Probing the correlation between insulin activity and structural stability through introduction of the rigid A6–A11 bond

Shee Chee Ong1, Alessia Belgi2, Bianca van Lierop1, Carlie Delaine3, Sofianos Andrikopoulos4, Christopher A. MacRaid2, Raymond S. Norton2, Naomi L. Haworth1, Andrea J. Robinson5 and Briony E. Forbes2 3

From the 3 College of Medicine & Public Health, Flinders University of South Australia, Bedford Park, 5042, Australia. 
2 School of Chemistry, Monash University, Clayton, Victoria, 3800, Australia. 
4 Department of Medicine, University of Melbourne, Parkville, Victoria, 3010, Australia. 
5 Medicinal Chemistry, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville 3052, Australia. 
7 Research School of Chemistry, Australian National University, Acton, ACT 2601, Australia. 
6 School of Life and Environmental Sciences, Deakin University, Warrnambool, Victoria, 3226, Australia. 

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The development of fast-acting and highly stable insulin analogues is challenging. Insulin undergoes structural transitions essential for binding and activation of the insulin receptor (IR), but these conformational changes can also affect insulin stability. Previously, we substituted the insulin A6–A11 cystine with a rigid, non-reducible C=C linkage (“dicarba” linkage). A cis alkene permitted the conformational flexibility of the A-chain N-terminal helix necessary for high affinity IR binding resulting in surprisingly rapid activity in vivo. Here, we show that, unlike the rapidly acting Lys828Pro829 insulin analogue (KP insulin), cis dicarba insulin is not inherently monomeric. We also show that cis dicarba KP insulin lowers blood glucose levels even more rapidly than KP insulin, suggesting that an inability to oligomerize is not responsible for the observed rapid activity onset of cis dicarba analogues. While rapid-acting, neither dicarba species is stable, as assessed by fibrillation and thermodynamics assays. MALDI analyses and molecular dynamics simulations of cis dicarba insulin revealed a previously unidentified role of the A6–A11 linkage in insulin conformational dynamics. By controlling the conformational flexibility of the insulin B-chain helix, this linkage affects overall insulin structural stability. This effect is independent of its regulation of the A-chain N-terminal helix flexibility necessary for IR engagement. We conclude that high affinity IR binding, rapid in vivo activity, and insulin stability can be regulated by the specific conformational arrangement of the A6–A11 linkage. This detailed understanding of insulin’s structural dynamics may aid in the future design of rapid-acting insulin analogues with improved stability.

Since the discovery of insulin by Banting and Best, insulin therapy remains the primary treatment for type 1 (T1D) and late stage type 2 diabetes (T2D), being prescribed to effectively lower blood glucose levels (1–3). Long-acting (eg insulin glargine; Lantus®, Sanofi) and rapid-acting insulin analogues (eg lispro insulin or KP insulin®); Humalog®, Eli Lilly) currently in clinical use were developed to mimic the physiological basal-bolus insulin profile (recently reviewed in Mathieu et al. (3)). Although these analogues are undoubtedly life-savers for diabetic patients, their pharmacokinetic and pharmacodynamic performance remains suboptimal for some patients (3). Of high priority is the development of a new rapid-acting insulin analogue possessing faster onset of action and greater structural stability. To achieve this, a deeper understanding is required of the structural determinants of insulin necessary for receptor binding and function.

Insulin is a small globular protein synthesized in the pancreatic β-cells and secreted as a two-chain polypeptide comprising a 21-residue A chain and a 30-residue B chain. The secondary structure of insulin consists of three α-helices, two within the A chain (A1 to A8 and A12 to A18, respectively) and a single α-helix within the central segment of B chain (B9 to B19) (Fig. 1, A and B) (4). It is stored in pancreatic β-cells as 2Zn-coordinated hexamers that, when released into the blood stream, rapidly dissociate into active monomers (5). The monomeric form then adopts the active conformation, and in doing so reveals essential residues within the hydrophobic core for binding (6) but also primes the molecule to the formation of higher order oligomers (7–9). This is highlighted in the rapid-acting KP insulin, an analogue possessing an inversion of Pro828Lys829 in the B chain of native insulin to render the molecule essentially monomeric through disruption of the dimer interface (Fig. 1A) (10,11). As
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A consequence, KP insulin is rapid acting, but concomitant exposure of core hydrophobic residues means that KP insulin readily forms fibrils (9,12,13). This highlights the fine balance between the competing requirements for stability and conformational plasticity needed for optimal activity.

High affinity binding to the insulin receptor (IR; a tyrosine kinase receptor (14)) is achieved by interaction through two distinct binding surfaces (1 and 2) on the ligand (6,15-17). Insulin site I binding residues (also known as the “classical binding site”) are also involved in insulin dimerization (18,19) while site 2 binding residues overlap with the hexamer-forming surfaces within a 2Zn-insulin hexamer (Fig. 1A) (6,16,20). Recent crystallographic studies of an IR fragment comprised of the ectodomain and the αCT segment in complex with insulin through site I residues provided key insights into the mechanism of insulin:IR interaction (15,21). Key conformational changes in insulin required for effective IR binding were identified. Movement of the C-terminal segment (B24-B30) of the B chain from the hormone core is required for engagement with the IR αCT and L1 domains (22). It is likely that the end of the B chain opens in a zipper-like fashion (23) to allow accessibility to insulin site I-binding residues that otherwise remain buried within the hydrophobic core (21,22). Evident in our previous findings, insulin also undergoes further conformational change within the first A chain helix to enable binding. The helix rotates in order to avoid a steric clash between the first four residue sidechains and the αCT segment (24). In this conformation, residues A1 to A5 form a single α-helical turn while residues A3 to A9 adopt a wider helix conformation, approximating an (i, i+5) π-helix (24). This conformation is also evident in the μIR: insulin crystal structure (pdb entries 4oga) (21,24) and can be artificially achieved by a mutation of residue B26 that also promotes opening of the B chain (25,26).

The correct folding and stabilization of the unique three-dimensional structure of mature insulin is supported by the three disulfide linkages: two interchain (Cys³⁶-Cys⁷₀ and Cys²₀⁶-Cys¹₀⁵) and one intra-chain (Cys⁴⁶-Cys¹₁¹) (Fig. 1, A and B) (5,27). Correct disulfide combination is essential for function (27-30). The Cys³⁶-Cys⁴⁰ bond is surface exposed and holds the N-termini of the two chains together. Both Cys⁴⁶-Cys¹₁¹ and Cys²₀⁶-Cys¹₀⁵ linkages are buried within the hydrophobic core. Once folded, the Cys²₀⁶-Cys¹₀⁵ disulfide is constrained in a fixed configuration and buried deeply within the core, suggesting its most likely function is to maintain structural stability. The intra-chain Cys⁴⁶-Cys¹₁¹ cystine, although buried, is relatively flexible and can adopt several different configurations (30-32). In our recent study, we identified that the Cys⁴⁶-Cys¹₁¹ linkage is an important modulator of the structural transitions of the N-terminal A chain helix required for insulin activity. Introduction of two isomeric fixed A6-A11 dicarba bridges in insulin did not perturb the overall structure but resulted in strikingly different receptor potencies, where the cis isomer permits high affinity IR binding and the trans isomer does not (Figs. 1C, 2A and (24)).

Arising from our study of the cis dicarba insulin was the observation that this analogue promotes a more rapid lowering of blood glucose levels than native insulin. It is also less thermodynamically and chemically stable than native insulin (24). As these properties are reminiscent of the monomeric KP insulin (7,9,33), we investigate here whether the A6-A11 linkage not only controls A chain flexibility but also influences the B chain conformation, leading to the monomeric state required for rapid receptor engagement.

In this study, through a combination of biophysical analyses of cis dicarba insulin and the monomeric cis dicarba KP insulin counterpart, we show that, despite its rapid action in vivo and its accelerated fibril formation relative to native insulin, the cis dicarba insulin is not inherently monomeric. Limited proteolysis studies alluded to an unexpected conformational change in the B-chain helix. Such a link between the A6-A11 disulfide and the conformational dynamics of the B chain has not been previously described. These findings suggest a key role for the A6-A11 linkage, not only in regulating A chain flexibility that primes insulin for receptor engagement, but also in influencing the B chain conformation and regulating insulin’s stability.

RESULTS

Chemical synthesis of dicarba insulins – We aimed to understand the mechanism by which the cis dicarba insulin was apparently rapid acting in vivo by directly comparing its in vitro and in vivo biological and biophysical activities with the monomeric c[Δ⁴A6,11]-dicarba human lispro insulin (cis- and trans dicarba KP insulins). The cis- and trans-configured dicarba insulin A chains, in which a C=C dicarba bond replaces the A6-A11 intra-chain S-S bond (Fig. 1C), were synthesised as previously described using a RCM and SPPS-catalysis approach (24,34). The modified dicarba insulin A chains were then combined with requisite insulin B chains to provide cis and trans isomers of c[Δ⁴A6,11]-dicarba human insulin and c[Δ⁴A6,11]-dicarba KP insulin. The dicarba analogues were purified by RP-HPLC (see Figs. 1, D and E, and S1) before being subjected to biological and biophysical analyses.

Cis dicarba insulin and cis dicarba KP insulin are equally potent to native insulin in receptor binding and activation – The binding affinities of the dicarba insulin analogues for the IR-B and IGF-1R were determined using competition binding assays (Figs. 2A, S2 and Table S1). Notably, the restrained A6-A11 cis and trans dicarba C=C bonds had the same effect when introduced into the monomeric KP insulin analogue as was previously seen with the cis and trans dicarba analogues of human insulin (24). Cis dicarba insulin and cis dicarba KP insulin were equipotent to native insulin in binding and activation of both the IR-B (Fig. 2, A and B) and the IGF-1R (Fig. S1, A and B) (Table S1), suggesting that the restrained cis dicarba...
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C=C bond allows both analogues to adopt a conformation that engages with both receptors in a manner similar to insulin. Conversely, trans dicarba insulin and trans dicarba KP insulin bind poorly to both the IR-B (Fig. 2A) and IGF-1R (Fig. S1A) (Table S1), indicating that the trans dicarba configuration restricts both analogues from forming a high affinity interaction with these receptors. The trans dicarba insulin analogues were subsequently excluded in this and further activity assays due to their poor receptor binding affinities.

The cis dicarba KP insulin promotes in vitro DNA synthesis and glucose uptake with equal potency to native insulin – Corresponding with its IR-B binding and activation potency, the cis dicarba KP insulin was equipotent with native insulin in promoting DNA synthesis in L6 rat skeletal myoblast overexpressing IR-A. This is in contrast to the cis dicarba insulin, which was 5 to 10-fold less potent than insulin in promoting mitogenic activity (Fig. 2C and (24)). The cis dicarba KP insulin was equipotent with cis dicarba insulin and insulin in promoting glucose uptake in cultured NIH3T3-L1 adipocytes (Fig. 2D). There is a trend of lower activities for the cis dicarba insulin and cis dicarba KP insulin, however the effect was not significantly different.

The cis dicarba insulin demonstrates identical self-association behaviour to native insulin – AUC was performed in order to determine whether the cis dicarba insulin is monomeric as suggested by its rapid action in vivo (Fig. 2, E and F, and (24)). In the presence of Zn²⁺, sedimentation equilibrium data for the cis dicarba insulin was fitted to a single species of apparent mass 34,500 ± 400 Da (Fig. 3A), consistent with the expected mass of a 2-Zn²⁺ human insulin hexamer (34,726 Da). At higher concentrations the fit was not perfect (reduced χ² ~ 4), suggesting the presence of other high molecular weight species, consistent with previous observations for native mammalian insulins (35).

In contrast, the shape of the equilibrium concentration distributions in the absence of Zn²⁺ were clearly concentration dependent, implying reversible self-association (Fig. 3B). Accordingly, these data could not be fit as a single species, in sharp contrast to those we have recently described for the strictly monomeric venom insulin of Conus geographus (Fig. S11) (36). Native insulin shows similar concentration dependence in the shape of its equilibrium concentration distributions (Fig. S11), and also fails to fit to single-species models, consistent with its expected tendency for self-association. For an initial model-free assessment of the self-association of Zn²⁺-free cis dicarba and native insulin, the data were plotted as the square of the radial position scaled by rotor speed [α²(τ²-τ₀²)] vs the logarithm of the equilibrium concentration (Fig. 3C). The slope of such a plot is proportional to the weight-average molecular weight of all species present at each point in the cell, and the observed non-linearity confirms the presence of multiple species in the sample. Over much of the accessible concentration range, the slopes of the cis-dicarba and native insulin plots are similar, implying that the self-association behaviour of the two insulins are similar. At low concentration (< 10 μM), the slope was consistent with that expected for monomeric insulin, suggesting that monomer dominated at these concentrations. The slope increased with increasing concentration, indicating that oligomeric species were dominant over much of the experimental concentration range. Only at the highest concentrations is there evidence of some divergence between the two curves, suggesting the possibility of some difference in the tendency of cis dicarba insulin to form higher-order oligomers.

Attempts to fit the two datasets to a specific, consistent model of self-association were unsuccessful, due to numerical instabilities in relevant models (37), and perhaps also reflecting the putative subtle difference in higher-order oligomerisation. Nonetheless, under the conditions studied, the self-association behaviour of the cis dicarba insulin is qualitatively similar to that of native mammalian insulins. Like native insulin, cis dicarba insulin is only monomeric in the absence of Zn²⁺, and only at low concentration.

Cis dicarba KP insulin lowers blood glucose levels more effectively and more rapidly compared to the cis dicarba insulin, KP insulin and native human insulin – Having established that the cis dicarba insulin is not monomeric we sought to compare the in vivo activities of this analogue with cis dicarba KP insulin, which we can assume is monomeric as per KP insulin. The cis dicarba KP insulin lowered blood glucose levels more effectively and more rapidly compared to native insulin and KP insulin when mice were treated with 0.75 IU/kg insulin or analogue under non-fasting conditions (Fig. 2, E and F). Notably, the cis dicarba KP insulin was even more effective and rapid acting than the cis dicarba insulin. The glucose-lowering effect was most evident and significant in insulin-resistant mice fed on high fat diet (Fig. 2F). The difference in activities between native and cis dicarba KP insulin, which are both expected to be monomeric, implies that the improved activity of the cis dicarba analogues is not the result of a change in self-association. This is consistent with the above observation that native and cis dicarba insulin show similar self-association behaviour.

Cis dicarba insulin and cis dicarba KP insulin more rapidly form fibrils than native human insulin – While the rapid action of the cis dicarba insulin compared to insulin is not due to this analogue being monomeric, it must have different biophysical properties to insulin that lead to this difference in biological activity. To explore this further we next investigated the ability to form fibrils in an AFM fibrillation assay. The cis dicarba insulin formed fibrils more rapidly than native insulin, with fibrils first detected after 2 h compared to 6 h for native insulin at the same temperature and concentration (60 °C and 1.16 mg/mL, respectively, Fig. 4). Consistent with being monomeric, KP insulin also rapidly formed fibrils, with fibrils first detectable at 2 h. The cis dicarba KP insulin fibrillation is evident between t = 6 – 8 h. Interestingly, the fibrils formed by both cis
dicarba insulin and cis dicarba KP insulin appeared shorter, thicker and of different morphology to those arising from native insulin and KP insulin (Fig. 4; see cis dicarba insulin at t = 15 h and cis dicarba KP insulin at t = 8 h). These observations are consistent with the fact that thioflavin T (ThT), a dye commonly used to detect insulin fibrils, did not bind cis dicarba insulin fibrils (data not shown). ThT normally binds to insulin fibrils at two sites (between fibers and/or between protofilaments) (38). Our data suggest that while cis dicarba insulin is not inherently monomeric it is conformationally different to native insulin.

Cis dicarba insulin and cis dicarba KP insulin are thermodynamically less stable than native insulin – Next, we compared the thermodynamic stability of the cis dicarba insulin with the cis dicarba KP insulin. Introduction of a dicarba A6-A11 bond into KP insulin also led to a decrease in thermal and chemical denaturation stabilities. The far-UV CD spectra (190 – 260 nm) of Zn2+-free cis- and trans dicarba KP insulin exhibited lower helical content (34 % and 17 %, respectively) compared to KP insulin (44 %) (see Fig. S4 and Table 1). The [0]222 values are directly proportional to the helical content of the proteins. As seen with the cis dicarba insulin (24), the cis dicarba KP insulin (Fig. 5B) exhibited smaller [0]222 negative magnitudes measured at 20 °C consistent with their lower initial helical content (Table 1). Denaturation induced by increasing temperature (20 – 70°C) (Fig. 5B) or GdnHCl concentrations (0 – 8M) (Fig. 5C) was monitored at wavelength 222 nm using CD. The relative thermodynamic stabilities of KP insulin were determined by comparing the relative change of ellipticity with increasing temperature at 1 °C intervals, i.e. by comparing the slope of temperature denaturation curves. Native and KP insulins exhibited similar thermal denaturation curves, whereas the cis dicarba KP insulin exhibited relatively small changes in ellipticity due to its significantly lower starting helical content (Fig. 5B and Table 1). The cis dicarba KP insulin is also considerably less stable upon GdnHCl denaturation, with ∆G°u = 1.71 kcal mol−1, as was seen with the dicarba insulin isomers (Fig. 5C, Table 1 and (24)).

The effect of introduction of the cis dicarba bond on thermodynamic stability is striking and much greater than the introduction of the KP mutation (Fig. 5, B and C, and (24)). The observed reduction in stability is thus independent of being monomeric.

The cis dicarba A6-A11 linkage promotes a structural change in the B chain helix, which leads to instability – In order to explain why the cis dicarba peptides are less thermodynamically stable, we performed a limited proteolysis study that allowed us to detect structural differences between the cis dicarba peptides, native insulin and KP insulin. This involved RP-HPLC separation of fragments generated by chymotrypsin proteolysis and subsequent mass spectrometry. Firstly, we observed that cis- and trans dicarba insulin isomers eluted later (15.8 and 14.6 min, respectively) than native insulin (13.3 min) when separated by RP-HPLC (Fig. 1D). Similarly, the cis- and trans KP isomers also eluted with delayed retention times (16.3 and 15.1 min, respectively) compared to KP insulin (13.5 min) (Fig. 1E). The relative delay in the retention times of dicarba insulins is an indication of an apparent increase in hydrophobicity compared to the native forms, with cis dicarba insulins being more surface hydrophobic. This was the first indication that the cis A6-A11 dicarba linkage induces a more open conformation of insulin than in the native hormone, and that it is likely that hydrophobic residues of the core are more exposed.

The enzymatic stability of dicarba insulin analogues was investigated through limited proteolysis by chymotrypsin under non-reducing conditions. The rate and kinetics of proteolysis were monitored through RP-HPLC as described in the Experimental Procedures. Our results show that cis dicarba insulin is significantly more rapidly cleaved by chymotrypsin compared to native insulin (Fig. 6A). At a protein: enzyme ratio of 86: 0.078 µM, the native insulin remained almost completely undigested after 3 h of proteolysis (> 95 % undigested peptide remaining), whereas almost no intact cis dicarba insulin remained. The monomeric KP insulin is more susceptible to proteolysis than insulin with ~ 65% undigested peptide remaining after 3 h (~ 30% reduction compared to native insulin). However, the cis dicarba KP insulin is rapidly cleaved with only ~ 30% of undigested peptide remaining at 3 h (~ 30% reduction compared to KP insulin). As different batches of chymotrypsin were used in the cis dicarba insulin and cis dicarba KP insulin experiments (Fig. 6A vs Fig. S4) we cannot determine if the difference in cleavage rates between the two cis dicarba analogues is significant. Clearly both are much more rapidly cleaved than native insulin and KP insulin, respectively.

Our RP-HPLC chromatograms of a chymotrypsin-cleaved insulin (Fig. 6B; also see Fig. S3E) and the cis dicarba insulin (Fig. 6C; also see Fig. S3F) were comparable to previously reported data, which identified 4 non-reduced metabolites (termed A, B, C, D) post-cleavage (39). Through positive ion mode of MALDI mass analysis of the entire digest, we detected native insulin metabolites A, C and D following the chymotrypsin digest (protein: enzyme ratio of 86: 0.078 µM; t = 60 min) but metabolite B was not detected (see chromatograms in Figs. 6B and S3E, MALDI analysis in Fig. S5 and schematic diagram in Fig. S7). By analysis of individual RP-HPLC fractions we could assign the different masses to individual peaks (Fig. S5 and S6; tables in bottom panel). Metabolite A eluted ≅ 1 min after the undigested peptide (Fig. 6, B and C), as was seen for all digested insulins (native and dicarba analogues) (labelled as 2 (grey) in Figs. S3 and S4, E – G). Peptides equivalent to metabolite C and metabolite D eluted at tq ≅ 3.5 min and ≅ 9.5 min respectively for all insulins (see Fig. 6, B, C and F; labelled as 3 (Metabolite C, orange) and 4 (Metabolite D, green) in Figs. S3 and S4, E – G).
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Close comparison of the RP-HPLC chromatograms revealed the presence of an apparently unique metabolite in chymotrypsin digests of cis dicarba insulins (Metabolite E in Fig. 6C; Metabolite 5, cis dicarba KP insulin Fig. S4F) that was not detected in native insulin (Fig. 6B) or KP insulin (Fig. S4E). Metabolite E was detected in proteolytic samples of cis dicarba insulin as early as \( t = 1 \) h (kinetics of cleavage shown in Fig. S3C and F). Analysis from MALDI data in negative ion mode identified the cis dicarba insulin metabolite E from a fractionated and reduced sample (Fig. 6D). As shown in the simplified schematic diagram, Figure 6F (orange box), the new metabolite is a full-length two-peptide (with intact A6-A11 dicarba bond and A7-B7 and A19-B20 disulfide bonds) with a single cleavage at the C-terminal end of Tyr\(^{B16}\) of B chain (indicated in cis dicarba insulin crystal structure in Fig. 6E, blue circled 1). This new metabolite E was not identified in native insulin (Figs. 6B, S3 and S5) or KP insulin (Fig. S4) cleaved with chymotrypsin, suggesting that chymotrypsin is able to access Tyr\(^{B16}\) in cis dicarba insulin more readily than in insulin or KP insulin.

In order to understand why only cis dicarba insulin is cleaved at this new site, we superimposed insulin structures on the active site of chymotrypsin, ensuring that the tyrosine residue that is recognised by the enzyme was localised in the appropriate binding pocket (Fig. 7). Significant steric interactions between the insulin structure and chymotrypsin (Fig. 7B) revealed that it would be necessary for the B chain helix of insulin to bend significantly to allow Tyr\(^{B16}\) to engage with the chymotrypsin enzyme. Such bending behaviour is evident in our previously reported MD simulations of insulin and its cis dicarba analogue (24). It is significant that this bending motion occurs more frequently and persists longer in cis dicarba insulin (see below). Hydrogen bonds between Val\(^{B12}\) and Tyr\(^{B16}\), Glu\(^{B13}\) and Leu\(^{B17}\), and Tyr\(^{B16}\) and Gly\(^{B22}\) must all be broken, allowing the Ca-Ca distances between Glu\(^{B13}\) and Tyr\(^{B16}\) and between Tyr\(^{B16}\) and Gly\(^{B22}\) to increase from their unperturbed values of 5.5 Å to ≥ 7 Å (Fig. 7A). This allows Tyr\(^{B16}\) to occupy the chymotrypsin binding pocket, with the two neighbouring loops of the helix wrapping around the binding pocket walls (compare Fig. 7B vs 7C). These changes in the B chain helix structure are concomitant with the twisting of Glu\(^{B13}\) - Tyr\(^{B16}\) Ca-Ca distance (green). The corresponding Cys\(^{A7}\) - Cys\(^{B7}\) Ca-Ca distances (purple) and the strain in the A7-B7 disulfide linkages (blue) are also shown. It is clear that, while bending of the B chain helix can occur in both insulin and the cis dicarba insulin, it occurs far more frequently in the cis dicarba analogue (Fig. 7A, A and B, outlined in black boxes), with the bulging events being more prolonged and showing increased spreading of the loops of the helix (Fig. 7A). As noted previously, the A chain N-terminal helix is far more labile in the cis dicarba insulin than in insulin (24). It appears that in the more stable insulin structure, the A6-A11 disulfide linkage rapidly damps the bending of the B chain helix and restores its helical structure. In contrast, this conformational lability of αAN in the cis dicarba insulin allows the bulge in the B chain helix to form more readily and to persist long enough to allow engagement with and digestion by chymotrypsin. It is important to note that there does not appear to be any correlation between bulging of the B chain and the unwinding motion of the A chain necessary for complexation of the insulin receptor (Fig. S9).

A second important consequence of enhanced B chain bulging for the cis dicarba insulin is that the hydrophobic core of the hormone is opened up, resulting in increased exposure to solvent (Fig. 7A, red). This is consistent with the longer observed elution time of the cis analogue in the RP-HPLC experiments (Fig. 1D).

In summary, the limited proteolytic-MS integrated analyses revealed that the kinetics of dicarba insulins proteolysis is different from those of native insulin, leading to the formation of new metabolites. This supports the notion that installation of the intra-chain dicarba bridge enhances structural perturbation near Tyr\(^{B16}\) to permit access of chymotrypsin to this site.

DISCUSSION

For the last decade, insulin analogue design has focused on improving insulin efficacy and stability. Ideally, we require new rapid-acting insulin analogues that perfectly mimic the normal rapid onset of bolus insulin action. Desirably, insulin analogues would also be physically and chemically stable during pump delivery or at sites of injection. The ‘bottleneck’ to creating the perfect insulin arises from our incomplete understanding of the relationship between insulin’s structure and function, particularly with respect to the fine balance between activity and stability.

Previously, we reported the chemical synthesis of two A6-A11 dicarba insulin analogues (cis- and trans dicarba insulins). Using these stereoisomers, we obtained remarkable insight into the previously unexplored function of the insulin A6-A11 disulfide bond in modulating insulin activity. Unique to these insulin analogues, only cis dicarba insulin is biologically active while trans dicarba insulin is inactive. We proposed that the underlying cause of this difference lies in the structural dynamics of the A6-A11 linkage, and that this dictates insulin’s ability to transition into its active conformation. We demonstrated that the configuration of the A6-A11...
linkage could modulate insulin’s ability to engage with the receptor through its influence on the conformational flexibility of the N-terminal A chain helix (24).

In the current study, we seek to explain why in vivo the cis dicarba insulin promotes more rapid lowering of blood glucose than native insulin. Taken together with our observation of reduced thermal and chemical denaturation stabilities we hypothesised the rapid action might be attributed to the cis dicarba insulin being monomeric. To address this, we undertook biophysical analyses of the cis dicarba insulin in comparison to the cis isomer of dicarba KP insulin, which we assume is monomeric as per KP insulin.

We first compared receptor binding and biological activity of the dicarba KP insulins with the cis- and trans dicarba insulins and native insulin. Consistent with the unique biological characteristics of cis- and trans dicarba isomers of native insulin, the cis dicarba KP insulin was also equipotent to native insulin (receptor binding, receptor activation, DNA synthesis in myoblasts and glucose uptake by adipocytes) while the trans dicarba KP insulin was inactive. This confirms the functional role of the native A6-A11 cystine bridge. Even in the context of a disrupted dimer interface induced by the B chain C-terminal KP mutation, the A6-A11 bond still influences the ability of insulin to engage with the receptor.

Next, we determined that the cis dicarba insulin is not inherently monomeric. The AUC results clearly showed that the distribution of cis dicarba insulin into monomeric and dimeric forms under zinc free conditions was similar to that of native insulin. This was surprising and prompted us to further explore the biophysical differences between the cis dicarba insulin and native insulin that might account for the more rapid action of the cis dicarba analogue.

Further evidence of structural differences between the dicarba insulins and native insulin was provided by our fibrillation assays. The qualitative differences in fibrillation rate and fibril conformation between the cis dicarba insulin and native insulin are additional indicators of conformational differences in their structures. It is well established that the movement of the B chain C-terminus away from the B chain helix and hydrophobic core promotes fibrillation, as is seen with KP insulin (Fig. 4 and (8,33,40,41)). The AUC results show that the cis dicarba insulin is not inherently monomeric, suggesting that the dimer interface is not disrupted, and hence this is not the source of the increased fibrillation rate of the cis dicarba insulin. Another key feature of fibril formation is the transition of the A chain N-terminal helix to a β-sheet, a process which requires displacement of the A chain away from the B chain helix and from the hormone core (9,33). Therefore, we postulated that cis dicarba insulin’s increased rate of fibrillation compared to native insulin was likely to be connected to its increased flexibility and altered helicity (more π-like) at the N-terminus of the A chain.

However, upon further investigation using limited chymotrypsin proteolysis we were able to detect an unexpected difference in the structure of the B chain between the cis dicarba insulin and native insulin. The initial cleavage in the cis dicarba insulin occurs at the C-terminal end of TyrB16 of B chain. This site is not the first site of cleavage in native insulin (the product was not detected), suggesting that the enzyme is unable to readily access this site in fully intact native insulin; cleavage at TyrB16 only occurs after the molecule has been cleaved at other sites. In the cis dicarba insulin, initial cleavage at TyrA16 indicates that chymotrypsin can readily access this site, implying that the C-terminal end of the B chain has a tendency to be in a non-native, partially open, or bulged, conformation, thereby allowing enzyme access. Interestingly, this bulged conformation does not affect the ability of the cis dicarba insulin to bind the insulin receptor. Previous mutation studies at TyrB16 (e.g. to His or Ala) highlighted the importance of this residue in receptor binding as well as being involved in the dimer interface. Interestingly, the TyrB16-Ala insulin mutant behaves as a monomer on size exclusion chromatography (42).

Previously, MD simulations have captured insulin in both “open” and “wide open (receptor bound)” states (23). In that study, the open state referred to a zipper-like opening from the end of the B chain. This state was also observed in our MD investigations of native insulin and its cis dicarba analogue. However, here we additionally observe that simulations of the cis dicarba insulin show a significantly enhanced propensity for outward bulging of the B chain helix. This opens up the helix loop between ValB12 and LeuB17, exposing TyrB16 for chymotrypsin cleavage (see Fig. 7). Hence, the cis dicarba linkage causes two fundamental changes to the dynamics of the insulin structure. On the one hand, it increases the mobility of the A chain N-terminal helix, enhancing its ability to engage favourably with the insulin receptor. On the other, it decreases the stability of the overall structure. This is seen in both the destabilisation of the B chain helix, increasing its susceptibility to chymotrypsin digestion, and in an increase in the solvent exposure of the hydrophobic core of insulin, leaving the hormone vulnerable to degradation via the formation of fibrils.

Recently Wade et al reported that the dicarba substitution of the intra-A-chain disulfide bond of the insulin-like peptide H2 relaxin resulted in significantly reduced stability to enzyme degradation in plasma, despite maintaining the ability to bind relaxin’s cognate receptor RXFP1. The mechanisms underlying the instability were not explored. Similar to dicarba insulins, dicarba H2 relaxins also displayed structural differences to the native peptide (43). We postulate that the role of intra-A-chain disulfide bond in regulating peptide stability is conserved across the insulin-relaxin superfamily. However, further investigation is required to confirm this.

The rapid action of both cis dicarba insulin and cis dicarba KP insulin in lowering blood glucose (in comparison with native insulin) is most likely
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explained by the increased mobility of the A chain N-terminal helix, as observed in our MD simulations. This enhances the ability of cis dicarba insulin to adopt a conformation consistent with IR engagement. By restraining the A6-A11 linkage through introduction of a cis dicarba bond, the bioactive conformation of the molecule is favoured, and hence the in vivo metabolism of glucose is promoted. It is as yet unclear whether the accompanying destabilisation of the B chain helix also contributes to the enhanced glucose consumption.

In conclusion, through introduction of a non-reducible A6-A11 dicarba bridge of fixed configuration into insulin and KP insulin we reveal the functional and structural roles of this linkage. It not only regulates structural flexibility at the N-terminus of the A chain helix, which is necessary for receptor binding, but also influences the frequency at which the B-chain helix “bulges”, thereby increasing insulin mimetic’s vulnerability to heat, chemical and enzymatic degradation.

Our findings suggest there is potential for the development of ultra-rapid insulin analogues through only minimal manipulation of native insulins or existing insulin analogues. Importantly, this study provides a deeper insight into the function of the A6-A11 bond in regulating the balance between optimal insulin potency and structural stability. We anticipate that this detailed understanding of the structural dynamics of insulin will aid in future design of rapid-acting insulin analogues with improved stability. Defining the determinants of receptor binding (including mechanisms driving necessary A- and B-chain flexibility), stability and fribillation will allow us to design analogues that permit high affinity binding but avoid instability and fibrillation.

EXPERIMENTAL PROCEDURES

Materials – Actrapid® insulin was purchased from Novo Nordisk Pharmaceuticals Pty Ltd. Humalog® lispro (KP insulin) was obtained from Eli Lilly Australia. Hybridoma cells expressing antibodies specific for the IR alpha subunit (83-7) and the insulin-like growth factor 1 receptor (IGF-1R) a-subunit (24-31) were a kind gift from Prof. K Siddle (44-46). [3H]-Thymidine and Eu-PY20 were purchased from Perkin Elmer Life Sciences. Sequencing grade chymotrypsin was purchased from Promega. The sodium salt of iodoacetic acid (IAA), HPLC grade acetonitrile (CH3CN) and TFA and DTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). Iodoacetamide (IAM) was a product of Bio-Rad (Hercules, CA, USA). All antibodies were purchased from Cell Signalling Technology (Danvers, MA) unless specified. The matrices for MALDI-TOF-MS (sinapinic acid (SA) and α-cyano-4-hydroxycinnamic acid (HCCA)) were products of Bruker Daltonics (Leipzig, Germany). All other reagents used were analytical grade.

Cell lines and cultures conditions – hIR-A and hIR-B overexpressing cells (RIR-A and RIR-B, respectively) were constructed as described in (47) using R fibroblasts (derived from IGF-1R knockout mouse embryonic fibroblasts) - a kind gift from Professor R. Baserga (Philadelphia, PA)(48). L6 rat skeletal myoblasts overexpressing human IR-A (hIR-A L6) were kindly provided by Dr B.F. Hansen (Novo Nordisk A/S, Denmark) (49). P6 cells (BALB/c3T3 cells overexpressing the human IGF-1R) were from Professor R. Baserga (50). All cells were maintained at 37 °C; 5% CO2. R IR-A, R IR-B, hIR-A L6 and P6 cells were maintained in Dulbecco’s minimal essential medium (DMEM) High Glucose (4.5 g/mL) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/l penicillin and 100 µg/L streptomycin. All cell culture media and supplements were purchased from Thermo Fisher Scientific Australia.

Chemical synthesis of dicarba insulin – The synthesis of c(A6A6,11)-dicarba insulin (cis- and trans dicarba insulins) (24) and c(A6A6,11)-dicarba KP insulins (cis- and trans dicarba KP insulins) were essentially the same. Synthesis of dicarba A chain was achieved through an interrupted solid-phase peptide synthesis (SPPS)-catalysis and ring-closing metathesis (RCM) procedures (24,34). Construction of insulin and KP insulin B-chain was achieved through microwave-accelerated SPPS. The monocyclic A-B conjugates were prepared by combination of the dicarba A chains with the insulin (or KP insulin) B chain under basic conditions resulting in spontaneous oxidation of the liberated free thiol groups generating cis- and trans dicarba insulins (or cis- and trans dicarba KP insulins). The details of the synthesis method for the dicarba KP insulins are provided in the Supporting Information. The synthesis method for dicarba insulins have been previously described in (24).

Immunocaptured-receptor binding assay – IR-A, IR-B and IGF-1R binding was measured essentially as described by Denley et al. (47). Human IR isoform A (IR-A), isoform B (IR-B) and IGF-1R were solubilized from R IR-A, R IR-B and P6 cells, respectively. Briefly, cells were serum-starved in serum free media (SM) containing 1% BSA for 4 h before lysis in ice-cold lysis buffer (20 mM HEpes, 150 mM NaCl, 1.5 mM MgCl2, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride, pH 7.5) for 1 h at 4 °C. Lysates were centrifuged for 10 min at 2,200 × g, then 100 µL lysate was added per well to a white Greiner Luminetra 600 96-well plate previously coated with anti-IR antibody 83-7 or anti-IGF-1R antibody 24-31 (250 ng/well in bicarbonate buffer pH 9.2). Approximately 500,000 fluorescent counts of europium-labelled insulin (Eu-insulin, prepared in-house) were added to each well along with increasing concentrations of unlabelled competitor in a final volume of 100 µL and incubated for 16 h at 4 °C. Wells were washed four times with 20 mM Tris pH 7.4, 150 mM NaCl and 0.1% (v/v) Tween-20 (TBST). Then 100 µL/well DELFIA enhancement solution (PerkinElmer Life Sciences) was added. After 10 min time-resolved fluorescence was measured using 340 nm excitation and 612 nm emission filters with a BMG Lab Technologies Polarstar fluorometer (Mornington, Australia). Assays were performed in triplicate in at least three independent experiments.
Kinase receptor activation assay (KIRA) – IR-A, IR-B and IGF-1R phosphorylation was detected essentially as described by Denley et al. (47). Briefly, R IR-A, R IR-B or P6 cells (5 x 10^4 cells/well) were plated in a 96-well flat bottom plate and grown overnight at 37 °C, 5% CO₂. Cells were serum-starved for 4 h before treatment with insulin or dicarba insulins in 100 µL of SFM / 1% BSA for 10 min or in a time course (0, 2, 5, 8, 12, 20, 30 min) at 37 °C, 5% CO₂. Cells were lysed with ice-cold lysis buffer containing 2 mM Na₂VO₄ and 100 mM NaF, and receptors were captured onto white Greiner Lumitrac 600 96-well plates pre-coated with anti-IR antibody 83-7 or anti-IGF-1R antibody 24-31 (250 ng/well) (44) and blocked with 20 mM Tris-HCl pH 7.4, 150 mM NaCl, and 0.1% (v/v) Tween-20 (TBST) / 0.5% BSA. Following overnight incubation at 4 °C, the plates were washed three times with TBST. Phosphorylated receptor was detected by incubation with Eu-PY20 (76 ng/well) at room temperature for 2 h. Wells were washed four times with TBST; and time-resolved fluorescence was detected as described above. Assays were performed in triplicate in at least three independent experiments.

DNA synthesis assay – DNA synthesis was carried out as described in Gaugain et al. (51). Briefly, hIR-A L6 myoblasts were plated in a 96-well flat bottom plate (1.5 x 10^4 cells/well) and grown overnight at 37 °C, 5% CO₂. Cells were starved in SFM / 1% BSA for 4 h before treatment with increasing ligand concentrations for 18 h in SFM / 1% BSA. The cells were incubated with 0.13 µCi/well [³H]-thymidine for 4 h, shaken for 2 h with 50 µL lysis buffer (40 mM Tris pH 7.5, 10 mM EDTA and 150 mM NaCl) and then harvested onto glass fibre filters (Millipore®) using a MICRO 96™ Skatron harvester (Molecular Devices). The filters were counted in a Wallac MicroBeta counter (PerkinElmer Life Sciences). Assays were performed in triplicate in at least three independent experiments.

Glucose uptake assay – Briefly, NIH3T3-L1 myoblasts (up to passage 20) grown in DMEM supplemented with 10% newborn calf serum, 2mM L-glutamine, 100 U/ml penicillin and 100 µg/L streptomycin at 37 °C were seeded into 24-well plates at 5 x 10^3 cells/well and grown for 8 days to confluence and were then differentiated into adipocytes as described (52). Glucose uptake in response to insulin and dicarba insulin analogues was measured essentially as described (53). Glucose uptake assay was performed in triplicate in at least three independent experiments.

Sedimentation equilibrium analytical ultracentrifugation (SE-AUC) – Analytical ultracentrifugation was conducted at 20 °C using a Beckman XLI analytical centrifuge in 12 mm path-length cells. Cis dicarba insulin was diluted from a 10 mg / mL stock in 10 mM HCl into 100 µL 10 mM sodium phosphate, 100 mM NaCl, pH 7.4, with or without 0.2 mM ZnCl₂ to a final concentration of 100 or 300 µg/mL. Radial concentration distributions were measured by absorbance at 230 nm. Sedimentation equilibrium was established at 25,000 and 40,000 rpm, as assessed by sequential absorbance scans 2 h apart. Data at both speeds were jointly fit to a single ideal diffusion model. Mass conservation was applied as a constraint on the fits by floating the position of the bottom of the cell. Reported errors describe the precision of the fit at 0.68 confidence level, estimated from Monte Carlo simulations as implemented in SEDPHAT.

Atomic force microscopy (AFM) – Analysis of fibrillation was monitored by AFM essentially as described (57). Briefly, lyophilized insulin, insulin lispro and dicarba isomers were re-suspended in 200 mM KCl-HCl in Milli-Q water, pH 1.6 at 200 µM (1.16 mg/mL) and incubated at 60 °C with gentle agitation. Samples (5 µL) were taken at different times, diluted 1:10 in the same buffer and immediately frozen. 30 µL of the insulin solution was dropped onto
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a freshly cleaved mica substrate. Once dried, the sample was washed dropwise with Milli-Q water, and then dried with a gentle stream of dry nitrogen. Images of the protein aggregates were recorded with a Multimode Nanoscope IV Atomic Force Microscope (Veeco Instruments, Santa Barbara, CA, USA), operating in Tapping Mode. Rigid cantilevers with resonance frequencies of 325 kHz and equipped with silicon tips (Mikromasch HQ: NSC15, Bulgaria) with nominal spring constant of 40 N/m and a nominal tip diameter of 16 nm were used. Typical scan size was 5 μm (512 x 512 points), and scan rate was 1-2 Hz. The scanner was calibrated in the x, y and z axes using silicon calibration grids (Bruker model numbers PG: 1 μm pitch, 110 nm depth and VGRP: 10 μm pitch, 180 nm depth). Images were analysed using the Nanoscope analysis program version 1.40.

Reversed-phase High Performance Liquid Chromatography (RP-HPLC) – Purification of insulin analogues was performed on a Vydac C4 analytical column (214TP5210; 5 μm, 2.1 x 100 mm, 300 Å) connected to the Agilent 1260 Infinity Quaternary LC system. Buffer A: 0.1% aqueous TFA. Buffer B: 80% CH3CN in 0.08% TFA. Peptides were eluted using a linear gradient of 20 – 25% CH3CN for 5 min, followed by a 25 – 38% CH3CN gradient over 13 min at flow rate of 0.5 mL/min. UV detection was at 280 nm and peak areas were used for quantitation of peptides.

Circular Dichroism (CD) – CD was carried out as previously described (24). Briefly, CD spectra were recorded on a Jasco J-815 CD spectrometer. Spectra were from 260 to 190 nm with a 1.0 nm step size using a 1.0 s response time and 1.0 nm bandwidth in a quartz cuvette with a 0.1 cm path length. Insulin and insulin analogues were incubated in 10 mM phosphate buffer (pH 7.4) to a concentration of 0.22 mg/mL (38 μM). Spectra were background-corrected by subtraction of the spectrum of buffer alone. Temperature denaturation was achieved by automated thermal control increasing by 2 °C/min at 1 °C intervals. Samples were diluted to 10 μM for equilibrium denaturation studies in guanidine hydrochloride (GdnHCl) (1 – 8 M). The machine unit collected, 0 in milliQ, is converted to mean residue ellipticity (MRE), [0] in degrees.cm2.dmole−1 residue−1, as follows:

\[
[0] = \theta \times \frac{(0.1 \times MRW)}{(P \times Conc.)}
\]

where MRW is the protein mean weight ((atomic mass units/daltons) / number of residues), P is pathlength (cm) and Conc is protein concentration in mg/mL. [0]222 is the molar ellipticity per residue at wavelength 222 nm.

In-solution Time-course Limited Proteolysis Assay – Limited proteolysis analyses were performed in 100 mM Tris-HCl pH 7.5 using sequencing grade chymotrypsin at final protease: peptide ratio of 1: 50 or 1: 250 (w/w); incubated at 37 °C. The final concentration of peptide was 0.5 mg/mL. The proteolytic reactions were analysed on a time-course basis by sampling 10 μL of proteolytic mixture (equivalent to 5 μg of insulin) into 100 μL 0.5% (v/v) TFA at t = 0, 1, 2 and 3 h. Digested samples were analysed and fractionated (fraction size: 0.25 mL or 0.5 mL) using RP-HPLC and lyophilised. The extents of proteolysis were analysed as follows:

\[
\text{% of Undigested Peptide} = \frac{\text{Mass of Undigested Peptide at } T = x}{\text{Mass of Undigested Peptide at } T = 0} \times 100\%
\]

Preparation of proteolysed insulin samples for mass spectrometry – Lyophilised fractions were resuspended in 20 μL of 100 mM ammonium bicarbonate (NH4HCO3). Half of the volume was kept separately as non-reducing samples for mass spectrometry analysis. The other half was reduced in 5 mM DTT for 45 min at 56 °C and alkylated with 14 mM IAM (for positive mode) or IAA (for negative mode) for 30 minutes in the dark at room temperature.

Matrix-Assisted Laser Desorption and Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) analysis – The molecular weights of intact insulin analogues and the proteolytically cleaved metabolites were identified using MALDI-TOF Autoflex™ III (Bruker Daltonics, Leipzig, Germany) equipped with a Nd: YAG laser (wavelength: 355 nm). Either the entire cleavage mixture or isolated peaks separated by RP-HPLC were subjected to mass spectral analysis using combinations of positive or negative ion with linear or reflectron mode. Insulin digest samples intended for mass analysis using positive mode were desalted using Zip-Tip C18 (Millipore®, Billerica, MA, USA), eluted with 2 μL of 0.1% TFA / CH3CN (70: 30, v/v). Samples intended for negative mode analysis were desalted and eluted with no TFA. 1 μL of each sample was spotted onto the MTP 384 ground steel target plate (Bruker Daltonics), air dried, then 1 μL of SA matrix is spotted on top of each dried sample. Calibrations were performed using Bruker Daltonics’ Peptide Calibration Standard II and Protein Calibration Standard I according to manufacturer’s instructions.

Molecular Dynamics, MD – Previously, MD simulations of insulin and its cis dicarba analogue were performed, and the different behaviours of the N-terminal A chain helix analysed in detail (24). Here, these simulation data are further analysed to give insight into the effect of the A6-A11 linkage on insulin structural stability. Details of the simulation and analysis protocols are provided in our previous report (24), and in the Supplementary Material. In brief, the AMBER14 program package (58) in conjunction with Amber ff14SB force field parameters (59) (along with calculated parameters for the dicarba linkage) were used to perform 200 ns room-temperature MD simulations for insulin and its dicarba isomers based on four different starting structures derived from high-resolution X-ray crystallography experiments. Twelve independent 200 ns simulations were performed for insulin and 16 simulations for the cis dicarba insulin. Simulation frames recorded at 200 ps intervals were used for the analysis in this paper (1000 frames per
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simulation). Data were processed and analysed using CPTRAJ, (60) along with custom computer scripts. The torsional strain in the A7-B7 disulfide linkages was estimated based on the data reported in references (61,62). Insulin structures were superimposed on the crystal structure of a chymotrypsin-inhibitor complex (PDB: 4h4f) using Swiss-PDBViewer (63) and VMD (64).

Statistical Analyses – Statistical analysis of receptor binding, receptor activation and DNA synthesis assays were performed using a 2-way ANOVA with a Dunnett’s multiple comparison. Insulin tolerance test and glucose uptake assay data were analysed with a paired t-test. Significance was accepted at P < 0.05.
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Conflict of interest: none

Author contributions: SCO performed in vitro activity assays, biophysical analyses and mass spectrometry analysis. AB and BVL conducted chemical syntheses, CAM and RSN performed the ultracentrifugation assay, CD performed the AFM assay, SA performed the insulin tolerance test, NLH conducted the MD analyses. AJR and BEF conceived and coordinated the study. SCO, RSN, NLH, AJR and BEF wrote the paper. All authors reviewed and approved the final version of manuscript.
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FOOTNOTES

This article contains supporting materials and methods, Table S1 and Figs. S1-S11.

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4 The abbreviations used are: KP insulin, insulin analogue containing reversed amino acids of Pro\(^{B28}\)Lys\(^{B29}\)→Lys\(^{B28}\)Pro\(^{B29}\); IR, insulin receptor; C=C or dicarba, unsaturated carbon-carbon bond; S-S bond, disulfide bond, T1D, type 1 diabetes; T2D, type 2 diabetes, αCT, C-terminus of the IR alpha chain; L1, leucine-rich repeat domain of IR (residues 1-157); IAA, iodoacetic acid; IAM, iodoacetamide; CH\(_3\)CN, acetonitrile; SA, sinapinic acid; HCCA, α-cyano-4-hydroxycinnamic acid; R fibroblasts, IGF-1R knockout mouse embryonic fibroblasts; hIR-A or hIR-B, human insulin receptor exon 11- or exon 11+ isoform; IGF-1R, insulin-like growth factor 1 receptor; hIR-A L6, L6 rat skeletal myoblasts overexpressing human IR-A; DMEM, Dulbecco’s minimal essential medium; FCS, fetal calf serum; SPPS, solid-phase peptide synthesis; RCM, ring-closing metathesis; SFM, serum-free media; Eu, europium; TBST, tris-buffered saline containing 0.1% (v/v) Tween 20; KIRA, kinase receptor activation assay; PY20, anti-phosphotyrosine antibody; KRP, Krebs-Ringer phosphate buffer; ip, intraperitoneal injection; 2-DOG, 2-deoxyglucose; HFD, high-fat diet; AFM, atomic force microscopy; GdnHCl, guanidine hydrochloride; MD, molecular dynamics; SE-AUC, Sedimentation equilibrium analytical ultracentrifugation; t, time; t\(_R\), retention time; [CME], carboxymethylcystine.
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**TABLES**

Table 1. Structural analyses of insulin, KP insulin and dicarba insulins using circular dichroism. Helical content calculated using the CONTINLL algorithm for deconvolution against the protein database reference set SP43. The program is available on the DICROWEB website (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml). ΔG° values were derived from guanidine denaturation studies. † Data previously reported in (24).

| Analogues                      | Secondary Structures Content (%) | Guanidine Denaturation |
|--------------------------------|----------------------------------|------------------------|
|                                | Helix   | Sheet | Turn | Unordered | ΔG° \_ U (kcal.mole \^-1) |
| Insulin †                      | 48%     | 12%   | 14%  | 26%       | 4.74                   |
| Cis dicarba insulin †          | 37%     | 16%   | 20%  | 27%       | 1.98                   |
| Trans dicarba insulin †        | 23%     | 24%   | 22%  | 31%       | 1.61                   |
| KPinsulin                      | 44%     | 14%   | 14%  | 28%       | n.a.                   |
| Cis dicarba KP insulin         | 34%     | 19%   | 20%  | 27%       | 1.71                   |
| Trans dicarba KP insulin       | 17%     | 31%   | 22%  | 30%       | n.a.                   |
**FIGURES**

**A**

**HUMAN INSULIN**  
Mw = 5807.3 Da; molecular formula: C_{255}H_{351}N_{59}O_{77}S_{6}  

\[ \text{GIVEQCTSICSLYLENYCN} \]  

**LISPRO INSULIN (KP insulin)**  
Mw = 5807.3 Da; molecular formula: C_{255}H_{351}N_{59}O_{77}S_{6}  

\[ \text{GIVEQCTSICSLYLENYCN} \]

**B**

**Ribbon diagram of human insulin (2-Zn-coordinated T_{6} conformation)**  
PDB entry 1MSO) showing the location of the three \( \alpha \)-helices (A chain: blue; B chain: grey) and the three disulfide bonds (yellow). (C) Schematic diagram of native cystine and isomeric \( \text{cis} \)- and \( \text{trans} \)-dicarba bridges. RP-HPLC chromatograms of (D) native insulin, \( \text{cis} \)- and \( \text{trans} \)-dicarba insulins; (E) KP insulin, \( \text{cis} \)- and \( \text{trans} \)-dicarba KP insulins.

**Figure 1. Insulin, KP insulin and dicarba insulin analogues.** (A) Primary sequence comparison of human insulin (top) and KP insulin (bottom). Both consist of A (blue) and B (grey) chains stabilized by three disulfide bridges (yellow). Underlined are site 1-binding residues and bold are site 2-binding residues (17). Rapid-acting KP insulin has a reversal of B28 and B29 amino acids (Lys^{B28}Pro^{B29}) (red) compared to insulin. (B) Ribbon diagram of human insulin (2-Zn-coordinated T_{6} conformation) showing the location of the three \( \alpha \)-helices (A chain: blue; B chain: grey) and the three disulfide bonds (yellow). (C) Schematic diagram of native cystine and isomeric \( \text{cis} \)- and \( \text{trans} \)-dicarba bridges. RP-HPLC chromatograms of (D) native insulin, \( \text{cis} \)- and \( \text{trans} \)-dicarba insulins; (E) KP insulin, \( \text{cis} \)- and \( \text{trans} \)-dicarba KP insulins.
Figure 2. *In vitro* (A – D) and *in vivo* (E – F) activity of insulin, KP insulin and their respective dicarba insulin analogues. (A) Competition binding of insulin and dicarba insulins with europium-labelled insulin. Results are expressed as a percentage of binding in the absence of competing ligand (% B/B₀). (B) Activation of IR-B by increasing concentration of dicarba insulins (10 min stimulation) is expressed as receptor phosphorylation as a percentage of the maximal phosphorylation induced by insulin. Insulin vs cis dicarba insulin (ns); insulin vs trans dicarba insulin **** (P ≤ 0.0001) (2-way ANOVA; Dunnett’s multiple comparison) (C) DNA synthesis in response to increasing concentrations of dicarba insulins is shown as percentage incorporation of [³H]-thymidine ([³H-Thy]) above basal. All data in (A – D) are the mean ± S.E.M. n = at least 3 independent experiments. (D) Glucose uptake stimulated by increasing concentrations of insulin, cis dicarba insulin or cis dicarba KP insulin is expressed as fold glucose uptake (pmol/min/mg) above basal. Insulin vs cis dicarba insulin vs cis dicarba KP insulin (ns) (paired t-test). (E) Insulin tolerance test in mice fed on a normal diet (chow), or on a high fat diet (HFD) were administered through intraperitoneal injection (ip) with 0.75 IU/kg insulin, KP insulin, cis dicarba insulin or cis dicarba KP insulin under non-fasting conditions and tail vein blood glucose was measured via glucose meter at indicated times. n = 5 – 6 per group. Blood glucose levels are expressed as change over basal levels (mmol/L). Chow diet: insulin vs cis dicarba insulin ** (P ≤ 0.01); KP insulin vs cis dicarba KP insulin ** (P ≤ 0.01) (paired t-test). High fat diet: insulin vs cis dicarba insulin ** (P ≤ 0.01); KP insulin vs cis dicarba KP insulin ** (P ≤ 0.01) (paired t-test). Significance of the change in blood glucose levels at each time-point was also determined by two-way ANOVA followed by Holm-Sidak’s multiple comparison test. Chow diet: KP insulin vs cis dicarba KP insulin at t = 60 min * (p ≤ 0.05). High fat diet: KP insulin vs cis dicarba KP insulin at t = 30, 45 and 60 min *** (p ≤ 0.001); t = 90 and 120 min ** (p ≤ 0.01).
Figure 3. Sedimentation equilibrium data for cis dicarba insulin in the presence and absence of zinc ion (Zn$^{2+}$). Radial concentration distributions at sedimentation equilibrium for cis dicarba insulin in the (A) presence and (B) absence of 0.2 mM Zn$^{2+}$ at loading concentrations of 100 (open symbols) and 300 µg/ml (closed symbols) at 25,000 (blue) and 40,000 rpm (red). In (A), data were globally fitted to a single species of 34,500 ± 400 Da (solid lines). Residuals to the fit (bottom) show some systematic deviation from an ideal fit. (C) Sedimentation equilibrium data for Zn-free cis dicarba (green) and native (magenta) insulin at each speed and loading concentration is plotted together as the square of the radial position scaled by rotor speed vs concentration on a logarithmic scale. The slopes expected for monomeric, dimeric and tetrameric insulin are shown for reference.
Figure 4. Time-course of fibril formation detected by AFM. Insulin fibrillation is first evident at $t = 6$ h. After $t = 15$ h, insulin has formed aggregates and fibrils are no longer easily detectable. Fibrillation of cis dicarba insulin is first evident after $t = 2$ h and is clearly detectable by $t = 6$ h. Fibrils formed by the cis dicarba insulin are of a different structure compared to insulin, particularly evident at $t = 15$ h. Fibrillation of the monomeric KP insulin is evident at earlier time-points across a wider incubation range ($t = 2 – 15$ h) compared to insulin. At $t = 24$ h, KP insulin fibrils are no longer easily detectable. Surprisingly, cis dicarba KP insulin fibrillation is only evident at $t = 6 – 8$ h with a rapid increase in formation of shorter and thicker fibrils at $t = 6$ h. In summary, the cis dicarba insulin adopts a different fibrillar pattern compared to insulin with an apparent increased complexity of fibrillar topology. These experiments are representative of $n = 4$ experiments for insulin, $n = 3$ for cis dicarba insulin, $n = 5$ for KP insulin and $n = 3$ for cis dicarba KP insulin. First detection of fibrils for each analogue is indicated by white arrows. The scale bar (white) corresponds to 1 µm in all images.
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Figure 5. Thermal and chemical stability of cis dicarba insulin and cis dicarba KP insulin. (A) Circular dichroism far-UV spectrum of cis dicarba KP insulin suggests there is a difference in structure of the cis dicarba KP insulin compared to KP insulin and insulin, where lower helical propensities in the cis dicarba KP insulin is observed (see Table 1). $\theta$ = ellipticity. (B) Differences in thermal unfolding were monitored by ellipticity at $\lambda = 222$ nm and show both the cis- and trans dicarba insulins are considerably less stable than insulin. (C) Unfolding in the presence of guanidine hydrochloride demonstrates that both cis dicarba analogues are considerably destabilized compared to insulin. $\Delta G$ values derived from guanidine denaturation studies are listed in Table 1.
Figure 6. Limited proteolysis of insulin and cis dicarba insulin using chymotrypsin. (A) Rate of proteolysis plotted as percentage of undigested peptide over time. Non-reducing (B) insulin and (C) cis dicarba insulin digested for $t = 60$ min were analysed and fractionated using RP-HPLC. Metabolite A – E were identified via MALDI analyses in combinations of different conditions: whole sample vs RP-HPLC-fractionated samples; non-reducing vs reducing; positive vs negative detection mode (see Figs. S5 and S6). Indicated in (B), (C) and (F) are: full length, undigested peptide; metabolite A, non-reduced metabolite resulting from single cleavage at Tyr$^{B26}$; metabolite C, C-terminal- and metabolite D, N-terminal non-reduced metabolites resulting from cleavage at Tyr$^{B26}$ followed by cleavages at Tyr$^{A14}$ and Tyr$^{B16}$; metabolite E is only present in cis dicarba insulin cleavage reactions.
and resulted from single cleavage at TyrB16. (D) Metabolite E of cis dicarba insulin was identified in fractioned sample proteolysed with chymotrypsin for t = 60 min. The sample was treated with DTT (reduction) and IAA (alkylation) prior to MALDI analysis under negative mode detection. [CME], Carboxymethylcystine residues with monoisotopic mass of 58.005 Da. (E) Cis dicarba insulin crystal structure (reported in (24); model coordinates not in the PDB database) with circled numbers indicating the order in which cis dicarba insulin peptide bonds are cleaved by chymotrypsin. (F) A simplified chymotryptic digestion kinetics of cis dicarba insulin showing sequences of peptides arising from chymotrypsin cleavage. A6-A11 dicarba bond (blue solid line). Insulin is cleaved first to metabolite A and then into metabolites C and D, with no metabolite E being detected. The rate of synthesis of each metabolite is presented in Figure S3 and S4. Subspecies of each metabolite were also identified via MALDI analyses (see Figs. S5 - 7).
Figure 7. Bending of the B chain helix required to enable chymotrypsin proteolysis. (A) Overlay of the bent structure (red; MD simulation frame for cis dicarba insulin) with a reference insulin crystal structure (blue; PDB: 1MSO chains C and D). Residues A1 to A21 and B9 to B23 are superimposed. Hydrogen bonds in the B chain helix connecting residues Val\(^B\)\(^{12}\) and Tyr\(^B\)\(^{16}\), Glu\(^B\)\(^{13}\) and Leu\(^B\)\(^{17}\) and Tyr\(^B\)\(^{16}\), and Gly\(^B\)\(^{20}\) are broken, allowing the Ca-Ca distances between Glu\(^B\)\(^{13}\) and Tyr\(^B\)\(^{16}\) and between Tyr\(^B\)\(^{16}\) and Gly\(^B\)\(^{20}\) to increase from \(\sim 5.5\) Å to \(> 7\) Å. The bending rotates and compresses the A7-B7 disulfide bond and increases the distance between the N-terminal A chain and B chain helices (as measured by the Ca-Ca distance between residues A6 and B11). (B) Superimposition of the B chain helix of the un-bent insulin crystal structure (stick model; PDB: 1MSO chains C and D) on the active site of chymotrypsin (dark green; PDB: 4h4f – chymotrypsin in complex with inhibitor eglin C). For proteolysis to occur, Tyr\(^B\)\(^{16}\) must be recognized by the active site of the enzyme. Superimposition of the backbone atoms of Tyr\(^B\)\(^{16}\) as well as N and CA of Leu\(^B\)\(^{17}\) on those of the corresponding residues in the inhibitor reveals that the un-bent structure cannot engage correctly with the peptidase: residues B12, B13 and all residues beyond B18 overlap significantly with the chymotrypsin structure, and the sidechain of Tyr\(^B\)\(^{16}\) cannot sit properly in the active site cavity. (B) Superimposition of the B chain helix of the bent cis dicarba insulin simulation frame on the active site of chymotrypsin. Bulging of the B chain helix allows both the B12-B16 and B16-B20 loops to fit over the surface of the chymotrypsin molecule, with Tyr\(^B\)\(^{16}\) sitting in the middle of the binding pocket. (Note: in both B and C the sidechains of B16, B17 and chymotrypsin residues 143 and 192 have been rotated to give the best possible engagement of the two molecules.) Loops of chymotrypsin which must move out of the way to allow insulin engagement are shown as dark green ribbons.
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Figure 8. Variations in structural parameters associated with B chain helix bending from MD simulations. **Green:** $r(Cα-Cα)$ between Glu$^{B13}$ and Tyr$^{B16}$; this is a direct measure of helix bending. **Red:** $r(Cα-Cα)$ between residue A6 and B11; a measure of the distance between the N-terminal A chain and B chain helices. **Purple:** $r(Cα-Cα)$ between Cys$^A7$ and Cys$^B7$; the length of the A7-B7 interchain linker. **Blue:** the relative torsional energy of the A7-B7 disulfide linkage. Data are presented as 50 period moving averages to highlight trends. Full data are presented in Figure S8. Overall averages for the purple and blue traces are shown with dashed and dotted lines, respectively. Bond distances are given in Å and relative torsional energies in kJ mol$^{-1}$. Bending events in the B chain helix are outlined in black boxes. Note that each bin on the horizontal axis represents an independent 200 ns simulations; traces are therefore not continuous between bins.
Probing the correlation between insulin activity and structural stability through introduction of the rigid A6–A11 bond

Shee Chee Ong, Alessia Belgi, Bianca van Lierop, Carlie Delaine, Sofianos Andrikopoulos, Christopher A. MacRaild, Raymond S. Norton, Naomi L. Haworth, Andrea J. Robinson and Briony E. Forbes

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