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Characterization of Sizes of Aggregates of Insulin Analogs and the Conformations of the Constituent Protein Molecules: A Concomitant Dynamic Light Scattering and Raman Spectroscopy Study

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ABSTRACT

To generate aggregates, 3 insulin analogs, lispro, aspart, and glulisine, were incubated without phenolic preservatives for 30 days at 37°C. As a function of incubation time, aggregation was quantified with size exclusion chromatography, and the sizes of aggregates and the conformations of the constituent molecules were characterized with concomitant dynamic light scattering and Raman spectroscopy. During incubation, lispro was progressively converted into soluble aggregates with hydrodynamic diameters of circa 15 nm, and 95% of the native protein had aggregated at day 30. Raman spectroscopy documented that aggregation resulted in conversion of a large fraction of native alpha helix into nonnative beta sheet structure and a distortion of disulfide bonds. In contrast, for aspart and glulisine only 20% of the native proteins aggregated after 30 days, and minimal structural perturbations were detected. In addition, consistent with the relative aggregation rates during isothermal incubation, Raman spectroscopy showed that during heating the onset temperature for secondary structural perturbations of lispro occurred 7°C-10°C lower than those for aspart or glulisine. Overall the results of this study demonstrated that—as in the case during formation of amyloid fibrils from insulin—formation of soluble aggregates of lispro resulted in a high level of conversion of alpha helix into beta sheet.

Introduction

Currently, the fast-acting insulin analogs lispro, aspart, and glulisine are the most widely used insulins in diabetic patients.[1] In contrast to native human insulin, the analogs have weaker monomer-monomer interactions, which on administration allow for quicker dissociation from the oligomeric state to the biologically active monomer. As a result, in patients, the analogs have a quicker onset of activity than does human insulin.[2,3] For each of the analogs a specific mutation is responsible for the weaker intermolecular interactions. Lispro has the penultimate 2 amino acids in the B-chain reversed compared with insulin (from Pro28-Lys29 to Lys28-Pro29). In the B-chain, aspart has 1 substitution, Pro28 to Asp; and glulisine has 2 substitutions, Asn3 to Lys and Lys29 to Glu.

In commercial formulations of lispro and aspart, metacresol and/or phenol and zinc ions promote native state assembly of the insulin molecules, which in turn increases protein storage stability.[4,5] In addition, metacresol and/or phenol also serve as antimicrobials.[4,6] In the commercial formulation of glulisine, metacresol is included as a stabilizer and antimicrobial preservative, but the formulation does not include zinc ions.[6,7]

There can be a gradual loss of metacresol and phenol from the formulations[3,8-13] during administration of the analogs into patients with pump and catheter systems. The reduction in the concentration of these stabilizers can lead to aggregation and even precipitation of insulins in the pump reservoir and tubing.[11,13,14] Published laboratory studies have characterized the aggregation of insulin analogs resulting from preservative depletion.[6,15] In one study, it was found that heating and agitation of lispro, aspart, and glulisine—in the absence of metacresol or phenol—caused all the analogs to precipitate via fibril formation.[6] Although the structural changes resulting from fibril formation were not characterized, it has commonly been observed that the secondary structure of insulin molecules is converted from native alpha helix to nonnative

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intermolecular beta sheet during fibril formation.[16-20] In another study, this structural transition was observed for lispro when solutions were seeded with preformed aggregates to induce fibril formation.[21] In more recent work, it was observed that quiescent incubation of lispro or aspart samples, which had been depleted of metacresol and phenol, at 37°C caused formation of soluble aggregates without observable fibrils.[15] However, the conformations of insulin analog molecules in the soluble aggregates were not studied. Therefore, in contrast to the wealth of published information on structural changes in insulin molecules on conversion to insoluble fibrils, there are not similar published insights into the structural changes arising when insulin analogs are converted into soluble aggregates.

In the present study, we addressed this issue by analyzing samples of the lispro, aspart, and glulisine with concomitant Raman spectroscopy and dynamic light scattering (DLS).[22] After removal of preservatives, 20 mg/mL solutions of each of the 3 insulin analogs were incubated quiescently at 37°C for 30 days. At certain time points during the incubation, samples were removed and characterized for protein secondary and tertiary structure with Raman spectroscopy. Concomitantly in the same sample cuvette, protein and aggregate sizes were determined with DLS.[22,23] In addition, size exclusion chromatography (SEC) was used to quantify the levels of native insulin and aggregates in the incubated samples. Finally, the relative thermal stabilities of the insulin analogs at 20 mg/mL were evaluated during heating studies with concomitant Raman spectroscopy and DLS.

Materials and Methods

Materials

Insulin analogs lispro (Humalog®-Eli Lilly and Company), aspart (Novolog®-Novo Nordisk), and glulisine (Apridra®- Sanofi-Aventis) were purchased from the University of Colorado at Boulder's Wardenburg pharmacy. Lispro is formulated in 1.88 mg/mL sodium phosphate buffer with 16 mg/mL glycerol, 3.15 mg/mL metacresol, and 0.0197 mg/mL zinc (pH 7.0-7.8). Aspart is formulated in 1.25 mg/mL phosphate buffer with 16 mg/mL glycerol, 1.5 mg/mL phenol, 1.72 mg/mL metacresol, 0.58 mg/mL sodium chloride, and 0.0196 mg/mL zinc (pH 7.2-7.6). Glulisine is formulated in 6 mg/mL Tris buffer, 3.15 mg/mL metacresol, 5 mg/mL sodium chloride, and 0.01 mg/mL Tween 20 (pH 7.0-7.8). All other chemicals were purchased from Fisher Scientific (Hampton, NH) and were of reagent grade or higher quality.

Methods

Preparation of Insulin Analog Solutions

Preservatives (metacresol and/or phenol) in the insulin formulations were removed with Zeba desalting columns (Thermo Scientific, Rockford, IL), as described previously.[15] As a result of this process, each insulin analog was in a solution with the respective original formulation composition but without preservative. The protein was concentrated to 20 mg/mL with Amicon Ultra 3000 MWCO centrifugal filters (Millipore, Cork, Ireland). Insulin concentration was determined by UV absorbance at 277.5 nm with an extinction coefficient of 0.9521 $cm^{-1}/mL/mg^{-1}$,[6] assuming the same extinction coefficient for insulin monomer, dimer, tetramer, and hexamer. Aliquots (200 μL) of each insulin solution were incubated quiescently at 37°C in 1.3-mL glass vials (West Pharmaceuticals, Exton, PA), and analyses were performed at days 0, 4, 10, 20, and 30 by ultra performance liquid chromatography–size exclusion chromatography and concomitant Raman spectroscopy or DLS. For each insulin analog solution, 3 sample vials were prepared for each time point. Thus, for each time point, independent triplicate samples were analyzed.

Size-Exclusion Chromatography

Sample aliquots were removed from the vials, placed into Eppendorf tubes, and centrifuged at 14,100 × *g* for 10 min. Aliquots of the "supernatants" were analyzed. It should be noted, however, that no pellet was observed for any of the centrifuged samples.

Native insulin and soluble aggregate levels were quantified with SEC using Waters Acquity UPLC BEH200 (Waters Corp., Milford, MA) SEC column (4.6 × 150 mm^2) on an Agilent 1100 HPLC (Santa Clara, CA). UV detection was at 280 nm. The mobile phase consisted of 100-mM sodium phosphate (pH 7.0), 100-mM sodium sulfate, and 0.05% wt/vol NaN_3, with a flow rate of 0.208 mL/min and run time of 37 min. For the purpose of quantification, the total peak area before incubation was considered as 100%. The peak areas for native species and soluble aggregates in samples taken at various incubation time points were calculated based on the initial total peak area.

Concomitant Raman Spectroscopy and DLS

Raman spectroscopy-DLS measurements were performed on a Zetasizer Helix system (Malvern Instruments, Malvern, United Kingdom).[22] For a typical experiment, a ~120 μL sample was loaded into a 3 × 3 mm quartz cuvette which was placed into a Peltier temperature-controlled sample compartment. Raman and DLS data were acquired sequentially.[21] More detailed information on the instrument can be found in previous study.[22] Raman scattering was excited by a 785 nm laser with approximately 280 mW power. Raman spectra of corresponding buffers for the protein samples were acquired under the identical conditions and subtracted from the spectra for the protein solutions. Unless otherwise noted, Raman spectra were collected with 10 coadditions of a 20-s exposure. DLS data were collected at a 173-degree backscattering angle from a 632-nm laser.

Insulin Analog Conformational Stability During Heating

Solutions (20 mg/mL) of each insulin analog were heated from 20°C to 90°C with data acquisitions at a 1°C increment. During heating, Raman spectra were collected at each temperature, as described previously.[1] For each analog, the heating study was repeated 3 times, with a fresh solution used each time.

Results and Discussion

Quantitation of Levels of Native Insulins and Aggregate by Size-Exclusion Chromatography

Figure 1a shows the SEC chromatograms of insulin lispro samples that were analyzed during the time course of the incubation. Native insulin eluted at around 12 min whereas the high molecular weight species (HMWS) eluted from 4 min to 10 min. The gradual loss of the peak for native insulin and gain of the HMWS peak indicated the conversion of native insulin species into higher-order oligomers as a function of incubation time.

Furthermore, there was a progressive reduction in total peak area in the chromatograms for lispro samples, reflective of a reduction in recovery of protein during sample processing and/or chromatography. When there is such a reduction in protein recovery, it may be ascribed to pelleting of so-called "insoluble aggregates" during the prechromatography centrifugation step. Also, there can be loss of protein during an SEC run (e.g., due to adsorption to the column matrix).[24] We did not observe a pellet for any of the samples; even those that had more than 30% reduction in total peak area on the SEC chromatograms. If, for example, 20% of