Design of ultra-stable insulin analogues for the developing world

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ABSTRACT
The engineering of insulin analogues illustrates the application of structure-based protein design to clinical medicine. Such design has traditionally been based on structures of wild-type insulin hexamers in an effort to optimize the pharmacokinetic (PK) and pharmacodynamic properties of the hormone. Rapid-acting insulin analogues (in chronological order of their clinical introduction, Humalog® [Eli Lilly & Co.], Novolog® [Novo-Nordisk], and Apidra® [Sanofi-Aventis]) exploit the targeted destabilization of subunit interfaces to facilitate capillary absorption. Conversely, long-acting insulin analogues exploit the stability of the insulin hexamer and its higher-order self-assembly within the subcutaneous depot to enhance basal glycemic control. Current products either operate through isoelectric precipitation (insulin glargine, the active component of Lantus®; Sanofi-Aventis) or employ an albumin-binding acyl tether (insulin detemir, the active component of Levemir®; Novo-Nordisk).

Such molecular engineering has often encountered a trade-off between PK goals and product stability. Given the global dimensions of the diabetes pandemic and complexity of an associated cold chain of insulin distribution, we envisage that concurrent engineering of ultra-stable protein analogue formulations would benefit the developing world, especially for patients exposed to high temperatures with inconsistent access to refrigeration. We review the principal mechanisms of insulin degradation above room temperature and novel molecular approaches toward the design of ultra-stable rapid-acting and basal formulations.

Keywords: Diabetes mellitus, nanotechnology, protein engineering, protein stability, protein degradation

INTRODUCTION
Elucidation of the crystal structure of the insulin hexamer in 1969 by the late Hodgkin DC et al., was a landmark in structural biology [Figure 1].[1,2] The first depiction of a protein homo-oligomer and associated zinc (Zn) binding site, the three-dimensional structure of insulin continues to provide rich insight into multiple biological and pharmacologic processes, ranging from the biosynthesis and storage of insulin in pancreatic β-cells,[3] to analogue design to enhance the treatment of diabetes mellitus (DM).[4-6] Although academic
studies of proteins have ordinarily focused on their native properties, the clinical importance of insulin has motivated detailed study of molecular mechanisms of chemical and physical degradation.[7,8] Such studies foreshadowed the recognition of analogous principles underlying diverse diseases of protein misfolding[9,10]

Although the insulin monomer (51 amino acids) represents a small motif of protein folding, the Zn hexamer exhibits key features of globular proteins in general: Canonical elements of secondary structure (the α-helix, 3_{10}-helix, β-sheet and β-turn), tertiary organization of a hydrophobic core, specific interfaces for self-assembly, and allosteric regulation through ligand-dependent long-range conformational changes.[11] The crystal structure of insulin underlies modern understanding of how small perturbations in chemical and physical properties influence biological activity. Overall, the key features of folding and assembly of insulin closely parallel these of canonical globular proteins.[12]

The overall goal of insulin products, singly or in combination, is to help patients with DM to mimic the physiologic pattern of insulin secretion by the β-cells and so achieve, at least approximately, metabolic homeostasis. The importance of tight glycemic control in Type 1 DM was shown by the classical Diabetes Control and Complications Trial study,[17] and the subsequent Epidemiology of Diabetes Interventions & Complications Study.[18] In long-established Type 2 DM; subsequent randomization of a hydrophobic core, specific interfaces for self-assembly, and allosteric regulation through ligand-dependent long-range conformational changes.[11] The crystal structure of insulin underlies modern understanding of how small perturbations in chemical and physical properties influence biological activity. Overall, the key features of folding and assembly of insulin closely parallel these of canonical globular proteins.[12]

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Weiss: Ultra-stable insulin analogues

### Table 1: Current insulin analogues and modes of action

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<td>Lispro (Humalog®)</td>
<td>Pro^{1}Lys</td>
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<td>Aspart (NovoLog®)</td>
<td>Pro^{1}Asp</td>
<td>Charge repulsion at dimer interface</td>
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<td>Novo-Nordisk</td>
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<td>Glulisine (Apidra®)</td>
<td>Asn^{1}Lys</td>
<td>Decreased zinc-free self-association</td>
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<td>Lys^{2}Glu</td>
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*Panel A describes rapid-acting analogues employed in prandial regimens and in insulin pumps whereas B lists basal insulin analogues with protracted action. Table is reprinted from Berenson et al. with permission of the authors.[9]*

### Prandial insulin analogues

Design of insulin analogues has followed the development and application of general principles of protein folding and assembly. These principles were first applied to accelerate the rate of disassembly of Zn hexamers.[32,33] Such efforts posited that more rapid disassembly in the subcutaneous depot would enhance capillary absorption.[34,35] Extensive collections of amino-acid substitutions at subunit interfaces were synthesized and characterized. Whereas, mutational disruption of structure is generally straightforward (in contrast to mutational optimization of structure), favourable substitutions were sought based on three functional criteria: Compatibility with high-affinity binding to the insulin receptor (IR), native biological activity, and feasibility of stable pharmaceutical formulation as defined by national regulatory agencies. There are three such rapid-acting analogues in current use [Table 1, Panel A]; in chronological order of clinical introduction, these are insulin lispro (the active component of Humalog®; Eli Lilly), insulin aspart (Novolog®; Novo-Nordisk), and insulin glulisine (Apidra®; Sanofi-Aventis). These products have been proven safe and effective in multi-injection regimens,[14,40] and for use in continuous subcutaneous insulin infusion devices (CSII; “insulin pumps”).[41] The different molecular changes employed in these three products confer more
rapid insulin absorption, reflecting the many ways that the elegantly crafted assembly of wild-type insulin may be destabilized with maintenance of biological activity. Although current rapid-acting insulin analogue products have met the threshold criteria for chemical and physical stability as set by the United States Food & Drug Administration, these formulations are in general more susceptible to physical and chemical degradation above room temperature than are comparable wild-type formulations. This trade-off in principle poses a dilemma for patients and physicians in the developing world as the therapeutic goal of tight glycemic control may come into conflict with the reality of conditions of daily living in the absence of electrification.

X-ray crystallographic studies of insulin lispro and aspart, have revealed native-like assemblies. The details of these structures have nonetheless provided insight into their respective mechanisms of rapid action, leading in turn to deeper insight into the structural determinants of classical dimerization. Hexamer assembly in each case requires the allosteric binding of phenol or the related ligand meta-cresol, commonly employed for their anti-microbial properties. Such ligands were previously found to induce a large-scale allosteric reorganization of the Zn insulin hexamer designated the TR transition. Three families of structures have thus been defined: T6 (the original Hodgkin DC structure of 1969), TRf3 (first observed under high-salt conditions as a rhombohedral transformation of Zn insulin crystals), and R6 (containing six bound ligands per hexamer). Whereas, the T-state protomer resembles the solution structure of insulin as a monomer in solution, in the R-state the B-chain exhibits a change in secondary structure to form an elongated B1 - B19 α-helix; an isolated R-like conformation has not to date been observed. Comparison of these structures by Hodgkin DC et al., provided an informative model for the transmission of conformational change in globular proteins. The crystal structure of insulin lispro was determined as a TRf3 Zn hexamer containing three bound phenolic ligands [Figure 3a], whereas the structure of insulin aspart was determined as an R6 hexamer containing six bound ligands [Figure 3b]. Comparison of their dimer contacts, including dimer-related anti-parallel β-sheets (residues B24 - B28), revealed subtle distortions [Figures 3c and d].

The dual role of phenolic ligands (i.e., the contingent function of anti-microbial preservatives as allosteric effectors) was essential to stabilize the pharmaceutical formulations of insulins lispro and aspart. Although dissociation of these ligands occurs on the millisecond time scale, the TR transition stabilizes Zn binding and profoundly retards rates of subunit exchange between hexamers. On subcutaneous injection, the
ligands presumably diffuse from the injected insulin depot, entering cellular membranes. The unliganded hexamers then rapidly disassemble, facilitating capillary absorption. How these mechanisms of assembly and disassembly differ from those of wild-type insulin is not well-understood, but may be pertinent to their increased susceptibility to thermal degradation. Although wild-type insulin forms monodisperse solutions of Zn hexamers in the presence or absence of phenolic ligands, ligand binding is required for monodisperse hexamer assembly of the analogues. Because, the binding sites are distant from the amino-acid substitutions at positions B28 and/or B29, it is not known whether the structural communication occurs between these sites or whether such ligand-dependent assembly reflects a purely formal thermodynamic linkage. While inter-strand hydrogen bonds in the R₆ aspart hexamer are similar to those of wild-type insulin in the same crystal form [Figure 3d], corresponding inter-strand hydrogen bonds in the T₃R₄ lispro hexamer are in part lengthened [Figure 3c], which might contribute to accelerated disassembly. Because, subsequent spectroscopic studies have suggested that the actual structure of insulin lispro in a formulation is R₆ and not T₃R₄ (based on cobalt models), it is possible that the T₃R₄ crystal structure represents an intermediate state of the subcutaneous depot on partial dissociation of the phenolic ligands.

Although the original ideas underlying the design of insulin aspart envisaged electrostatic repulsion at the dimer interface, mutagenesis studies highlighted the key role played by the absence of ProB28 and so highlighted its implicit contribution to the wild-type interface. This proline, although conserved at this position among vertebrate insulins, had not previously attracted attention, in part because des-tripeptide (B28 - B30)-insulin, des-tetrapeptide (B27 - B30)-insulin, and des-pentapeptide (B26 - B30)-insulin-amide each exhibit native activity. Comparison of the crystal structures of insulins lispro and aspart with wild-type insulin in corresponding crystal forms demonstrated a local distortion of the dimer interface associated with the absence of ProB28. The wild-type pyrrolidine ring contacts GlyB23 in the opposite β-turn [Figures 3a and b]. Thus, informed by the structures of the analogues, this perspective highlighted for the first time the importance of the same contact in the wild-type dimer contact [Figure 3c]. This insight exemplifies the power of mutagenesis to decipher wild-type structure-function relationships even after high-resolution crystallographic analysis.

Advances in closed-loop systems in which control of the pump is controlled by an algorithm based on feedback from a continuous glucose monitor, (i.e., “smart” pumps) have highlighted the need for insulin analogues whose absorption is even faster than current products [Table 1, Panel A]. Diverse technologies have been considered, including injection-site heating to increase blood flow, co-injection of an enzyme (hyaluronidase) to break down connective-tissue barriers, micro-needle patches, needle-free jet injection, and additives (excipients) that might enhance rates of disassembly. The diversity of these approaches highlights the clinical need as perceived in affluent societies. Even as, we anticipate that continuing structural analysis of insulin is likely to provide guidance for future design of ultra-fast analogues synergistic with one or more of these approaches, the trade-off described above between structural strategies for rapid action and protein stability may further limit the practicality of closed-loop systems in the developing world, especially in indigent communities.

**Basal insulin analogues**

A complementary therapeutic objective in the treatment of DM is provided by basal insulin analogue formulations. Whereas, fast-acting analogues are essential for the management of Type 1 DM (including CSII pumps), basal insulin analogues contribute to glycemic control as part of multi-injection regimens and are of fundamental importance in Type 2 DM.

The latter patients, when controllable by either prandial or basal analogues alone, prefer a basal regimen to its simplicity and reduced association with weight gain. The global need for ultra-stable basal products may exceed that of rapid-acting formulations given the emerging pandemic of the metabolic syndrome and Type 2 DM in the developing world. Such epidemiological trends are particularly pronounced in the Arabian Gulf region, wherein temperatures in excess of 45°C (even as high as 50 - 60°C) are routinely encountered, including during the Hajj (pilgrimage).

Despite the global need for ultra-stable basal insulin analogues, the goal of targeted stabilization of the insulin hexamer poses a more subtle challenge to the protein engineer than does the converse goal of targeted destabilization as pertinent to rapid-acting analogues. Evolutionary optimization of insulin and its elegant self-assembly surfaces (conserved among mammalian insulins) obscures possible structural routes to further improvement. Although pioneering efforts in this direction were anticipated by Dodson EJ et al., design of current products has circumvented such detailed structural analysis. Insulin glargine (the active component of Lantus®; Sanofi-Aventis) exploits isoelectric precipitation, a reversible transition to insolubility that classically occurs between pH 5.
and 6 (under which conditions wild-type insulin exhibits little or no net charge).[78,79] This phenomenon is robust to the details of molecular structure. Insulin glargine contains a two-residue basic extension of the B-chain (ArgB31 and ArgB32) whose positive charges result in a shift in the isoelectric point to neutrality. Injection of an unbuffered pH 4 formulation; thus, leads to subcutaneous precipitation and in turn protracted absorption.[80] The B-chain extension is disordered and largely removed by endogenous exopeptidases. Lantus® is the most widely-used long-acting insulin currently on the market.[81] Related analogues containing additional basic residues elsewhere in the insulin molecule have also been described, but are not in clinical use.[82] Insulin detemir (the active component of Levemir®; Novo-Nordisk) contains a fatty acyl group attached to the side chain of LysB29. This prosthetic group mediates binding to serum albumin to provide a circulating depot.[83] Although receptor binding is partially impaired, this decrease in affinity for the insulin receptor (IR) can be overcome by injecting a higher dose (in nanomoles), rendered convenient to patients by a redefinition of units in a U-100 formulation. The tethered moiety serendipitously enhances the stability of hexamers in the subcutaneous depot, further extending its PK properties.[84] Levemir® is nonetheless ordinarily administered twice a day as its duration of action is less prolonged than that of Lantus®.

**MECHANISMS OF INSULIN DEGRADATION**

Future progress toward the design of ultra-stable rapid-acting and basal insulin analogues requires analysis of molecular mechanisms of physical and chemical degradation at elevated temperatures. Physical degradation refers to an irreversible change in the physical state of the protein with no change in its covalent structure. This process—designated fibrillation—is now appreciated to be a universal property of polypeptides leading to cross-β assembly of linear polymers.[85] Because analogous misfolding underlies diverse human diseases, including Alzheimer’s Disease, Parkinson’s Disease, systemic amyloidoses and prion-related encephalopathies,[86-88] the mechanism of protein fibrillation is not well-understood and poses a problem of enormous medical significance.[89] Chemical degradation by contrast refers to a change in the covalent structure of the protein. Mechanisms of chemical degradation have been well-characterized: Principal routes involve deamidation of asparagine, transamidation reactions leading to covalent dimers, and disulfide exchange leading to covalent polymers.[90-94] The relative susceptibility of insulin analogues to chemical degradation correlates with their thermodynamic stabilities and propensity to undergo transient conformational excursions.[7]

To limit degradation, insulin formulations are shipped and stored at 4 - 8°C at which temperature shelf-life is 2 years. Physical and chemical degradation accelerates rapidly with increasing temperature, especially above 30°C. Vials, once used, must be kept below 30°C and discarded after 30 days (15 days in the case of diluted solutions of Humalog® or Novalog®). A general principle is that regions of the molecule that are flexible are most susceptible to degradation and as a corollary conditions that damp conformational fluctuations enhance resistance to degradation. Principal mechanisms of chemical degradation are as follows.[90-95]

**Hydrolitic reactions**

Acid-catalyzed deamidation of AsnB21 and base-catalyzed deamidation of AsnB3 are the principal hydrolytic products in acidic and neutral solutions, respectively. Deamidation of AsnB3 can also lead to the formation of iso-AspB3 with change in main-chain structure. In neutral solutions phenol retards the rate of B3 deamidation by reducing the conformational flexibility of the B1 - B6 segment.[46,90,96] Deamidation products retain essentially native activity and are not associated with adverse immunogenicity. Substitution of A21 (as in insulin glargine) or B3 (as in insulin glulisine, the active component of Apidra®; Sanofi-Aventis) prevents such deamidation.[5]

**Formation of covalent dimers and oligomers**

Covalent insulin dimers and oligomers are formed by transpeptidation between amine groups on one molecule (predominantly the B1 α-amino group) and side-chain carboxyamide groups on another (in the A chain). All types of formulations are susceptible. Heterodimers also are formed in neutral-protamine Hagedorn (NPH) insulin between insulin and protamine by the same mechanism. B1-mediated transpeptidation can be avoided by blocking its α-amino group as is under investigation in analogues containing B1 adducts with polyethylene glycol or thyronine.[5] Covalent dimerization can also be initiated by reaction of insulin with aldehyde impurities of glycerol; these can be avoided by using glycerol of high purity or omitting glycerol from the formulation.

**Formation of disulfide-linked polymers**

Disulfide interchange reactions are initiated by cleavage of cystine A7 - B7 by hydrolysis or β-elimination,[90]; because intermolecular disulfide exchange requires juxtaposition between disulfide bridges between different molecules. Such polymers are detectable in regular formulations, but not in micro-crystalline formulations (such as NPH formulations). Their formation is 5-10-fold faster at 37°C than at 4°C.[90-95]
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The above degradative mechanisms reflect general properties of proteins. There is an additional mechanism of chemical degradation that is unique to classical Ultralente and Lente formulations. Although these long-acting formulations are generally more robust (due to the tight packing and conformational rigidity of the Zn-stabilized rhombohedral crystals), Ultralente and Lente insulins degrade by specific cleavage of the A8 - A9 peptide bond to yield an inactive but immunogenic three-chain species. Remarkably, in this crystal form insulin acts as a specific endopeptidase: A Zn-binding site between hexamers in the crystal lattice mimics the active site of a metalloproteinase, activating a solvent-derived hydroxide molecule for nucleophile attack on the A8 - A9 scissile bond. This mechanism is only of historical interest in light of the limited use of such micro-crystalline suspensions.

In contrast to the well-characterized mechanisms of chemical degradation, how insulin forms fibrils is enigmatic. Such fibrillation poses a significant issue in the production and storage of formulations.[12,97,98] Patients are instructed to inspect vials for signs of precipitation or frosting of the glass, an indication of surface denaturation and fibrillation. Such vials must be discarded. Although rare, obstruction of insulin pumps due to insulin aggregation can be life-threatening for patients with Type 1 DM due to the rapid onset of ketoacidosis.[99,100]

Insulin is a “grandfather” of amyloidogenic proteins. In the late 1920s, it was observed that heating insulin in acidic solutions yielded an inactive precipitate.[101-103] In the 1940s and 1950s Waugh realized that electron microscopy (EM) observable fibrillation precedes precipitation and defined a nucleation-growth mechanism involving three or four insulin monomers.[104-106] Whereas, elevated temperatures are required for the formation of a nucleus, subsequent growth into fibrils can occur at low temperature and is highly cooperative.[107,108] The danger posed by even transient exposure of insulin formulations to elevated temperatures exacerbates the challenges posed by interruptions in the global cold chain of delivery, distribution, and storage in the developing world.

The molecular structure of an insulin proto-filament and mature fibril defines an active field of research. EM studies between 1950 and 1970 defined fibril dimensions and morphological variability.[111-113] Infrared spectra in 1951 first suggested layers of extended β-strands perpendicular to the fibril axis.[114] Raman studies by Yu NT et al. in the 1970s demonstrated that fibrillation is accompanied by an α→β conformational change.[115-117] Insulin fibrillation is accompanied by formation of β-sheet,[118] as characterized by cross-β

X-ray diffraction,[119] a general feature of protein fibrils.[120] The molecular basis of fibrillation is poorly understood. Fibrillation is delayed by classical self-association and by osmolytes like sucrose.[120] Fibrillation is promoted by factors that destabilize the classical self-association pathway from monomer → dimer → tetramer → hexamer → higher-order native assemblies, presumably by increasing the availability of the susceptible monomer [Figure 4].[123] Although relative susceptibilities to fibrillation among analogues do not correlate with their global thermodynamic stabilities, fibrillation is also enhanced by partial unfolding of the monomer by a variety of perturbations, including urea, guanidine, co-solvents (such as ethanol), hydrophobic surfaces, stirring, and high temperature.[120] Insulin fibrillation is accelerated under acidic conditions (pH 1.5 - 2.5), presumably due to dissociation of classical oligomers. The kinetics of fibril formation follows a nucleation-elongation process wherein nucleation involves non-native self-assembly of several partially unfolded monomers; elongation is proposed to occur through subsequent addition of monomers. Nucleation and elongation are manifest as a lag period prior to onset of fibrillation, followed by apparent first-order kinetics in a growth period.[122] The structure of the conformationally altered monomer [purple rhombus in Figure 4] and the nature of its interactions to form a nucleus are poorly understood.[123,124] Although five in equivalent structural models of insulin fibrils have been proposed,[121,125-128] no biophysical data are presently available that discriminate between these proposals.

**NEXT-GENERATION INSULIN ANALOGUES**

Insulin analogues in current clinical use [Table 1] were

![Figure 4: Pathway of insulin fibrillation. Native self-assembly (left) protects insulin from fibrillation through sequestration of monomer (triangle).][120-122] Partial unfolding of monomer (rhombus) exposes non-polar surfaces, leading to an amyloidogenic nucleus and fibril (right). Susceptibility of the insulin monomer to conformational distortion may facilitate receptor binding (bottom) and is distinct from the unfolded state (top); the three disulfide bridges tethering the unfolded state are shown in yellow. Figure is adapted from Fink AL et al.[69] and reprinted from Yang Y et al.[49]
Three novel approaches promise to provide a rational basis for design of innovative ultra-stable insulin analogues. The first, based on biophysical analysis of the native state, seeks to define “Achilles’ heels” whose conformational distortion underlies formation of an amyloidogenic nucleus. An example of such analysis was provided by 13C-NMR studies of an engineered insulin monomer.[69] This approach exploited an empirical relationship (designated the chemical shift index [CSI]) between Cα and Cβ 13C-NMR chemical shifts and elements of secondary structure, such as α-helix and β-sheet.[135,136] Surprisingly, Yang Y et al., observed that the A1 - A8 segment of an insulin monomer, presumed to be α-helical based on crystal structures of insulin dimers and hexamers, exhibits CSI scores indicative of exchange among α-helical and substantially populated non-helical conformations [Figure 5a]. The anomalous attenuation of CSI scores in the A1 - A8 segment correlated with the conformational variability of this segment among multiple independent crystallographic protomers [Figure 5b], suggesting that in each crystal form the α-helix, when considered in detail, is “frozen” in a distinct segmental conformation. Evidence was presented that such segmental instability reflects the presence of multiple β-branched residues in this segment (Ile A2, Val A3, and Thr A8), intrinsically unfavourable to α-helical folding.[137] Although Ile A2 and Val A3 are required for biological activity,[138] substitution of Thr A8 by non-β-branched side chains confers enhanced stability; dampens conformational fluctuations, and augments resistance to fibrillation.[69]

The second approach to obtaining analogues resistant to fibrillation at elevated temperatures exploits models of cross-β assembly.[121,125-128] In such models, the distance between the C-terminus of the B-chain and N-terminus of the A-chain (ordinarily in close proximity in the native state) are generally > 30 Å.[139,140] Imposition of a short peptide tether between these termini (5 - 10 residues in length) may therefore restrict the splaying of A and B chains in the fibrillar state. This idea has led to the design of active single-chain insulin analogues with intrinsic resistance to fibrillation [Figure 6a].[69] Clinical application of such designs will require optimization of their PK/PD properties as well as assessment of potential carcinogenicity in animal models. Recently, investigators at Novo-Nordisk have generalized this strategy by the introduction of a fourth disulfide bridge between A and B chains of an otherwise standard two-chain analogue [Figure 6b]. Tethering novel cysteines at positions B4 and A10 (sites ordinarily nearby in the native state), this cross-linked molecule retains biological activity and also exhibits marked resistance to fibrillation. The analogue retains competence to form T6 Zn hexamers, but not the more stable R6 structures preferred in pharmaceutical formulations. The feasibility of introducing additional disulfide bridges into insulin was motivated by the finding of such non-canonical cysteines within an invertebrate superfamily of insulin-related polypeptides.[141,142]
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CONCLUSION

The discovery of insulin in 1922, a landmark in molecular medicine, elicited its broad and enduring public support of biomedical research in the western world.[144] The pioneering efforts of the late Hodgkin DC[11] and Liang DC[11] (her former student who led the independent studies of the Peking Insulin Structure Group,[144,145] to decipher the atomic structure of insulin and its conformational repertoire extended over six decades and engaged an international network of laboratories. Given this epic history, it seems remarkable that this small globular protein continues to inspire molecular innovation, motivated by clinical needs in the developed and developing worlds. Patients with DM daily navigate between treatment-related hypoglycemia on one hand and hyperglycemic excursions on the other. Whereas, acute metabolic decompensation (diabetic ketoacidosis in Type 1 DM and hyperosmolar coma in Type 2) imposes short-term risks, chronic episodic hyperglycemic excursions impose baseline long-term risks of microvascular, macrovascular, and neurologic complications. Next-generation insulin analogues in affluent societies seek to enable patients to achieve glycemic targets and at the same time enhance the convenience and safety with which patients can manage their daily lives. Ongoing efforts to develop next-generation ultra-rapid insulin analogue formulations and ultra-flat basal insulin analogue formulations promise to permit patients to mimic with greater precision endogenous mechanisms of hormonal regulation.

Principles of protein dynamics and stability will come to the fore in yet another dimension of the diabetes challenge: The emerging pandemic of DM in the developing world.[146] The structural principles discussed in this review promise to enable design of novel insulin analogues to meet the higher standards of stability appropriate in the developed world. Whereas for patients in affluent western societies thermal degradation of insulin and insulin analogues is uncommon, the majority of patients in the coming decades will be living in the developing world, including regions (such as in the Arabian Gulf) routinely exposed to summertime temperatures > 45°C. In such regions intertwined scientific, technical, and societal challenges are posed by the cold chain of insulin delivery, storage, and use in the absence of refrigeration or a reliable electrical grid.[147-149]

The growing prevalence of DM in Saudi Arabia and elsewhere in the Muslim world poses unique challenges given even more extreme temperatures in the Arabian Peninsula,[75] and the medical needs of those fulfilling the pilgrimage of the Hajj, one of the five pillars of Islam.[76] Given this need and its growing scale, we anticipate that third-generation insulin analogues must combine ultra-stability with optimized PK/PD properties. Such efforts are likely to require the present understanding of the native state of insulin to be extended to non-native states, including metastable partial folds and amyloid.[69]

At this frontier design of insulin analogues will require the integration of biochemical principles with ancillary technologies, including the emergent perspective of nanotechnology. We anticipate that such a confluence of technologies will enable diabetes patients in both developed and developing worlds to navigate with ever-increasing safety between the Scylla of hyperglycemia and Charybdis of hypoglycemic episodes.

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