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Research Article

Structure, Aggregation, and Activity of a Covalent Insulin Dimer Formed During Storage of Neutral Formulation of Human Insulin

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A specific covalently linked dimeric species of insulin high molecular weight products (HMWPs), formed during prolonged incubation of a neutral pharmaceutical formulation of human insulin, were characterized in terms of tertiary structure, self-association, biological activity, and fibrillation properties. The dimer was formed by a covalent link between A21Asn and B29Lys. It was analyzed using static and dynamic light scattering and small-angle X-ray scattering to evaluate its self-association behavior. The tertiary structure was obtained using nuclear magnetic resonance and X-ray crystallography. The biological activity of HMWP was determined using 2 *in vitro* assays, and its influence on fibrillation was investigated using Thioflavin T assays. The dimer's tertiary structure was nearly identical to that of the noncovalent insulin dimer, and it was able to form hexamers in the presence of zinc. The dimer exhibited reduced propensity for self-association in the absence of zinc but significantly postponed the onset of fibrillation in insulin formulations. Consistent with its dimeric state, the tested species of HMWP showed little to no biological activity in the used assays. This study is the first detailed characterization of a specific type of human insulin HMWP formed during storage of a marketed pharmaceutical formulation. These results indicate that this specific type of HMWP is unlikely to antagonize the physical stability of the formulation, as HMWP retained a tertiary structure similar to the noncovalent dimer and participated in hexamer assembly in the presence of zinc. In addition, increasing amounts of HMWP reduce the rate of insulin fibrillation.

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Introduction

Physical and chemical stability are critical objectives in the development of safe and potent pharmaceutical formulations of proteins. Degradation products can potentially have reduced biological activity and can promote unwanted side effects through toxic or immunogenic actions. Pharmaceutical formulations of human insulin and modern analogues are critical to the treatment of diabetes mellitus. The development of insulin pharmaceutics is challenged by the formation of high molecular weight products (HMWPs), which are defined by a pharmacopoeia assay as covalent impurities larger than the insulin monomer.[1] Human insulin HMWP is suspected of promoting immunogenic responses in patients, although a causal relationship with specific species of HMWP has not been established.[2-4] Protein aggregates and their immunogenic potential have recently been the focus of debate between academia and industry.[5-9]

Human insulin is a peptide composed of 51 amino acids (AAs) forming the A-chain (21 AAs) and B-chain (30 AAs) connected by 2 disulfide bridges. Human insulin HMWP has been investigated by Darrington et al. who identified insulin HMWP as mainly covalent

Abbreviations used: DLS, dynamic light scattering; HMWP, high molecular weight products; nOe, nuclear overhauser effect; NOESY, nuclear overhauser effect spectroscopy; PDB, Protein Data Bank (RCSB); SAXS, small-angle X-ray scattering; SLS, static light scattering; TOCSY, total correlation spectroscopy.

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dimers formed via the formation of a cyclic anhydride intermediate at the A21Asn C-terminus followed by nucleophilic attack by the B1Phe N-terminus.[10,11] Brange et al. also investigated HMWP formation in early modern formulations of human and porcine insulin and also observed covalent dimers cross-linked via the B1Phe N-terminal.[12,13] In an recent article, we have presented a novel primary structure of an insulin HMWP species formed in a marketed neutral formulation of human insulin[14]: more than 60% of the formed HMWP was identified as covalent dimers formed by A21Asn-B29Lys cross-links, with the cross-link in either the extended amide form (open) or succinimidyl form (closed).

Pharmaceutical formulations of human insulin often exploit the property of insulin to form hexamers in the presence of zinc, as the hexamer is favorable in terms of chemical and physical stability.[15-17] The hexamer is further stabilized by addition of phenolic ligands.[18,19] On injection, the hexamers dissociate to the biologically active monomers.[20,21] The crystal structure of porcine insulin was first determined using X-ray in 1969 by Hodgkin et al.,[22] and the 2-Zn hexamer structure was further detailed in the following years,[23-25] along with the structure of human insulin.[26,27] The tertiary structures of various insulin analogues have also been investigated by nuclear magnetic resonance (NMR).[28,29] The insulin hexamer consists of 3 noncovalent insulin dimers. The dimer forming interface is composed primarily of the residues B8, B9, B12, and B16 with B23-B26 in an antiparallel β-strand interface.[23,25] The B10 histidine from each monomer coordinates to 1 of 2 zinc atoms located above and below the 2-fold axis.[22] The 6 monomers composing a hexamer can be denoted R or T depending on the secondary structure of the B1-B8 segment. The T type is variable in conformation, whereas the R type is defined by a B1-B8 α-helix.[19,30] It is unknown if insulin HMWP is capable of participating in hexamer formation and if the covalent link has any effect on self-association at formulation conditions. Furthermore, it is unknown if the native fold of insulin is preserved for this HMWP species.

This study aims to further characterize the properties of the A21Asn-B29Lys HMWP species isolated and identified in our previous work.[14] The biological activity was investigated using a soluble insulin receptor assay and adipocyte assay. The secondary and tertiary structure of A21Asn-B29Lys HMWP was identified using far-UV CD, X-ray crystallography, and hydrogen nuclear magnetic resonance, whereas the self-association behavior was investigated using small-angle X-ray scattering (SAXS) and light scattering techniques. The overall structure of HMWP was found to be very similar to the noncovalent insulin dimer, and the covalent dimer was biologically inactive. Although HMWP exhibited a lower average association state in the absence of zinc than human insulin, it readily formed hexamers structurally similar to human insulin hexamers in the presence of zinc and *m*-cresol. The onset of fibrillation in samples of human insulin was found to be delayed by addition of HMWP. These results indicate that the formation of HMWP is unlikely to have a major negative impact on the physical stability of human insulin formulations during storage.

Materials and Methods

Zinc-free human insulin was provided by Novo Nordisk A/S. Specific species of HMWP were isolated from marketed formulation of human insulin stored for 1 year at 37°C (Actrapid®/Novolin® R 100 U/mL, 0.6-mM human insulin, 0.33-mM zinc, 28-mM *m*-cresol, 1.6% (w/v) glycerol, pH 7.4) as described by Hjorth et al.[14] All samples of HMWP were dialyzed in 10-mM phosphate buffer (pH 7.4) for 36 h at 5°C using a cellulose membrane cassette (Slide-A-Lyzer, 3K Da cutoff, Thermo Fisher Scientific) with gentle stirring. The dialysis buffer was completely exchanged 2 times during the process. Where appropriate, samples were diluted with dialysis buffer before analysis. Concentration of samples was accomplished using spin vials before dialysis (Amicon Ultra, 3K Da cutoff, Merck Millipore Ltd., Tullagreen, Carrigtwohill Co., Cork, Ireland). All chemicals were of analytical grade or higher purity. Two identified species of HMWP were used, each composed of 2 insulin monomers covalently linked via A21Asn to B29Lys with A21 in either the closed (succinimide) or open (Asp) state[14] (Supplementary Fig. S1).

Far-UV CD

Samples of HMWP (open state) and human insulin were prepared at a concentration of 0.8 mg/mL. Samples containing zinc at 0.25 zinc/monomer (mol/mol) and 0.55 zinc/monomer (mol/mol) ratios were prepared by addition of zinc acetate. The pH of the samples was adjusted to pH 7.4 using HCl and NaOH. The samples were allowed to rest for 1 h at room temperature before analysis. The samples were checked for zinc precipitation using UV-absorption at 200 nm, and no precipitation was observed.

Far-UV CD spectra were recorded using a JASCO J-815 (Jasco, Easton, MD) coupled to a Julabo F-250 thermostated water bath (Julabo USA Inc., Allentown, PA). Samples were scanned at 20°C in the range of 190-260 nm with 1 scan per 0.5 nm at a scanning speed of 50 nm/min. Measurements were carried out in 0.01-cm quartz cuvettes of 30 μL (Hellma Analytics, Müllheim, Germany). A buffer spectrum was subtracted from the sample spectra, and the baseline was corrected. Data are presented as average of 3 scans per sample using mean residue ellipticity according to Kelly et al.[31]:

$$[\theta]_{\mathrm{mrw},\,\lambda} = \theta_\lambda \times \frac{\mathrm{MRW}}{10 \times c \times d},$$

where mean residue ellipticity ($[\theta]_{(\mathrm{mrw},\lambda)}$) is calculated as a function of observed ellipticity at wavelength λ (θ_λ), the path length (d, cm), and the protein concentration (c, g/mL). Mean residue weight (MRW) of 113.88 Da was used for human insulin ($M_w = 5807.65$ Da) and 113.71 Da for HMWP ($M_w = 11598.28$ Da).

NMR Spectroscopy

A sample of zinc-free HMWP (open state) was prepared at 4.5 mg/mL. After NMR analysis of the zinc-free HMWP sample, zinc acetate was added to 0.55 zinc/monomer (mol/mol) equivalents, and *m*-cresol was added to 28-mM. NMR spectra were recorded on a Bruker Avance III 800 equipped with a TCI Z-gradient CryoProbe™ and a 18.7-T Oxford magnet and an Avance III 700 equipped with a TCI Z-gradient CryoProbe™ and a 16.4-T Bruker Ultrashield™ magnet. Homonuclear and heteronuclear spectra included a total correlation spectroscopy (TOCSY) with a spinlock of 60 ms and 4096 × 800 data points, a nuclear overhauser effect spectroscopy (NOESY) with mixing time of 120 ms and 4096 × 800 data points and a heteronuclear single quantum coherence with 2048 × 512 data points. In addition, a double quantum filter correlation spectroscopy was recorded with 4096 × 512 data points but with limited quality. The sample was dissolved in H_2O/D_2O 90%/10% in a 5-mm tube, and spectra were recorded at 318 K.

SAXS

Hexamer Formation

A sample of HMWP (open state) was dialyzed into 10-mM phosphate buffer pH 7.4 with 28-mM *m*-cresol for 30 h at 5°C with gentle stirring. The dialysis buffer was replaced with fresh buffer 2 times during the process. The final concentration of HMWP was analyzed using UPLC-SEC and diluted to 1 mg/mL using left-over dialysis buffer. Zinc acetate was added to a concentration of

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