília Antolíková<sup>‡</sup>, Lenka Žáková<sup>‡</sup>, Johan P. Turkenburg<sup>§</sup>, Christopher J. Watson<sup>§</sup>, Ivona Hančlová<sup>‡</sup>, Miloslav Šanda<sup>‡</sup>, Alan Cooper<sup>¶</sup>, Tomáš Kraus<sup>‡</sup>, A. Marek Brzozowski<sup>§1</sup>, and Jiří Jiráček<sup>‡2</sup>

From the <sup>‡</sup>Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, v.v.i., Flemingovo nám. 2, 166 10 Prague 6, Czech Republic, the <sup>§</sup>York Structural Biology Laboratory, Department of Chemistry, The University of York, Heslington, York YO10 5YW, United Kingdom, and the <sup>¶</sup>School of Chemistry, Glasgow University, College of Science and Engineering, Glasgow G12 8QQ, Scotland, United Kingdom

Apart from its role in insulin receptor (IR) activation, the C terminus of the B-chain of insulin is also responsible for the formation of insulin dimers. The dimerization of insulin plays an important role in the endogenous delivery of the hormone and in the administration of insulin to patients. Here, we investigated insulin analogues with selective N-methylations of peptide bond amides at positions B24, B25, or B26 to delineate their structural and functional contribution to the dimer interface. All N-methylated analogues showed impaired binding affinities to IR, which suggests a direct IR-interacting role for the respective amide hydrogens. The dimerization capabilities of analogues were investigated by isothermal microcalorimetry. Selective N-methylations of B24, B25, or B26 amides resulted in reduced dimerization abilities compared with native insulin  $(K_d = 8.8 \ \mu\text{M})$ . Interestingly, although the N-methylation in [NMeTyrB26]-insulin or [NMePheB24]-insulin resulted in  $K_d$ values of 142 and 587 µM, respectively, the [NMePheB25]-insulin did not form dimers even at high concentrations. This effect may be attributed to the loss of intramolecular hydrogen bonding between NHB25 and COA19, which connects the B-chain  $\beta$ -strand to the core of the molecule. The release of the B-chain  $\beta$ -strand from this hydrogen bond lock may result in its higher mobility, thereby shifting solution equilibrium toward the monomeric state of the hormone. The study was complemented by analyses of two novel analogue crystal structures. All examined analogues crystallized only in the most stable R<sub>6</sub> form of insulin oligomers (even if the dimer interface was totally disrupted), confirming the role of R<sub>6</sub>-specific intra/intermolecular interactions for hexamer stability.

Insulin is an important polypeptide hormone that controls a wide range of cellular processes such as the regulation of blood glucose uptake and has a large impact on protein and lipid metabolism. However, despite decades of intensive research, many questions about the structure of insulin and its mechanism of action remain. The solid state-based structural insight into the insulin molecule is limited to inactive dimeric or hexameric storage forms (1-3), whereas the insulin monomer represents the active form of the hormone when binding to the insulin receptor (IR).<sup>3</sup> It is also widely accepted that insulin undergoes a profound structural change during this process (4-6), a hypothesis supported by a plethora of highly dynamic hormone conformers identified by NMR studies (7-13). Attempts to determine the structure of the insulin-IR complex have been unsuccessful so far. However, the regions of the insulin molecule responsible for the interaction with the IR (3, 14) or for its dimerization and hexamerization (15, 16) have been functionally and structurally identified in a number of insulin analogues.

The insulin molecule consists of two peptide chains, a 21amino acid A-chain and a 30-amino acid B-chain, interconnected by two interchain and one intrachain disulfide bridges. The C terminus of the B-chain of insulin, particularly residues B24–B26, plays a substantial role in the initial contact with the receptor. It is believed that the C terminus of the B-chain of insulin must be detached away from the central B-chain  $\alpha$ -helix of insulin (2, 6). One of the main signatures of this so-called "active form" of insulin should be the exposure of the previously hidden amino acids Gly-A1, Ile-A2, and Val-A3, which are important for the interaction with IR (3). Recently, we described crystal structures of several shortened and full-length insulin analogues with modifications at the B26 position (17). The structural convergence of some of these highly active analogues (200 - 400%) enabled us to postulate that the active form of human insulin is characterized by a formation of a new type II  $\beta$ -turn at positions B24–B26.

Besides its role in IR activation and IR negative cooperativity (18, 19), the C terminus of the B-chain is also responsible for the formation and stabilization of the insulin dimers that are the building blocks of storage hexamers. In addition, the dimeriza-

<sup>\*</sup> This work was supported by Ministry of Education, Youth, and Sports of the Czech Republic Grant LC060777 (Research Centre for Chemical Genetics, to J. J.), Research Project of the Academy of Sciences of the Czech Republic Grant Z40550506 (to J. J.). This work was also supported by the Diamond Light Source (Didcot, United Kingdom) and European Synchrotron Radiation Facility (Grenoble, France).

The atomic coordinates and structure factors (codes 3ZQR and 3ZS2) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2 and Fig. S1.

<sup>&</sup>lt;sup>1</sup> To whom correspondence may be addressed. Tel.: 441904 328265; Fax: 441904-328366; E-mail: marek@ysbl.york.ac.uk.

<sup>&</sup>lt;sup>2</sup> To whom correspondence may be addressed: Tel.: 420220183441; Fax: 420220183571; E-mail: jiracek@uochb.cas.cz.

<sup>&</sup>lt;sup>3</sup> The abbreviations used are: IR, insulin receptor; DOI, des(B23–B30)octapeptide insulin; DTI; des(B27–B30)tetrapeptide insulin; Fmoc, fluorenylmethoxycarbonyl; ITC, isothermal titration microcalorimetry.

tion of insulin plays an important role in the endogenous delivery of the hormone from the pancreas to the circulatory system and in the administration of insulin to diabetic patients (3, 20-22). The structures of insulin dimers and hexamers have been determined from several crystal structures (e.g. Refs. 15, 23, 24). They revealed that the key interactions between insulin molecules I and II of the dimer are complementary and involve reciprocal hydrogen bonds between the carbonyl oxygen of Tyr-B26 (Phe-B24) of one molecule and the amide hydrogen of Phe-B24 (Tyr-B26) of the complementary second molecule of the dimer. Hence, antiparallel  $\beta$ -strands of the C termini of B-chains are connected by four hydrogen bonds. It has also been found that the amide hydrogen of Phe-B25 of molecule II forms an intramolecular hydrogen bond with the carbonyl of Tyr-A19. The side chains of Phe-B24, Tyr-B26, Tyr-B16, and Pro-B28 reinforce further the stabilization of the dimer by a network of Van der Waals contacts (15).

Our previous studies on the structural and functional relevance of the B26 (17, 25, 26) and B25 (27) positions of insulin focused on modulation of hormone conformation by the incorporation of an *N*-methyl group on the amide nitrogen of the peptide bond in shortened and full-length insulin analogues. Subsequently, we investigated the selective application of this *N*-methylation within the B24–B26 region of insulin by the systematic elimination of the inter/intramolecular hydrogen bonds, which are important for the stability and folding of the insulin monomer, dimer formation, and interaction with IR. Therefore, the approach reported here represents an application of a very specific, chemically controlled molecular tool for the functional assessment of the hydrogen bond network related to the dimer interface.

Here, we present the synthesis and characterization of several novel full-length insulin analogues with the selective N-methylation of the peptide bond at position B24, B25, or B26 to delineate the role of each of these particular peptide units in insulin dimer/hexamer assembly. In this process, we correlate the binding affinities of the analogues to IR (determined in rat adipose membranes) using isothermal titration microcalorimetry (ITC) dilution experiments, performed to assess the thermodynamic contributions toward hormone dissociation. The wild-type insulin (capable of dimer formation) and des(B23-B30)octapeptide insulin (DOI, not able to dimerize (28, 29)) were used as references for the dimeric and monomeric forms, respectively. This study is also complemented by the analysis of two novel crystal structures and two previously published analogue crystal structures that have not been hitherto discussed in the context presented here. This approach allowed us to shed light on the contributions of individual peptide bond amides to the dimerization and binding affinity of insulin.

#### MATERIALS AND METHODS

*Materials*—2-Chlorotrityl chloride resin, protected amino acids, and reagents for solid phase synthesis of peptides were purchased from Novabiochem Merck (Laufelfingen, Switzerland). Fmoc-Lys(phenylacetyl)-OH was prepared as described previously (30). Tosyl phenylalanyl chloromethyl ketonetreated trypsin was purchased from Sigma, and penicillin G acylase was from Fluka. Human [<sup>125</sup>I]monoiodotyrosylA14-insulin was purchased from PerkinElmer Life Sciences. Human and porcine insulins were purchased from Sigma. All other chemicals and solvents were obtained from Sigma-Aldrich.

*Peptide Synthesis and Enzymatic Semisynthesis*—The syntheses of peptides and the semisyntheses of analogues were performed according to Zakova *et al.* (30). The identity of peptides and analogues was confirmed with a Fourier transform mass spectrometer LTQ Orbitrap XL (Thermo Fisher).

*Receptor Binding Studies*—Receptor binding studies with plasma membranes prepared from epididymal adipose tissue of adult male Wistar rats were performed according to Zakova *et al.* (26).

*Isothermal Titration Microcalorimetry*—Human and porcine insulin (Sigma) were used without further purification. DOI was prepared in our laboratory from porcine insulin by tryptic cleavage (31). Purification was performed using reversephase HPLC, and identification was confirmed with electrospray ionization mass spectrometry. The analogues were prepared as described above.

Peptide solutions (~1 mM) were prepared in 0.1 M glycine/ HCl buffer, pH 2.5 (total volume of 1 ml). Peptide solutions were dialyzed in dialysis cassettes (Slide-A-Lyzer cut-off, molecular weight 3,500, Pierce) against 600 ml of the buffer at 4 °C. The buffer was changed three times (after 3, 20, and 26 h). Following dialysis, the concentrations of peptides were determined from UV absorbance at 280 nm (insulin,  $\epsilon = 5,840 \text{ M}^{-1} \text{ cm}^{-1}$ ; DOI,  $\epsilon = 4,560 \text{ M}^{-1} \text{ cm}^{-1}$ ). Concentrations were confirmed by amino acid analysis (± 10%). The final dialysis buffer and peptide solutions were briefly degassed prior to ITC dilution titration.

Calorimetric experiments were performed using a VP-ITC<sup>®</sup> apparatus (MicroCal, Inc.) operated in dilution/dissociation mode, with the reaction vessel (1.417 ml) held at 25 °C. The instrument was temperature-equilibrated prior to the start of injection. The sample contents were stirred at a speed of 307 rpm over the duration of the titration. A typical experiment comprised of 20 injections of 12.5  $\mu$ l (first injection of 2  $\mu$ l) of insulin solution into the reaction vessel, initially loaded with dialysis buffer. The duration of each injection was 25 s, with a time interval between injections of 300 s.

Integrated heat pulse data were corrected for small injection/ mixing effects from controls that were performed separately and analyzed under identical conditions by omitting the first injection and using MicroCal LLC Origin software (version 7). The software employed an updated and corrected (June 2008) version of the dissociation analysis procedure and was validated by comparison with earlier analysis methods (32, 33). For human insulin, the experimental values ( $\pm$  S.E.) were determined from eight parallel experiments. For porcine insulin, DOI, and insulin analogues, the experimental values ( $\pm$  range) were determined from two parallel experiments. The initial concentrations in the injection syringe ranged from 0.208 to 1.220 mM (supplemental Table S1).

*X-ray Studies*—Crystallization of the analogues ([*N*Me-PheB24]-insulin, [*N*MePheB25]-insulin, and [TyrB25,*N*Me-PheB26,LysB28,ProB29]-insulin) were performed with the inhouse insulin crystallization screens covering most of the reported crystal growth parameters of insulin. Crystallization



#### TABLE 1

Values of IC<sub>50</sub> and relative receptor binding affinities of human insulin and insulin analogues

Peptide	$IC_{50} \pm S.E.^{a}$	Potency <sup>b</sup>
	пм (п)	%
Human insulin	$0.89 \pm 0.06 (3)$	$100^{c}$
[NMePheB24]-insulin	$30.1 \pm 2.2 (3)$	2.96 <sup>c</sup>
[NMePheB25]-insulin	$515 \pm 32 (3)$	$0.17^{c}$
[ <i>N</i> MeTyrB26]-insulin	$4.30 \pm 0.46$ (3)	$20.7^{d}$
[PheB26]-insulin	$1.94 \pm 0.31$ (3)	$45.9^{c}$
[NMePheB26]-insulin	$21.3 \pm 2.7 (3)$	$4.17^{c}$
[NMePheB25,NMePheB26]-insulin	201 ± 39 (3)	$0.44^{c}$

<sup>a</sup> IC<sub>50</sub> values represent concentrations of insulin or the analogue that cause halfmaximal inhibition of binding of human [125]monoiodotyrosylA14-insulin to IR. Each value represents the mean  $\pm$  S.E. of multiple determinations (*n*).

 $^b$  Relative receptor binding affinity is defined as (IC  $_{50}$  of human insulin/IC  $_{50}$  of analogue)  $\times$  100. <sup>c</sup> This study

<sup>d</sup> From Ref. 17.

conditions, data collection, and refinement and model statistics are given in supplemental Table S2. X-ray data were processed by HKL2000 (34), and model building and refinement were performed by the CCP4 suite of programs (35) and COOT (36). Crystal structures were solved by Molrep (37) with B1-B6 and B23-B30-truncated insulin hexamer or monomer as a model (Protein Data Bank code 1MSO) (24) and refined by Refmac5.6 (38). All figures were made by CCP4mg (35). For structural comparisons of the dimer interfaces, the best defined (i.e. with the best electron density for typically very mobile Phe-B25 side chain and B28-B30 amino acids) dimers (referred here as chains AB and CD) from the relevant structures were superimposed on the B9–B19 and D9–D19 C $\alpha$  atoms by the LSQKAB program from the CCP4 suite. Both reported crystal structures contain one full hexamer in the asymmetric unit. As the nature of changes along the dimer interface observed here was virtually identical within each of the three dimers related by hexamer symmetry, only one dimer was selected for comparisons as a fully representative structural entity.

#### RESULTS

Binding Affinities-Insulin analogues were prepared by trypsin-catalyzed semisynthesis, and their binding affinities to the insulin receptor in membranes from rat adipose tissue were determined (Table 1 and Fig. 1). Four of these analogues are novel molecules, with [PheB26]-insulin having been already reported by Gauguin et al. (39). The crystal structure and binding affinity of [NMeTyrB26]-insulin was recently reported by us elsewhere (17). The first three analogues differ in the position of N-methylation of the peptide bond amide at the B24, B25, or B26 positions of human insulin. Additionally, two insulin analogues were also produced in which Tyr at the B26 position was replaced with either Phe or N-MePhe. The last analogue contains two mutations, i.e. N-MePhe residue in both B25 and B26 positions.

All analogues showed diminished binding affinity compared with human insulin. The most drastic reduction in binding was observed in the case of analogues N-methylated at the B25 position (<1%). The N-methylation of the B24 amide also had a highly negative effect on the binding affinity of the respective analogue (3%). In contrast, the B26 position was much more tolerant to the modifications, with [PheB26]-insulin displaying



FIGURE 1. Inhibition of binding of human [1251]-insulin to adipose plasma membranes by insulin and insulin analogues. A, human insulin (black circles), [NMePheB24]-insulin (blue triangles), and [NMePheB25]-insulin (red squares). B, [PheB26]-insulin (black circles), [NMePheB26]-insulin (blue triangles), and [NMePheB25,NMePheB26]-insulin (red squares). Quantitative information is provided in Table 1. See "Materials and Methods" for details.

only moderate reduction of the affinity (45.9%) (very similar to its binding affinity (47.6%), determined using an IM-9 cells binding assay (39)). N-Methylation of Tyr-B26 reduces the binding affinity of the analogue to 20.7%, and the combination of both N-methylation and the substitution of Phe for Tyr at B26 results in a very weak binding of this insulin analogue (4.2%).

ITC Measurements—The key part of our study was devoted to the investigation of the dimerization ability of insulin analogues by ITC dilution measurements that detect heat energy changes upon the dissociation of insulin dimers. The dimerization properties of the analogues were set against human/porcine insulin and DOI as benchmarks of regular and non-dimerization hormone behavior. The results of titration experiments are summarized in Table 2, and the complete data from all titration experiments used for the calculation of kinetic and thermodynamic parameters are shown in supplemental Table S1. The dilution experiments with human or porcine insulin yielded sequences of endothermic heat pulses characteristic of molecular dissociation as described previously (32). As the insulin concentration in the calorimeter cell increased with successive injections, dissociation decreased, and the magnitude of the heat uptake diminished accordingly, giving heat typical of dilution curves for human insulin, as shown in Fig. 2A. The example of the dilution process giving no heat effect is shown in Fig. 2B (dilution curve of [NMePheB25]-insulin). Dilution curves for porcine insulin, DOI, [NMePheB24]-insulin, [NMeTyrB26]-insulin, and [NMePheB26]-insulin are



#### TABLE 2

#### ITC analyses of dimerization capabilities of insulin and insulin analogues

The experimental data were analyzed without first injection using LLC ITC Origin 7 software. The experimental values for human insulin are means of eight parallel experiments  $\pm$  S.E. (n = 8). The experimental values for porcine insulin, DOI, and insulin analogues were determined from two parallel experiments and are provided as means  $\pm$  range (n = 2) with the exception of DOI and [*NMePheB25*]-insulin, where no heat effect was observed. The ITC experiments were performed at injection syringe concentrations of insulins ranging from 0.213 mM to 1.22 mM. The complete ITC data are shown in supplemental Table S1.



FIGURE 2. Representative dilution ITC curves for human insulin (A) and [NMePheB25]-insulin (B).

shown in supplemental Fig. S2. The curves can be fit in terms of a monomer-dimer equilibrium model  $(Ins_2 \leftrightarrow 2Ins; \Delta H^o_{d}; K_d = [Ins_{tot}]^2/[Ins_2]$ , where  $[Ins_{tot}]$  represents the total free insulin monomer concentration) yielding the apparent equilibrium constant of insulin dissociation  $K_d$  and enthalpy of dissociation  $\Delta H^o_d$  (per mol of dimer). The values of the thermodynamic parameters  $\Delta G^o_d$  and  $\Delta S^o_d$  were calculated from standard expressions:  $\Delta G^o_d = -RTlnK_d$  and  $\Delta G^o_d = \Delta H^o_d - T\Delta S^o_d$ , respectively.

The titration experiments with human insulin resulted in an apparent dissociation constant  $K_d$  of ~8.8  $\mu$ M. This value is in a

very close agreement with  $K_d$  of ~9  $\mu$ M, which we determined for porcine insulin (Table 2) and with the previous determinations of the  $K_d$  under similar conditions (32). Human and porcine insulin represent molecules with native and full dimerization potency. In contrast, the dilutions of DOI did not yield any heat effect, indicating that this (B23–B30)-truncated insulin analogue does not form dimers within the range of concentrations tested here.

All investigated analogues showed considerably reduced dimerization capabilities. The *N*-methylation of the B26 position resulted in an analogue ([*N*MeTyrB26]-insulin) with  $K_d$  of



~142  $\mu$ M, which was >15-fold higher than the  $K_d$  of native insulin. Moreover, the combination of *N*-methylation and substitution of Phe for Tyr at the B26 position ([*N*MePheB26]insulin) suppresses the dimerization capacity even further to a  $K_d$  of ~1.24 mM. The *N*-methylation of B24 amide nitrogen yielded the analogue ([*N*MePheB24]-insulin) with a low dimerization capability ( $K_d$  of ~587  $\mu$ M, which is 65-fold higher than the  $K_d$  of native insulin). However, the most drastic effect of the *N*-methylation on insulin dimerization was observed in the case of the modification at the B25 position. The [*N*MePheB25]-insulin analogue, similarly to DOI, gave no heat effect and behaved solely as a monomer, even at millimolar concentrations (Fig. 2*B*).

Structural Characterization of Analogues-The analogues [NMePheB24]-insulin and [NMePheB25]-insulin were subjected to intensive crystallization screening under a plethora of monomeric, dimeric, and hexameric conditions. Only R<sub>6</sub> hexameric crystals of [NMePheB25]-insulin were obtained. The monomeric and hexameric (R<sub>6</sub>) crystals of [NMeTyrB26]-insulin and hexameric (R<sub>6</sub>) of [TyrB25,NMePheB26]-insulin, also discussed in this work, were already reported by us (Refs. 17 and 27, respectively). However, their oligomeric organization was not described there in detail, as these studies were focused on the monomeric form of the hormone. In addition, the [TyrB25,NMePheB26,LysB28,ProB29]-insulin analogue, not investigated here by the ITC due to its Lys  $\leftrightarrow$  Pro sequence swap and, thus, likely monomeric properties, was also crystallized in the hexameric  $(R_6)$  form; we reported the synthesis and binding affinity (4%) of this analogue elsewhere (30).

*Crystal Structure of [NMePheB25]-Insulin*—This analogue yielded crystals only under hexameric conditions in the presence of  $Zn^{2+}$  and phenol (see supplemental Table S2). The analogue oligomer adopted a typical R<sub>6</sub> conformation with six phenol ligands at the so-called type I site (located at the dimer interface, with hydrogen bonds to COA6 and NHA11, loose van der Waals contacts (~3.7 Å) with imidazole of His-B5 (40)) and two  $Zn^{2+}$  cations and two  $Cl^-$  anions as their axial ligands. The overall structure of this hexamer followed general fold and most of the structural details of a typical R<sub>6</sub> hexamer such as that of the insulin structure of Protein Data Bank code 1ZNJ.

In the [*N*MePheB25]-insulin  $R_6$  hexamer, the *N*-methylation of the NHB25 did not result in significant disruption of the dimer interface hydrogen bonds motifs (Fig. 3*A*). All four symmetrical B24–B26 hydrogen bonds were preserved there despite a small weakening of the B24CO-NHD26 interaction by ~0.2 Å. The conservation of these interactions is likely due to an "outward" orientation of the *N*MeB25 groups, which point out away from the dimer interface (Fig. 3*A*) and are thus easily accommodated in the vicinity of the A19–A21 region. However, the introduction of two *N*MeB25 groups resulted in much greater mobility of the Phe-B25 side chains; only one B25 phenyl moiety (with poor definition in the electron density maps) could be located. Both C-terminal threonines B30 are not visible in the structure.

*Crystal Structure of [NMeTyrB26]-Insulin Hexamer*—Although we already reported this structure elsewhere (17), the nature of the dimer interface and its  $R_6$  hexamer structure was not discussed in that report. Remarkably, it can be crystallized



FIGURE 3. Main chain representation of the dimer (chains AB in molecule I and chains CD in molecule II) interface in [/M@PheB25]-insulin (Protein Data Bank code 3ZQR) (A), [/M@TyrB26]-insulin (Protein Data Bank code 2WS6) (B), [TyrB25,/M@PheB26]-insulin (Protein Data Bank code 1W8P) (C), and [TyrB25,/M@PheB26]-insulin (Protein Data Bank code 3ZS2) (D) analogues. The reference wild-type insulin (Protein Data Bank code 1ZNJ) is in white with its dimer hydrogen bonds as *black dashed lines*; hydrogen bonds of the analogues are colored *magenta*. The methyl groups at *N*-methylated atoms are indicted by *magenta asterisks*; hydrogen bond distances are in Å.

both as a monomer and  $R_6$  hexamer under appropriate conditions. The hexamer crystallization protocol, similar to conditions for [*N*MePheB25]-insulin (see supplemental Table S2), yielded crystals of the  $R_6$  oligomer very similar to that of [*N*MePheB25]-insulin. However, the methylation of NHB26 resulted in a much more disturbed dimer interface than in the [*N*MePheB25]-insulin structure. As both B26 *N*-methyl groups pointed into the dimer interface, its quartet of the hydrogen bonds was almost lost; only one of them, COB26-NHD24, remained in place (Fig. 3*B*). The disturbance created by *N*-MeB26 groups had a non-symmetrical character. Only one  $\beta$ -strand of the two-strand dimer  $\beta$ -sheet bulged out between B24–B26 to accommodate the more bulky substitutions of the NHB26 hydrogen atoms; the other D21–D27  $\beta$ -strand followed the typical fold of the wild-type insulin. However, it still exerted



some signs of induced structural stress, reflected in its higher mobility, with B28–B30 residues being fully disordered. Interestingly, the  $\beta$ -strand bulge in the B-chain brought the B25NH peptide unit closer to the COA19 group, resulting in the unusual, for the R<sub>6</sub> hexamer, B25NH-COA19 hydrogen (2.9 Å) bond.

Crystal Structure of [TyrB25,NMePheB26]-Insulin—The structure of our already reported (27) analogue with NHB26 *N*-methylation and the Tyr  $\leftrightarrow$  Phe B25–B26 swap was also analyzed here in more detail. The main focus of the previous discussion of this structure was the impact of the IGF-like swap of B25-B26 side chains, combined with simultaneous modification of the main chain, on insulin and IGF-I structure-function relationships. Here, we tried to extract the relevant structural features in the context of the dimer interface. [TyrB25, NMePheB26]-insulin formed an  $R_6$  hexamer that is quite similar to [NMeTyrB26]-insulin and [NMePheB25]-insulin oligomers. Although the bulging of the B24-B26 chain was "symmetrical" to that observed in the [NMeTyrB26]-insulin (Fig. 3C), its nature and structural meaning was practically identical to the [NMeTyrB26]-insulin change, considering the 32 symmetry of the hexamer and the relativity of its chain nomenclature.

Crystal Structure of [TyrB25,NMePheB26,LysB28,ProB29]-Insulin-The crystal structure of [TyrB25,NMePheB26,LysB28, ProB29]-insulin was obtained under hexameric conditions (supplemental Table S2) in the pursuit of a very extensive characterization of the dimer interface hydrogen bond network of the B25-B26 side chains for the stability of this region. The  $R_6$  hexamer was also very similar to all other  $R_6$  oligomers described here. However, the B28 – B29 Lys  $\leftrightarrow$  Pro and B25–B26 Tyr  $\leftrightarrow$  Phe swaps, combined with N-MeB26, fully disrupted the dimer interface, with none of its hydrogen bonds being preserved (Fig. 3D). Hence, the remarkable stability of these R<sub>1</sub>R<sub>1</sub> dimers depended entirely on the phenolinduced and R-form specific inter- and intramolecular interactions (see "Discussion"). The disruption of the dimer interface was also very asymmetrical, in a fashion similar to that observed in [NMeTyrB26]-insulin; one  $\beta$ -strand followed the wild-type conformation, whereas its counterpart systematically departed from the dimer interface from D23 to become fully disordered from D27 onwards.

#### DISCUSSION

Impact of Modifications on Binding Affinity of Analogues— Analogues N-methylated at the B24 position ([NMePheB24]insulin, 3%) or B25 ([NMePheB25]-insulin, 0.17%) showed drastically reduced binding potency. To our knowledge, [NMePheB24]-insulin represents the first insulin analogue with a backbone modification at the B23–B24 peptide bond.

The low binding activity of [*N*MePheB25]-insulin is in agreement with findings of Wollmer *et al.* (41) for an almost inactive (3–4%) insulin analogue ([depsi(B24–B25)]-insulin) in which the B24–B25 CO-NH peptide bond was replaced by a CO-O ester bond. It seems that the very low binding affinity of [*N*MePheB24]-insulin and [*N*MePheB25]-insulin results from the lack of the respective peptide bond amides that may be crucial for the direct interaction of insulin with IR. These obser-

## Dimerization of Insulin Analogues

vations are also supported by findings of Nakagawa *et al.* (42) concerning inactive insulin analogues in which peptide bonds at the B24–B25 or B25–B26 positions were replaced by reduced  $CH_2$ -NH groups. They attributed the extremely low activity of their analogues to the loss of respective carbonyl oxygens involved in H-bonding with IR. Another possible reason for the low binding affinity of [*N*MePheB24]-insulin and [*N*MePheB25]-insulin, *N*-methylation-induced conformational changes in the structures of their B24–B30 strands, will be discussed below.

Interestingly, the [NMeTyrB26]-insulin (17) also displayed lower binding potency toward IR (21%, Table 1) than human insulin, but significantly less reduced than that of analogues N-methylated at the B24 and B25 positions (3 and 0.17%, respectively). This may indicate that the NH of B26 is less important for IR binding than its counterparts at the B24 and B25 positions and/or has a different function. Our recent study (17) demonstrated that N-methylation of Tyr-B26 induces a new type II of  $\beta$ -turn at positions B24–B26 (B26 turn), which results in the departure of B-chain B22–B30  $\beta$ -strand from its typical hexamer/dimer conformation. The B26 turn is stabilized by a typical  $i+3 \beta$ -turn hydrogen bond between CO of Phe-B24 and NH of Thr-B27. These large conformational changes unmask previously buried amino acids Gly-A1, Ile-A2, and Val-A3, which are implicated in IR binding. We proposed (17) that similar conformational changes may occur in the native insulin molecule upon binding to IR. Hence, it is possible that the reduced binding affinity of [NMeTyrB26]-insulin (21%) may be caused by a non-optimal arrangement of residues B27–B30. However, the reduced affinity of this analogue may also result from the loss of NHB26 hydrogen bonding capabilities, required in IR interactions, or by a combination of these factors. The impact of some distinct (e.g. long range) N-methylation-induced conformational changes in the structures of B24-B30 strands in [NMePheB24]-insulin and [NMePheB25]insulin cannot be excluded as well. However, their existence still awaits structural proof as [NMePheB24]-insulin eluded all crystallization trials so far, and [NMePheB25]-insulin has been crystallized only in the form of a typical R<sub>6</sub> hexamer (see below).

The last three analogues in Table 1 represent molecules with single, double, or multiple modifications compared with native human insulin, respectively. The [PheB26]-insulin analogue was included in this study to examine the importance of the Tyr-B26 hydroxyl group for insulin affinity, as the next analogue studied here, [NMePheB26]-insulin, featured two combined modifications: the loss of phenolic character of the B26 side chain with N-methylation of the B26NH. The [PheB26]insulin showed 46% binding affinity that was in full agreement with data (47%) already reported for this analogue (39), clearly indicating that the loss of Tyr-B26 hydroxyl group is responsible for the reduced binding affinity of this analogue. Therefore, an even more drastic (actually to 4%) loss of affinity of the [NMePheB26]-insulin was expected, as it results from a double (side and main chain) modification of the hormone at this position. Subsequently, the practically total loss of affinity (0.44%) of the [NMePheB25,NMePheB26]-insulin analogue is not surprising, as it fosters the cumulative effect of three unfavorable



modifications (two *N*-methylations and loss of a phenolic B26 side chain).

ITC Measurements and Dimerization Capabilities of Analogues—ITC measurements of insulin dimer dissociation require relatively high amounts of protein material. Their optimum required concentration should be in the 0.2–1 mM range, but concentrations closer to the high end yield more reliable data (32). Hence, a typical ITC experiment in a 1.4-ml reaction vessel requires ~8 mg of insulin, which presents some experimental challenges due to the relatively low yields of semisynthetic reactions (30). Also, the recovery of analogues from reaction mixtures after ITC experiments is not very efficient. Therefore, we determined the dimerization capabilities of analogues only at two replicates at different protein concentrations. However, the complete ITC data presented in supplemental Table S1 indicate that duplicate measurements performed here produced very consistent and reliable results.

As some reports (43, 44) have shown, pH has a rather small effect on the outcomes of insulin-dimerization experiments; thus, all ITC experiments were performed here also at acidic pH 2.5 to avoid reduced insulin solubility at the physiological pH (32). As expected, our ITC experiments with human and porcine insulin gave almost identical dissociation constants of dimerization ( $K_d$ ) of ~9  $\mu$ M, and the changes in Gibbs free energies of dissociation ( $\Delta G^o_d$ ) of ~29 kJ/mol (Table 2). These results are close to the  $K_d$  and  $\Delta G^o_d$  of  $\sim 12 \ \mu\text{M}$  and 28 kJ/mol, respectively, measured by Lovatt et al. (32) for bovine insulin under similar ITC conditions. Strazza et al. (43) used concentration difference spectroscopy to determine the monomerdimer association constants of porcine insulin, finding only minor differences in  $K_d$  at pH 2 (15  $\mu$ M) and at pH 7 (18  $\mu$ M). Equilibrium sedimentation, an alternative assessment of insulin dimerization, provided  $K_d$  of  ${\sim}4.5~\mu{\rm M}$  for bovine insulin (at pH 8.0) (28), whereas this approach for porcine insulin gave  $K_d$ of  $\sim$ 7  $\mu$ M (at pH 7.0) (44). Altogether, the dimerization dissociation constants for human and porcine insulin determined in this study fall well into the range of  $K_d$  values determined experimentally by other groups and different techniques. The reliability of the approach presented here therefore allows a more systematic insight into the evaluation of the roles of individual hydrogen bonds involving B24-B26 peptide units in dimer stability. They are discussed below in the context of structural changes caused by their alternations.

Structure-Function Relationships in Insulin Analogue Dimers— The resultant minor differences in equilibrium constants and thermodynamic parameters of insulin dimerization at different pHs led to the conclusion that insulin association is mainly driven by the nonpolar interactions (44). However, Strazza *et al.* (43) also indicated that insulin dimerization is under enthalpic control and that the formation of four hydrogen bonds in the apolar protein environment is the main driving force in the assembly of insulin dimer. However, molecular dynamics simulations suggested that insulin dimerization results mainly from nonpolar interactions (contributed mostly by residues B24–B26) (45) and that the role of hydrogen bonds between monomers is to provide a correct directional and spatial guidance to prevailing non-polar interactions. Our experimental data do not exclude any of these hypotheses but indicate more



FIGURE 4. The B-A (*right panel*) and C-D (*left panel*) insulin intrachain coupling effect of the B25NH-COA19 hydrogen bond in the wild-type insulin T<sub>6</sub> dimer (Protein Data Bank code 1MSO) (in *dark gray*), wild-type R<sub>6</sub> dimer (Protein Data Bank code 1ZNJ) (*white*), [*NM*ePheB25]-insulin (Protein Data Bank code 3ZQR) (*green*), and [*NM*eTyrB26]-insulin (Protein Data Bank code 2WS6) (*yellow*). The T state characteristic B25NH-COA19 hydrogen bond in the T<sub>6</sub> dimer is in *red* on both panels (distances in Å); this AB monomer-only hydrogen bond in the [*NM*eTyrB26]-insulin analogue is in *magenta*. Phe-B25 side chains are disordered in [*NM*ePheB25]-insulin and are not represented here.

clearly the important individual contributions of B24–B26 hydrogen bonds toward the stability of the dimer.

The individual knock-outs of the NHB24 ([NMePheB24]insulin) and NHB26 ([NMeTyrB26]-insulin) hydrogen bond donors increased  $K_d$  from 9  $\mu$ M to 587 and 142  $\mu$ M, respectively (Table 2). As the role of the B24-B26 side chains can, to some extent, be decoupled in this process due to the 2-fold symmetry of these regions, the B24NH-COB26 hydrogen bond can be seen as a more important polar stabilizer of the insulin dimer. Its loss causes a much larger ( $\sim$ 4-fold) dimer disruptive effect than abolishing the B26NH-COB24 interaction. This can result from its flanking positions on the dimer interface, closer to the solvent exposed termini of the dimer  $\beta$ -sheet (Fig. 3, *A* and *B*). The lack of B24NH-COB26 interactions, linked with the mobility-prone character of the B27-B30 terminal residues (observed in many insulin crystal and NMR structures; see, for example, Fig. 3, B-D), can lead to easier solvent penetration through the dimer interface and to faster unzipping of the  $\beta$ -sheet via solvation/breaking of the remaining central pair of B26NH–COB24 hydrogen bonds (46). The lower  $K_d$  of the [NMeTyrB26]-insulin analogue, its higher dimeric stability than the [NMePheB24]-insulin, can also result from the nontypical (for the R-state) B25NH-COA19 hydrogen bond in one of its monomers. It seems that it is a side effect of the  $\beta$ -strand bulge that brings NHB25 into the proximity of the Tyr-A19 main chain, allowing the formation of an intramolecular hydrogen bond (2.9 Å, Fig. 4), which may be important for insulin dimerization (see "Discussion"). However, as this interaction is not symmetrical ( $\beta$ -strand bulge occurs only in one monomer), it cannot fully compensate for the instability of the interface in this analogue. Therefore, it is possible that the lack of positive crystallization results of the [NMePheB24]-insulin analogue



(significant but rather indirect evidence) may result from the rapid monomer-dimer kinetics that prevents a stable crystal nucleation process. Moreover, it is interesting that the higher stability of the [*N*MeTyrB26]-insulin dimer is also reflected in its remarkable availability to form monomeric and hexameric crystal structures (17); appropriate physicochemical solution parameters can shift the monomer-oligomer equilibrium toward one of the stable (crystallisable) forms of this analogue, especially if the most stable (>8 h (47)) phenol-induced R-form of the hexamer is assured. Although crystallization trials cannot be considered as an unambiguous experimental proof, the ~15-fold higher  $K_d$  of [*N*MeTyrB26]-insulin compared with native insulin (Table 2) also agrees with the lack of its dimer crystals, even under extensive dimer crystallization conditions (17).

Interestingly, the most dramatic/disruptive impact on insulin dimerization properties, evident in the ITC data, is not provided by the knock-out of each of the intermonomer  $\beta$ -sheet hydrogen bonds pairs, but by the N-methylation of the B25 amide unit in the [NMePheB25]-insulin analogue. Despite the almost wild-type nature of the dimer interface here (Fig. 3A) and an easy spatial accommodation of the outward (from the dimer  $\beta$ -sheet interface) pointing B25 *N*-Me groups, the lack of intramolecular hydrogen bond donor properties of B25NH group results in no-heat effects in the ITC measurements of this analogue, in a fashion similar to the monomeric-benchmark DOI insulin. The only direct hydrogen bond-related impact of the loss of the B25NH proton is the lack of the B25NH-COA19 hydrogen bond that connects B-chain  $\beta$ -strand with the core of the molecule in the T-forms (dominant insulin state in the metal/ligand-free ITC experiments) of the hormone (Fig. 4). The release of the C-terminal  $\beta$ -strand residues from this intramolecular hydrogen bond lock may result in its higher mobility shifting solution equilibrium toward the monomeric state. Additionally, increased dynamics of this region can contribute to, or be amplified further by, the Phe-B25 side chain mobility. Indeed, the B25 phenyl ring can be located with difficulty (poor electron density map definition) only for one of the B25 residues. Therefore, it is possible that the monomeric character of [NMePheB25]-insulin can be associated with the detachment of Phe-B25 (and surrounding B-chain) from the A-chain.

The  $\beta$ -strand detachment-driven monomeric behavior in the solution and formation of hexameric crystals of [NMePheB25]-insulin are somehow reflected in the crystal structure (and solution properties) of the [LysB28,ProB29]-insulin, an analogue also with a very substantially diminished  $(\sim 300 \times)$  dimerization capacity (48). Its monomeric behavior did not also prohibit its crystallization as the  $T_3R_3^{f}$  hexamer in the presence of zinc and phenol (16). The [NMePheB26]-insulin was an additional step in probing of the resistance of the dimer interface by prevention of the "central" B26NH-COB24 hydrogen bond of the dimer interface, combined with the loss of B26 phenolic character. It is surprising that, although the "peripheral" dimer-spanning B24NH-COD26 hydrogen bonds are preserved here (like in the [NMeTyrB26]-insulin analogue), the loss of the central pair (B26NH-COD24) of these interactions, combined with a simultaneous loss of a Tyr-B26 OH group, cannot prevent the further dramatic increase of the  $K_d$ into the mM range (1.240 mM). It seems then that the phenolic indirect hydrogen bonds of the Tyr-B26 side chain (*e.g.* to Tyr-D16) and its multiple van der Waals interactions (*e.g.* Phe-B24, Pro-B28, Ile-A2, Val-A3, Ile-B11, and Val-B12), multiple combined contributions, are rather important for monomer (and dimer) stability. This also indicates that the dimer-stabilizing role of B24–B26 hydrogen bonds cannot be unambiguously decoupled from the individual contributions of the associated individual side chains.

*Role of Hexameric Form in Dimer Stability*—It has to be stressed that all dimer-related comparisons and correlations of structural information with the solution (ITC) studies presented here are based on different quaternary forms of insulin. Structural data were derived exclusively from the R<sub>6</sub> hexamers (two Zn<sup>2+</sup>, six phenol ligands), and none of the analogues discussed in this report attained the conformation of  $T_3R_3$  ( $T_3R_3^f$ ) or  $T_6$  hexamers. In contrast, the ITC solution measurements were performed in a metal/ligand-free environment, with the T state being the likely form of the hormones.

Considering the dimer  $\beta$ -sheet disruptive character of the chemical changes in analogues studied here, it is not surprising that the most stable form of the hexamer (R<sub>6</sub>) (47, 49, 50) was found to be adopted by all modified insulin. The dominance of the R<sub>6</sub> hexameric form likely originates from the additional reinforcement of dimer stability provided the B1–B6 helical extension. It not only delivers typical phenol site I-generated interactions (40) but also a few new, strong (~2.7 Å) intermolecular dimer-spanning protein-protein hydrogen bonds such as Tyr-B16NH-COHis-D5. It seems that these interactions must also compensate for a loss of the B25NH-COA19 intramolecular hydrogen bond, which occurs only in the T state of insulin. Hence, the changes in dimer interfaces in various analogues are displayed here against virtually the same reference-like core of the R<sub>6</sub> oligomer.

It is interesting that even very disruptive effects on the dimer interface (i) resulting from the interference with the B24-B26 intermolecular hydrogen bonds, (ii) amplified further by some mutations in this region, and (iii) leading, for example, to a full detachment of dimer  $\beta$ -strands (*i.e.* [TyrB25,NMePheB26,LysB28,ProB29]-insulin, Fig. 3D), are still compensated and accommodated in a stable R<sub>6</sub> hexamer. Energy-driven superiority of the R<sub>6</sub> oligomer is especially visible in the already reported (17) crystal structure of the truncated [ProB26]-DTI analogue, in which the dimer interface is totally disrupted beyond B/D21-22 residues (Fig. 5). The maintenance of the hexameric form there, despite the lack of the entire dimer  $\beta$ -strand interface, is somehow compatible with the association of DOI into a R<sub>6</sub> hexamer observed in the presence of  $Zn^{2+}$  and cyclohexanol (29). Although the  $T_6 \rightarrow T_3 R_3 \rightarrow R_6$  dynamic transitions are rather well described by the SMB model (51-56), the conformational events on the monomer  $\rightarrow$  dimer  $\rightarrow$  hexamer pathway are much less understood. Our results presented here provide further evidence that the nature of the changes on the insulin dimer interfaces (and associated other parts of the insulin molecule) is quite asymmetrical (Fig. 3, B-D); thus, they fit and support well the occurrence of structural asymmetries that are important features of a SMB model for the allosteric behavior of insulin (57).





FIGURE 5. General fold of the B-chain, including the dimer interface, in the [ProB26]-DTI dimer interface-deprived analogue (Protein Data Bank code 2WS7) (in *red*) and in the wild-type insulin (Protein Data Bank code 1ZNJ) (in *white*).

Undoubtedly, the formation and dissociation of insulin hexamers and dimers into active monomers plays an essential role in the endogenous delivery of the hormone (20, 22). First, the release of insulin hexamers from secretory granules (pH 5.5) into the bloodstream (pH 7.4) results in the loss of zinc (due to the higher pH) and in the dissociation of hexamers to insulin dimers (21). The process of dimer dissociation is likely concentration-driven ( $K_d$  of insulin dimerization is ~9  $\mu$ M). Therefore, the dissociation of insulin dimers into monomers in the bloodstream is a dilution-driven phenomenon. The clinically used and fast acting [LysB28,ProB29]-insulin (Humalog<sup>®</sup>) (58) or [AspB28]-insulin (Novolog<sup>®</sup>) (59, 60) are not able to form dimers, but still associate into hexamers (16), providing evidence that the disruption of insulin dimerization has a fundamental impact on the endogenous action of this hormone.

## CONCLUSIONS

The C terminus of the B-chain, namely, residues B24–B26 and B28–B29, is a crucial part of insulin for its interaction with IR and for its self-association into dimers. The importance of Phe-B24, Phe-B25, and Tyr-B26 residues in the formation of dimers is well established; however, the contribution of their respective individual amide hydrogens for the stability of this aggregates has not been characterized. Here, we delineated their roles by preparation of a series of insulin analogues with selective *N*-methylations of peptide bond amides at positions B24, B25, or B26. All of these *N*-methylated insulin analogues showed significantly impaired binding affinities to the receptor, confirming also the role of respective amide hydrogens in the direct interaction/involvement with insulin receptor. System-

atic, individual N-methylations of B24, B25, and B26 peptide bonds in studied analogue amides resulted in a wide spectrum of reduced dimerization abilities of the analogues. Surprisingly, the most dramatic impact on insulin dimerization capability was exerted by the N-methylation at the B25 position, likely due to a loss of the intramolecular  $\beta$ -strand-hormone-core connecting NHB25-COA19 hydrogen bond. The subsequent higher mobility of the  $\beta$ -strand in this analogue severely shifts the solution equilibrium toward the monomeric state. All examined analogues were crystallized only as R<sub>6</sub> hexamers, which is the most stable form of insulin oligomers and the only oligomeric form capable to accommodate/withstand disrupted insulin  $\beta$ -strands. This study helped to understand the importance and contribution of the B24NH-COB26, B26NH-COB24, and, especially, B25NH-COA19 hydrogen bonds in the formation and stabilization of insulin dimers. The behavioral, functional, and structural correlations presented here provide much evidence that clearly supports and further validates the SMB model of insulin allostery. A better understanding of the structure-function relationships in the association-dissociation pathways of this hormone is important for the understanding of its physiology but may also facilitate development of better, more controlled insulin for clinical applications.

#### REFERENCES

- 1. Derewenda, U., Derewenda, Z., Dodson, E. J., Dodson, G. G., Bing, X., and Markussen, J. (1991) *J. Mol. Biol.* **220**, 425–433
- 2. Weiss, M. A. (2009) in *Insulin and IGFs* (Litwack, G., Ed.) pp. 33–49, Elsevier Academic Press, Inc., San Diego, CA
- 3. Mayer, J. P., Zhang, F., and DiMarchi, R. D. (2007) *Biopolymers* 88, 687-713
- 4. Hua, Q. X., Shoelson, S. E., Kochoyan, M., and Weiss, M. A. (1991) *Nature* **354**, 238–241
- Ludvigsen, S., Olsen, H. B., and Kaarsholm, N. C. (1998) J. Mol. Biol. 279, 1–7
- Xu, B., Huang, K., Chu, Y. C., Hu, S. Q., Nakagawa, S., Wang, S., Wang, R. Y., Whittaker, J., Katsoyannis, P. G., and Weiss, M. A. (2009) *J. Biol. Chem.* 284, 14597–14608
- 7. Kaarsholm, N. C., and Ludvigsen, S. (1995) Receptor 5, 1-8
- Keller, D., Clausen, R., Josefsen, K., and Led, J. J. (2001) *Biochemistry* 40, 10732–10740
- Ludvigsen, S., Roy, M., Thøgersen, H., and Kaarsholm, N. C. (1994) *Bio-chemistry* 33, 7998–8006
- Olsen, H. B., Ludvigsen, S., and Kaarsholm, N. C. (1996) *Biochemistry* 35, 8836–8845
- 11. Hua, Q. X., and Weiss, M. A. (1991) Biochemistry 30, 5505-5515
- Huang, K., Xu, B., Hu, S. Q., Chu, Y. C., Hua, Q. X., Qu, Y., Li, B., Wang, S., Wang, R. Y., Nakagawa, S. H., Theede, A. M., Whittaker, J., De Meyts, P., Katsoyannis, P. G., and Weiss, M. A. (2004) *J. Mol. Biol.* **341**, 529–550
- Hua, Q. X., Xu, B., Huang, K., Hu, S. Q., Nakagawa, S., Jia, W., Wang, S., Whittaker, J., Katsoyannis, P. G., and Weiss, M. A. (2009) *J. Biol. Chem.* 284, 14586-14596
- Kristensen, C., Kjeldsen, T., Wiberg, F. C., Schäffer, L., Hach, M., Havelund, S., Bass, J., Steiner, D. F., and Andersen, A. S. (1997) *J. Biol. Chem.* 272, 12978–12983
- Baker, E. N., Blundell, T. L., Cutfield, J. F., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Hodgkin, D. M., Hubbard, R. E., Isaacs, N. W., Reynolds, C. D., Sakabe, K., Sakabe, N., and Vijayan, N. M. (1988) *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **319**, 369–456
- Ciszak, E., Beals, J. M., Frank, B. H., Baker, J. C., Carter, N. D., and Smith, G. D. (1995) *Structure* 3, 615–622
- Jirácek, J., Záková, L., Antolíková, E., Watson, C. J., Turkenburg, J. P., Dodson, G. G., and Brzozowski, A. M. (2010) *Proc. Natl. Acad. Sci. U.S.A.* 107, 1966–1970



- 18. De Meyts, P., Van Obberghen, E., and Roth, J. (1978) Nature 273, 504-509
- Keefer, L. M., Piron, M. A., De Meyts, P., Gattner, H. G., Diaconescu, C., Saunders, D., and Brandenburg, D. (1981) *Biochem. Biophys. Res. Commun.* 100, 1229–1236
- DeFelippis, M. R., Chance, R. E., and Frank, B. H. (2001) Crit. Rev. Ther. Drug Carrier Syst. 18, 201–264
- 21. Dodson, G., and Steiner, D. (1998) Curr. Opin. Struct. Biol. 8, 189-194
- 22. Brange, J., Owens, D. R., Kang, S., and Vølund, A. (1990) *Diabetes Care* 13, 923–954
- Adams, M. J., Blundell, T. L., Dodson, E. J., Dodson, G. G., Vijayan, M., Baker, E. N., Harding, M. M., Hodgkin, D. C., Rimmer, B., and Sheat, S. (1969) *Nature* 224, 491–495
- Smith, G. D., Pangborn, W. A., and Blessing, R. H. (2003) Acta Crystallogr. D 59, 474–482
- Záková, L., Barth, T., Jirácek, J., Barthová, J., and Zórad, S. (2004) *Biochem*istry 43, 2323–2331
- Záková, L., Kazdová, L., Hanclová, I., Protivínská, E., Sanda, M., Budesínský, M., and Jirácek, J. (2008) *Biochemistry* 47, 5858–5868
- Záková, L., Brynda, J., Au-Alvarez, O., Dodson, E. J., Dodson, G. G., Whittingham, J. L., and Brzozowski, A. M. (2004) *Biochemistry* 43, 16293–16300
- 28. Goldman, J., and Carpenter, F. H. (1974) Biochemistry 13, 4566-4574
- 29. Pittman, I., 4th, and Tager, H. S. (1995) Biochemistry 34, 10578-10590
- Záková, L., Zyka, D., Jezek, J., Hanclová, I., Sanda, M., Brzozowski, A. M., and Jirácek, J. (2007) J. Pept. Sci. 13, 334–341
- Bromer, W. W., and Chance, R. E. (1967) *Biochim. Biophys. Acta* 133, 219–223
- 32. Lovatt, A., Cooper, A., and Camilleri, P. (1996) *Eur. Biophys. J.* 24, 354-357
- McPhail, D., and Cooper, A. (1997) J. Chem. Soc. Faraday Trans. 93, 2283–2289
- 34. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307-326
- 35. Bailey, S. (1994) Acta Crystallogr. D 50, 760-763
- 36. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr. D 60, 2126-2132
- 37. Vagin, A., and Teplyakov, A. (1997) J. Appl. Crystallogr. 30, 1022-1025
- Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. D 53, 240–255
- Gauguin, L., Klaproth, B., Sajid, W., Andersen, A. S., McNeil, K. A., Forbes, B. E., and De Meyts, P. (2008) *J. Biol. Chem.* 283, 2604–2613
- 40. Whittingham, J. L., Edwards, D. J., Antson, A. A., Clarkson, J. M., and

Dodson, G. G. (1998) Biochemistry 37, 11516-11523

- Wollmer, A., Gilge, G., Brandenburg, D., and Gattner, H. G. (1994) *Biol.* Chem. Hoppe-Seyler 375, 219–222
- Nakagawa, S. H., Johansen, N. L., Madsen, K., Schwartz, T. W., and Tager, H. S. (1993) Int. J. Pept. Protein Res. 42, 578–584
- Strazza, S., Hunter, R., Walker, E., and Darnall, D. W. (1985) Arch. Biochem. Biophys. 238, 30–42
- 44. Pekar, A. H., and Frank, B. H. (1972) Biochemistry 11, 4013-4016
- 45. Zoete, V., Meuwly, M., and Karplus, M. (2004) J. Mol. Biol. 342, 913-929
- Ganim, Z., Jones, K. C., and Tokmakoff, A. (2010) *Phys. Chem. Chem. Phys.* 12, 3579–3588
- Hassiepen, U., Federwisch, M., Mülders, T., and Wollmer, A. (1999) *Bio-phys. J.* 77, 1638–1654
- Brems, D. N., Alter, L. A., Beckage, M. J., Chance, R. E., DiMarchi, R. D., Green, L. K., Long, H. B., Pekar, A. H., Shields, J. E., and Frank, B. H. (1992) *Protein Eng.* 5, 527–533
- Rahuel-Clermont, S., French, C. A., Kaarsholm, N. C., Dunn, M. F., and Chou, C. I. (1997) *Biochemistry* 36, 5837–5845
- Huus, K., Havelund, S., Olsen, H. B., Sigurskjold, B. W., van de Weert, M., and Frokjaer, S. (2006) *Biochemistry* 45, 4014–4024
- Seydoux, F., Malhotra, O. P., and Bernhard, S. A. (1974) CRC Crit. Rev. Biochem. Mol. Biol. 2, 227–257
- Roy, M., Brader, M. L., Lee, R. W., Kaarsholm, N. C., Hansen, J. F., and Dunn, M. F. (1989) *J. Biol. Chem.* 264, 19081–19085
- Brader, M. L., Kaarsholm, N. C., Lee, R. W., and Dunn, M. F. (1991) Biochemistry 30, 6636-6645
- Choi, W. E., Brader, M. L., Aguilar, V., Kaarsholm, N. C., and Dunn, M. F. (1993) *Biochemistry* 32, 11638–11645
- Bloom, C. R., Choi, W. E., Brzovic, P. S., Ha, J. J., Huang, S. T., Kaarsholm, N. C., and Dunn, M. F. (1995) *J. Mol. Biol.* 245, 324–330
- 56. Dunn, M. F. (2005) Biometals 18, 295-303
- Brzovic, P. S., Choi, W. E., Borchardt, D., Kaarsholm, N. C., and Dunn, M. F. (1994) *Biochemistry* 23, 13057–13069
- DiMarchi, R. D., Chance, R. E., Long, H. B., Shields, J. E., and Slieker, L. J. (1994) *Horm. Res.* 41, 93–96
- Hedman, C. A., Lindström, T., and Arnqvist, H. J. (2001) *Diabetes Care* 24, 1120–1121
- Homko, C., Deluzio, A., Jimenez, C., Kolaczynski, J. W., and Boden, G. (2003) *Diabetes Care* 26, 2027–2031

## Dimerization of Insulin Analogues

