A publication of the American Pharmaceutical Association and the American Chemical Society

JOURNAL OF Pharmaceutical Sciences

May 1997
Volume 86, Number 5

REVIEW ARTICLE

Toward Understanding Insulin Fibrillation

JENS BRANGE[X], LENNART ANDERSEN, ERIK D. LAURSEN, GIORGIO MEYN, AND EIGIL RASMUSSEN

Received July 18, 1996, from the *Novo Nordisk A/S, Novo Alle, DK-2880 Bagsvaerd, Denmark.* Final revised manuscript received December 31, 1996. Accepted for publication January 20, 1997[⊗].

Abstract □ Formation of insulin fibrils is a physical process by which partially unfolded insulin molecules interact with each other to form linear aggregates. Shielding of hydrophobic domains is the main driving force for this process, but formation of intermolecular β-sheet may further stabilize the fibrillar structure. Conformational displacement of the B-chain C-terminal with exposure of nonpolar, aliphatic core residues, including A2, A3, B11, and B15, plays a crucial role in the fibrillation process. Recent crystal analyses and molecular modeling studies have suggested that when insulin fibrillates this exposed domain interacts with a hydrophobic surface domain formed by the aliphatic residues A13, B6, B14, B17, and B18, normally buried when three insulin dimers form a hexamer. In rabbit immunization experiments, insulin fibrils did not elicit an increased immune response with respect to formation of IgG insulin ant bodies when compared with native insulin. In contrast, the IgE response increased with increasing content of insulin in fibrillar form. Strategies and practical approaches to prevent insulin from forming f brils are reviewed. Stabilization of the insulin hexameric structure and blockage of hydrophobic interfaces by addition of surfactants are the most effective means of counteracting insulin fibrillation.

1. Introduction

Like other globular proteins, insulin tends to adopt and maintain a three-dimensional structure in which its hydrophobic surfaces are buried by folding and assembly of individual molecules. However, changes of the native conformation or of its "normal" pattern of assembly can be provoked by a variety of factors. To assure insulin stability the processes that cause insulin deterioration must be understood. The propensity of insulin, under the influence of heat and exposure to hydrophobic surfaces, to undergo conformational changes, resulting in successive, linear aggregation and formation of a viscous gel or insoluble precipitates, has been one of the most intriguing and widely studied phenomena in relation to insulin stability.

Unfortunately this aggregation process has often in the literature been mistermed with phrases like "association", "polymerization", or even "crystal formation". In the following these terms are defined as used in this paper: The marked tendency of insulin to undergo self-association into soluble dimers and hexamers is a natural process which, for example, occurs in vivo in the B-cell as a consequence of processing and storage requirements. Association involves the interaction of two or more insulin monomers in their native conformation and in a spheric (globular) arrangement, and this process is easily reversible by reducing the insulin concentration. Insulin aggregation (fibrillation) also involves non-covalent interaction of several insulin molecules but these are to some extent unfolded (partially denatured) compared with their normal globular structure. This physical process creates long fibers which eventually form large, insoluble aggregates. Insulin fibrillation is normally not reversible without dramatic changes in the solvent environment. Insulin is also known to aggregate and precipitate in the presence of excess Zn^{2+} at pH values above 4, but the tertiary structure is not thought to be significantly affected by this process. Denaturation (complete unfolding) of insulin requires organic solvents. Under such conditions insulin will sometimes precipitate, possibly as unfolded aggregates, but there is no convincing evidence as to its loss of structure. The term insulin polymerization should preferably be used to designate a chemical process whereby two or more insulin molecules are covalently linked together.

The objective of this paper is to survey the biochemical literature on insulin fibril formation in order to present an overview of this subject. Particular attention will be given to studies aimed at identifying the interacting domains in insulin fibrils and elucidating the molecular mechanism of insulin fibril formation. Strategies and means to reduce insulin's tendency to form fibers are also discussed.

2. Early Observations

The original investigators already noticed the irreversible inactivation of insulin that occurred in response to heat treatment,[1] and at the time when crystalline and, therefore,

[⊗] Abstract published in *Advance ACS Abstracts,* March 1, 1997.

© 1997, American Chemical Society and American Pharmaceutical Association

S0022-3549(96)00297-3 CCC: $14.00

Regeneron Pharmaceuticals, Inc. Exhibit 2063 Page 1
Celltrion, Inc. v. Regeneron Pharmaceuticals, Inc. IPR2023-00462

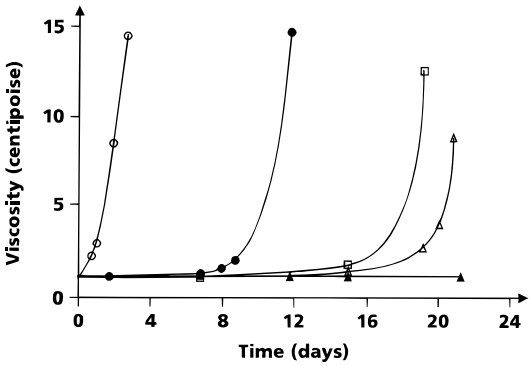

Figure 1—Insulin fibrillation monitored by an increase in viscosity on standing of acid solu ions of different insulins (1.5% insulin, 0.025 M HCl, pH 2.1, 45 mM NaCl, 21 °C): 10 times crystallized bovine insulin (○); Monocomponent bovine insulin (●); 10 times crystallized bovine insulin (lab. preparation) (□); Monocomponent porcine insulin (△); 5 times crystallized porcine insulin (▲).

relatively pure insulin preparations were becoming available, it was found that heating insulin in acid provoked insulin to precipitate in an inactive form.[2−4] The phenomenon was termed heat denaturation or coagulation, but it was shown later that heat precipitation and insulin fibrillation are directly related.[5] The precipitate was shown to be insoluble in acid but to resume its original solubility and activity in dilute alkali.[3,6,7]

The first systematic study of the heat precipitation of insulin was published by Krogh and Hemmingsen,[4] who found the rate of inactivation at pH 3 to be proportional to the insulin concentration and to closely follow the law of Arrhenius in the temperature interval 50−117 °C. Subsequent studies showed the rate of reaction in acid to increase with acidity in the pH range from 3 to 1 and to be dependent of the type of acid (H_2SO_4 > HCl > H_3PO_4 ≫ HAc),[8,9] probably as a consequence of the promoting effect of ion strength on insulin fibrillation (see section 3.1.3).

Waugh[10,11] was the first to realize that formation of insulin fibers precedes precipitation and that the heat treatment does not radically alter the globular nature of the insulin molecule. He later showed that heat precipitation involves three reactions: formation of active centers (nucleation), elongation of these centers to fibrils (growth), and floccule formation (precipitation).[5,12,13]

The nucleation reaction, which is the slowest process, requires the nearly simultaneous interaction of 3 to 4 insulin molecules.[14] Essentially undistorted insulin is involved in these interactions, which are mainly between nonpolar side chains (hydrophobic interactions).[5,13,15] The subunits of these interactions have been suggested to be either the dimer or the monomer of insulin.[13,16] Whereas nucleation seems to require temperatures above normal, the growth into fibrils can proceed at ambient or even lower temperatures and, depending on the conditions, the growth leads to long fibers resulting in a thixotropic gel or to shorter fibers with a tendency to arrange radially to spherolites with precipitation as the consequence.[13,14] The growth of fibrils has been found to be a function of the surface area of the fibril population and the concentration of insulin in solution,[17] and thus it is a highly cooperative process able to quantitatively remove the insulin from solution in the fibrous form. An increase in ionic strength increases the rate of growth and, in particular, nucleation[14] whereas high concentrations of organic acids, urea and phenol suppress the nucleation reaction while allowing the growth reaction to proceed.[13]

Table 1—Fibrillation and Species of Insulin

Species of Insulin	Hours to fibrillation[a] (Mean ± SEM) aqueous, pH 2.6 (40 °C) (3% insulin)	60% ethanol, pH 7.4 (30 °C) (1% insulin)
Porcine	474 ± 79 (n = 32)	26 ± 2 (n = 9)
Bovine	123 ± 24(n = 26)	17 ± 1 (n = 11)

[a] Time to 50% fibrillation of different batches of porcine and bovine insulin dissolved in different media. Samples of insulin were dissolved in hydrochloric acid, 0.05% phenol and the pH was adjusted to 2.6 (aqueous) or dissolved in 0.1 M tris buffer (pH 7.4) containing 2 mM EDTA (60% ethanol). Aqueous solutions were stored wi hout agitation, ethanol solutions were agitated in 10 mL vials (amplitude 55 mm, frequency 110/min).

3. Formation of Insulin Fibrils

The classical method to induce insulin fibrillation has been to heat a concentrated acid solution.[12] The formation of the fibrils can be observed as an increase in viscosity or precipitate formation. A more recent method is agitation of neutral solutions of insulin under the presence of a hydrophobic surface (air or polymeric material) at ambient or slightly increased temperature. Precipitation of fibrils is monitored by the increasing turbidity (nephelometry) of the shaken solution, as compared with that of an unshaken control,[18] or by different light-scattering techniques.[19−21]

3.1. Factors Affecting Fibril Formation.—*3.1.1. Origin, Species, and Purity of Insulin*—The propensity to fibrillate varies from batch to batch of the insulin, even when it is isolated from the same source and purified to the same extent (Figure 1). Different batches come with different histories associated with them, probably including a slightly varying content of fibril nuclei which are able to seed growth of fibrils. Evidence for such aseeding effect comes from the fact that crystallization of insulin, followed by careful removal of noncrystalline (amorphous) protein by washing or centrifugation, is able to reduce the inherent fibrillation tendency significantly. Further support has been obtained by ultracentrifugation of a solution of a rapid fibrillating insulin batch. Following this treatment the tendency to fibrillation of the upper layer of the insulin solution was greatly reduced as compared to that of the lower layer (unpublished observation).

Apart from the inherent batch variation, there seems to be no significant difference between the tendency to fibrillation of human and porcine insulins.[18] In contrast, bovine insulin is significantly more prone to fibrillation than the other two species of insulin (Figure 1, Table 1). Apparently the hydrophobicity of the A8 residue (Ala in bovine and Thr in porcine and human insulin), which is on the surface of the molecule, plays a role in the fibrillation process by directing the interactions of insulin with hydrophobic surfaces. The presence of certain types of inert protein was early shown to retard the rate of fibril formation, although no conclusive evidence of the influence of purity could be obtained.[8] Later, following the introduction of highly purified insulins in the 1970s, a strong reverse relationship between the tendency to fibrillation and the content of a few percent of insulin-related impurities was demonstrated. It was also shown that this effect was not a result of the purification processes as such. If the impurities were reintroduced into monocomponent insulin, the former propensity to less fibrillation could be reestablished. Higher molecular weight contaminants such as covalent dimer and proinsulin were active in counteracting fibrillation when present in amounts corresponding to a few percent of the total protein material, whereas more simple derivatives like desamidoinsulins were ineffective even at 5−10% concentration.[22] Presumably the covalent dimer or proinsulin is able to block further linear aggregation by

Regeneron Pharmaceuticals, Inc. Exhibit 2063 Page 2
Celltrion, Inc. v. Regeneron Pharmaceuticals, Inc. IPR2023-00462

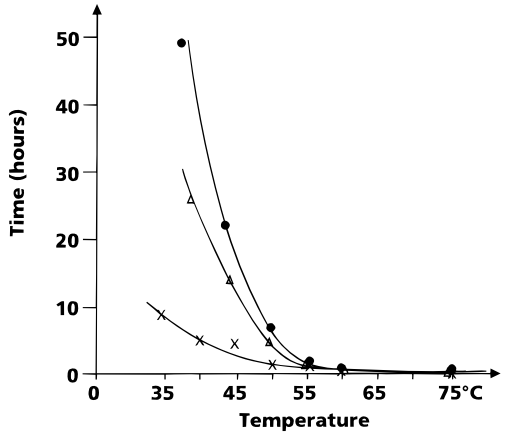

Figure 2—Time to a 4-fold increase in viscosity of various insulin solutions at different temperatures (3% insulin, pH 2.5): 10 times crystallized bovine insulin (×); Monocomponent porcine insulin (△); Monocomponent bovine insulin (●).

providing only one of the two complementary interacting surfaces in the insulin fibril.

3.1.2. Temperature—The effect of temperature on insulin fibrillation in acid solution is illustrated in Figure 2. The results also demonstrate that the inherent differences in the tendency to fibrillation between insulins becomes less apparent with increasing temperature. Apparently the rate of nucleus formation at temperatures above 60 °C is so fast that initiation of fibril growth by the inherent content of fibril nuclei becomes insignificant compared with that of freshly formed nuclei. It is noteworthy that the fastest fibrillating insulins in acid solution form fibrils within a few hours at 37 °C. By contrast, the same insulin in aqueous solution at pH 7 can be stored (without agitation) at 37 °C for many months without any fibril formation. At neutrality, however, formation of covalent polymerization products via thiol-catalyzed disulfide exchange becomes significant and increases exponentially with increasing temperature.[23] As this chemical process eventually leads to insoluble covalent products, great care should be taken not to confuse such precipitates with insulin fibrillation.[24]

3.1.3. Ion Strength—The pronounced influence of ion strength on the rate of insulin fibril formation was demonstrated by Waugh et al.,[13] who compared fibrillation in 8 M acetic acid solutions (pH 1.6) with different ion strength, obtained by addition of sodium chloride. The growth reaction was examined by seeding preformed insulin fibrils into the organic solutions of insulin, and the overall reaction (nucleation + growth) was examined by heating 1−2% solutions of insulin in the organic medium. At the lowest ion strength of 0.04, the growth was substantially retarded in acetic acid as compared to a hydrochloric acid solution with the same pH, but this effect was neutralized by an increase in ion strength. Whereas the overall reaction, which is dependent on nucleation, was virtually absent after 18 h at 50 °C and ion strength 0.04, the fibril growth after addition of fibril seeds was 52% complete after less than 4 h under the same conditions. However, increasing the ion strength to 0.11 increased the rate of nucleation substantially (84% fibrillation after 16 h at 50 °C). The promoting influence of ion strength on fibril formation is probably due to its neutralizing effect on repulsive forces between similarly charged groups. This is supported by the observation that sodium chloride inhibits renaturation of insulin fibrils in alkali (see section 4.2).

3.1.4. Concentration of Insulin—Rate of insulin fibrillation increases in acid solution, as would be expected, with an increase in insulin concentration[14] (Figure 3). In neutral solutions of insulin the behavior is more complicated. In

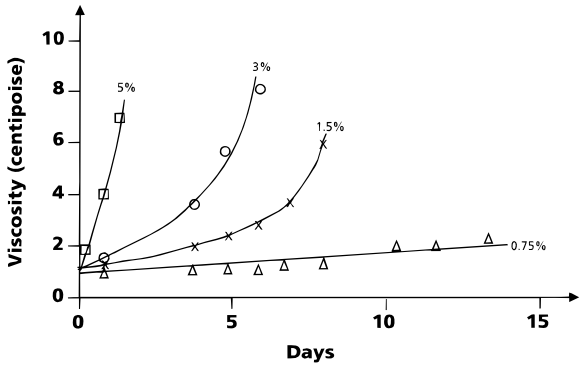

Figure 3—Rate of insulin fibrillation as a function of insulin concentration (bovine insulin, pH 2.5, 21 °C).

Table 2—Influence of Insulin Association State on Fibrillation Tendency

	Days[a]		
	Monomer (Zn-free, 60% ethanol)		Hexamer (4 Zn ions per hexamer, aqueous)
	Standing		Agitation[b] at 37 °C
Insulin Concn (mM)	23 °C	37 °C	
0.1	8	1.5	1.5
0.6	2.3	0.5	8.0
3	0.8	<0.1	25

[a] Days to first sign of fibrilla ion (visual precipitation) in solutions at pH 7.4. [b] Shaking test as descibed in ref 18.

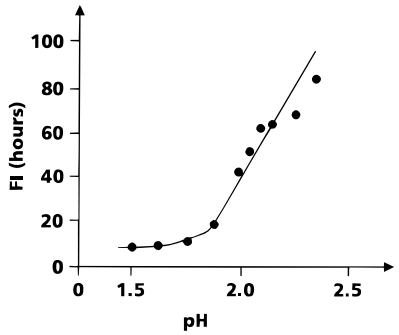

Figure 4—Influence of pH on the fibrillation tendency (1% bovine insulin, 35 °C). FI = time to a 4-fold increase of viscosity.

aqueous solutions of Zn−insulin at pH 7, insulin is predominantly present as hexameric associates. In such solutions insulin exhibits a quite anomalous aggregation behavior, being more prone to fibrillation at reduced concentrations of insulin.[19,20,25] By contrast, if zinc-free insulin (or Zn−insulin + EDTA) is dissolved in organic solvent (e.g., 60% ethanol) in which the insulin will be present as monomers, the extent and rate of aggregation is proportional to insulin concentration[26] (Table 2). These results indicate that the initial formation of fibril nuclei probably proceeds via monomerization of oligomeric insulin.

3.2. Significance of pH and Insulin Association State—The prominent effect of pH on insulin fibrillation is mainly a consequence of the influence of pH on the association behavior of insulin. In acid solutions insulin maintains its tertiary structure but is mainly monomeric below pH 2 whereas an increasing population of dimers dominates the mixture of monomers and dimers with increasing pH above 2.[27] Investigation of the influence of pH on fibrillation in acid (Figure 4) reveals a decreasing tendency with increasing pH above pH 2, which supports the idea that fibril formation requires an interaction between monomers. By accepting this

Regeneron Pharmaceuticals, Inc. Exhibit 2063 Page 3
Celltrion, Inc. v. Regeneron Pharmaceuticals, Inc. IPR2023-00462

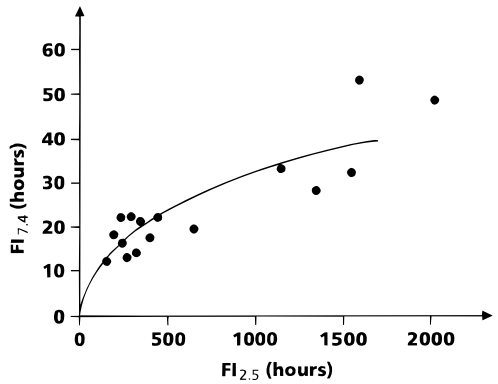

Figure 5—Correlation between fibrillation tendency of different insulins in aqueous acidic solution (3% insulin, pH 2.5, 40 °C) and in 60% ethanol at neutral pH (1% insulin, pH 7.4, 30 °C).

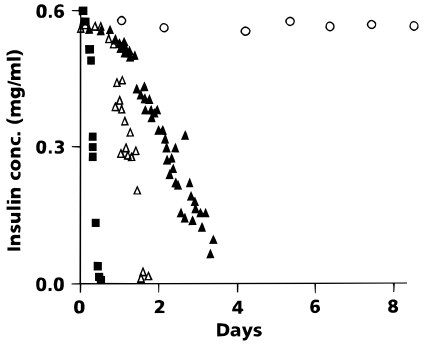

Figure 6—Effect of surface hydrophobicity on agitation-induced insulin fibrillation. Insulin in solution in samples agitated at 160 rpm at 37 °C in the presence of teflon (■), polypropylene (▲), siliconized glass (△), and unmodified glass spheres (○). (Reproduced from ref 21, copywright 1992, Wiley.)

theory, it is not surprising that insulin fibrillation in aqueous solutions at pH 7 is a much slower and more complicated process than in acid solution. In neutral, aqueous solution the insulin molecule is typically associated into mainly hexameric units. Only by 10 times dilution of the normal, therapeutic concentration of insulin (0.6 mM) does a significant dissociation into tetramers and dimers occur, which increases the propensity for non-native aggregation. However, insulin fibrillation does still not occur to any significant extent in aqueous solution at moderate temperatures in the absence of hydrophobic surfaces[22] or agitation.[20] By contrast, fibrillation readily occurs at neutrality, even in the absence of agitation, in organic media that are able to dissociate the insulin into monomers (Table 2).

Although the charge distribution on the insulin molecule differs in acid and neutral solution, the inherent tendency of different insulins to fibrillate in aqueous solution at pH 2.5 correlates well with that of the same insulins dissolved in 60% ethanol at pH 7 (Figure 5). In both cases, however, the normal hexameric units are dissociated into smaller units with an increase of exposed hydrophobic surface area. The more rapid fibrillation in ethanol, despite the lower temperature and insulin concentration, can be explained by the monomeric state of the insulin with more exposed hydrophobic area as compared to the mainly dimeric state in acidic aqueous solution. Therefore, these results support the theory that fibril formation is mainly driven by nonpolar and entropic effects.

3.3. Effect of Surface Interaction—The agitation-provoked fibrillation of insulin in neutral solution is now widely accepted as being mediated by insulin's interaction with hydrophobic surfaces, and a model has been proposed for the mechanism.[20] According to this model, monomers as well as dimers and hexamers can reversibly adsorb to the surface. However, monomers are the least stable species and therefore more likely than dimers and hexamers to undergo conformational changes at hydrophobic interfaces. The partially unfolded monomers can now either refold to the native conformation or combine with other unfolded monomers, initiating nucleation (formation of intermediate aggregates). The intermediates can either fall apart or interact with other unfolded species. Once a critical size is reached, the intermediates have sufficient surface area for stability and can start reacting with native molecules. The slow formation of stable intermediates explained the lag phase often observed in insulin fibrillation experiments, and the fast insulin depletion following the formation of stable nucleus implied that native molecules participate in the final aggregation steps.

Insulin aggregation at solid−water interfaces has been studied by several groups.[22,21,28,29] In systematic studies on the influence of different materials, Sluzky et al.[21] showed the fibrillation tendency to increase with the surface area of hydrophobic material, and fibrillation tended to proceed faster with increasing hydrophobicity of the material (Figure 6). They also provided evidence that aggregation caused by agitation is the result of both the interfacial and shear forces on the protein. However, because an insulin solution can be rotated in a glass vial (without any air present) for several months[22] or agitated with glass beads for many days[21] (Figure 6) without any fibrillation, the shear forces probably play a minor role compared with the surface interactions.

The air−water interface is hydrophobic, and therefore, agitation of insulin solutions with an air surface causes adsorption of insulin and subsequent surface-induced denaturation and aggregation. Shaking allows a continuous creation of new interface and thus provides a large surface area for adsorption of insulin. Insulin fibrillation at the agitated air−water interface increases with increasing rate of agitation.[20] The insulin monomer, with its exposed hydrophobic surfaces normally buried by dimer and hexamer formation, has potentially higher binding affinity for a hydrophobic interface than the dimer and hexamer. However, at high insulin concentration, the equilibrium is shifted toward the hexamer which may populate the interface and reduce access for the monomeric species. This may explain the atypical inverse relationship between insulin concentration and tendency to surface-induced fibrillation at neutral pH.

4. Morphology and Properties of Insulin Fibrils

4.1. Structure of Fibrous Insulin—In the electron microscope insulin fibrils are seen as long fibers with the smallest diameters ranging from 3 to 15 nm[5,30−32] and lengths up to several microns.[30,31] Sometimes the individual fibrils are seen as bundles of two to five fibers twisted together at 120−160 nm intervals into filaments with a gross diameter of 10−20 nm.[30−32]

Fibrous insulin has been studied by various other techniques, including spectroscopic and diffraction techniques. Using an infrared technique, insulin fibrils were shown to consist of extended β-chains lying perpendicular to the fibril axis, with a layer structure involving interchain hydrogen bonds.[33] In contrast, from an X-ray diffraction investigation, it was suggested that insulin enters the fibril structure with, at most, slight distortion and without the large changes accompanying transformation to the β-form. It was also suggested that the polypeptide chains in insulin fibrils retain their normal folded condition, with axes predominantly parallel to the fibril axis.[34] Raman spectra of insulin fibrils[35] and studies of the binding of Congo Red to fibrillar insulin[36] gave

Regeneron Pharmaceuticals, Inc. Exhibit 2063 Page 4
Celltrion, Inc. v. Regeneron Pharmaceuticals, Inc. IPR2023-00462

Table 3—Renaturation of Insulin Fibrils

Solvent[b]	% dissolved after treatment for 5 min at 0 °C	10 °C	25 °C	45 °C	75 °C
DMSO				0	2
DMSO + HCl[c]				50	100
DMF			0	0	15
Glycerin			0	0	30[d]
Formic acid		100	100		
Phenol 10% (pH 1.6)					1
NaOH, 0.05 M	100				

[a] Fibrils of bovine and porcine insulin were prepared by storage of a 5% insulin solution (60% ethanol, 0.1 M Tris buffer, 2 mM EDTA, pH 7.4) for 2 days at 37 °C corresponding to >99% fibrillation. Fibrils were washed several times with 60% ethanol and dried in vacuum; 10 mg fibril material was suspended in 1 mL of solvent and incubated under frequent stirring. [b] Solvents without effect included tetramethylurea, dioxane, acetic acid, and 0.1 M HCl. DMSO = Dimethyl sulfoxide; DMF = Dimethylformamide. [c] 2 μL of undiluted HCl acid was added to 1 mL of DMSO. [d] Only porcine insulin fibrils were partially dissolved.

evidence of an antiparallel β-structure, whereas infrared spectroscopy[31] and X-ray diffraction data[16] pointed to the parallel pleated-sheet type. The latter investigators also suggested that the fibrils had a uniform cross section with dimensions of about 3 × 5 nm. Using a dynamic light scattering technique it has more recently been found that insulin fibrils are of a narrow size distribution with a mean diameter of 200 nm.[19]

Fibrillation experiments at lower (e.g., 10 °C) and higher temperatures (e.g., 80 °C) indicate that the mechanism of fibril formation is independent of temperature within this range.[14] Thus, whereas the initial steps in aggregation of insulin monomers into fibrils probably are common for any insulin fibril formation, the further growth to form lateral aggregates or tightly packed deposits will depend on the experimental conditions by which they are produced.[37,38]

4.2. Renaturation—Insulin fibrils are insoluble in most aqueous media, including diluted mineral acids, but can be dissolved and renatured into essentially fully biologically active insulin in alkaline medium at pH > 11, followed by neutralization,[39] in 20% HCl,[40] in at least 10% phenol[14,41] or by using strong organic acids.[13] In contrast, insulin fibrils are not dissolved in 7 M urea from pH 2 to 8[42] or in 5 M guanidine hydrochloride, 50% acetonitrile, and nonionic and anionic detergents.[43] Waugh[39] found the optimal conditions for regeneration in alkali to be treatment in 0.03 M NaOH at 0 °C for 45 min. He also showed that NaCl inhibits reversion, which strongly suggests that repulsive forces between similarly charged groups play an important role in the mechanism of disaggregation. Our own results of regeneration of insulin from insulin fibrils with different solvents are summarized in Table 3. In all cases with complete dissolution of the fibrils, the isolated insulin exhibited full biological activity.

It is striking that full recovery of native insulin is only obtained at extremes of pH. Thus, renaturation by DMSO was only achieved by addition of hydrochloric acid. Apparently, breakage of the intermolecular interactions in the fibril is dependent on the introduction of charge repulsion between the molecules. The inability of urea, guanidine, detergents, etc. to dissolve insulin fibrils probably reflects that the interfaces between molecules is poorly accessible for hydrophilic substances due to tight hydrophobic interactions between units in the fibril.

4.3. Immunogenicity—When a protein chemically or physically degrades, its immunogenicity may be altered. Studies indicating an increased immune response of aggregated protein have been performed with bovine serum albumin[44] and human growth hormone.[45] Even when treated with homologous human insulin, circulating IgG insulin antibodies are found in detectable levels in 40–50% of diabetics patients whereas local allergy due to IgE insulin antibody formation is much less frequent. The reasons why subcutaneously administered human insulin induces antibody production in contrast to the endogenous hormone are not well-understood. However, some of the immunological side effects associated with insulin therapy seem to be due to a minor content of covalent dimerization and polymerization products of insulin. Thus, specific IgG antibodies against covalent insulin dimer have been identified in 30% of insulin-treated diabetic patients,[46] and cutaneous insulin allergy has been associated with a cell-mediated hypersensitivity response to the covalent dimer.[47] Whether physical aggregates of insulin also are able to elicit an immune response is uncertain, but the observation that a high percentage of patients treated with continuous subcutaneous insulin infusion developed insulin allergy after initiation of pump therapy[48] may suggest

Table 4—Immunogenicity of Porcine Insulin Fibrils

Sample No.	Purity of Insulin[a]	Treatment at 39 °C	Fibrillated Insulin[b] (%)	IgG Antibody Formation: Area Response[c] (Relative to Reference)	IgE Antibody Formation: Allergy Response[d] (nmol of histamine/mL of whole blood/per h)	IgE Antibody Formation: Number of responding animals
1	MC insulin	Untreated (reference)	0	≡1	0	0
2		Storage for 5 days	0	1.03	0	0
3		Agitation	1.5	1.17	0	0
4			2.4	1.33	3	1
5			9	0.89	4	3
6			32	0.46	392	10
7		Sample 6 diluted 5× with sample 1	~6	1.17	21	10
8	Insulin cryst 5×	Untreated	0	2.06	4	1

[a] MC insulin = monocomponent insulin (<1 ppm proinsulin, < 0.2% covalent insulin dimer); cryst 5× = conventionally purified insulin (2–4% proinsulin, 2–3% covalent insulin dimer). Insulins were dissolved at pH 7.4 with 0.2% phenol and 1.6% glycerol in a concentration of 40 IU/mL. [b] The neutral insulin solutions were agitated at 39 °C for various dura ions as described earlier[18]. The extent of fibrillation was evaluated by filtration through a 0.2 μm filter and UV absorbance of the filtrate at 276 nm. [c] Groups of 10 rabbits were immuninized twice weekly for 3 months with 20 IU as descibed earlier,[49] with the exception that complete Freund's adjuvant was used at the first injection. Blood samples were drawn every 2 weeks. Area response is the mean percent binding (area under the curve) to added [^{125}I]insulin for each group of rabbits. Sta istical analysis showed significantly higher response of the conventionally purified insulin (sample 8), reflecting its content of immunogenic proinsulin and covalent insulin dimers, when compared with samples 1–3 and samples 5–7 (p < 0.01–0.05). In addition, the response of sample 6 was significantly lower than that of sample 1 (p < 0.05). Differences between other samples were not sta is ically significant. [d] Blood cells were isolated from each rabbit after 3 months of immunization. The histamine release after one hour incubation at 37 °C of blood cells mixed with a fixed amount of the sample used for the immunization was analyzed in accordance with the method described earlier.[50] The allergy response is expressed as the mean histamine release from blood cells of 10 rabbits.

Regeneron Pharmaceuticals, Inc. Exhibit 2063 Page 5
Celltrion, Inc. v. Regeneron Pharmaceuticals, Inc. IPR2023-00462

a possible connection with the propensity of insulin to fibrillate in the pump systems (see section 6).

In rabbit immunization experiments, partially fibrillated insulin samples exhibited a tendency to increasing formation of insulin IgG antibodies with increasing content of fibrillar insulin when compared with a reference sample of native, unfibrillated insulin (Table 4). However, the differences were not statistically significant. The most fibrillated sample actually showed a significant decrease in immune response compared with the reference sample but a 5 times dilution of this sample with the reference sample rather showed an increase in response (not significant) compared with the reference sample. In contrast, the formation of IgE antibodies seems to correlate with the degree of fibrillation of insulin in the sample. It is interesting that the IgE response mediated by sample 7 (5 times dilution of sample 6 corresponding to 6% fibrillation) was 5-fold higher than that of the more fibrillated sample 5 (9% fibrillation). This may indicate that the size of the fibrils rather than the quantity of insulin fibrils is the stimulating factor for the allergic response.

5. Molecular Mechanism of Insulin Fibrillation

5.1. The Interacting Domains—The specific amino acid residues involved in the interactions between units in fibrillar insulin have been searched for in a number of studies. Fibrillation experiments on different insulin derivatives have shown that fibril formation is not dependent on the presence of carboxyl groups,[13,51] whereas a marked decrease in the tendency to fibrillation is observed when the amino groups are acetylated.[13,52] Studies on chemical reactivity of functional groups have revealed that about half of the imidazolyl[42,53] and half of the tyrosyl groups[42] have reduced reactivity in fibrillar as compared to native insulin. As mentioned earlier (section 2), the interactions in fibrillar insulin are supposed mainly to be between nonpolar side chains (hydrophobic interactions), and the fact that growth of fibrillar insulin can proceed at close to 0 °C[14] strongly indicates that these interactions occur without extensive unfolding of the globular insulin.

5.2. Significance of the B-Chain C-Terminal—In native insulin an antiparallel β-sheet at the B-chain C-terminal plays an important role in the interaction between monomers in the insulin dimer.[54] Raman spectra[35] and studies of the binding of Congo Red to insulin fibrils[55] support the presence of an antiparallel β-sheet also in the insulin fiber. According to spectroscopic observations, this sheet is perpendicular to the fiber axis.[33]

Fibrillation experiments on B-chain C-terminal truncated insulins have shown that the contacts which dominate fibril formation are made by those residues which are not in the B-chain C terminus since both desoctapeptide-(B23−B30)-insulin (DOI) and despentapeptide-(B26−B30)-insulin (DPI) are able to form fibrils. In fact, the propensity to fibril formation increases with increasing truncation. If, on the other hand, mobility at the C-terminus of the B-chain is restricted by cross-linking with the nearby N-terminus of the A-chain, fibrillation proceeds much more slowly. Proinsulin, in which A1−B30 is cross-linked by more than 30 residues, fibrillates to a greatly reduced extent, and a dipeptide cross-linked miniproinsulin fibrillates even less if at all (Table 5). These results strongly suggest that fibril nucleation requires exposure of amino acid residues, normally buried in the insulin monomer, to the surface of the insulin molecule by displacement of the B-chain C-terminal from its normal position.[26]

On the basis of X-ray diffraction studies[56] and NMR structural studies[57] this C-terminal segment has been shown to be highly flexible and easily displaced, and an equilibrium unfolding intermediate involving such displacement has recently been proposed[58] (Figure 7).

Table 5—Resistance to Fibrillation of Different Insulin Derivatives

Insulin	Aqueous (agitation at 37 °C) pH 2	Aqueous (agitation at 37 °C) pH 7.4	60% ethanol, pH 7.4 (25 °C without agitation)
Porcine insulin	0.46 ± 0.04	0.30 ± 0.04	~2
Proinsulin (porcine)	16 ± 2		~12
Mini-proinsulin[b]	>100		>50
DPI[c]	0 29 ± 0.06		
DOI[d]	0.10 ± 0.03	<0.04	

[a] The values are the mean number of days (±SD, $n = 5$) until to first sign of fibrillation (precipitation or turbidity). All insulins contained <0.02% Zn^{2+}. [b] "Proinsulin" with only two amino acid residues in the connecting peptide compared with the 33 residues in porcine proinsulin. [c] DPI = despentapeptide-(B26–B30)-insulin (porcine). [d] DOI = desoctapeptide-(B23–B30)-insulin (porcine).

5.3. Modeling Insulin Fibrillation—The extra domain exposed when the B-chain C-terminus is displaced includes the hydrophobic, side chains of residues A2 (Ile), A3 (Val), B11 (Leu), and B15 (Leu).[26] On the basis of the theory that this domain must be one of the hydrophobic surfaces involved in fibril interactions, the crystal structure of despentapeptide-(B26−B30)-insulin (DPI), in which these residues are on the surface, has been used to predict and model the organization of insulin molecules in the insulin fibril.[59] Apart from the loss of the β-sheet of residues B26−B30, the DPI molecule retains all the secondary and tertiary structure characteristics of the native insulin, including an intact hydrophobic surface domain normally buried when dimers form a hexamer [A13 (Leu), B14 (Ala), B17 (Leu), and B18 (Val)]. In the model presented these nonpolar, aliphatic residues constitute the complementary surface to that exposed by B-chain C-terminal displacement. Shielding of the nonpolar surfaces on one monomer requires the simultaneous assembly of three insulin molecules, which is consistent with previous observations.[14] The dimensions of the model fibril correspond well with those measured experimentally by electron microscopy, and the monomer−monomer contacts within the fibril are in accordance with the known physical chemistry of insulin fibrils.

The presence of an antiparallel β-sheet in insulin fibrils (see section 5.2), can in the model be explained by a β-sheet contact made by the B-chain N-terminal residues. This sheet is perpendicular to the principal fiber axis, as required from spectroscopic observations,[33] and provides a favorable contact through which bundles of fibrils may develop from individual fibrils.[30] The location of the B-chain C-terminal residues when the native insulin forms a fiber has not been identified. However, these residues may themselves pack favorably against the remaining exposed, hydrophobic monomer surface (Figure 8).

6. Stabilization Strategies and Prevention of Insulin Fibrillation

During the first 60 years of insulin therapy, fibrillation-related stability problems during normal handling, storage, or use of insulin preparations were rarely encountered. In the late 1970s, however, the endeavors to obtain normoglycemia in diabetes treatment resulted in the introduction of devices for continuous insulin infusion (insulin pumps), and it soon became evident that commercial insulin formulations were not sufficiently stable for long-term use in infusion pumps.[60−63] Precipitation problems were of major concern and a comprehensive search for more stable formulations was initiated, mainly as empirical approaches (for review and a comprehensive reference list, see ref 64).

It is now obvious that rational strategies to improve the practical stability of insulin include the following ap-

Regeneron Pharmaceuticals, Inc. Exhibit 2063 Page 6
Celltrion, Inc. v. Regeneron Pharmaceuticals, Inc. IPR2023-00462

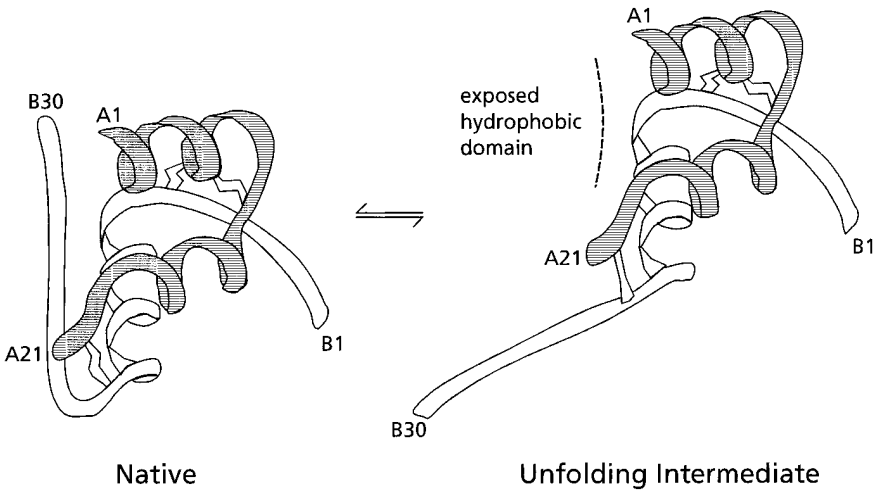

Figure 7—Unfolding of native insulin (as proposed in ref 59) with exposure of a hydrophobic domain including residues A2 (Ile), A3 (Val), B11 (Leu), and B15 (Leu).

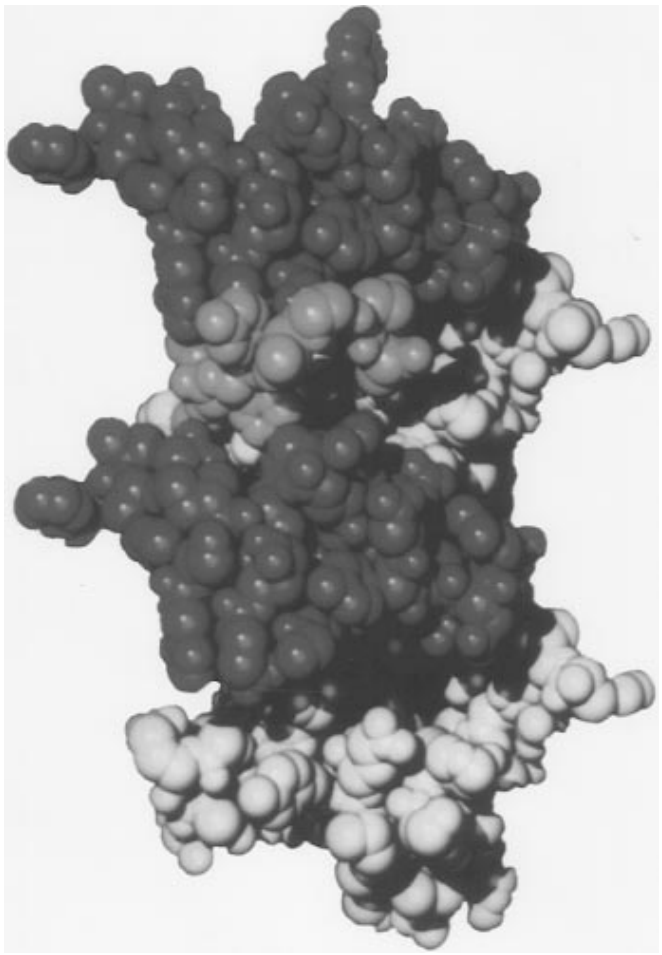

Figure 8—Four adjacent despentapeptide-(B26–B30)-insulin molecules (shown in dark gray and white) of the model fibril showing the B-chain C-terminus (residues B26–B30 shown in light grey) modeled onto the hydrophobic surface in a remaining cavity in one of the possible three interfaces between molecules.

proaches: (1) promote self-association, (2) stabilize the native structure by (a) preventing unfolding (displacement of the C-terminus of the B-chain) and (b) enhancing native hydrophobic interactions, (3) avoid or block unwanted hydrophobic interfaces, (4) reduce shear forces, (5) modify the molecule (derivatization or protein engineering).

Retrospectively, several of the more effective means to counteract fibrillation can be related to one or more of these categories. Thus, calcium[22,65] or zinc ions[18] most likely exert their fibrillation-inhibitory effect by neutralizing negative charges in the center of the insulin hexamer, whereby the hexameric assembly is stabilized. These metal ions probably also play an important role for the storage stability of insulin in the β-cells of the pancreas.[66] The most effective prevention of fibrillation is probably achieved by preventing displacement of the B-chain C-terminus by cross-linking it to the A-chain N-terminus (Table 5). Unfortunately restricted mobility at the C-terminus of the B-chain is also associated with complete loss of biological activity.[67] Enhancement of the hydrophobic interactions within proteins can be achieved by addition of polyhydric alcohols that reduce the interaction of the protein with solute and the penetration of water into the hydrophobic interior. Hence carbohydrates[68,69] as well as glycerol[68,70] have been found to increase the physical stability of neutral insulin solutions, but the physical stabilization is often accompanied by an unacceptable reduction in the chemical and biological stability of the preparations due to the capacity of some of these products to react chemically with the insulin amino groups.[64,68]

The marked fibrillation-inhibitory effect obtained by adding surfactants like block copolymers of ethylene oxide and propyl oxide,[71,72] and lecithins[73] is due to their ability to bind to hydrophobic interfaces and block unfavorable insulin interactions at container surfaces, air–liquid interfaces, and perhaps also exposed hydrophobic surfaces on the insulin monomer. Cyclodextrins, with an ability to microencapsulate small molecules, have also been claimed to bind to hydrophobic residues exposed on protein surfaces and thereby decrease aggregation.[74] Recently such compounds, in a concentration of 1.5%, have been shown to minimize agitation-induced fibrillation of insulin to some degree.[75]

Shear forces can be reduced by increasing the viscosity of the solution, and it has been demonstrated that addition of 15% (w/w) of polygeline, a partially hydrolyzed and subsequently cross-linked gelatine product, counteracts the promoting effect of shear forces on insulin fibrillation.[76]

Sulfated insulin, in which two to four of the hydroxy groups of Ser and Thr have been converted into negatively charged groups, is an example of chemical modification capable of reducing the tendency toward fibrillation.[26,63,77] This positive effect is unexpected because the derivatization renders the molecule monomeric and consequently more likely to partially unfold and fibrillate. However, the extra negative charges which counteract self-association apparently are also able, by increasing the repulsive forces between the molecules, to

Regeneron Pharmaceuticals, Inc. Exhibit 2063 Page 7
Celltrion, Inc. v. Regeneron Pharmaceuticals, Inc. IPR2023-00462

Table 6—Stabilization of Neutral Insulin Solutions against Fibrillation[a]

Concentration of Lecithin (DOPC[b]) (μg/mL)	Zinc ions per Insulin Hexamer	Days to Precipitation in Shaking Test (37 °C)
0	2.2	1
	4.3	30
30	2.2	18
	2.9	32
	3.9	40
	4.6	>100

[a] Neutral solutions of porcine insulin (100 IU/ml) in 10 mL vials were agitated horizontally as described earlier.[18] Data are from ref 18 and 78. [b] DOPC = dioctanoylphosphatidylcholine.

inhibit the hydrophobic interactions responsible for the aggregation into fibrils.

A combination of excipients is used to take advantage of the characteristics of each excipient. Thus, most effective stabilization of insulin is achieved when surfactant additives are combined with elevation of the viscosity of the solution[76] or with metal ion stabilization[78] (Table 6).

7. Conclusions

A number of studies over the last 50 years have shown that fibrillation of insulin is an entropically driven physical process by which insulin molecules with nearly complete nativelike globular structure aggregate in a linear fashion. The tendency to fibrillation increases with temperature and is influenced by species, purity, concentration, and association state of the insulin and interaction with hydrophobic surfaces including the air−water interface. Fibrillation studies on B-chain-truncated insulins and on proinsulins with restricted mobility of the C-terminus of the B-chain have provided strong evidence that the linear aggregation requires a displacement of the C-terminal segment of the B-chain with exposure of a hydrophobic domain normally buried in the insulin monomer. Recently the crystal structure of despentapeptide insulin has been used to predict and model the contacts this exposed domain make in the fibril, and it is proposed that the complementary domain is composed by the hydrophobic surface normally buried when insulin dimers form hexamers. The dimensions and characteristics of this model insulin fibril are in accordance with those measured experimentally.

Acknowledgments

The authors want to thank Professor Guy Dodson, University of York, for stimulating discussions.

References and Notes

1. Banting, F. G.; Best, C. H. *J. Lab. Clin. Med.* **1922**, *7*, 464−472.
2. Bischoff, F.; Sahyun, M. *J. Biol. Chem.* **1929**, *81*, 167−173.
3. Blatherwick, N. R.; Bischoff, F.; Maxwell, L. C.; Berger, J.; Sahyun, M. *J. Biol. Chem.* **1927**, *72*, 57−89.
4. Krogh, A.; Hemmingsen, A. M. *Biochem. J.* **1928**, *22*, 1231−1238.
5. Waugh, D. F. *J. Am. Chem. Soc.* **1946**, *68*, 247−250.
6. Dickens, F.; Dodds, E. C.; Lawson, W.; Maclagan, N. F. *Biochem. J.* **1927**, *21*, 560−571.
7. Vigneaud, V.,du; Geiling, E. M. K.; Eddy, C. A. *J. Pharmacol. Exp. Ther.* **1928**, *33*, 497−509.
8. Gerlough, T. D.; Bates, R. W. *J. Pharmacol. Exp. Ther.* **1932**, *45*, 19−51.
9. Vigneaud, V.,du; Sifferd, R. H.; Sealock, R. R. *J. Biol. Chem.* **1933**, *102*, 521−533.
10. Langmuir, I.; Waugh, D. F. *J. Am. Chem. Soc.* **1940**, *62*, 2771−2793.
11. Waugh, D. F. *Am. J. Physiol.* **1941**, *133*, P484-P485.
12. Waugh, D. F. *Fed. Proc.* **1946**, *5*, 111.
13. Waugh, D. F.; Wilhelmson, D. F.; Commerford, S. L.; Sackler, M. L. *J. Am. Chem. Soc.* **1953**, *75*, 2592−2600.
14. Waugh, D. F. *J. Cell. Comp. Physiol.* **1957**, *49 (Suppl.1)*, 145−164.
15. Waugh, D. F. *J. Am. Chem. Soc.* **1944**, *66*, 663.
16. Burke, M. J.; Rougvie, M. A. *Biochemistry* **1972**, *11*, 2435−2439.
17. Waugh, D. F. *Adv. Protein Chem.* **1954**, *9*, 325−437.
18. Brange, J.; Havelund, S.; Hommel, E.; Sørensen, E.; Kühl, C. *Diabetic Med.* **1986**, *3*, 532−536.
19. Dathe, M.; Gast K.; Zirwer, D.; Welfle, H.; Mehlis, B. *Int.J. Pept. Protein Res.* **1990**, *36*, 344−349.
20. Sluzky, V.; Tamada, J. A.; Klibanov, A. M.; Langer, R. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 9377−9381, 1991.
21. Sluzky, V.; Klibanov, A. M.; Langer, R. *Biotechnol. Bioeng.* **1992**, *40*, 895−903.
22. Brange, J.; Havelund, S.; Hansen, P.; Langkjaer, L.; Sørensen, E.; Hildebrandt, P. In *Hormone Drugs*, Gueriguian, J. L., Bransome, E. D., Outschoorn, A. S., Eds.; US Pharmacopoeial Convention: Rockville, MD, 1982; pp 96−105.
23. Brange, J.; Havelund, S.; Hougaard, P. *Pharm. Res.* **1992**, *9*, 727−734.
24. Costantino, H. R.; Langer, R.; Klibanov, A. M. *Pharm. Res.* **1994**, *11*, 21−29.
25. Brange, J.; Havelund, S. In *Artificial Systems for Insulin Delivery;* Brunetti, P., Alberti, K. G. M. M., Albisser, A. M., Hepp, K. D., Benedetti, M. M., Eds.; Raven: New York, 1983; pp 83−88.
26. Brange, J.; Hansen, J. F.; Havelund, S.; Melberg, S. G. In *Advanced Models for the Therapy of Insulin-Dependent Diabetes*; Brunetti, P., Waldhäusl, W. K., Eds.; Raven: New York, 1987; pp 85−90.
27. Blundell, T.; Dodson, G.; Hodgkin, D.; Mercola, D. *Adv. Protein Chem.* **1972**, *26*,279−402.
28. Chawla, A. S.; Hinberg, I.; Blais, P.; Johnson, D. *Diabetes* **1985**, *34*, 420−424.
29. Feingold, V.; Jenkins, A. B.; Kraegen, E. W. *Diabetologia* **1984**, *27*, 373−378.
30. Farrant, J. L.; Mercer, E. H. *Biochim. Biophys. Acta* **1952**, *8*, 355−359.
31. Glenner, G. G.; Eanes, E. D.; Bladen, H. A.; Linke, R. P.; Termine, J. D. *J. Histochem. Cytochem.* **1974**, *22*, 1141−1158.
32. Kung, T. H.; Tsao, T. C. *Sci. Sin.* **1964**, *13*, 471−478.
33. Ambrose, E. J.; Elliott, A. *Proc. R. Soc. (London)* **1951**, *208*, 75−90.
34. Koltun, W. L.; Waugh, D. F.; Bear, R. S. *J. Am. Chem. Soc.* **1954**, *76*, 413−417.
35. Yu, N. T.; Jo, B. H.; Chang, R. C. C.; Huber, J. D. *Arch. Biochem. Biophys.* **1974**, *160*, 614−622.
36. Turnell, W. G.; Finch, J. T. *J. Mol. Biol.* **1992**, *227*, 1205−1223.
37. Brange, J.; Skelbaek-Pedersen, B.; Langkjaer, L.; Damgaard, U.; Ege, H.; Havelund, S.; Heding, L. G.; Jørgensen, K. H.; Lykkeberg, J.; Markussen, J.; Pingel, M.; Rasmussen, E. *Galenics of Insulin: The Physico-chemical and Pharmaceutical Aspects of Insulin and Insulin Preparations*, Springer: Berlin, 1987.
38. Hutchison, K. G. *J. Pharm. Pharmacol.* **1985**, *37*, 528−531.
39. Waugh, D. F.; Smith, M. J.; Fearing, D. F. *Fed. Proc.* **1948**, *7*, 131.
40. Vigneaud, V.,du *J. Biol. Chem.* **1931,** *92*, liv-llv.
41. Lens, J. *J. Biol. Chem.* **1947**, *169*, 313−322.
42. Pettinga, C. W. *Biochem. Prep.* **1958**, *6*, 28−31.
43. Perry, E. K.; Oakley, A. E.; Candy, J. M.; Perry, R. H. *Neurosci. Lett.* **1981**, *25*, 321−325.
44. Pinckard, R. N.; Weir, D. M.; McBride, W. H. *Clin. Exp. Immunol.* **1967**, *2*, 331−341.
45. Moore, W. V.; Leppert, P. *J. Clin. Endocrinol. Metab.* **1980**, *51*, 691−697.
46. Robbins, D. C.; Cooper, S. M.; Fineberg, S. E.; Mead, P. M. *Diabetes* **1987**, *36*, 838−841.
47. Ratner, R. E.; Phillips, T. M.; Steiner, M. *Diabetes* **1990**, *39*, 728−733.
48. Pietri, A.; Raskin, P. *Diabetes Care*, **1981**, *4*, 624−626.
49. Schlichtkrull, J.; Brange, J.; Christiansen, A. H.; Hallund, O.; Heding L. G.; Jørgensen, K. H. *Horm. Metab. Res.* **1974**, *5 (Suppl. Ser.)* 134−143.
50. Diamant, B.; Shamkant, P. *Int. Archs. Allergy Appl. Immun.* **1982**, *67*, 13−17.
51. Mommaerts, W. F. H. M.; Neurath, H. *J. Biol. Chem.* **1950**, *185*, 909−917.
52. Jensen, H.; Evans, E. A., Jr.; Pennington, W. D.; Schock, E. D. *J. Biol.Chem.* **1936**, *114*, 199−208.
53. Porter, R. R. *Biochem. J.* **1950**, *46*, 304−307.
54. Baker, E. N.; Blundell, T. L.; Cutfield, J. F.; Cutfield, S. M.; Dodson, E. J.; Dodson, G. G.; Hodgkin, D. M. C.; Hubbard, R.

Regeneron Pharmaceuticals, Inc. Exhibit 2063 Page 8
Celltrion, Inc. v. Regeneron Pharmaceuticals, Inc. IPR2023-00462

E.; Isaacs, N. W.; Reynolds, C. D.; Sakabe, K.; Sakabe, N. Vijayan, N. M. *Phil. Trans. R. Soc.* **1988**, *319*, 369−456.
55. Turnell, W. G.; Finch, J. T. *J. Mol. Biol.* **1992**, *227*, 1205−1223.
56. Derewenda, U.; Derewenda, Z.; Dodson, E.; Dodson, G. G.; Reynolds, C. D.; Smith, G. D.; Sparks, C.; Swenson, D. *Nature* **1989**, *338*, 594−596.
57. Hua, Q.; Weiss, M. A. *Biochemistry* **1991**, *30*, 5505−5515.
58. Millican, R. L.; Brems, D. N. *Biochemistry* **1994**, *33*, 1116−1124.
59. Brange, J.; Dodson, G. G.; Edwards, D. J.; Holden, P. H.; Whittingham, J. L. *Proteins*, in press.
60. Buchwald, H.; Varco, R. L.; Rupp, R. L.; Goldenberg, F. J.; Barbosa, J.; Rohde, T. D.; Schwartz, R. A.; Rublein, T. G.; Blackshear, P. J. *Lancet* **1981**, *1*, 1233−1235.
61. Irsigler, K.; Kritz, H. *Diabetes* **1979**, *28*, 196−203.
62. James, D. E.; Jenkins, A. B.; Kraegen, E. W.; Chisholm, D. J. *Diabetologia* **1981**, *21*, 554−557.
63. Lougheed, W. D.; Woulfe-Flanagan, H.; Clement, J. R.; Albisser, A. M. *Diabetologia* **1980**, *19*, 1−9.
64. Brange, J. *Stability of Insulin. Studies on the Physical and Chemical Stability of Insulin in Pharmaceutical Formulation*; Plenum: London, 1994.
65. Brange, J.; Havelund, S. In *Artificial Systems for Insulin Delivery;* Brunetti, P., Alberti, K. G. M. M., Albisser, A. M., Hepp, K. D., Benedetti, M. M., Eds.; Raven: New York, 1983; pp 83−88.
66. Howell, S. L.; Tyhurst, M.; Duvefelt, H.; Andersson, A.; Hellerström, C. *Cell. Tissue Res.* **1978**, *188*, 107−118.
67. Derewenda, U.; Derewenda, Z.; Dodson, E. J.; Dodson, G. G.; Bing, X.; Markussen, J. *J. Mol. Biol.* **1991**, *220*, 425−433.
68. Brange, J.; Havelund, S. *Acta Med. Scand.* **1983**, *671 (Suppl.)*, 135−138.
69. Katakam, M.,; Banga, A. J.; *PDA J. Pharm. Sci. Technol.* **1995**, *49*, 160−165.
70. Blackshear, P. J.; Rohde, T. D.; Palmer, J. L.; Wigness, B. D.; Rupp, W. M.; Buchwald, H. *Diabetes Care* **1983**, *6*, 387−392.
71. Grau, U.; Saudek, C. D. *Diabetes* **1987**, *36*, 1453−1459.
72. Thurow, H.; Geisen, K. *Diabetologia* **1984**, *27*, 212−218.
73. Hansen, P. E.; Brange, J.; Havelund, S. US patent 4,614,730, 1986.
74. Brewster, M. E.; Hora, M. S.; Simkins, J. W.; Bodor, N. *Pharm. Res.* **1991**, *8*, 792−795.
75. Banga, A. K.; Mitra, R. *J. Drug Targeting* **1993**, *1*, 341−345.
76. Grau, U.; Geisen, K.; Jährling, P. *Diabetes Nutr. Metab.* **1989**, *2*, 43−52.
77. Pongor, S.; Brownlee, M.; Cerami, A. *Diabetes* **1983**, *32*, 1087−1091.
78. Brange, J.; Langkjær, L. In *Stability and Characterization of Protein and Peptide Drugs: Case Histories*; Wang, Y. J., Pearlman, R., Eds.; Plenum: New York, 1993; pp 315−350.

JS960297S

Regeneron Pharmaceuticals, Inc. Exhibit 2063 Page 9
Celltrion, Inc. v. Regeneron Pharmaceuticals, Inc. IPR2023-00462