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Can aggregation of insulin govern its fate in the intestine? Implications for oral delivery of the drug

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ABSTRACT

The objective of this study is to elucidate the role of low-molecular weight biogenic agents, resembling dietary-derived products naturally occurring in the intestine, in the regulation of transformations of soluble aggregation-prone insulin into aggregates of higher order. In the course of model experiments, a striking potential of the amino acids L-arginine (Arg) and L-lysine (Lys) and a number of positively charged peptides to induce formation of heterogenic supramolecular structures of insulin was demonstrated under environment conditions where the protein aggregation in their absence was not observed. This phenomenon is assumed to be essential for elaboration of strategies of oral delivery of insulin to diabetic patients supplemented by controlling the pH values of the intestinal environment where the drug is released.

An obvious scientific challenge for oral delivery of proteins is various physiological barriers in the gastrointestinal tract (GIT), mainly the aggressive acidic environment in the stomach, different digestive enzymes and limited intestinal absorption. A great number of protective carrier systems for oral delivery of insulin have been developed, containing protease inhibitors, absorption enhancers, and other components designed for co-administration with insulin (Chen et al., 2011; Sonia and Sharma, 2012; Yaturu, 2013). Although certain progress has been achieved in the past few years, the fate of the protein on its "dangerous" pathway through GIT still seems to be quite uncertain, as many hidden rocks lying in the course of the insulin vehicles are very difficult to avoid. Among them, the structural transformation and aggregation of insulin remain a great challenge.

Studies on insulin aggregation are going on from the late twenties of 20th century. It is widely known that aggregation of insulin may occur at almost every stage of the pharmaceutical process, from production and storage to delivery and absorption (Brange et al., 1997; Dische et al., 1988). In particular, the ingested insulin, for the most part, is not absorbed and may accumulate in the intestine, as the wide range of formulations of the delivery systems provide prolonged retention time and high local drug concentrations in the vicinity of hydrophobic surfaces of the intestinal epithelium. In a heterogeneous environment, under extreme conditions insulin can become highly reactive with other components of the surroundings that can potentially induce physical–chemical destabilization and aggregation of the protein (Ahmad et al., 2005; Grabovac et al., 2008; Li and Leblanc, 2014; Torosantucci et al., 2014). These interactions may represent a more significant potential obstacle to oral delivery of insulin, than previously suspected. Therefore, estimation of consequences of the measures that are currently taken to enhance absorption of insulin, in particular high concentrations of the protein, protease inhibitors, and permeation enhancers deserves more attention, as under certain conditions, all these measures may induce the aggregation process and a decrease in absorption of the drug.

Among numerous carrier systems for insulin delivery, the particles sensitive to changes in the pH values of the environment appear to have considerable promise (Lowman et al., 1999; Peppas and Kavimandan, 2006). Under acidic conditions, these carriers are stable and thus can protect insulin from degradation in the stomach, whereas under slightly alkaline pH of the intestine, they are able to disintegrate and release insulin.

These observations have awakened interest in low-molecular weight biogenic agents, resembling dietary-derived products naturally occurring in the intestine, as effectors involved in transformation of soluble aggregation-prone proteins into structures of higher order. Amino acids and short peptides can be considered for this role. Taking into consideration that the net negative charge of insulin increased, as its amino acids undergo

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deprotonation at pH greater than the pI value (5.4), we assumed that the positively charged amino acids L-arginine (Arg) and L-lysine (Lys) can interact with oppositely charged protein domains under conditions, mimicking those occurring at pH in the range of 7.0–8.0 in the intestine. The complementary peptides that survive proteolysis in the intestinal microenvironment, where oral insulin is released, being accompanied by protease inhibitors, were also suggested to possess this property. In developing these assumptions, we have initiated a series of model experiments *in vitro* on the aggregation of human recombinant insulin under destabilizing conditions.

Although the experimental conditions presented below may be considered exaggerated, physiologically irrelevant, and unlikely to occur *in vivo* as simultaneously developing events, we chose them mainly to get an overall picture of great many low-molecular weight substances surrounding insulin in a heterogeneous intestinal environment containing destabilizing factors, digested food products and food additives or drugs, especially in cases of gastrointestinal dysfunction.

The experiments were mainly based on dynamic light scattering allowing to measure the time-course of the increase in the light scattering intensity and the distribution of aggregates by their size expressed by the hydrodynamic radius (R_h), as described in our previous studies of aggregation of proteins (Artemova et al., 2012; Smirnova et al., 2013). The formation of the aggregates was followed in the process of incubation of 0.15 mg/ml insulin (Sigma) at 37 °C in 25 mM sodium-phosphate buffer, pH 8.0, containing 0.15 M NaCl and 5 mM dithiothreitol (DTT). Though the latter was added to the incubation mixture to mimic effects of destabilizing factors of the environment that can transform the insulin molecule into the aggregation-prone state, under these experimental conditions, the aggregation of insulin alone was not observed during incubation for at least 3 h (Fig. 1a and b, curves 1). However, upon addition of Arg with the concentrations of 10–100 mM, the aggregation was induced and developed very rapidly in a concentration-dependent manner, contrary to the commonly accepted property of Arg to suppress protein aggregation (Tsumoto et al., 2004). Lys appeared to be more potent than Arg (Fig. 1a and b, curves 2 and 3). This phenomenon was also observed in the presence of the Lys–Leu and Arg–Phe dipeptides at concentrations of 0.5–2 mM (Fig. 1c and d, curves 2 and 3). However, no effect of the negatively charged Asp–Phe dipeptide on the aggregation of insulin could be observed (Fig. 1c and d, curves 5). The peptide fragment of human adrenocorticotropic hormone ACTH (1–24), containing 8 positively charged amino acid residues (Fig. 1c, inset), appeared to exert the stimulatory effect on the insulin aggregation at 5–70 μM (curves 4). Though survival of such long peptides in GIT seems highly improbable, ACTH (1–24) is used as a model of peptides that may withstand digestion in the presence of protease inhibitors.

To study morphological characteristics of the insulin aggregates formed in the presence of Arg, the samples of the incubation mixture were subjected to atomic force and transmission electron microscopy analyses. Typical images revealed formation of morphologically distinct supramolecular structures generated at the initial stage of the aggregation process. Along with small-sized asymmetric granular units of 3–8 nm in apparent diameter, being associated into clusters or short chains of 20–50 nm in length, the larger chains of 100–200 nm were formed (Fig. 2a–c). The heterogeneity of the protein species was also revealed by electron

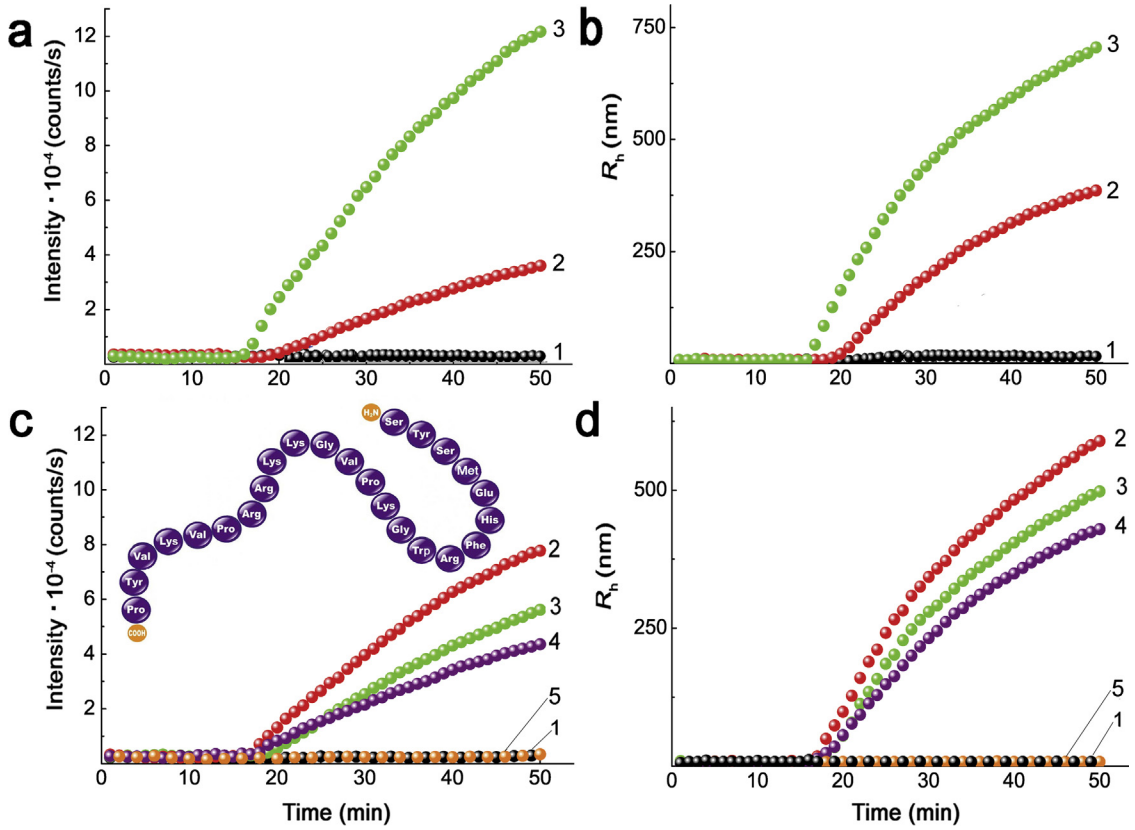

Fig. 1. The representative kinetic curves of insulin aggregation: dependences of the light scattering intensity (a) and the hydrodynamic radius (R_h) values (b) on time of the aggregates formed during the incubation of insulin in the absence (curves 1) or presence of the amino acids Arg (curves 2) or Lys (curves 3), both at the concentration of 100 mM, the Lys–Leu dipeptide (c and d, curves 2), the Arg–Phe dipeptide (curves 3), both at the concentration of 1 mM, or ACTH (1–24) at the concentration of 35 μM (curves 4). Schematic representation of ACTH (1–24) illustrating the sequence of the amino acid residues of the peptide chain (c, inset). The negatively charged Asp–Phe dipeptide exerted no effect on the aggregation of insulin (curves 5).