



## Review

## Diabetes mellitus due to the toxic misfolding of proinsulin variants



Michael A. Weiss

Department of Biochemistry, Case Western Reserve University, Cleveland, OH 44106, United States

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## ABSTRACT

**Dominant mutations in the human insulin gene can lead to pancreatic  $\beta$ -cell dysfunction and diabetes mellitus due to toxic folding of a mutant proinsulin. Analogous to a classical mouse model (the Akita mouse), this monogenic syndrome highlights the susceptibility of human  $\beta$ -cells to endoreticular stress due to protein misfolding and aberrant aggregation. The clinical mutations directly or indirectly perturb native disulfide pairing. Whereas the majority of mutations introduce or remove a cysteine (leading in either case to an unpaired residue), non-cysteine-related mutations identify key determinants of folding efficiency. Studies of such mutations suggest that the evolution of insulin has been constrained not only by its structure and function, but also by the susceptibility of its single-chain precursor to impaired foldability.**

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### 1. Introduction

Insulin plays a central role in the regulation of vertebrate metabolism. The mature hormone, the post-translational product of a single-chain precursor, is a globular protein containing two chains, A (21 residues) and B (30 residues). Advances in human genetics over the past five years have identified dominant mutations in the insulin gene associated with diabetes mellitus (DM) (for reviews, see [1–3]). This syndrome arises from the toxic misfolding: the clinical mutations impair the folding of proinsulin in the endoplasmic reticulum (ER) of pancreatic  $\beta$ -cells [4,5]. Originally described in relation to neonatal-onset DM [6–9], DM may also present in childhood [10] or early adulthood [11]. Age of onset is proposed to reflect mutational differences in the degree of impaired folding and its cellular consequences [5,12]. Phenotypic variation may also arise due to polygenic differences in  $\beta$ -cell susceptibility to ER stress [13].

Patients with the mutant proinsulin syndrome are heterozygous. Although expression of one wild-type insulin allele would in other circumstances be sufficient to maintain metabolic homeostasis, studies of a corresponding mouse model (the Akita mouse [14–16]) have demonstrated that misfolding of the variant proinsulin perturbs the biosynthesis of wild-type insulin [17,18]. In a

survey of human mutations similar perturbations in trans have been demonstrated in  $\beta$ -cell lines [4,5,12]. Impaired  $\beta$ -cell secretion is associated with ER stress, distorted organelle architecture, and eventual cell death [19,20]. These findings have stimulated renewed interest in the biosynthesis of insulin [21–23] and the structural basis of disulfide pairing [24–29]. The foundational importance of this problem and its clinical relevance have motivated construction of novel molecular reagents and animal models [30–33]. Such studies have led in parallel to an enhanced understanding of structure–function relationships [13] and cellular mechanisms of proinsulin folding and insulin biosynthesis [1].

Clinical mutations in the insulin gene provide probes into evolutionary constraints shared by a family of single-chain factors (such as mammalian insulin-like growth factors) and in part by a superfamily of invertebrate insulin-like proteins [34–36]. Such relationships have motivated studies of the mitogenic properties of proinsulin, revealing preferential signaling through the A isoform of the insulin receptor (IR) [35]. Although beyond the scope of this review, these activities may be relevant to carcinogenesis [37]. Therapeutic use of proinsulin in the treatment of human DM has been investigated but would have unclear clinical benefits [38].

### 2. Biosynthesis of insulin

The insulin gene encodes a single-chain precursor polypeptide, designated *preproinsulin* (Fig. 1A, top). The signal peptide (gray bar in Fig. 1A) is cleaved on translocation into the ER to yield *proinsulin*.

Abbreviations: DM, diabetes mellitus; ER, endoplasmic reticulum; GA, Golgi apparatus; MODY, maturity-onset diabetes of the young; NMR, nuclear magnetic resonance

E-mail address: [michael.weiss@case.edu](mailto:michael.weiss@case.edu)

Reduced and unfolded, the translocated polypeptide contains a connecting domain (black in Fig. 1A) between the C-terminal residue of the B domain (blue) and N-terminus of the A domain (red) [39]. Its folding in the ER is coupled to the specific pairing of three cystines (Fig. 1A, center). The disulfide bridges (A6–A11, A7–B7, and A20–B19; shown in gold in Fig. 1B) contribute to protein stability and biological activity [27,40–48]. The solution structure of proinsulin as an engineered monomer consists of a folded insulin-like moiety and a flexible C domain (black segment in Fig. 1B) [49]. The overall structure is in accordance with prior biochemical evidence [50–55]. The structure, stability, and receptor-binding activity of insulin (Fig. 1A, bottom) require maintenance of each disulfide bridge [27,40–42,44–48]. Conserved among insulin-related ligands, the cystines provide interior struts (A19–B20 and A6–A11) and an external staple between chains (A7–B7). Insulin disulfide isomers exhibit molten structures of marginal stability and low activity [56–58].

Proinsulin binds only weakly to the insulin receptor; proteolytic processing liberates the mature hormone [22,38]. On transit through the Golgi apparatus (GA) and immature secretory granules [59], the C-peptide is excised by prohormone convertases [60], which cleave conserved dibasic sites flanking the C domain (BC and CA junctions; green in Fig. 1A, B). Insulin is stored as  $Zn^{2+}$ -stabilized hexamers as microcrystalline arrays [61–63] within specialized secretory granules [64]. The hexamers dissociate on secretion into the portal circulation, enabling the circulating hormone to function as a  $Zn^{2+}$ -free monomer. Because the insulin monomer is exquisitely susceptible to fibrillation at millimolar concentration and body temperature [65], its zinc-mediated assembly within  $\beta$ -cells may represent a defense against toxic misfolding [66] during storage in the secretory granule [23]. Evidence that such assembly contributes to insulin storage and efficient secretion has recently been obtained through studies of a mouse model lacking the  $\beta$ -cell zinc transporter [67].

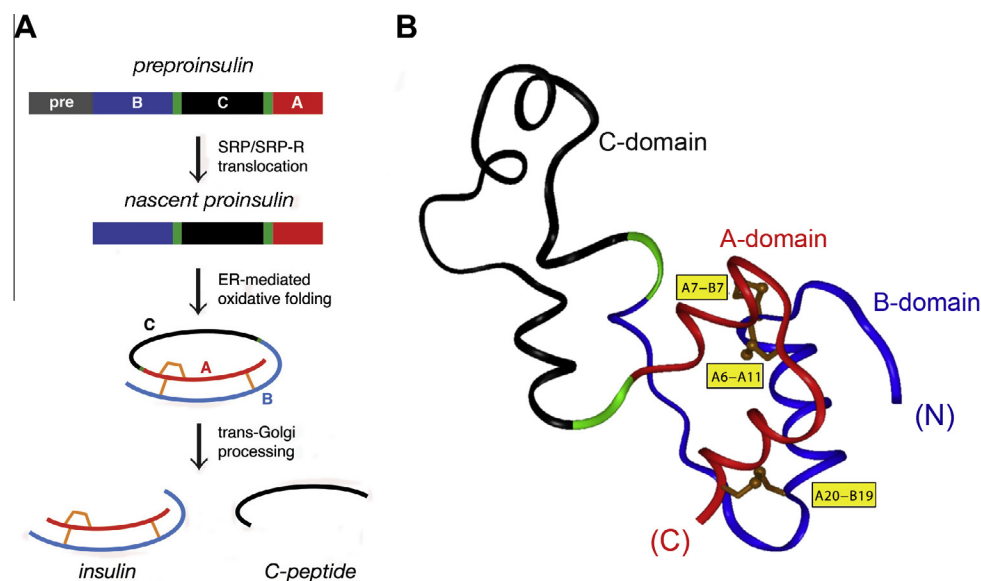
Although insulin biosynthesis occurs via a single-chain precursor, its chemical synthesis has traditionally employed isolated A- and B-chain peptides (for review see [68]). The success of insulin

chain combination implies that chemical information required for folding is contained within the sequences of the A- and B domains [69,70]. A variety of analogs have been prepared by this protocol, facilitating their pharmaceutical development for treatment of DM [71,72]. Despite the general robustness of insulin chain combination, synthesis of certain analogs has been confounded by low yields [26,73–79]. Such synthetic failures provide models of impaired folding, providing insight into structural mechanisms that underlie the mutant proinsulin syndrome.

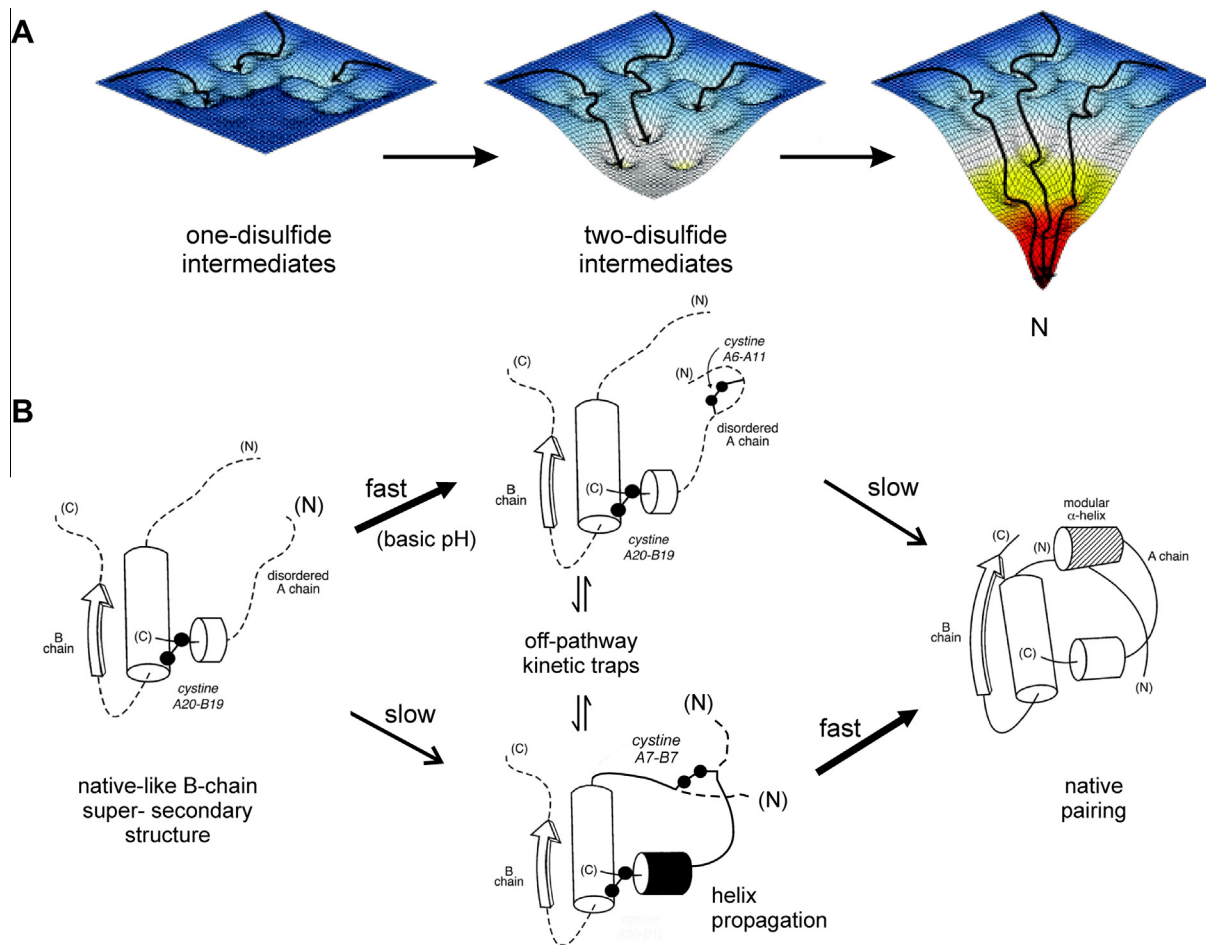
### 3. Mechanism of disulfide pairing

Oxidative folding of globular proteins has traditionally been investigated by chemical trapping of populated disulfide intermediates [80]. Such studies of proinsulin and related polypeptides – foreshortened single-chain analogs (mini-proinsulin [81]) and insulin-like growth factors – are notable for the transient accumulation of one- and two-disulfide intermediates [24,25,82]. Successive steps of partial folding define a series of trajectories on a sequence of free-energy landscapes (Fig. 2A). Each landscape underlies the dynamics of an ensemble of accessible polypeptide conformations in the presence of a specific subset of disulfide bridges. The landscapes proceed from shallow to steep, enabling the folding chain to acquire structure stepwise on successive disulfide pairing. The preferred sequence of disulfide intermediates, as defined by chemical trapping, thus corresponds to a progression of multiple folding trajectories on funnel-shaped landscapes. This perspective extends the classical disulfide-centered view of protein-folding intermediates [83] to encompass general biophysical paradigms of protein folding [84–86].

Although refolding studies of proinsulin are limited by aggregation (imposing a requirement for pH conditions  $>9$ , confounding interpretation [87]), the disulfide pathways of mini-proinsulin and IGF-I are well characterized near neutral pH [24,25,40,43,82,88,89]. A structural pathway is suggested by spectroscopic studies of equilibrium models (Fig. 2B) [27,40–42,44–48,90]. In this pathway a key role is played by initial formation



**Fig. 1.** Biosynthesis of proinsulin. (A) Pathway begins with preproinsulin (top): signal peptide (gray), B-domain (blue), dibasic BC junction (green), C-domain (black), dibasic CA junction (green), and A-domain (red). Specific disulfide pairing in the ER yields native proinsulin (middle panels). BC- and CA cleavage (mediated by prohormone convertases PC1 and PC2) releases insulin and C-peptide (bottom). (B) Solution structure of proinsulin: insulin-like moiety and disordered connecting peptide (dashed line). A- and B-domains are shown in red and blue, respectively; C-domain contains a nascent  $\alpha$ -helical turn near the CA junction [49]. Cystines are labeled in yellow boxes. The solution structure exploited an engineered monomer (DKP-proinsulin) as characterized by multi-dimensional  $^1H$ - $^{13}C$ - $^{15}N$  NMR. Panel A is adapted from Ref [13] panel B depicts a representative member of an ensemble of solution structures (Protein Databank entry 2KQP).



**Fig. 2.** Energy landscape paradigm. (A) Successive disulfide pairing enables a sequence of folding trajectories on ever-steeper funnel-shaped free-energy landscapes. (B) Preferred pathway of disulfide pairing. Initial formation of cystine A20–B19 (left) is directed by a nascent hydrophobic core comprising the central B-domain  $\alpha$ -helix (residues B9–B19), part of the C-terminal B-chain  $\beta$ -strand (B24–B26), and part of the C-terminal A-domain  $\alpha$ -helix (A16–A20). Alternative pathways mediate successive disulfide pairing (middle panel) leading in turn to the native state (right). The mechanism of disulfide pairing is perturbed by clinical mutations associated with a monogenic syndrome of DM due to toxic misfolding of the variant proinsulin in the ER. Figure is adapted from Ref. [49]; panel A is adapted from an image kindly provided by J. Williamson.

of cystine A20–B19. In the native state this disulfide bridge packs within a cluster of conserved aliphatic and aromatic side chains in the hydrophobic core to connect the C-terminal  $\alpha$ -helix of the A domain to the central  $\alpha$ -helix of the B domain [57,58,75]. Because cystine A20–B19 is the only one-disulfide species to accumulate in the refolding of mini-proinsulin and IGF-I [24,25,89], its initial pairing is proposed to contribute to the stabilization of a specific folding nucleus [27,90,91].

The structural role of cystine A20–B19 in populated one- and two-disulfide intermediates motivated construction of analogs (of insulin, mini-proinsulin, and IGF-I) containing pairwise substitution of the other cysteines by Ala or Ser [27,40–48]. Such analogs exhibit attenuated  $\alpha$ -helix content. Nucleation of native-like structure was observed in a disulfide-linked peptide fragment of IGF-I containing cystine A20–B19: at low temperature the disulfide bridge native tethers  $\alpha$ -helices within a cluster of non-polar side chains resembling the native core [27]. Mutations within this A20–B19-associated subdomain impair disulfide pairing in insulin chain combination and the biosynthesis of single-chain precursors in *Saccharomyces cerevisiae* [44,77,91–93].

$^1\text{H}$  NMR spectra of the above equilibrium models exhibit a progressive increase in chemical-shift dispersion with successive disulfide pairing, suggesting stepwise stabilization of structure. These findings are in accordance with the landscape perspective [40,90]. After A20–B19 pairing, folding can proceed through multiple alternative channels. Folding of mini-proinsulin near neutral

pH exhibits subsequent rapid formation of cystine A7–B7 (lower pathway in Fig. 2B) or slow pairing of A6–A11 (upper pathway). Although it is not intuitive why pairing of cysteines distant in the sequence (A7 and B7) is favored relative to pairing of proximate cysteines (A6 and A11), spectroscopic studies have demonstrated that removal of cystine A7–B7 (by pairwise substitution) destabilizes the structure of insulin more markedly than does removal of A6–A11 [91]. These findings suggest that nascent structure in the one-disulfide [A20–B19] intermediate either more effectively aligns Cys<sup>A7</sup> and Cys<sup>B7</sup> or more significantly impairs pairing of Cys<sup>A6</sup> and Cys<sup>A11</sup>. Either of these on-pathway two-disulfide intermediate may interconvert with non-native disulfide isomers as off-pathway kinetic traps (central panel of Fig. 2B).

The hazard of competing traps in the refolding of proinsulin is suggested by the refolding of an homologous polypeptide (IGF-I) to form native and non-native disulfide isomers as alternative ground states (native IGF-I and IGF-*swap*) [24,82]. Containing respective pairings [A20–B19, A7–B7, A6–A11] (native) and [A20–B19, A6–B7, A7–A11] (*swap*), the isomers exhibit similar core structures near the shared A20–B19 disulfide bridge [24,94]. The relative stabilities of these isomers in a mini-IGF model is controlled by the N-terminal segment of the B domain [95,96]. Although proinsulin has a unique disulfide-specific ground state, this and a second isomer (pairings [A20–B19, A11–B7, A6–A7]) readily form following disulfide exchange in the presence of a chemical denaturant [57]. Whereas the stability of IGF-*swap* is

similar to that of native IGF-I, the accessible non-native isomers of insulin and proinsulin are molten globules of marginal stability [58].

Evidence for the pathological relevance of non-native disulfide isomers of proinsulin and related polypeptides has been obtained in eukaryotic cell culture [20,26,97–99]. An assay for non-native isomers has been developed based on native polyacrylamide gel electrophoresis. This assay exploits the slower mobilities of misfolded proteins, presumably reflecting their less compact structures. Formation of non-native proinsulin isomers has thus been observed on transfection of expression constructs in both *S. cerevisiae* and rodent neurosecretory cell lines. Although non-native isomers are generally not secreted, amino-acid substitutions in human proinsulin can enhance the fraction of mispairing in the ER [98,99]. Because propensity to misfold in this assay does not correlate with effects of the substitutions on thermodynamic stability in vitro, its mechanism is not well understood.

Studies of proinsulin variants containing substitutions or deletions in the N-terminal segment of the B chain suggest that the contribution of specific side chains to foldability may not be apparent in the native state [100]. We imagine that the substituted side chains perturb the relative stabilities or kinetic accessibility of disulfide intermediates disproportionately to their effects on the native state, once achieved. Alternatively, it is possible that these residues contribute to interactions of the nascent polypeptide with ER chaperones and the oxidative machinery of disulfide pairing [101]. The ER of  $\beta$ -cells may contain a lineage-specific set of chaperones and foldases required in combination for insulin biosynthesis. Defining such an “ER proteome” of  $\beta$ -cell-specific foldases represents a major goal of current studies. Altered ER proteomes in non-neurosecretory cell lines is likely to underlie their impaired ability to support the efficient folding and secretion of proinsulin [102].

Remarkably, mini-proinsulin (which refolds in vitro more efficiently than proinsulin [103]) misfolds in *S. cerevisiae* to form a metastable disulfide isomer as the predominant product. This non-native protein is secreted, indicating that its globular structure can resemble a native state sufficiently to fulfill quality-control checkpoints. Quantitative misfolding of mini-proinsulin in yeast demonstrates that the ER folding machinery of a eukaryotic cell can selectively target folding into a non-ground-state conformation. It would be of future interest to characterize this alternative pairing scheme and determine the extent of structural resemblance to the native fold. Because the aberrant protein is not degraded prior to exit through the ER and GA, mini-proinsulin provides an example of “stealth” misfolding, leading to secretion of a protein caught in a kinetic trap.

#### 4. Diabetes-associated mutations

The majority of mutations in the insulin gene lead to permanent neonatal-onset DM [8]. Impaired  $\beta$ -cell function develops prior to maturation of the immune system and so presents as an auto-antigen negative form of apparent Type 1 DM. This presentation may be due to mutations in genes other than the insulin gene [104]. The most common cause is a heterozygous activating mutation in a subunit of the  $\beta$ -cell voltage-gated potassium channel, either *KCNJ11* (encoding the Kr6.2 subunit) or *ABCC8* (encoding the Sur1 subunit) [105,106]. The resulting diabetic phenotype may be transient or permanent. In favorable cases recognition of this syndrome enables patients to be treated with oral agents that inhibit the channel (sulfonylureas) rather than insulin [104].

Dominant mutations in the insulin gene define the second most common genetic cause of permanent neonatal DM [6–8,10]. Such mutations occur in each region of preproinsulin: its signal peptide,

A-, B- and C domains (Fig. 1A). The majority of such mutations result in the addition or removal of a cysteine, leading in either case to an odd number of potential pairing sites. This imbalance leads in general to misfolding and aggregation [4,5,12]. Remarkably, the catalogue of human mutations encodes the same “Akita” substitution (Cys<sup>A7</sup> → Tyr) as in the *Ins2* gene of the *Mody4* mouse [14–16]; this dominant murine substitution thus provides an attractive model of progressive postnatal  $\beta$ -cell failure [18–20]. The variant murine proinsulin in vitro undergoes partial unfolding with increased aggregation [107]. Analogous perturbations were characterized in human insulin- and proinsulin analogs lacking cysteine A7–B7 [47,91]. Heterozygous expression of a variant *Ins2* allele encoding Cys<sup>A6</sup> → Ser (identified in an N-ethyl-N-nitrosourea screen) likewise induces DM [108].

The identification of identical human and murine mutations at position A7 suggests that the pathogenesis of neonatal DM in these patients is likewise similar to that of the Akita mouse, which has been extensively characterized [14–16,18–20]. Although the mechanism of  $\beta$ -cell degeneration in the Akita mouse remains incompletely understood, its  $\beta$ -cells exhibit an early defect in the folding and trafficking of both wild-type and variant proinsulins. Such impaired biosynthesis is accompanied by elevated markers of ER stress, electron-dense deposits in abnormal ER and GA, mitochondria swelling, and progressive loss of  $\beta$ -cell mass [18–20]. Extension of such findings to human variants has been aided by the construction of innovative fluorescent proinsulin fusion proteins and their use in cell lines and transgenic mice as probes of subcellular localization and aggregation [30–33].

Biochemical studies of clinical variants in  $\beta$ -cells or neurosecretory cell lines have revealed perturbations in disulfide pairing, which range from severe or mild depending on the site of mutation and the properties of the substituted side chain [1–3]. Whereas an odd number of cysteines presumably induces a severe block to folding, better-tolerated mutations present later in life as auto-antibody-negative presumed Type 1 DM or Type 2 DM. One such mutation, presenting in the second decade as maturity-onset diabetes of the young (MODY), is due to substitution of Arg<sup>B22</sup> by Gln. This mutation alters a solvent-exposed site in the B20–B23  $\beta$ -turn not required for receptor binding [6–8,10]. DM presenting in the third decade of life is associated with substitution of Phe<sup>B24</sup> by Ser [109]. This mutation causes only a mild impairing of receptor binding [110] but like Gln<sup>B22</sup> imposes ER stress at a level intermediate between over-expression of wild-type proinsulin and neonatal variants [5]. Chronic elevation of moderate ER stress in  $\beta$ -cells presumably leads to a slow but progressive loss of  $\beta$ -cell mass.

Evidence is accumulating that subtle perturbations of insulin biosynthesis (due either to variant insulin genes or mutations in the ER folding machinery) may contribute to the pathogenesis of non-syndromic Type 2 DM in humans [111,112] as in the Akita mouse [17,113]. A newly recognized biophysical contribution to such  $\beta$ -cell dysfunction is “molecular crowding” in the ER due to over-expression of nascent proinsulin in the face of peripheral insulin resistance [114], a concept that may have therapeutic implications [33].

Mutations in the insulin gene unassociated with the defective folding of proinsulin can also be associated with adult-onset phenotypes of variable penetrance [115]. The diversity of genetic syndromes reflects a range of molecular mechanisms in accord with Murphy’s Law of genetics. Classical insulinopathies (Leu<sup>A3</sup> and Leu<sup>B25</sup>), for example, markedly perturb receptor binding [109], leading to reduced clearance and hence mutant hyperinsulinemia [110]. Substitution of His<sup>B10</sup> by Asp by contrast would enhance the receptor-binding activity of the variant insulin if secreted [116], but within  $\beta$ -cells the substitution leads to aberrant sorting of the mutant proinsulin to a constitutive (*i.e.*, non-glucose-regulated) granule

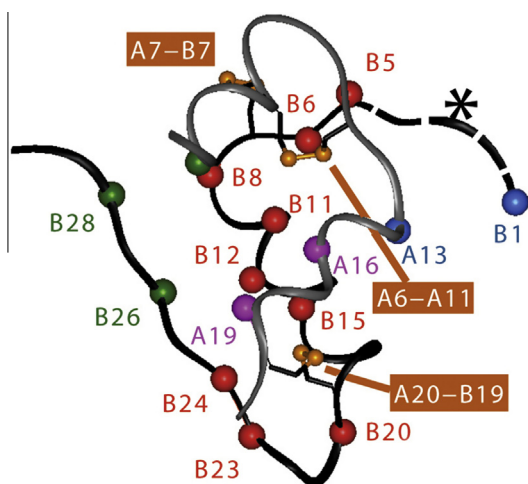
[117,118]. Because constitutive granules lack prohormone convertases, the patients exhibit mutant hyperproinsulinemia. Yet another syndrome is caused by a mutation at the CA junction [119]. Impaired prohormone processing leads to the secretion of a split proinsulin, which has reduced biological activity. Secretion of mutant insulins or proinsulins in the above patients does not exclude concomitant induction of chronic ER stress or perturbation of other organelles as a contributing pathobiological mechanism. Indeed, we speculate that the variable genetic penetrance of the above syndromes may be the hallmark of a genetic variant that modifies long-term survival of  $\beta$ -cells as one component of a multi-genic trait.

## 5. Structural implications

Mutations in proinsulin associated with neonatal DM can encode amino-acid substitutions not involving cysteine (for review, see [13]). Within the insulin moiety such mutations have been found only in the B domain. These substitutions are of structural interest in relation to the mechanism of protein folding. The mutations occur within the N-terminal segment of the B-chain (residues B1–B8), central  $\alpha$ -helix (B9–B19), and adjoining  $\beta$ -turn (B20–B23). An overview of key positions in these elements is provided in Fig. 3B. We discuss these structural elements in turn.

### 5.1. N-terminal segment

In both the solution structure of insulin [41,120,121] and its crystallographic T-state [122,123] residues B1–B6 are extended (asterisk in Fig. 3) whereas B7–B10 comprise a  $\beta$ -turn adjoining the central  $\alpha$ -helix. N-terminal residues contribute to the specification of the A7–B7 disulfide bridge in vitro [95,96] and to the overall efficiency of proinsulin folding in cell lines [100]. Sites of clinical mutation (His<sup>B5</sup> and Gly<sup>B8</sup>; broadly conserved among vertebrate insulins) have been well characterized [26,76,78,79]. (i) In the



**Fig. 3.** Critical sites governing the foldability of proinsulin are widely distributed in insulin. Asterisk indicates N-terminal segment of the B chain, which promotes foldability but is dispensable in the mature hormone [100]. Conserved side chains in or adjoining the C-terminal  $\alpha$ -helix of the A chain (Leu<sup>A16</sup> and Tyr<sup>A19</sup>; C<sub>α</sub> purple spheres) and at multiple sites in the B chain (C<sub>α</sub> red spheres) impair insulin chain combination in accord with studies of mutant proinsulins in mammalian cell lines and the distribution of clinical non-cysteine mutations in the insulin gene. Contacts between the side chains Phe<sup>B1</sup> and Ile<sup>A13</sup> (C<sub>α</sub> blue spheres), although not well ordered in the native state, contribute to the cellular foldability of proinsulin. Residues Ile<sup>A2</sup>, Tyr<sup>B26</sup>, and Pro<sup>B28</sup> (C<sub>α</sub> green spheres) contribute to the structure and stability of the native state but are not required for efficient disulfide pairing in chain combination. Disulfide bridges are as indicated (gold). Coordinates were obtained from Protein Databank file 4INS and correspond to molecule 1 of the classical 2-Zn insulin hexamer [123].

native state His<sup>B5</sup> packs within an inter-chain crevice, making one or more hydrogen bonds to carbonyl oxygens in the A chain. Diverse substitutions impair chain combination [26]; Arg<sup>B5</sup> (which is observed in some non-mammalian insulins) is by contrast well tolerated, presumably through formation of analogous inter-chain hydrogen bonds as visualized in its crystal structure [79]. In mammalian cell culture substitution of His<sup>B5</sup> by Asp blocks the folding and secretion of human proinsulin [26]. (ii) Gly<sup>B8</sup> exhibits a positive  $\phi$  dihedral angle in an insulin monomer and so occupies a position in the Ramachandran plane ordinarily forbidden to L-amino acids. Any L-substitution at this site impairs chain combination whereas yield is enhanced by D-substitutions [76]. Such stereospecificity implies that the sign of the B8  $\phi$  angle contributes to the alignment or misalignment of neighboring Cys<sup>B7</sup> for disulfide pairing.

The solution structure of Ala<sup>B5</sup> and Ser<sup>B8</sup> insulin analogs retain native-like features and exhibit substantial biological activity despite their reduced foldabilities [26,78]. Decreased thermodynamic stabilities are observed, presumably due to altered inter-chain packing (Ala<sup>B5</sup>) or an unfavorable main-chain conformation (Ser<sup>B8</sup>). Whereas decreased native-state stability can in principle contribute to misfolding, it is likely that such perturbations also impose a kinetic block to disulfide pairing and concomitantly decrease the stability of populated folding intermediates. Evidence for such kinetic blocks has been provided by cellular studies in which these and related mutations lead to formation of non-native disulfide isomers [26,78]. Interestingly, the substantial biological activities of such “non-foldable” analogs [26,78,100] indicate that residues required for efficient folding may be dispensable in the native state [124,125].

### 5.2. Central $\alpha$ -helix

Nascent folding of the B-chain  $\alpha$ -helix is observed in equilibrium models of the key [A20–B19] folding intermediate [27,40] and subsequent two-disulfide intermediates [44,47,48,91]. In these models Leu<sup>B11</sup> and Leu<sup>B15</sup> each contribute to nascent clustering of non-polar residues. It is thus plausible that clinical mutations at these sites would impede initial disulfide pairing and, should native disulfide pairing be achieved, perturb native structure and assembly. Ala substitutions at B11 and B15 (although helicogenic in model peptides) impair the expression and secretion of mini-proinsulin in *S. cerevisiae* [93]. Similarly, insulin chain combination is markedly impaired by interchange of Leu<sup>B11</sup> and Val<sup>B12</sup>, which would be predicted to perturb tertiary structure without net change in intrinsic helical potential [73]. It is not clear why substitution of Tyr<sup>B16</sup> by His, a clinical mutation that is predicted to project at the surface of this  $\alpha$ -helix [7], may impair folding.

### 5.3. B20–B23 $\beta$ -Turn

B-chain super-secondary structure requires a  $\beta$ -turn between the central  $\alpha$ -helix and the neighboring  $\beta$ -strand (residues B24–B28). This turn contains conserved glycines at B20 and B23, each of which exhibits a positive  $\phi$  dihedral angle. Whereas Ala substitutions impair the expression of mini-proinsulin in *S. cerevisiae* and impede chain combination, efficient disulfide pairing (like that at B8 above) can be rescued by D-amino acid substitutions. A clinical mutation has been found at B23 and is expected to perturb pairing of cystine A20–B19. The mechanism by which a mutation at B22 (Arg → Gln; see above) causes MODY is not apparent.

The absence of clinical mutations in the A domain may reflect incomplete sampling of patients, or alternatively, may indicate that non-cysteine residues in the A domain play a less critical role in disulfide pairing than the B domain. We believe that both explanations have partial validity. On the one hand, studies of the

mechanism of chain combination have shown that the N-terminal A-chain  $\alpha$ -helix (residues A1–A8) is not required for disulfide pairing [75]. Such dispensability is in accord with a putative structural pathway in which segmental folding of this  $\alpha$ -helix is a late event (Fig. 2B). On the other hand, the efficiency of chain combination is exquisitely sensitive to substitutions of core residue in the C-terminal A-chain  $\alpha$ -helix (Leu<sup>A16</sup> and Tyr<sup>A19</sup>; purple spheres in Fig. 3) [74,126]. Accordingly, we anticipate that continuing genetic screening of infants and children with antibody-negative DM will uncover analogous A-domain mutations.

## 6. Protein folding and induced fit

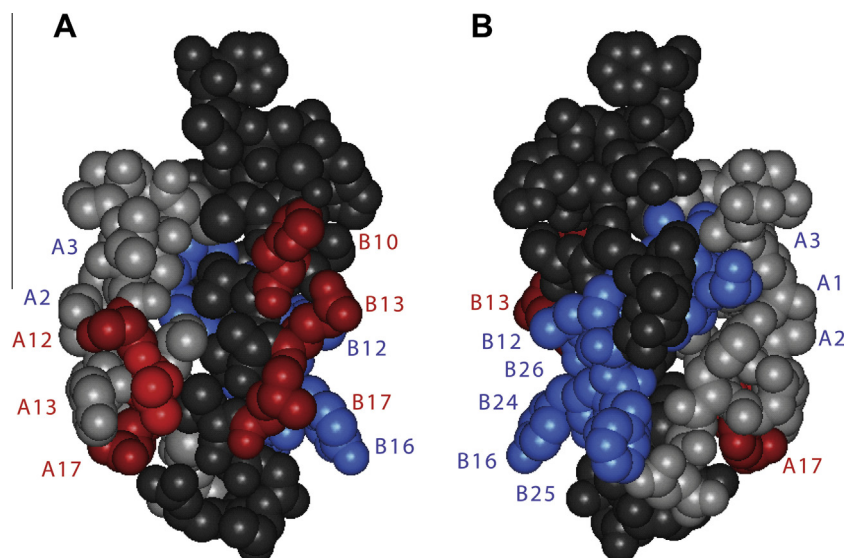
The evolution of insulin is enjoined by multiple biological constraints, including biosynthesis, structure, and function. We imagine that conserved residues may contribute to one or more essential requirements of viability: foldability in the  $\beta$ -cell, protection from intra- or extracellular toxic misfolding, self-assembly within the secretory granule, and receptor binding. The overlapping nature of these constraints may account for the limited sequence diversity among vertebrate insulins [123].

Evolutionary constraints may be coincident at some positions but opposing at others. Studies of stereospecific B8 analogs, for example, suggest that at this site kinetic determinants of foldability are at odds with conformational requirements of receptor binding [76,78]. Whereas a positive  $\phi$  dihedral angle (like that of a D-amino acid) facilitates disulfide pairing, a negative dihedral angle (like that of an L-amino acid) is required for receptor binding [76,78]. These opposing requirements presumably underlie the invariance of glycine – the only achiral amino acid – at a site of conformational change. Indeed, a classical model for the induced fit of insulin on receptor binding is provided by the TR transition among zinc insulin hexamers [127]. Although the choreography of conformational changes seen in the TR transition may pertain only to hexamers [79], the transition is remarkable for a change in the sign of the B8 dihedral angle from positive (the foldable state) to negative (the active state). That B8 provides a hot spot for mutations causing neonatal DM in turn suggests that induced fit provides protective mechanism: a premature setting of the B8

dihedral angle in its active conformation within the  $\beta$ -cell may lead to toxic misfolding.

The recent co-crystal structure of insulin bound to a fragment of the IR ectodomain has provided evidence for induced fit within the C-terminal segment B-chain [128]. As predicted based on studies of anomalous insulin analogs [120,129,130], detachment of the C-terminal  $\beta$ -strand (residues B24–B28) enables docking of a receptor  $\alpha$ -helix (denoted  $\alpha$ CT) against the N-terminal A-chain  $\alpha$ -helix. Such induced fit of the B chain may likewise represent an evolutionary response to the danger of proteotoxicity. Indeed, pre-detachment of the B-chain  $\beta$ -strand by a chiral perturbation (substitution by Phe<sup>B24</sup> by D-Ala) enhances receptor binding at the expense of increased susceptibility to fibrillation [130]. The aromatic ring of Phe<sup>B24</sup> anchors the native  $\beta$ -strand, stabilizes the hydrophobic core, contributes to dimerization and hexamer formation [123], enables induced fit on receptor binding [130], and even further, is likely to contact the receptor itself [131]. Much remains to be learned. How induced fit of the B chain is triggered on IR binding, the nature of the switch at (or near) residue B24, and the contacts subsequently made by Phe<sup>B24</sup>, Phe<sup>B25</sup>, and Tyr<sup>B26</sup> were not addressed in the recent co-crystal structure of a “micro-receptor” model [128].

Understanding the co-evolution of insulin and the IR will require further structural analysis of their binding interfaces. A framework is provided by the elegant classification of hormone-binding surfaces in the receptor as Site 1 and Site 2 [132,133]. The cognate IR-binding surfaces of insulin correspond in large part to its respective dimerization and hexamer-forming surfaces (Fig. 4) (for review, see [134]). Because the recent co-crystal structure was restricted to Site 1, it will be of particular interest to extend such analysis to larger fragments of the ectodomain containing Site 2 surfaces. Given the ancestral status of insulin signaling in both vertebrates and invertebrates [34–36], an intriguing possibility exists that distinct evolutionary constraints and histories are associated with these complementary interfaces. Such an historical understanding would highlight the relationship between proinsulin and single-chain homologues (including mammalian IGF-I and IGF-II), which may have translational implications in cancer biology [37,135].



**Fig. 4.** Proposed binding surfaces of insulin. (A) Front view and (B) back view. Whereas the classical receptor-binding surface of insulin engages IR Site 1 (blue; Ref. [132,133]), its Site 2-related surface includes hexamer contacts His<sup>B10</sup>, Val<sup>B18</sup>, Ser<sup>A12</sup>, Leu<sup>A13</sup> and Glu<sup>A17</sup> (red). The A- and B chains are otherwise shown in light gray and dark gray, respectively. The structures shown are based on an R crystallographic protomer, which contains a receptor-active positive  $\phi$  angle at Gly<sup>B24</sup> (in accordance with the results of Ref. [76]) but is otherwise unlikely to represent the receptor-bound conformation of insulin [128].

We imagine in general that conserved side chains in insulin play multiple distinct roles at each stage of its conformational “life cycle,” representing a remarkable compression of information within a short protein sequence. It would be of future interest to probe the range of structures within this life cycle through crystal structures of insulin–holoreceptor complexes and through solid-state NMR-based models of non-native insulin aggregates and fibrils.

## 7. Concluding remarks

The mutant proinsulin syndrome provides an opportunity to investigate principles of protein folding at the intersection of cell biology and human genetics. Consistent with classical diffusion-collision and framework models [136], folding of a globular protein represents the coalescence of discrete subdomains [137,138]. Whereas funnel-like energy landscapes highlight the role of parallel events in folding [84], evidence for preferred trajectories [139] has been provided by disulfide trapping studies of insulin-related polypeptides. Characterization of predominant intermediates promises to enable structural interpretation of many of the clinical mutations. Sites of mutation reflect mechanisms of folding or misfolding that may not be apparent in the native state [13].

Foldability of proteins is an evolved property of polypeptide sequences [140]. The general threat of toxic misfolding provides a hidden constraint underlying such evolution. Application of these principles to insulin promises to connect bench to bedside. An intrinsic tension between folding-competent and active conformations of insulin, only partially resolved in the wild-type hormone by induced fit, may underlie the role of chronic ER stress in the progression of  $\beta$ -cell dysfunction in Type 2 DM. Molecular dissection of this progression promises to provide novel therapeutic targets.

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## References

- [1] Liu, M., Hodish, I., Haataja, L., Lara-Lemus, R., Rajpal, G., Wright, J. and Arvan, P. (2010) Proinsulin misfolding and diabetes: mutant INS gene-induced diabetes of youth. *Trends Endocrinol. Metab.* 21, 652–659.
- [2] Greeley, S.A., Tucker, S.E., Naylor, R.N., Bell, G.I. and Philipson, L.H. (2010) Neonatal diabetes mellitus: a model for personalized medicine. *Trends Endocrinol. Metab.* 21, 464–472.
- [3] Greeley, S.A., Naylor, R.N., Philipson, L.H. and Bell, G.I. (2011) Neonatal diabetes: an expanding list of genes allows for improved diagnosis and treatment. *Curr. Diab. Rep.* 11, 519–532.
- [4] Park, S.Y., Ye, H., Steiner, D.F. and Bell, G.I. (2010) Mutant proinsulin proteins associated with neonatal diabetes are retained in the endoplasmic reticulum and not efficiently secreted. *Biochem. Biophys. Res. Commun.* 391, 1449–1454.
- [5] Liu, M., Haataja, L., Wright, J., Wickramasinghe, D.N., Hua, Q.X., Phillips, N.B., Barbetti, F., Weiss, M.A. and Arvan, P. (2010) Mutant INS-gene induced diabetes of youth: proinsulin cysteine residues impose dominant-negative inhibition on nonmutant proinsulin transport. *PLoS ONE* 5, e13333.
- [6] Stoy, J., Edghill, E.L., Flanagan, S.E., Ye, H., Paz, V.P., Pluzhnikov, A., Below, J.E., Hayes, M.G., Cox, N.J., Lipkind, G.M., et al. (2007) Insulin gene mutations as a cause of permanent neonatal diabetes. *Proc. Natl. Acad. Sci. USA* 104, 15040–15044.
- [7] Colombo, C., Porzio, O., Liu, M., Massa, O., Vasta, M., Salsardi, S., Beccaria, L., Monciotti, C., Toni, S., Pedersen, O., et al. (2008) Seven mutations in the human insulin gene linked to permanent neonatal/infancy-onset diabetes mellitus. *J. Clin. Invest.* 118, 2148–2156.
- [8] Edghill, E.L., Flanagan, S.E., Patch, A.M., Boustred, C., Parrish, A., Shields, B., Shepherd, M.H., Hussain, K., Kapoor, R.R., Malecki, M., et al. (2008) Insulin mutation screening in 1044 patients with diabetes: mutations in the INS gene are a common cause of neonatal diabetes but a rare cause of diabetes diagnosed in childhood or adulthood. *Diabetes* 57, 1034–1042.
- [9] Polak, M., Dechaume, A., Cave, H., Rivital, N., Crosnier, H., Sulmont, V., De Kerdanet, M., Scharfmann, R., Lebenthal, U., Froguel, P., et al. (2008) Heterozygous missense mutations in the insulin gene are linked to permanent diabetes appearing in the neonatal period or in early infancy. *Diabetes* 57, 1115–1119.
- [10] Molven, A., Ringdal, M., Nordbo, A.M., Raeder, H., Stoy, J., Lipkind, G.M., Steiner, D.F., Philipson, L.H., Bergmann, I., Aarskog, D., et al. (2008) Mutations in the insulin gene can cause MODY and autoantibody-negative type 1 diabetes. *Diabetes* 57, 1131–1135.
- [11] Boesgaard, T.W., Pruhova, S., Andersson, E.A., Cinek, O., Obermannova, B., Lauenborg, J., Damm, P., Bergholdt, R., Pociot, F., Pisinger, C., et al. (2010) Further evidence that mutations in *INS* can be a rare cause of maturity-onset diabetes of the young (MODY). *BMC Med. Genet.* 12, 42.
- [12] Meur, G., Simon, A., Harun, N., Virally, M., Dechaume, A., Bonnefond, A., Fetita, S., Tarasov, A.I., Guillausseau, P.J., Boesgaard, T.W., et al. (2010) Insulin gene mutations resulting in early-onset diabetes: marked differences in clinical presentation, metabolic status, and pathogenic effect through endoplasmic reticulum retention. *Diabetes* 59, 653–661.
- [13] Weiss, M.A. (2009) Proinsulin and the genetics of diabetes mellitus. *J. Biol. Chem.* 284, 19159–19163.
- [14] Yoshioka, M., Kayo, T., Ikeda, T. and Koizumi, A. (1997) A novel locus, *Mody4*, distal to D7Mit189 on chromosome 7 determines early-onset NIDDM in nonobese C57BL/6 (Akita) mutant mice. *Diabetes* 46, 887–894.
- [15] Wang, J., Takeuchi, T., Tanaka, S., Kubo, S.K., Kayo, T., Lu, D., Takata, K., Koizumi, A. and Izumi, T. (1999) A mutation in the insulin 2 gene induces diabetes with severe pancreatic  $\beta$ -cell dysfunction in the *Mody* mouse. *J. Clin. Invest.* 103, 27–37.
- [16] Oyadomari, S., Koizumi, A., Takeda, K., Gotoh, T., Akira, S., Araki, E. and Mori, M. (2002) Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *J. Clin. Invest.* 109, 525–532.
- [17] Ron, D. (2002) Proteotoxicity in the endoplasmic reticulum: lessons from the Akita diabetic mouse. *J. Clin. Invest.* 109, 443–445.
- [18] Izumi, T., Yokota-Hashimoto, H., Zhao, S., Wang, J., Halban, P.A. and Takeuchi, T. (2003) Dominant negative pathogenesis by mutant proinsulin in the Akita diabetic mouse. *Diabetes* 52, 409–416.
- [19] Zuber, C., Fan, J.Y., Guhl, B. and Roth, J. (2004) Misfolded proinsulin accumulates in expanded pre-Golgi intermediates and endoplasmic reticulum subdomains in pancreatic  $\beta$  cells of Akita mice. *FASEB J.* 18, 917–919.
- [20] Liu, M., Hodish, I., Rhodes, C.J. and Arvan, P. (2007) Proinsulin maturation, misfolding, and proteotoxicity. *Proc. Natl. Acad. Sci. USA* 104, 15841–15846.
- [21] Steiner, D.F., Cunningham, D., Spigelman, L. and Aten, B. (1967) Insulin biosynthesis: evidence for a precursor. *Science* 157, 697–700.
- [22] Steiner, D.F., Clark, J.L., Nolan, C., Rubenstein, A.H., Margoliash, E., Aten, B. and Oyer, P.E. (1969) Proinsulin and the biosynthesis of insulin. *Recent Prog. Horm. Res.* 25, 207–282.
- [23] Dodson, G. and Steiner, D. (1998) The role of assembly in insulin's biosynthesis. *Curr. Opin. Struct. Biol.* 8, 189–194.
- [24] Miller, J.A., Narhi, L.O., Hua, Q.X., Rosenfeld, R., Arakawa, T., Rohde, M., Prestrelski, S., Lauren, S., Stoney, K.S., Tsai, L., et al. (1993) Oxidative refolding of insulin-like growth factor 1 yields two products of similar thermodynamic stability: a bifurcating protein-folding pathway. *Biochemistry* 32, 5203–5213.
- [25] Qiao, Z.S., Guo, Z.Y. and Feng, Y.M. (2001) Putative disulfide-forming pathway of porcine insulin precursor during its refolding in vitro. *Biochemistry* 40, 2662–2668.
- [26] Hua, Q.X., Liu, M., Hu, S.Q., Jia, W., Arvan, P. and Weiss, M.A. (2006) A conserved histidine in insulin is required for the foldability of human proinsulin. Structure and function of an Ala<sup>B5</sup> analog. *J. Biol. Chem.* 281, 24889–24899.
- [27] Hua, Q.X., Mayer, J., Jia, W., Zhang, J. and Weiss, M.A. (2006) The folding nucleus of the insulin superfamily: a flexible peptide model foreshadows the native state. *J. Biol. Chem.* 281, 28131–28142.
- [28] Qiao, Z.S., Guo, Z.Y. and Feng, Y.M. (2006) In vitro folding/unfolding of insulin/single-chain insulin. *Protein Pept. Lett.* 13, 423–429.
- [29] Guo, Z.Y., Qiao, Z.S. and Feng, Y.M. (2008) The in vitro oxidative folding of the insulin superfamily. *Antioxid. Redox Signal.* 10, 127–140.
- [30] Hodish, I., Liu, M., Rajpal, G., Larkin, D., Holz, R.W., Adams, A., Liu, L. and Arvan, P. (2010) Misfolded proinsulin affects bystander proinsulin in neonatal diabetes. *J. Biol. Chem.* 285, 685–694.
- [31] Hodish, I., Absood, A., Liu, L., Liu, M., Haataja, L., Larkin, D., Al-Khafaji, A., Zaki, A. and Arvan, P. (2011) In vivo misfolding of proinsulin below the threshold of frank diabetes. *Diabetes* 60, 2092–2101.
- [32] Haataja, L., Snapp, E., Wright, J., Liu, M., Hardy, A.B., Wheeler, M.B., Markwardt, M.L., Rizzo, M. and Arvan, P. (2013) Proinsulin intermolecular interactions during secretory trafficking in pancreatic  $\beta$  cells. *J. Biol. Chem.* 288, 1896–1906.
- [33] Absood, A., Gandomani, B., Zaki, A., Nasta, V., Michail, A., Habib, P.M. and Hodish, I. (2013) Insulin therapy for pre-hyperglycemic  $\beta$ -cell endoplasmic reticulum crowding. *PLoS ONE* 8, e54351.
- [34] Pierce, S.B., Costa, M., Wisotzkey, R., Devadhar, S., Homburger, S.A., Buchman, A.R., Ferguson, K.C., Heller, J., Platt, D.M., Pasquinelli, A.A., et al. (2001) Regulation of DAF-2 receptor signaling by human insulin and ins-1, a

- member of the unusually large and diverse *C. elegans* insulin gene family. *Genes Dev.* 15, 672–686.
- [35] Hua, Q.X., Nakagawa, S.H., Wilken, J., Ramos, R.R., Jia, W., Bass, J. and Weiss, M.A. (2003) A divergent INS protein in *Caenorhabditis elegans* structurally resembles human insulin and activates the human insulin receptor. *Genes Dev.* 17, 826–831.
- [36] Sajid, W., Kulahin, N., Schluckebier, G., Ribbel, U., Henderson, H.R., Tatar, M., Hansen, B.F., Svendsen, A.M., Kiselyov, V.V., Norgaard, P., et al. (2011) Structural and biological properties of the *Drosophila* insulin-like peptide 5 show evolutionary conservation. *J. Biol. Chem.* 286, 661–673.
- [37] Malaguarnera, R., Sacco, A., Voci, C., Pandini, G., Vigneri, R. and Belfiore, A. (2012) Proinsulin binds with high affinity the insulin receptor isoform A and predominantly activates the mitogenic pathway. *Endocrinology* 153, 2152–2163.
- [38] Galloway, J.A., Hooper, S.A., Spradlin, C.T., Howey, D.C., Frank, B.H., Bowsher, R.R. and Anderson, J.H. (1992) Biosynthetic human proinsulin. Review of chemistry, in vitro and in vivo receptor binding, animal and human pharmacology studies, and clinical trial experience. *Diabetes Care* 15, 666–692.
- [39] Steiner, D.F. (1967) Evidence for a precursor in the biosynthesis of insulin. *Trans. N.Y. Acad. Sci.* 30, 60–68.
- [40] Narhi, L.O., Hua, Q.X., Arakawa, T., Fox, G.M., Tsai, L., Rosenfeld, R., Holst, P., Miller, J.A. and Weiss, M.A. (1993) Role of native disulfide bonds in the structure and activity of insulin-like growth factor 1: genetic models of protein-folding intermediates. *Biochemistry* 32, 5214–5221.
- [41] Hua, Q.X., Hu, S.Q., Frank, B.H., Jia, W., Chu, Y.C., Wang, S.H., Burke, G.T., Katsyannis, P.G. and Weiss, M.A. (1996) Mapping the functional surface of insulin by design: structure and function of a novel A-chain analogue. *J. Mol. Biol.* 264, 390–403.
- [42] Dai, Y. and Tang, J.G. (1996) Characteristic, activity and conformational studies of [A6-Ser, A11-Ser]-insulin. *Biochim. Biophys. Acta* 1296, 63–68.
- [43] Hober, S., Uhlen, M. and Nilsson, B. (1997) Disulfide exchange folding of disulfide mutants of insulin-like growth factor I in vitro. *Biochemistry* 36, 4616–4622.
- [44] Weiss, M.A., Hua, Q.X., Jia, W., Chu, Y.C., Wang, R.Y. and Katsyannis, P.G. (2000) Hierarchical protein “un-design”: insulin’s intrachain disulfide bridge tethers a recognition  $\alpha$ -helix. *Biochemistry* 39, 15429–15440.
- [45] Guo, Z.Y. and Feng, Y.M. (2001) Effects of cysteine to serine substitutions in the two intra-A-chain disulfide bonds of insulin. *Biol. Chem.* 382, 443–448.
- [46] Feng, Y., Liu, D. and Wang, J. (2003) Native-like partially folded conformations and folding process revealed in the N-terminal large fragments of staphylococcal nuclease: a study by NMR spectroscopy. *J. Mol. Biol.* 330, 821–837.
- [47] Jia, X.Y., Guo, Z.Y., Wang, Y., Xu, Y., Duan, S.S. and Feng, Y.M. (2003) Peptide models of four possible insulin folding intermediates with two disulfides. *Protein Sci.* 12, 2412–2419.
- [48] Yan, H., Guo, Z.Y., Gong, X.W., Xi, D. and Feng, Y.M. (2003) A peptide model of insulin folding intermediate with one disulfide. *Protein Sci.* 12, 768–775.
- [49] Yang, Y., Hua, Q.X., Liu, J., Shimizu, E.H., Choquette, M.H., Mackin, R.B. and Weiss, M.A. (2010) Solution structure of proinsulin: connecting domain flexibility and prohormone processing. *J. Biol. Chem.* 285, 7847–7851.
- [50] Frank, B.H. and Veros, A.J. (1968) Physical studies on proinsulin-association behavior and conformation in solution. *Biochem. Biophys. Res. Commun.* 32, 155–160.
- [51] Pekar, A.H. and Frank, B.H. (1972) Conformation of proinsulin. A comparison of insulin and proinsulin self-association at neutral pH. *Biochemistry* 11, 4013–4016.
- [52] Frank, B.H., Pekar, A.H. and Veros, A.J. (1972) Insulin and proinsulin conformation in solution. *Diabetes* 21, 486–491.
- [53] Snell, C.R. and Smyth, D.G. (1975) Proinsulin: a proposed three-dimensional structure. *J. Biol. Chem.* 250, 6291–6295.
- [54] Brems, D.N., Brown, P.L., Heckenlaible, L.A. and Frank, B.H. (1990) Equilibrium denaturation of insulin and proinsulin. *Biochemistry* 29, 9289–9293.
- [55] Weiss, M.A., Frank, B.H., Khait, I., Pekar, A., Heiney, R., Shoelson, S.E. and Neuringer, L.J. (1990) NMR and photo-CIDNP studies of human proinsulin and prohormone processing intermediates with application to endopeptidase recognition. *Biochemistry* 29, 8389–8401.
- [56] Sieber, P.S., Eisler, K., Kamber, B., Riniker, B., Rittel, W., Marki, F. and deGasparo, M. (1978) Synthesis and biological activity of two disulfide bond isomers of human insulin: [A7–A11, A6–B7-cystine]- and [A6–A7, A11–B7-cystine] insulin (human). *Hoppe Seylers Z. Physiol. Chem.* 359, 113–123.
- [57] Hua, Q.X., Gozani, S.N., Chance, R.E., Hoffmann, J.A., Frank, B.H. and Weiss, M.A. (1995) Structure of a protein in a kinetic trap. *Nat. Struct. Biol.* 2, 129–138.
- [58] Hua, Q.X., Jia, W., Frank, B.H., Phillips, N.B. and Weiss, M.A. (2002) A protein caught in a kinetic trap: structures and stabilities of insulin disulfide isomers. *Biochemistry* 41, 14700–14715.
- [59] Huang, X.F. and Arvan, P. (1994) Formation of the insulin-containing secretory granule core occurs within immature  $\beta$ -granules. *J. Biol. Chem.* 269, 20838–20844.
- [60] Steiner, D.F. (1998) The proprotein convertases. *Curr. Opin. Chem. Biol.* 2, 31–39.
- [61] Greider, M.H., Howell, S.L. and Lacy, P.E. (1969) Isolation and properties of secretory granules from rat islets of Langerhans. II: ultrastructure of the  $\beta$  granule. *J. Cell Biol.* 41, 162–166.
- [62] Lange, R.H. (1974) Crystalline islet B-granules in the grass snake (*Natrix natrix* (L.)): tilting experiments in the electron microscope. *J. Ultrastruct. Res.* 46, 301–307.
- [63] Michael, J., Carroll, R., Swift, H.H. and Steiner, D.F. (1987) Studies on the molecular organization of rat insulin secretory granules. *J. Biol. Chem.* 262, 16531–16535.
- [64] Huang, X.F. and Arvan, P. (1995) Intracellular transport of proinsulin in pancreatic  $\beta$ -cells. Structural maturation probed by disulfide accessibility. *J. Biol. Chem.* 270, 20417–20423.
- [65] Brange, J. and Langkjaer, L. (1997) Insulin formation and delivery in: Protein Delivery: Physical Systems (Sanders, L.M. and Hendren, R.W., Eds.), pp. 343–410, Plenum Press, New York.
- [66] Dobson, C.M. (2003) Protein folding and misfolding. *Nature* 426, 884–890.
- [67] Lemaire, K., Ravier, M.A., Schraenen, A., Creemers, J.W., Van de Plas, R., Granvik, M., Van Lommel, L., Waekens, E., Chimienti, F., Rutter, G.A., et al. (2009) Insulin crystallization depends on zinc transporter ZnT8 expression, but is not required for normal glucose homeostasis in mice. *Proc. Natl. Acad. Sci. USA* 106, 14872–14877.
- [68] Katsyannis, P.G. (1966) Synthesis of insulin. *Science* 154, 1509–1514.
- [69] Tang, J.G. and Tsou, C.L. (1990) The insulin A and B chains contain structural information for the formation of the native molecule. Studies with protein disulfide-isomerase. *Biochem. J.* 268, 429–435.
- [70] Wang, C.C. and Tsou, C.L. (1991) The insulin A and B chains contain sufficient structural information to form the native molecule. *Trends Biochem. Sci.* 16, 279–281.
- [71] Brange, J. (1997) The new era of biotech insulin analogues. *Diabetologia* 40, S48–S53.
- [72] Hirsch, I.B. (2005) Insulin analogues. *N. Engl. J. Med.* 352, 174–183.
- [73] Hu, S.Q., Burke, G.T., Schwartz, G.P., Ferderigos, N., Ross, J.B. and Katsyannis, P.G. (1993) Steric requirements at position B12 for high biological activity in insulin. *Biochemistry* 32, 2631–2635.
- [74] Weiss, M.A., Nakagawa, S.H., Jia, W., Xu, B., Hua, Q.X., Chu, Y.C., Wang, R.Y. and Katsyannis, P.G. (2002) Protein structure and the spandrels of San Marco: insulin’s receptor-binding surface is buttressed by an invariant leucine essential for protein stability. *Biochemistry* 41, 809–819.
- [75] Hua, Q.X., Chu, Y.C., Jia, W., Phillips, N.B., Wang, R.Y., Katsyannis, P.G. and Weiss, M.A. (2002) Mechanism of insulin chain combination. Asymmetric roles of A-chain  $\alpha$ -helices in disulfide pairing. *J. Biol. Chem.* 277, 43443–43453.
- [76] Nakagawa, S.H., Zhao, M., Hua, Q.X., Hu, S.Q., Wan, Z.L., Jia, W. and Weiss, M.A. (2005) Chiral mutagenesis of insulin. Foldability and function are inversely regulated by a stereospecific switch in the B chain. *Biochemistry* 44, 4984–4999.
- [77] Nakagawa, S.H., Hua, Q.X., Hu, S.Q., Jia, W., Wang, S., Katsyannis, P.G. and Weiss, M.A. (2006) Chiral mutagenesis of insulin. Contribution of the B20–B23  $\beta$ -turn to activity and stability. *J. Biol. Chem.* 281, 22386–22396.
- [78] Hua, Q.X., Nakagawa, S.H., Hu, S.Q., Jia, W., Wang, S. and Weiss, M.A. (2006) Toward the active conformation of insulin. Stereospecific modulation of a structural switch in the B chain. *J. Biol. Chem.* 281, 24900–24909.
- [79] Wan, Z., Huang, K., Whittaker, J. and Weiss, M.A. (2008) The structure of a mutant insulin uncouples receptor binding from protein allostery. An electrostatic block to the TR transition. *J. Biol. Chem.* 283, 21198–21210.
- [80] Baldwin, T.O., Ziegler, M.M., Chaffotte, A.F. and Goldberg, M.E. (1993) Contribution of folding steps involving the individual subunits of bacterial luciferase to the assembly of the active heterodimeric enzyme. *J. Biol. Chem.* 268, 10766–10772.
- [81] Huang, Y., Liang, Z. and Feng, Y. (2001) The relationship between the connecting peptide of recombinant single chain insulin and its biological function. *Sci. China* 44, 593–600.
- [82] Hober, S., Forsberg, G., Palm, G., Hartmanis, M. and Nilsson, B. (1992) Disulfide exchange folding of insulin-like growth factor I. *Biochemistry* 31, 1749–1756.
- [83] Creighton, T.E. (1997) Protein folding coupled to disulfide bond formation. *Biol. Chem.* 378, 731–744.
- [84] Dill, K.A. and Chan, H.S. (1997) From Levinthal to pathways to funnels. *Nat. Struct. Biol.* 4, 10–19.
- [85] Onuchic, J.N., Luthey-Schulten, Z. and Wolynes, P.G. (1997) Theory of protein folding: the energy landscape perspective. *Annu. Rev. Phys. Chem.* 48, 545–600.
- [86] Oliveberg, M. and Wolynes, P.G. (2005) The experimental survey of protein-folding energy landscapes. *Q. Rev. Biophys.* 38, 245–288.
- [87] Qiao, Z.S., Min, C.Y., Hua, Q.X., Weiss, M.A. and Feng, Y.M. (2003) In vitro refolding of human proinsulin. Kinetic intermediates, putative disulfide-forming pathway, folding initiation site, and potential role of C-peptide in folding process. *J. Biol. Chem.* 278, 17800–17809.
- [88] Hober, S., Hansson, A., Uhlen, M. and Nilsson, B. (1994) Folding of insulin-like growth factor I is thermodynamically controlled by insulin-like growth factor binding protein. *Biochemistry* 33, 6758–6761.
- [89] Milner, S.J., Carver, J.A., Ballard, F.J. and Francis, G.L. (1999) Probing the disulfide folding pathway of insulin-like growth factor-I. *Biotechnol. Bioeng.* 62, 693–703.
- [90] Hua, Q.X., Narhi, L., Jia, W., Arakawa, T., Rosenfeld, R., Hawkins, N., Miller, J.A. and Weiss, M.A. (1996) Native and non-native structure in a protein-folding intermediate: spectroscopic studies of partially reduced IGF-I and an engineered alanine model. *J. Mol. Biol.* 259, 297–313.

- [91] Hua, Q.X., Nakagawa, S.H., Jia, W., Hu, S.Q., Chu, Y.C., Katsoyannis, P.G. and Weiss, M.A. (2001) Hierarchical protein folding: asymmetric unfolding of an insulin analogue lacking the A7–B7 interchain disulfide bridge. *Biochemistry* 40, 12299–12311.
- [92] Chu, Y.C., Burke, G.T., Chanley, J.D. and Katsoyannis, P.G. (1987) Possible involvement of the A20–A21 peptide bond in the expression of the biological activity of insulin. 2. [21-Asparagine diethylamide-A]insulin. *Biochemistry* 26, 6972–6975.
- [93] Kristensen, C., Kjeldsen, T., Wiberg, F.C., Schaffer, L., Hach, M., Havelund, S., Bass, J., Steiner, D.F. and Andersen, A.S. (1997) Alanine scanning mutagenesis of insulin. *J. Biol. Chem.* 272, 12978–12983.
- [94] Gill, R., Verma, C., Wallach, B., Urso, B., Pitts, J., Wollmer, A., De Meyts, P. and Wood, S. (1999) Modelling of the disulphide-swapped isomer of human insulin-like growth factor-1: implications for receptor binding. *Protein Eng.* 12, 297–303.
- [95] Chen, Y., You, Y., Jin, R., Guo, Z.Y. and Feng, Y.M. (2004) Sequences of B-chain/domain 1–10/1–9 of insulin and insulin-like growth factor 1 determine their different folding behavior. *Biochemistry* 43, 9225–9233.
- [96] Huang, Q.L., Zhao, J., Tang, Y.H., Shao, S.Q., Xu, G.J. and Feng, Y.M. (2007) The sequence determinant causing different folding behaviors of insulin and insulin-like growth factor-1. *Biochemistry* 46, 218–224.
- [97] Liu, M., Ramos-Castaneda, J. and Arvan, P. (2003) Role of the connecting peptide in insulin biosynthesis. *J. Biol. Chem.* 278, 14798–14805.
- [98] Zhang, B.Y., Liu, M. and Arvan, P. (2003) Behavior in the eukaryotic secretory pathway of insulin-containing fusion proteins and single-chain insulins bearing various B-chain mutations. *J. Biol. Chem.* 278, 3687–3693.
- [99] Liu, M., Li, Y., Cavener, D. and Arvan, P. (2005) Proinsulin disulfide maturation and misfolding in the endoplasmic reticulum. *J. Biol. Chem.* 280, 13209–13212.
- [100] Liu, M., Hua, Q.-X., Hu, S.-Q., Jia, W., Yang, Y., Saith, S.E., Whittaker, J., Arvan, P. and Weiss, M.A. (2010) Deciphering the hidden informational content of protein sequences: foldability of proinsulin hinges on a flexible arm that is dispensable in the mature hormone. *J. Biol. Chem.* 285, 30989–31001.
- [101] Noiva, R. (1999) Protein disulfide isomerase: the multifunctional redox chaperone of the endoplasmic reticulum. *Semin. Cell Dev. Biol.* 10, 481–493.
- [102] Zhu, Y.L., Abdo, A., Gesmonde, J.F., Zawalich, K.C., Zawalich, W. and Dannies, P.S. (2004) Aggregation and lack of secretion of most newly synthesized proinsulin in non- $\beta$ -cell lines. *Endocrinology* 145, 3840–3849.
- [103] Markussen, J. (1985) Comparative reduction/oxidation studies with single chain des-(B30) insulin and porcine proinsulin. *Int. J. Pept. Protein Res.* 25, 431–434.
- [104] Murphy, R., Ellard, S. and Hattersley, A.T. (2008) Clinical implications of a molecular genetic classification of monogenic  $\beta$ -cell diabetes. *Nat. Clin. Pract. Endocrinol. Metab.* 4, 200–213.
- [105] Slingerland, A.S. and Hattersley, A.T. (2005) Mutations in the Kir6.2 subunit of the KATP channel and permanent neonatal diabetes: new insights and new treatment. *Ann. Med.* 37, 186–195.
- [106] Babenko, A.P., Polak, M., Cave, H., Busiah, K., Czernichow, P., Scharfmann, R., Bryan, J., Aguilar-Bryan, L., Vaxillaire, M. and Froguel, P. (2006) Activating mutations in the ABCC8 gene in neonatal diabetes mellitus. *N. Engl. J. Med.* 355, 456–466.
- [107] Yoshinaga, T., Nakatome, K., Nozaki, J., Naitoh, M., Hoseki, J., Kubota, H., Nagata, K. and Koizumi, A. (2005) Proinsulin lacking the A7–B7 disulfide bond, Ins2Akita, tends to aggregate due to the exposed hydrophobic surface. *Biol. Chem.* 386, 1077–1085.
- [108] Herbach, N., Rahtkolb, B., Kemter, E., Pichl, L., Klafien, M., De Angelis, M.H., Halban, P., Wolf, E., Aigner, B. and Wanker, R. (2007) Dominant-negative effects of a novel mutated *Ins2* allele causes early-onset diabetes and severe  $\beta$ -cell loss in Munich *Ins2*<sup>C95S</sup> mutant mice. *Diabetes* 56, 1268–1276.
- [109] Shoelson, S., Haneda, M., Blix, P., Nanjo, A., Sanke, T., Inouye, K., Steiner, D., Rubenstein, A. and Tager, H. (1983) Three mutant insulins in man. *Nature* 302, 540–543.
- [110] Shoelson, S.E., Polonsky, K.S., Zedler, A., Rubenstein, A.H. and Tager, H.S. (1984) Human insullin B24 (Phe→Ser), secretion and metabolic clearance of the abnormal insulin in man and in a dog model. *J. Clin. Invest.* 73, 1351–1358.
- [111] Fonseca, S.G., Gromada, J. and Urano, F. (2011) Endoplasmic reticulum stress and pancreatic  $\beta$ -cell death. *Trends Endocrinol. Metab.* 22, 266–274.
- [112] Thomas, S.E., Dalton, L., Malzer, E. and Marciniak, S.J. (2011) Unravelling the story of protein misfolding in diabetes mellitus. *World J. Diab.* 2, 114–118.
- [113] Yuan, Q., Tang, W., Zhang, X., Hinson, J.A., Liu, C., Osei, K. and Wang, J. (2012) Proinsulin atypical maturation and disposal induces extensive defects in mouse *Ins2*<sup>+/Akita</sup>  $\beta$ -cells. *PLoS ONE* 7, e35098.
- [114] Despa, F. (2010) Endoplasmic reticulum overcrowding as a mechanism of  $\beta$ -cell dysfunction in diabetes. *Biophys. J.* 98, 1641–1648.
- [115] Steiner, D.F., Tager, H.S., Chan, S.J., Nanjo, K., Sanke, T. and Rubenstein, A.H. (1990) Lessons learned from molecular biology of insulin-gene mutations. *Diabetes Care* 13, 600–609.
- [116] Schwartz, G.P., Burke, G.T. and Katsoyannis, P.G. (1987) A superactive insulin: [B10-aspartic acid]insulin(human). *Proc. Natl. Acad. Sci. USA* 84, 6408–6411.
- [117] Carroll, R.J., Hammer, R.E., Chan, S.J., Swift, H.H., Rubenstein, A.H. and Steiner, D.F. (1988) A mutant human proinsulin is secreted from islets of Langerhans in increased amounts via an unregulated pathway. *Proc. Natl. Acad. Sci. USA* 85, 8943–8947.
- [118] Gross, D.J., Halban, P.A., Kahn, C.R., Weir, G.C. and Villa-Komaroff, L. (1989) Partial diversion of a mutant proinsulin (B10 aspartic acid) from the regulated to the constitutive secretory pathway in transfected AtT-20 cells. *Proc. Natl. Acad. Sci. USA* 86, 4107–4111.
- [119] Chan, S.J., Seino, S., Gruppuso, P.A., Schwartz, R. and Steiner, D.F. (1987) A mutation in the B chain coding region is associated with impaired proinsulin conversion in a family with hyperproinsulinemia. *Proc. Natl. Acad. Sci. USA* 84, 2194–2197.
- [120] Hua, Q.X., Shoelson, S.E., Kochoyan, M. and Weiss, M.A. (1991) Receptor binding redefined by a structural switch in a mutant human insulin. *Nature* 354, 238–241.
- [121] Olsen, H.B., Ludvigsen, S. and Kaarsholm, N.C. (1996) Solution structure of an engineered insulin monomer at neutral pH. *Biochemistry* 35, 8836–8845.
- [122] Blundell, T.L., Cutfield, J.F., Cutfield, S.M., Dodson, E.J., Dodson, G.G., Hodgkin, D.C., Mercola, D.A. and Vijayan, M. (1971) Atomic positions in rhombohedral 2-zinc insulin crystals. *Nature* 231, 506–511.
- [123] Baker, E.N., Blundell, T.L., Cutfield, J.F., Cutfield, S.M., Dodson, E.J., Dodson, G.G., Hodgkin, D.M., Hubbard, R.E., Isaacs, N.W. and Reynolds, C.D. (1988) The structure of 2Zn pig insulin crystals at 1.5 Å resolution. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 319, 369–456.
- [124] Betts, S. and King, J. (1999) There's a right way and a wrong way: in vivo and in vitro folding, misfolding and subunit assembly of the P22 tailspike. *Structure* 7, R131–R139.
- [125] Weigle, P.R., Haase-Pettingell, C., Campbell, P.G., Gossard, D.C. and King, J. (2005) Stalled folding mutants in the triple  $\beta$ -helix domain of the phage P22 tailspike adhesin. *J. Mol. Biol.* 354, 1103–1117.
- [126] Liu, M., Wan, Z.L., Chu, Y.C., Aladdin, H., Klaproth, B., Choquette, M.H., Hua, Q.X., Mackin, R.B., Rao, J.S., De Meyts, P., et al. (2009) Crystal structure of a “non-foldable” insulin: impaired folding efficiency and ER stress despite native activity. *J. Biol. Chem.* 284, 35259–35272.
- [127] Brader, M.L. and Dunn, M.F. (1991) Insulin hexamers: new conformations and applications. *Trends Biochem. Sci.* 16, 341–345.
- [128] Menting, J.G., Whittaker, J., Margetts, M.B., Whittaker, L.J., Kong, G.K.-W., Smith, B.J., Watson, C.J., Žáková, L., Kletvikova, E., Jiráček, J., et al. (2013) How insulin engages its primary binding site on the insulin receptor surface. *Nature* 493, 241–245.
- [129] Mirmira, R.G., Nakagawa, S.H. and Tager, H.S. (1991) Importance of the character and configuration of residues B24, B25, and B26 in insulin–receptor interactions. *J. Biol. Chem.* 266, 1428–1436.
- [130] Hua, Q.X., Xu, B., Huang, K., Hu, S.Q., Nakagawa, S., Jia, W., Wang, S., Whittaker, J., Katsoyannis, P.G. and Weiss, M.A. (2009) Enhancing the activity of insulin by stereospecific unfolding. *Conformational life cycle of insulin and its evolutionary origins.* *J. Biol. Chem.* 284, 14586–14596.
- [131] Xu, B., Hu, S.Q., Chu, Y.C., Wang, S., Wang, R.Y., Nakagawa, S.H., Katsoyannis, P.G. and Weiss, M.A. (2004) Diabetes-associated mutations in insulin identify invariant receptor contacts. *Diabetes* 53, 1599–1602.
- [132] De Meyts, P. (1994) The structural basis of insulin and insulin-like growth factor-I receptor binding and negative co-operativity, and its relevance to mitogenic versus metabolic signaling. *Diabetologia* 37, S135–S148.
- [133] Schäffer, L. (1994) A model for insulin binding to the insulin receptor. *Eur. J. Biochem.* 221, 1127–1132.
- [134] De Meyts, P. and Whittaker, J. (2002) Structural biology of insulin and IGF1 receptors: implications for drug design. *Nat. Rev. Drug Discov.* 1, 769–783.
- [135] Malaguarnera, R. and Belfiore, A. (2011) The insulin receptor: a new target for cancer therapy. *Front. Endocrinol. (Lausanne)* 2, 93.
- [136] Ptitsyn, O.B. (1991) How does protein synthesis give rise to the 3D-structure. *FEBS Lett.* 285, 176–181.
- [137] Matagne, A., Radford, S.E. and Dobson, C.M. (1997) Fast and slow tracks in lysozyme folding: insight into the role of domains in the folding process. *J. Mol. Biol.* 267, 1068–1074.
- [138] Yokota, A., Izutani, K., Takai, M., Kubo, Y., Noda, Y., Koumoto, Y., Tachibana, H. and Segawa, S. (2000) The transition state in the folding-unfolding reaction of four species of three-disulfide variant of hen lysozyme: the role of each disulfide bridge. *J. Mol. Biol.* 295, 1275–1288.
- [139] Lazaridis, T. and Karplus, M. (1997) “New view” of protein folding reconciled with the old through multiple unfolding simulations. *Science* 278, 1928–1931.
- [140] Mirny, L.A., Abkevich, V.I. and Shakhnovich, E.I. (1998) How evolution makes proteins fold quickly. *Proc. Natl. Acad. Sci. USA* 95, 4976–4981.