Structural Integrity of the B24 Site in Human Insulin Is Important for Hormone Functionality

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The atomic coordinates and structure factors (codes 2m2m, 2m2n, 2m2o, 2m2p, and 2m2q) have been deposited in the Protein Data Bank (http://wwwpdb.org).

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Background: The structure of the C-terminal B21-B30 chain of insulin bound to the insulin receptor remains undetermined.

Results: The structures of B24-modified insulins were determined.

Conclusion: The structural integrity of PheB24 but flexibility of B25-B30 insulin residues are important for receptor binding.

Significance: The knowledge of the receptor-bound structure of insulin is important for the design of new insulin receptor agonists.

Despite the recent first structural insight into the insulin–insulin receptor complex, the role of the C terminus of the B-chain of insulin in this assembly remains unresolved. Previous studies have suggested that this part of insulin must rearrange to reveal amino acids crucial for interaction with the receptor. The role of the invariant PheB24, one of the key residues of the hormone, in this process remains unclear. For example, the B24 site functionally tolerates substitutions to d-amino acids but not to L-amino acids. Here, we prepared and characterized a series of B24-modified insulin analogues, also determining the structures of [D-HisB24]-insulin and [HisB24]-insulin. The inactive [HisB24]-insulin molecule is remarkably rigid due to a tight accommodation of the i-His side chain in the B24 binding pocket that results in the stronger tethering of B25-B28 residues to the protein core. In contrast, the highly active [d-HisB24]-insulin and [HisB24]-insulin. The B25→D-B24 downshift results in a subsequent downshift of TyrB24 into the B25 site and the departure of B26-B30 residues away from the insulin core. Our data indicate the importance of the aromatic L-amino acid at the B24 site and the structural invariance/integrity of this position for an effective binding of insulin to its receptor. Moreover, they also suggest limited, B25-B30 only, unfolding of the C terminus of the B-chain upon insulin activation.

Human insulin is a 51-amino acid hormone that regulates blood glucose levels and affects lipid/protein metabolism and, to some extent, the life span. Insulin exerts its actions through binding as a monomer to the (αB)-homodimeric, tyrosine kinase-type insulin receptor (IR) (1).

Insulin is the final product of an extensive processing from single-chain preproinsulin (2) and consists of an A-chain (GlyA1–AsnA21) and B-chain (PheB1–ThrB30) that are linked by two interchain disulfide bridges (CysA17–CysA7 and CysB20–CysB19). In addition, the A chain includes an intrachain CysA6–CysA11 disulfide bond. Insulin is capable of dimerization at low micromolar concentrations (3), and the presence of divalent metal ions leads to the formation of insulin hexamers that represent the storage form of this hormone (2). There is overwhelming evidence that monomeric insulin, which dissociates from its storage forms, must undergo structural changes upon binding to IR. Mainly, the C-terminal ~B22-B30 part of the B-chain has to rearrange to reveal amino acids that are crucial for the effective formation of the hormone-receptor interface (LeuB11, ValB12, and LeuB15 on the B-chain and GlyA1, IleA2, LeuA2, and GlNA5 on the A-chain) (4, 5) and that are obstructed by the C-terminal β-strand in the dimeric and hexameric forms of the hormone (6, 7). The importance of the B-chain C-terminal residues in IR binding has also been demonstrated by their critical role in negative cooperativity (8). The changes at the C terminus are likely accompanied by the reorganization of the N terminus of the B-chain (B1–B8), which adopts one of the conformations ranging from the so-called T-state (extended) to the R-state (α-helical) folds (9). The current understanding of the three-dimensional insulin structure upon binding to the IR is based mostly on inactive storage (hexamers, dimers) states of the hormone (9–11). Our recent structural and functional study of the insulin-IR complex (5) provided the first insight into the mode of insulin binding to its receptor, although the...
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The insulin molecule can be considered the result of complex functional and evolutionary development. The small size of this hormone, the proximity of its key side chains, and the compactness of the long range (allosteric) intramolecular interactions combined with the complex behavior of this protein (challenging bio-synthesis, processing, storage, activation of the monomer, “induced fit” etc.), gives each of its amino acids a multifaceted and convoluted function. Therefore, the role of the invariant Phe$_{B24}$ should be considered in a wider context of its molecular contributions, for example, toward conformational switching during hormone activation, facilitation of proper disulfide pairing, and hormone self-assembly (17).

Here, we provide an explanation of the role for the Phe$_{B24}$ site in the context of insulin structure and function by studying the effects of its substitutions by a combination of functional, structural (NMR, x-ray), and computational (molecular dynamics, MD) approaches. Systematic substitutions of chiral (L/D) amino acids and structural main chain constraints inducing (Pro and Sar$^3$) amino acids resulted in four novel B24-substituted analogues, whose functional and structural relationships have been established. Furthermore, the possible cooperative character of B24 and B26 sites in insulin-IR interactions and their impact on insulin affinity was investigated in three double (D-His$_{B24}$, Glu$_{B26}$) insulin mutants.

EXPERIMENTAL PROCEDURES

Materials—2-Chlorotryptil chloride resin, protected amino acids, and reagents for solid-phase synthesis of peptides were purchased from Novabiochem Merck (Laufelfingen, Switzerland). Fmoc-Lys(Pac)-OH (fluorenylmethoxycarbonyl-Lys[phenylacetyl]-OH) was prepared as described previously (34). TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone)-treated trypsin was purchased from Sigma, and penicillin G acylase was from Fluka. Human [125I]moniodotyrosylA14-insulin was purchased from Perkin Elmer Life Sciences. Human insulin was purchased from Sigma. All other chemicals and solvents were obtained from Sigma.

Peptide Synthesis and Enzymatic Semisynthesis—The syntheses of peptides and the semisyntheses of analogues were performed according to Záková et al. (34). The identity of the peptides and analogues was confirmed with an FTMS 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 Záková et al. (35). Competitive binding curves were plotted using GraphPad (San Diego, CA) Prism 5, comparing the best fits for single-binding site models. The half-maximal inhibition values of binding of $^{125}$I-insulin to the receptor (IC$_{50}$) were obtained from nonlinear regression analysis. The only exception was [ProB24]-insulin, which was tested for binding to the insulin receptor in membranes in IM-9 lymphocytes according to the procedure that we described recently in Morcavallo et al. (36). The binding data for [ProB24]-insulin were analyzed with Excel software using a one-site fitting program developed in the lab-

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3 The abbreviations used are: Sar, sarcosine; r.m.s.d., root-mean-square deviations.
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oratory of Dr. Pierre De Meyts (A. V. Groth and R. M. Shymko, Hagedorn Research Institute, Denmark, a kind gift of Pierre De Meyts) and GraphPad Prism 5, and the dissociation constant was determined. The dissociation constant of human $^{125}$I-insulin was set up to 0.3 nM. Receptor binding assays were performed under conditions excluding the depletion of free ligand.

X-ray Studies—Crystallization of [HisB24]-insulin was performed using in-house insulin crystallization screens covering most of the reported crystal insulin growth parameters. Crystallization conditions, data collections, and refinement and models statistics are given in supplemental Table S1. X-ray data were processed by HKL2000 (37), and model building and refinement were performed using the CCP4 suite of programs (38) and COOT (39). Crystal structures were solved using Mole rep (40) with B1-B6- and B23-B30-truncated insulin hexamer or hexamer-derived monomer as a model (PDB code 1mso) (41) and refined by Refmac 5.6 (42). The figures were made using CCP4 mg (38). For structural comparisions of the dimer interfaces, the best defined dimers (referred here as monomers A-B and C-D) from the relevant structures were superimposed on the B9-B19 and D9-D19 C$^\alpha$ atoms using the LSQKAB program from the CCP4 suite.

NMR Spectroscopy and Structure Calculations—NMR spectra were acquired from 0.35-ml samples of 0.2–0.5 mM insulin analogues in a 20% d$_3$-acetic acid (pH 1.9) or 25 mM deuterated-lin was set up to 0.3 nM. Receptor binding assays were per formed under conditions excluding the depletion of free ligand.

Molecular Dynamics Simulations—Two starting structures of the analogues obtained in this work. Details of the structure preparation before MD simulation runs are shown in the supplemental information. The insulin monomers were solvated in a box of TIP3P water molecules (48) extending 11 Å from the protein. Two Na$^+$ counterions were added to neutralize the charge. The systems were relaxed in several steps before MD (for details, see the supplemental information). The MD was run in periodic boundary conditions using AMBER (45) and comprised three stages: warming, equilibration, and production runs. In the first stage, the temperature was raised gradually from 10 to 300 K over 25 ps. The equilibration stage was run for 200 ps, and a production of 50 ns length ensued at 300 K and 1 atm. Three trajectories for each case were simulated using different starting conditions (random number generator seed for assigning the initial velocities), and only the most structurally stable ones according to the root mean square deviations (r.m.s.d.) were chosen for further analyses. All the bond lengths were constrained using the SHAKE algorithm (49), which allowed a time step of 2 fs. The frames were saved every 5 ps. The analyses of the MD trajectories included measurements of the positional fluctuations of insulin residues, selected interatomic distances, and backbone r.m.s.d.

RESULTS

Binding Affinities of Analogues

Seven new insulin analogues have been prepared by enzymatic semisynthesis. Their binding affinities, presented here in two groups for single-B24 and double-B24/B26-substituted analogues, are shown in Table 1, with the corresponding binding curves provided in supplemental Fig. S1. We consider here the [D-HisB24, GluB26, LysB28, ProB29]-insulin as a “double-mutant” as the LysB28 ↔ ProB29 swap does not have any impact on binding affinity (50). This swap was introduced into this analogue to promote its monomeric behavior (51) for solution NMR studies.

In the group of B24-single substituted analogues, [D-HisB24]-insulin displays the highest binding affinity of ~212% compared with wild-type insulin. In contrast, its l-amino acid analogue, [HisB24]-insulin, has an only very low IR affinity of 1.5%. Mutations with an expected impact on conformation of the main chain yielded a [SarB24]-insulin analogue with highly reduced affinity (6.3%), whereas [ProB24]-insulin was inactive (0.16%).

In the second group of analogues, two “positive” hormone mutations (D-His$^{B24}$ and Glu$^{B26}$, each individually with higher IR activity) were combined to elucidate their simultaneous effect on binding affinity. The high biological activity of the [GluB26]-insulin was already reported (52), but the actual binding affinity data were not available. Therefore, the [GluB26, LysB28, ProB29]-insulin was prepared as a reference molecule, and it confirmed the high affinity (193%) of this analogue. Surprisingly, the simultaneous combination of the positive mutations of the B24 and B26 sites, [D-His$^{B24}$ and Glu$^{B26}$], respectively, abolished the individual affinity gains, having a detrimental effect on
TABLE 1

Values of IC<sub>50</sub>, K<sub>d</sub>, and the relative binding affinities of human insulin and insulin analogues

| Analogue | IC<sub>50</sub> ± S.E., (n) | K<sub>d</sub> ± S.E., (n) | Potency<sup>a</sup> |
|----------|-----------------------------|--------------------------|------------------|
| Single B24-substituted analogues | | | |
| Human insulin | 0.89 ± 0.06 (3) | ND<sup>b</sup> | 100 ± 7 |
| Human insulin | ND<sup>b</sup> | 0.39 ± 0.01 (6) | 100 ± 3 |
| [HisB24]-insulin | 59.5 ± 6.2 (3) | ND<sup>b</sup> | 1.5 ± 0.16 |
| [D-HisB24]-insulin | 42.4 ± 6.0 (3) | ND<sup>b</sup> | 21.2 ± 20 |
| [ProB24]-insulin | 14.1 ± 2.4 (5) | ND<sup>b</sup> | 6.3 ± 1.1 |
| [SarB24]-insulin | 240 ± 7 (5) | ND<sup>b</sup> | 0.16 ± 0.005 |

<sup>a</sup> IC<sub>50</sub> values represent the concentrations of insulin or the analogues that cause half-maximal inhibition of binding of human [125I]monoiodotyrosylA14-insulin to IR. Each value represents the mean ± S.E. of multiple determinations (n).

<sup>b</sup>Relative receptor binding affinity (potency) is defined as (IC<sub>50</sub> or K<sub>d</sub> of human insulin)/IC<sub>50</sub> or K<sub>d</sub> of analogue) × 100.

<sup>c</sup>ND, Not determined.

the affinities of both double analogues ([D-HisB24, GluB26]-insulin with 2.8% affinity and [D-HisB24, GluB26, LysB28, ProB29]-insulin with 1.8% binding affinity).

**X-ray Structure of [HisB24]-insulin**

This analogue gave crystals only under monomeric conditions (pH 3.0, see supplemental Table S1) with one molecule in the asymmetric unit. Despite relatively high resolution data, the monomer of this analogue displayed a high degree of disorder (supplemental Fig. S2). Only the Val<sup>B2</sup>–Glu<sup>B21</sup> part of the B-chain was defined in the electron density maps with the Glu<sup>B21</sup> side chain being fully disordered. The position of the A-chain C-terminal Asn<sup>A21</sup> also could not be allocated. The B2-B6 B-chain N terminus adopts a T-R “intermediate” conformation observed in other monomeric x-ray structures of insulin analogues (19) and some of its dimeric forms (53) as well. The total disorder of B22–B30 chain (hence the lack of B25NH–COA19 hydrogen bond) results in the conformation of the A-chain observed in some other monomeric analogues (19, 54) (movement from the wild-type insulin T-state conformation, i.e. away from the B24–B30 chain, and ~38° rotation of the A1–A9 helix around A2C<sup>W</sup> axis). In addition, the lack of the B23CO–NHA21 hydrogen bond did not allow clear localization of the A-chain C-terminus or the adjacent disulfide bonds in the NMR time scale. Because of limited solubility and susceptibility to aggregation, it was not possible to obtain reasonable quality NMR data for L- and D-His<sup>B24</sup> analogues in aqueous solution at pH 7.

However, the analogues were fully soluble and predominantly monomeric at pH 8.0 up to a concentration of 0.5 mM. Nevertheless, a significant proportion of the H<sup>2</sup>N signals were not present in the NMR spectra due to a rapid exchange of the amide hydrogens with the solvent at basic pH. Consequently, the number of assigned resonances for both analogues was significantly lower at this pH. In particular, 91.0% (95.1% excluding H<sup>2</sup>CO) resonances could be assigned for [HisB24]-insulin and 86.5% (93.0% excluding H<sup>2</sup>CO) resonances for [D-HisB24]-insulin.

The assignments obtained for analogues at pH 8.0 are shown in supplemental Tables S4 and S5. Because of the extensive overlaps of the NMR signals in certain spectral regions, the number of resonances that could not be unambiguously assigned was higher in the [D-HisB24]-insulin analogue. The chemical shift values of the B24 histidine side chains for both analogues suggest their mono-protonated (neutral) state under basic conditions.

The 1H resonance assignments obtained for [HisB24]-insulin and [D-HisB24]-insulin under acidic and basic conditions allowed automated assignment of the NOEs identified in two-dimensional homonuclear experiments. The detailed resonance assignments are shown in supplemental Tables S2 and S3. The chemical shift values for H<sup>2</sup>CO from the side chains of three histidines (at positions B5, B10, and B24) in the acidic pH were indicative of their double-protonated (charged) state (56). In addition, a significant line-broadening of the H<sup>2</sup>CO NMR signals of several residues (Thr<sup>A8</sup>, Ser<sup>A9</sup>, Cys<sup>A11</sup>, Leu<sup>B6</sup>, and Gly<sup>B8</sup>) was observed, suggesting the presence of chemical exchange due to the isomerization of the adjacent disulfide bonds in the NMR time scale.
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The side chains of the aromatic residues from the C-terminal loop of B-chain re-establish the interaction with the methyl groups from the protein core under basic conditions. Panel A shows a region from the two-dimensional NOESY spectrum of [HisB24]-insulin acquired at pH 1.9 showing NOE cross-peaks between aromatic and methyl hydrogens. The limited number of NOEs originates mainly from the contacts involving TyrB25, TyrA19, HisB5, and HisB24 with the adjacent methyl side chains. Panel B shows the data obtained for [HisB24]-insulin at pH 8.0. The additional NOE cross-peaks are from the contacts between the aromatic rings (HisB24 and TyrB25) and methyl groups (LeuB11 and LeuB15).

Figure 1. The side chains of the aromatic residues from the C-terminal loop of B-chain re-establish the interaction with the methyl groups from the protein core under basic conditions. Panel A shows a region from the two-dimensional NOESY spectrum of [HisB24]-insulin acquired at pH 1.9 showing NOE cross-peaks between aromatic and methyl hydrogens. The limited number of NOEs originates mainly from the contacts involving TyrB25, TyrA19, HisB5, and HisB24 with the adjacent methyl side chains. Panel B shows the data obtained for [HisB24]-insulin at pH 8.0. The additional NOE cross-peaks are from the contacts between the aromatic rings (HisB24 and TyrB25) and methyl groups (LeuB11 and LeuB15).

Trated by the calculated chemical shift differences for HN and Hα at pH 1.9 and 8.0 shown in supplemental Table S7. The significant differences were found for the HN chemical shift values from residues at positions B20-B26 at pH 1.9 and B23-B27 at pH 8.0. Interestingly, only a very limited number of the NOE contacts between the side chains of aromatic residues (HisB24, PheB25, TyrB26) from the C-terminal part of the B-chain and hydrophobic core of insulin were observed in the NOESY spectra acquired at pH 1.9. However, an extensive network of these contacts re-emerged in the NOESY spectra obtained at pH 8 (Fig. 1).

The solution structures calculated from the NMR data for [HisB24]-insulin and [D-HisB24]-insulin at pH 1.9 and pH 8.0 are shown in Fig. 2. Detailed analysis revealed that A-chain of both analogues adopts a characteristic helix-turn-helix conformation independently of the pH. As expected, the N-terminal α-helix of A-chain (A1-A6) is not very well defined at pH 8.0 due to an insufficient number of NOE contacts involving backbone amide hydrogens, which are key for stabilization of the local helical conformation in proteins. The effect is more evident for [D-HisB24]-insulin (Fig. 2D). In addition, the overall appearance of the [D-HisB24]-insulin structure was affected by a significantly lower number of NOE peaks observed in the NOESY spectrum, which resulted in a reduced number of the non-redundant distance constraints (supplemental Table S7).

Under acidic conditions (pH 1.9), the C-terminal parts of the B-chain (B21-B30) were found disordered in both analogues despite a well defined central α-helix (B9-B20). Hence, medium to long range NOE contacts were not identified between residues B21 and B30 and the rest of the molecule (Fig. 2, A and C).

At pH 8.0, the NOE pattern indicating the reestablishment of the contacts between the core hydrophobic residues and the C-terminal loop of the B-chain was identified in the NOESY spectra of both analogues (Fig. 1) before the structure calculation. However, the overall organization of the pH 8.0 NMR structures (Fig. 2, B and D) of these analogues revealed significant differences in the conformations of their respective B-chains (Fig. 3).

The overall feature of the [HisB24]-insulin pH 8.0 NMR structure is its well ordered and defined character. Detailed analysis of the [HisB24]-insulin structure revealed that the histidine side chain is very well accommodated and stabilized in the B24 pocket not only by typical, wild-type-like nonpolar interactions (with ValB12 and LeuB15) but by new hydrogen bonds as well (Fig. 3A). The protonated B24 imidazole Nε2 atom forms a strong (2.9–3.2 Å) hydrogen bond (calculated between heteroatoms) with ValB12 CO, whereas the deprotonated imidazole Nδ1 atom forms a weaker and transient hydrogen bond (3.4–3.9 Å) with TyrB26 NH. Subsequently, the TyrB26 side chain is shifted closer to the core of the molecule compared with its position in human insulin, and subsequently its OH group is engaged in hydrogen bonds with GlyB8 CO and/or GlyB8 NH (3.8–8.8 and 3.3–9.9 Å, respectively). However, the observed ranges suggest that these hydrogen bonds may be transient or mediated by water molecules, as observed in human insulin (57).

In contrast to the ordered and compacted structure of the [His]-insulin analog, parts of the [D-His]-insulin structure at pH 8.0 are not fully defined. This is despite similar and clear reestablishment of contacts between the insulin hydrophobic core and the C-terminal loop of B-chain at pH 8.0, which were also undefined in this analogue at pH 1.9. The incorporation of the D-form of the His residue at the B24 site had a profound impact on the organization of the main chain and side chains in this region (Fig. 3B). This substitution changed the local B24-backbone topology, where the D-chirality of the B24Cε swayed the D-HisB24 away from the insulin core. The uprooting of the His...
side chain from the Phe$_{B24}$ pocket had, in turn, profound consequences on the B24 down- and upstream parts of the B-chain. Most remarkably, the cavity of the His-depleted Phe$_{B24}$ hydrophobic pocket was filled again by the Phe$_{B25}$, which mimics the “missing” d-His side chain. This retractile change of the “register” along the B23-B30 strand results in subsequent structural downshift of the Tyr$_{B26}$ into the B25 site. Here, the Tyr$_{B26}$ follows the Phe$_{B25}$-typical outward conformation.

In addition, in contrast to the [His$_{B24}$] analogue pH 8.0 structure, the B27-B30 residues depart from the insulin molecule core revealing previously fully buried Val$_{B12}$ and Leu$_{B15}$ (supplemental Fig. S3). The N-terminal arms of the B-chains (B1-B6) were found in the T-like states in both analogues, as in solution structures of monomeric insulins (7).

**MD of the Wild-type Insulin and [His$_{B24}$]- and [d-His$_{B24}$]-Insulin Analogues**

**Overall Structural Stability**—The 50-ns MD trajectories of the A-B and C-D insulin monomers derived from the 1mso dimer of human insulin (41) became stable after 20 ns with a r.m.s.d. oscillation $\sim$2.5 or 3 Å from the starting x-ray structure (Fig. 4A).

The backbone r.m.s.d. of the [His$_{B24}$]-insulin 50-ns trajectory was more stable with r.m.s.d. oscillating $\sim$2 Å from the first NMR structure. The hydrogen bond pattern of [His$_{B24}$]-insulin observed in the NMR structure of this analogue (N$_{B2}^2$-Va$_{B12}$ CO of 2.9–3.2 Å and N$_{B1}$-Tyr$_{B26}$ HN of 3.4–3.9 Å) was fully preserved throughout the whole simulation run. In contrast, the [d-His$_{B24}$]-insulin trajectory departed very significantly from the starting NMR structure (up to r.m.s.d. of $\sim$5–6 Å) and appeared stable only after 40 ns at a higher plateau of 2.6 Å (Fig. 4B).

**Segment Movements**—To understand the movements of the C terminus of the B-chain (residues B24-B28) in relation to the insulin rigid core (B7-B19 central $\alpha$-helix), five representative distances (B24C$_{B-chain}$-B15C$_{B-chain}$, B25C$_{B-chain}$-B15C$_{B-chain}$, B26C$_{B-chain}$-B27C$_{B-chain}$, B27C$_{B-chain}$-B11C$_{B-chain}$, and B28C$_{B-chain}$-B8C$_{B-chain}$) between these structural elements were measured in [His$_{B24}$]-insulin, [d-His$_{B24}$]-insulin, and in A-B and C-D monomers of human insulin in the course of the MD production runs. The C$_\alpha$ atoms of residues B26-B28 in the wild-type A-B insulin monomer (supplemental Fig. S4A) displayed significant movements; however, the B24 and B25 C$_\alpha$ atoms remained relatively stable. Smaller shifts in the initial part of the simulation were observed in the case of wild-type C-D monomers (supplemental Fig. S4B); however, they tended to converge to the A-B-like r.m.s.d. after 20 ns. All selected distances in the [d-His$_{B24}$]-analog displayed relatively high flexibility in the course of MD runs (Fig. 5B). In contrast, the B24-B28 chain in [His$_{B24}$]-insulin was very rigid (Fig. 5A) without any significant movements (cf. Fig. 4B).

**Residue Flexibility**—Positional fluctuations of the wild-type insulin and analogue residues over the 50-ns MD simulation runs are shown in supplemental Fig. S5. The largest flexibility (>500) is concentrated on the N and C termini of the B-chain in both the A-B and C-D monomers of human insulin. The [His$_{B24}$]-insulin yielded the most stable structures (supplemental Fig. S5A). This trend, increased residue flexibility of the
human insulin and [d-His]-insulin (supplemental Fig. S5B) and lower flexibility of [His]-insulin, was observed throughout the entirety of the insulin molecule.

**DISCUSSION**

The three aromatic amino acids PheB24, PheB25, and TyrB26 from the C-terminal part of the B-chain are some of the key residues identified in human insulin. These residues are important for both formation of the dimer interface of the hormone (3) and its interactions with IR (18, 20). Considering the prolonged absence of structural description of the B22-B30-chain-IR interface (5), we focused here on a more systematic probing of the role of PheB24 aromatic side chain and chirality of its Cα atom for the structure and function of the hormone. Extensive and complex data emerging from other studies on the B24 site are summarized in supplemental Table S8. Focusing on the B24 site, we have tested its functional and structural response to (i) an alternative polar aromatic side chain (His), (ii) inverse Cα chirality (d-His), and (iii) structural main chain restraints (Pro, Sar) on hormone structure and function. The
histidine side chain was selected for its partially aromatic character, similar size to phenylalanine side chain, and its imidazole hydrophobic-bond formation potential. The possible functional cooperativity between B24 and B26 sites in insulin-IR interactions was further investigated in a double \([\text{[D-HisB24, GluB26]}]\)-insulin mutant and its variant \((\text{[D-HisB24, GluB26, LysB28, ProB29]}-\text{insulin})\) (Table 1).

The replacement of Phe\textsubscript{B24} by any \(\text{L}\)-amino acid disrupts insulin biological activity and affinity (supplemental Table S8). The binding affinities of non-aromatic Leu\textsubscript{B24}, Ser\textsubscript{B24}, and Ala\textsubscript{B24} insulin analogues do not exceed 20\% in most cases. Interestingly, the simultaneous loss of the side chain and chirality at the B24 position \((i.e.\ \text{GlyB24]-insulin})\) is relatively well tolerated, yielding affinities in the range of 22–78\%. In contrast, the replacement of Phe\textsubscript{B24} by \(\text{D}\)-amino acids enhances binding affinity in most of the analogues \((i.e.\ \text{[D-AlaB24]}-\text{insulin}, 150\%; \text{[D-PheB24]}-\text{insulin}, 140–180\%), with an exception for the \(\text{[D-ProB24]}\) analogue. Double B24/B26 mutants followed this trend as the \([\text{D-TyrB24, PheB26]}\)-insulin possesses 163\% affinity \((\text{[PheB26]}-\text{insulin itself displays 45\% binding affinity (3, 58)})\), whereas its \(\text{L}\)-amino acid variant \([\text{TyrB24, PheB26]}\)-insulin is inactive \((2\%)\). Interestingly, the incorporation of an achiral amino acid derivative with a pair of methyl groups attached to the \(\text{C}^\alpha\) carbon atom, \(\alpha\)-aminoisobutyric acid, at the B24 site results in an \([\text{AibB24]}\)-insulin analogue with no potency \((0.3\%)\).

Therefore, the high affinity \((212\%)\) of the \([\text{[HisB24]}-\text{insulin and weak binding of [HisB24]}-\text{insulin (1.5\% affinity) obtained here were in agreement with trends observed in available data. However, they did not provide immediate explanation of the binding pattern observed in B24-site insulin mutants. Therefore, a comprehensive structural characterization of \([\text{HisB24]}\)- and \([\text{[HisB24]}\)-insulins was undertaken in this study. NMR structures were determined in parallel at acidic and basic pH values \((i.e. \text{pH} \sim 1.9 \text{ versus } \text{pH} 8.0)\) to avoid possible bias resulting from the pH and buffer effects on insulin conformation \((55)\). Additional insights were obtained from theoretical MD simulations.

Indeed, the crystal and NMR structures of \([\text{HisB24]}\)-insulin and \([\text{[HisB24]}\)-insulin analogues obtained here provide rational explanation of the importance of an aromatic invariance of the B24 site and underline the conservation of the structural integrity of the Phe\textsubscript{B24} and its pocket for an effective hormone functionality.

The striking feature of both the crystal and NMR structures of \([\text{HisB24]}\)- and \([\text{[HisB24]}\)-insulins is the widespread disorder of B20-B30 chains at pH 1.9 compared with their well- and reasonably ordered conformations at pH 8.0 (Fig. 2). A similar disorder of the B20-B30 part of insulin has been observed in acidic pH NMR structures of other B24 mutants such as \([\text{GlyB24]}\)-insulin \((7, 59)\), \([\text{GlyB24, GluB16, desB30]}\)-insulin \((55)\) and in \([\text{[AlaB24]}-\text{insulin})\) \((17)\). Hence, it is possible that this phenomenon arises from the structurally deleterious effects of low pH and/or acetic acid on some insulin analogues \((55)\). However, it cannot be excluded that disorder of \([\text{HisB24]}\)-insulin and \([\text{[HisB24]}\)-insulin analogues at pH 1.9 arises from the repulsive effect of the doubly protonated His\textsubscript{B24} that cannot be accommodated in the B24 hydrophobic cavity. Therefore, we focused here on analysis of more physiological pH 8.0-derived insulin structures and their defined conformations.

The NMR spectra at pH 8.0 yielded a well ordered and compact structure of \([\text{HisB24]}-\text{insulin (Fig. 2B)})\). Its main feature is a stable accommodation of the N\textsubscript{des}-monoprotonated imidazole ring inserted in the B24 hydrophobic cavity. Interestingly, the histidine side chain is accommodated there even more firmly than the phenyl moiety of Phe\textsubscript{B24} as it thrusts into the cavity \(\sim 1.2\) Å deeper than the Phe\textsubscript{B24} phenyl ring of the wild-type insulin. This His\textsubscript{B24}-related compactness of the structure is a likely result of the combination of the combined effects of the still-present aromatic character of the B24 imidazole with its hydrogen bonding capability. In addition to close van der Waals imidazole B24-Leu\textsubscript{B15} contacts, the side chain of His\textsubscript{B24} is anchored firmly by strong B24N\textsubscript{H}2-COVal\textsubscript{B12} and weaker B24N\textsubscript{A1}-NHTyr\textsubscript{B26} hydrogen bonds (Fig. 3A). This tight engagement of His\textsubscript{B24} within the B24 cavity has a precipitious effect on the remaining part of the B25-B30 chain, which is also pulled back close to the core of the hormone. This insulin wrapping conformation of the B-chain is further stabilized by Gly\textsubscript{B16}CO/\text{NH}-OH-Tyr\textsubscript{B26} direct hydrogen bonds and wedging of the Tyr\textsubscript{B26} phenyl ring between Pro\textsubscript{B28} and Ile\textsubscript{A12}. This “immobilization” and tethering of the B24-B30 chain into the [HisB24]-insulin core at pH 8.0 are likely reasons for the low affinity of this analogue and of its monomeric behavior.

The “over-conservation” of the B24-B30-fold observed in [HisB24]-insulin is in contrast with the radical rearrangement of the B20-B30 chain in the [HisB24]-insulin at the same, high pH 8.0 (Fig. 2D). Such rearrangement is a result of the pull-out of the histidine side chain from the Phe B24 pocket by the inverse chirality of the B-[HisB24] C\textsubscript{\alpha} atom and subsequent/simultaneous filling-up of the emerging empty cavity by another available aromatic side chain that is provided here by Phe\textsubscript{B25}. The locations of Phe\textsubscript{B24} and Phe\textsubscript{B25} aromatic rings in the wild-type and [HisB24]-insulins, respectively, are very similar, with a remarkable conservation of the position of the C\textsubscript{\alpha} atoms that differ by only \(\sim 0.55\) Å in both analogues (Fig. 3B).

The compensatory capability of the Phe\textsubscript{B25} to act as Phe\textsubscript{B24} structural substitute has also been observed in the pH 8.0 NMR structure of [GlyB24, GluB16, desB30]-insulin in which both the side chain of the B24 residue and the chirality of its C\textsubscript{\alpha} atom have been removed \((55)\). In contrast, the NMR structure of [AlaB24]-insulin \((17)\) indicated disorder of the B20-B30 part of the molecule. However, due to a lack of some NMR experimental detail there \((e.g. \text{pH at which the NMR spectra were measured})\), we suppose that the significant mobility of this chain in [AlaB24]-insulin arise from the use of low pH (deuteroacetic acid) in these NMR experiments \((17)\). The “down-register” shift of the B24-B30 chain in [HisB24]-insulin is structurally propagated there on both sides of the B24 site. For example, the B19-B22 \(\beta\)-turn is lost in favor of a larger bulge, Tyr\textsubscript{B26} occupies Phe\textsubscript{B25} position, and the B26-B30 chain departs from the insulin core. Importantly, these large scale structural rearrangements are correlated with the high affinity of this analogue.

Furthermore, it is not excluded that the B26\rightarrow B25 downshift observed in [HisB24]-insulin contributes also to the high affinity of this analogue because Glendorf
et al. (60) reported [TyrB25]-insulin with a high binding affinity (285%).

The NMR/x-ray experimental structural evidence about [HisB24]- and [D-HisB24]-insulins was confirmed by MD simulations. First, it was established that the overall mobility and dynamics of the wild-type insulin does not depend on slightly different starting models (i.e. A-B versus C-D monomers derived from the human insulin dimer, PDB code 1mso) (41). Although the C terminus of the B-chain detached sooner from the insulin core in the A-B monomer compared with the C-D one (cf. the overall r.m.s.d. in Fig. 4A), both simulations converged to stable plateaus of similar r.m.s.d. after an ∼20-ns long run. The reason for this delayed detachment in the C-D monomer is possibly due to the salt bridge between the Glyα1 N and Thrβ30 O atoms present in the x-ray structure only in this monomer and persisting in the MD up to four ns. In addition, our simulations agreed with previous insulin MD studies (61, 62); all of the simulations indicate the same range of insulin mobility and partial, approximately B25-B30, detachment of the B-chain from the hormone core. Second, the MD simulations also fully agreed with the distinct NMR conformational stabilities of [HisB24]- and [D-HisB24]-insulins. The r.m.s.d. for [HisB24]-insulin are strikingly invariant and steady, in contrast with the higher r.m.s.d. of [D-HisB24]-insulin that indicates its increased mobility that exceeds the dynamics of the wild-type insulin (Fig. 4B). Third, the analysis of selected distances between the key regions of the hormone indicates the detachment of the B25-B30 B-chain in the wild-type and [D-HisB24]-insulin but conservation of the B24 Cα region (Fig. 5).

Direct correlation of the affinities of [HisB24]- and [D-HisB24]-insulin with their contrasting structural features observed here, i.e. “gluing” of the B24–B30 chain to insulin core versus “liberation” of the B25–B30 chain, respectively, confirm the established consensus about the detachment of the B-chain needed for insulin activation upon its binding to the IR. However, the scale of the detachment of the B20–B30 chain in this process is still not determined. It is debatable whether a full unfolding of the approximately B22–B30 chain is needed for insulin activation (18) or whether the effective engagement of the hormone with IR can be achieved by a more modest reshaping of the B25–B30 residues in a form similar to that observed in B26-turn containing high affinity analogues (19). The high affinity of the [D-HisB24]-insulin and its structural persistence for conservation and occupation of the PheB24 cavity by an aromatic moiety suggest that the PheB24 side chain may remain in place upon insulin activation. The maintenance of the B24 “aromatic” cavity in a very mobile and reshaped, but still highly active, analogue is in concordance with the strict preservation of the B24 side chain in high affinity analogues in which it was considered as one of the crucial invariant side chain pivots (19). In addition, it appears that [D-HisB24]-insulin, with its less rigid and more flexible structure, is better primed than native insulin for the detachment and activation of the approximately B25–B30 C-terminal β-strand upon binding to the IR.

The need for the structural invariance of the B24 Cα atoms is supported further by the low affinities of [ProB24]-insulin and [SarB24]-insulin analogues (Table 1 and supplemental Table S8) that contain the main chain constraining amino acids (63). The ProB24 and SarB24 substitutions not only result in the absence of aromatic side chains, but their specific dihedral angles impose restrictions on moderate flexibility at the B24 site, likely excluding any B25→B24-like compensatory structural downshift observed in highly active [D-HisB24]-insulin. In contrast, the dihedral ϕ/ψ angles of glycine and d-amino acids at the B24 site allow for a rearrangement of insulin molecule that leads to an effective IR binding. The structural vulnerability of the main chain at the B24 site and the sensitivity of its Cα chirality are, therefore, expressed in both aromatic pulldown (B25→B24) structural rearrangements and maintenance of the position of this atom. These underline the need for an overall conservation of the phenylalanine-like character of this site. However, it cannot be excluded that the low affinities of [ProB24]-insulin and [SarB24]-insulin analogues may result from the loss of B24 amide hydrogens, which may be important for IR binding (3).

Despite the progress in understanding the roles of individual substitutions at the B24 site, the negative-cooperative effects of double B24/B26 mutations in [D-HisB24, GluB26]- and [D-HisB24, GluB26, LysB28, ProB29]-insulins, which abolish affinity gains of single site replacements at the B24/B26 sites, are less obvious. The only one available NMR structure of [HisB24, GluB26, LysB28, ProB29]-insulin determined at pH 1.9 that shows large scale disorder of the B-chain (data not shown) observed in “acidic” structures of [HisB24]- and [D-HisB24]-insulins, and the structural effect of double [D-HisB24,GluB26] mutation remains unclear. However, the low affinity of the [D-HisB24,GluB26]-mutants may result from the unfavorable replacement of PheB25 aromatic ring by glutamic acid side chain if the B25→B24 downshift takes place in this analogue as well as in [D-HisB24]-insulin. If such rearrangement does occur there, the GluB26-side chain should occupy the position of the B25 phenyl ring in wild-type insulin, hence mimicking the PheB25→GluB25 mutation, which is most likely fully detrimental as observed in the case of similar PheB25→AspB25 substitution (64). In contrast, the B25→B24 downshift in high affinity [D-HisB24]-insulin is compensated by the move of another aromatic TyrB26 side chain into the B25 position. It is also possible that the GluB26 side chain follows the TyrB26 solvent-pointing conformation observed in [D-HisB24]-insulin and, therefore, is capable of forming hydrogen bonds to also solvent-exposed D-HisB24 and neighboring Asnα21 or Thrβ30. These interactions altogether can have a negative effect on IR binding due to the over-stabilization of this region.

In summary, our data rationalize the positive effects of d-amino acid substitutions at the B24 site. They indicate the importance of the aromatic character of the B24 side chain for insulin activity, which is demonstrated by the B25→B24 “rescue” downshift of PheB25 into the B24 site. They also underline the invariance of the position of the B24Cα atom for effective IR binding. Moreover, they also provide supporting evidence for a limited, B25 onward, unfolding of the C terminus of the B-chain during hormone activation in disfavor of its full (B22–B30) detachment. However, the actual nature of PheB25 engagement with IR requires further insulin-IR complex studies.
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