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Structure and function of anhydride-modified forms of human insulin: In silico, in vitro and in vivo studies

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ABSTRACT

Insulin is a therapeutic protein whose amyloid formation is reported in diabetic patients. Four anhydride compounds were used in the current study in order to experiment their potential reducing effect on insulin propensity to form amyloid fibrils. The modified forms (obtained with succinic-, 3,3-dimethylglutaric, 2-phenylglutaric-, and (2-Dodecen-1-yl) succinic anhydride), were first characterized with regard to melting temperature (Tm), changes in secondary structure percentage and hydrophobic surface. Fibril formation was then assessed by Congo red absorbance kinetics and transmission electron microscopy. Functionality was investigated with the use of an insulin tolerance test in NMRI mice. Finally, 10 ns molecular dynamics simulations were performed during which structural changes, potential energy, gyration radius, RMSD, and accessible surface area were monitored.

In all cases, α-helical structure content of the modified forms was reduced, but thermal stability and structural compactness of modified insulin were increased except in case of the dodecenylated species. All modified insulin forms undergo amorphous aggregation instead of amyloid fibrils formation, and dodecenylated insulin makes the largest amorphous aggregates. In silico results were overall in accordance with in vitro studies. Finally, only succinylated insulin was functional, although dimethylglutaric-modified insulin started to show some activity after 2 h.

1. Introduction

Formation of insoluble, distinct amyloid fibrils from soluble proteins is related to the pathophysiology of several disorders including neurodegenerative ones (Skovronsky et al., 2006), systemic diseases such as AA and AL (amyloid light-chain) amyloidosis, and even type II diabetes (Chiti and Dobson, 2006). Many other proteins with various structures and function are able to convert to amyloid fibrils in vitro (Booth et al., 1997; Chinisaz et al., 2014a), and it is now widely believed that this process is a common, intrinsic property of proteins (Chiti et al., 2002).

Several proteins, including insulin have been used as models to investigate amyloid structures properties (Arora et al., 2004; Fodera and Donald, 2010; Loksztejn and Dzwolak, 2010). Insulin is a small peptide hormone, composed of two A and B polypeptide chains which are linked by two disulfide bonds (Baker et al., 1988). In the secretary vesicles of pancreas, the predominant form of insulin is a three-dimers hexamer containing 2–4 zinc ions, but in order to become biologically active, insulin has to take a monomeric form (Derewenda et al., 1991; Zoete et al., 2004). Similarly, amyloid formation of insulin (in vitro) occurs on monomers and is influenced by low pH, high temperature and increased ionic strength (Muzaffar and Ahmad, 2011; Sneideris et al., 2015).

In vivo, insulin amyloid fibrils have been observed in diabetic patients as part of cutaneous amyloid tumors at the sites where frequent insulin injections are made, in as many various locations as shoulders,

Abbreviations: SUC, [succinic anhydride]; SUCINS, Insulin modified by SUC; DMG, [3,3-Dimethylglutaric Anhydride]; DMGINS, Insulin modified by DMG; PHG, [2-phenylglutaric anhydride]; PHGINS, PHG1INS and PHG2INS, Insulin modified by PHG; DOD, [(2-Dodecen-1-yl)succinic anhydride]; DODINS, DOD1INS and DOD2INS, Insulin modified by DOD; HI, native human insulin.

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arms, thighs and abdomen (Störkel et al., 1983; Dische et al., 1988; Albert et al., 2007; Yumlu et al., 2009; Sie et al., 2010). These deposits results into poor absorption of subsequently injected insulin, leading to impaired control of hyperglycemia, and a need to increase insulin dosage (Nagase et al., 2014). However, hypoglycemia may also occur, due to a sudden change in injection location from the amyloid deposit location to another area (Nilsson, 2016). The number of reported cases have dramatically increased since 2012, compared to reports between 1983 and 2011, possibly due to patients improved care (Nilsson, 2016) and it has been suggested that the formation of insulin fibrils in diabetic patients may be in fact more common than generally thought (Gupta et al., 2015).

Subcutaneous injection of insulin fibrils results into a similar state in animal models (Chinisaz et al., 2014b), and could cause amyloid plaque formation when injected into rats brains (Kheirbakhsh et al., 2015).

Chemical modification of insulin can improve its stability and absorption and extend its duration of action in vivo (Myers et al., 1997; Heinemann et al., 1999; Uchio et al., 1999). The modification-available primary amino groups on insulin are located at the N-termini Gly^{A1}, Phe^{B1} and on the position 29 of the B-chain (Lys^{B29}) (Huang and Huang, 2005). It is known that Phe^{B1} and Lys^{B29} modification does not affect insulin function, while modifying Gly^{A1}causes a decrease in activity (Lindsay and Shall, 1971). So far, different types of insulin modifications have been reported, including glycation (O'Harte et al., 1996; Oliveira et al., 2011), lipid-modification (Hashimoto et al., 1989; Huang and Huang, 2005, 2006), and PEGylation (Hinds et al., 2000). Deamidation of Asn^{A21}, Asn^{B3} (which may occur in therapeutically used forms of the peptide), and Gln^{B4} promotes insulin amyloid fibril formation (Nilsson and Dobson, 2003) while glycation of Arg^{A18} by methylglyoxal inhibits the formation of fibrils and results in native-like aggregation (Oliveira et al., 2011).

Lysine residues have important roles in the structure of proteins, especially as a target for post-translational modification (Ikeda and Dikic, 2008; Kang et al., 2015). These residues may undergo glycation, acetylation, methylation or carboxymethylation which may affect (positively or negatively) the propensity of the protein to aggregate (Morshedi et al., 2010; DiMauro et al., 2014; Funk et al., 2014; Arndt et al., 2015). The single lysine residue of insulin, which is located in the C-terminal of B-chain, is very flexible (Babenko and Dzwolak, 2013) and previous studies have shown that conformational changes happening to the B-chain are important in insulin fibrillation (Muzaffar and Ahmad, 2011). The absence of five C-terminal residues of the B-chain results in an increase of the aggregation process (Brange et al., 1997a; Brange et al., 1997b), and comparison between molecular dynamics simulation made on truncated and intact forms of insulin has shown a higher stability of the latter (Chinisaz et al., 2014c).

In the present report, modification of the lysine residue of human insulin has been performed by the use of four anhydride compounds: succinic anhydride (SUC), 3,3-Dimethylglutaric Anhydride (DMG), 2-Phenylglutaric anhydride (PHG), and [(2-Dodecen-1-yl) succinic anhydride] (DOD). The effect of these modifications was then investigated on the structure, function and aggregation propensity of the modified forms.

2. Experimental

2.1. Materials

Human insulin, thioflavin T (ThT), Congo red (CR), SUC, DMG, PHG, and DOD were purchased from Sigma Chemical Co. (St. Louis, MO). 8-Anillino-1-naphthalene-sulfonic acid (ANS), and all salts and organic solvents were from Merck (Germany). Protein concentration was determined spectrophotometrically by Bradford assay.

2.2. Methods

2.2.1. In Vitro Methods

2.2.1.1. Modification of Lysine Residues. The protein was used at 3 mg/ml concentration in 50 mM sodium phosphate (pH 8.0). During addition of modifiers, reaction mixtures were well stirred (50 rpm), and the temperature and pH were maintained at 0–4 °C and 8.0 respectively. After protein modification, extensive dialysis was performed against 50 mM phosphate buffer (pH 7). Finally, 200 μl hydroxylamine (0.5 mM) was added gradually to the resulting solution for 30 min in order to ensure O-deacetylation of tyrosine residues (Riordan and Vallee, 1972).

To verify modification of lysine residues, protein solution (5 μl; approximately 2 mg/ml), 250 μl 100 mM Na_2HPO_4, 90 μl deionized water, and 100 μl fluorescamine reagent (1 mM fluorescamine in acetonitrile) were mixed and incubated in dark room for 10 min, after what fluorescence of the solution was measured using excitation/emission wavelengths of 390/490 nm (Schmitt et al., 2005).

2.2.1.2. Circular Dichroism Method. Far ultraviolet (UV) circular dichroism (CD) spectra were obtained using an AVIV 215 spectropolarimeter (Aviv Associates, Lakewood, NJ, USA) with 1 mm path length cell at room temperature. CD spectra were obtained from aliquots withdrawn from the aggregation mixtures and recorded between 200 and 260 nm. The mean residue ellipticity θ (deg cm^2 $dmol^{-1}$) was calculated from the formula:

$$\theta = (\theta_{obs}/10)(MRW/lc)$$

where θ_{obs} is the observed ellipticity in degree, MRW is the mean residue molecular mass, l is the optical path length (in centimeters), and c is the protein concentration (in g/ml). The protein concentration was 0.2 mg/ml. Protein secondary structure analysis was conducted using CDNN software (CD Spectra Deconvolution v 2.1) (Böhm et al., 1992).

2.2.1.3. Tm Measurement. Scanning microcalorimetric measurements of native and modified insulin were carried out at 0.9 mg/ml protein concentration with the use of a Cary100 Bio UV–visible spectrophotometer. A 0.3 ml cell was used and the heating rate was fixed at 1 K/min. The heating curves were corrected for an instrumental baseline obtained by heating the buffer (50 mM phosphate buffer, pH 7.4) alone.

2.2.1.4. ANS Fluorescence Assays. ANS was used at 100 μM concentration. Excitation wavelength was 365 nm and emission spectra were taken at 450–600 nm. Excitation and emission slit widths were both set at 5 nm. Final protein concentration in samples was 20 μg/ml.

2.2.1.5. Amyloid Preparation From Human Insulin. Native and modified insulin were dissolved at 0.5 mg/ml concentration in phosphate buffer, the final pH was adjusted at 7.4 by addition of 1 M HCl, and then incubated at 37 °C for the specified durations while being stirred gently (about 150 rpm) by Teflon magnetic bars (Rabiee et al., 2011).

2.2.1.6. Congo Red (CR) Absorbance Assays. CR was dissolved at 7 mg/ml in a buffer consisting of 0.15 M NaCl and 5 mM potassium phosphate (pH 7.4). The prepared solution was first filtered through a 0.2 μm filter. Subsequently, 5 μl of well-mixed incubation sample were added to 300 μl of the CR solution and incubated for 30 min. Absorbance spectra were recorded at 400–600 nm using a Shimadzu UV–visible spectrophotometer (Kyoto, Japan). Spectra in the presence of the dye were compared with those of the buffer containing CR in the absence of protein. As a mean to monitor the kinetics of fibril formation, absorbance intensity at 400–600 nm was followed over time, and the absorbance at 520 nm was taken as representative of amyloid formation (Klunk et al., 1989).