Supramolecular assembly of Cp1-11 peptide and insulin for rapid-acting formulation

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ABSTRACT

In order to improve the life quality of diabetic patients, it is very important to develop rapid-acting insulin formulations that can mimic the physiological meal-time secretion profile of insulin in healthy people. Although several insulin analogues have been designed to provide postprandial glycemic control, still there are some serious disadvantages. A supramolecular strategy is presented here to inhibit insulin aggregation and improve its bioactivity by using Cp1-11 peptide. As a fragment of C-peptide in proinsulin, Cp1-11 peptide was found to influence insulin oligomerization by supramolecular interactions. This work demonstrates that the Cp1-11 peptide can interact with oligomeric insulin and facilitate its disaggregation into the physiologically active monomeric form. Computer simulation indicates that Cp1-11 can insert into the space between the C-terminal tail and the N-terminal helix of the B-chain of insulin, causing dissociation of the insulin dimer. The supramolecular assembly of Cp1-11 and insulin can improve the bioavailability and therapeutic effect of insulin on the control of in vivo blood glucose levels. These results suggest that Cp1-11 peptide can modulate the intermolecular interaction of aggregated insulin and prevent the transition from monomeric to multimeric states, and shows great potential for the development of an effective rapid-acting strategy to treat diabetes.

Keywords: Supramolecular chemistry; Cp1-11 peptide; Drug delivery; Self-assembly; Rapid-acting insulin

1. INTRODUCTION

Diabetes mellitus (DM) has been one of the main threats to human health in the 21st century.1-4 It is among the most serious metabolic diseases and responsible for extensive deaths worldwide.5, 6 The economic impact of DM is even more striking since patients may live for many years with diabetes, accompanying with various complications such as cataract, diabetic foot and cardiovascular diseases.7 Therefore, a better control of blood glucose level and reduction of the complications occurrence risk have become the aim of diabetes treatment.

Insulin, which contains a 21-residue A chain and a 30-residue B chain and crosslinked by disulfide bonds, has so far been the most important pharmaceutical protein for the treatment of diabetes. Insulin secretion in healthy people includes basal insulin and bolus (mealtime) insulin. Insulin can self-assemble into dimers, three of which combine into a hexameric complex with the aid of two zinc ions, thus hindering the release of monomeric insulin and extending its release time. Bioinspired by these natural phenomena, some insulin analogues (e.g., glargine, detemir, and glulisine) have been designed with reduced protein-protein interactions between insulin monomers, mostly in the monomeric form without aggregation, to provide better postprandial glycemic control.16-22 However, the strategy of insulin analogues may also have some serious disadvantages, such as increasing cancer risk.23-26 Thus, we are interested in developing new strategies to prepare rapid-acting insulin formulation by the supramolecular method, as it can release monomeric insulin instead of insulin analogues to interact with the insulin receptor while controlling the blood glucose levels.

In nature, proteins play important roles in various biological processes at both cellular and systemic levels of living organisms.27 It has been revealed that most proteins function in the form of multimeric or supramolecular assemblies.28-32 These supramolecular self-assemblies are mainly based on the versatility of non-covalent interactions.33 Also, it is noted that diverse essential biological processes are carried out by molecular machines, which are composed of a large number of proteins organized by protein-protein interactions (PPIs).34, 35 Many studies have
indicated that PPIs are in fact dominated by the interactions between protein and peptide. Therefore, the essence of PPIs can be further understood by studying protein-peptide interactions. For example, peptides can bind to proteins named major histocompatibility complexes (MHC) and peptide-MHC complexes can be recognized by the antigen-specific receptor of T lymphocytes for the cellular immune system to distinguish self from nonself. Another example is that, antimicrobial peptides (AMPs) act as important mediators of innate immune responses. AMPs can interact with proteins of the innate immune system, such as G protein-coupled receptors, to indirectly eradicate pathogens.

C-peptide is the connecting peptide of proinsulin between its A and B chains (Scheme 1). It plays an important role in the biosynthesis of insulin as it facilitates the appropriate folding and formation of insulin SS bridges. C-peptide has been found to interact with insulin oligomers, influence their aggregation, and is thus capable of serving as a chaperone for insulin to promote activation by monomerization and prevention of aggregation. Recently, C-peptide fragments have been proved effective in preventing insulin aggregation, dependent on the amino acid sequence and chain length. Among them, the most effective is the EAEDLQVGQVE amino acid sequence (Cp1-11), which can decrease insulin oligomers, even better than the intact C-peptide. These insights shield lights on the bioinspired rapid-acting formulation for diabetes therapy.

2. EXPERIMENTAL

2.1 Materials

Porcine insulin was purchased from Xuzhou Wanbang Biological Pharmaceutical Enterprise (Jiangsu, China). Streptozotocin (STZ), heparin sodium, porcine insulin ELISA Kit, glucose and Dulbecco’s modified eagle medium (DMEM), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), fetal bovine serum (FBS, 10%), penicillin, streptomycin were purchased from Baoxin Biotechnology Co., Ltd. (Chengdu, China). Cp1-11 peptide (EAEDLQVGQVE, 99%) was purchased from Shanghai Botai Biological Pharmaceutical Enterprise (Shanghai, China). Cp1-11 was stored in a lyophilized state at −20°C until use. Ultrapure water produced from a Milli-pore system with a resistivity of 18.2 MΩ·cm was used throughout. Chemicals were purchased from Baoxin Biotechnology Co., Ltd. (Chengdu, China) if not otherwise stated. 100 mg insulin was dissolved and incubated in 100 mL HCl (pH 3.0) for 30 min and then the solution was adjusted to pH 7.0 using 0.1N NaOH to prepare the insulin solution (with aggregates). As for SACI-1/1 and SACI-2/1, the Cp1-11 powder with molar ratios of 1:1 and 2:1 to insulin was added with insulin powder in the above process. In this work, monomeric insulin solution was prepared in 20% acetic acid.

2.2 Dynamic light scattering (DLS)

The mean particle size distribution of the sample was determined by DLS (Malvern, UK) at a concentration of 1

Scheme 1. Supramolecular interaction between Cp1-11 and insulin. Cp1-11 is the EAEDLQVGQVE peptide fragment of the C-peptide of proinsulin. Cp1-11 can prevent the aggregation of insulin to form a supramolecular assembly of Cp1-11 and insulin (SACI), which will release monomeric insulin.

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mg/mL and pH 7.4. All measurements were carried out at 37 °C with a 90° scattering angle. The samples were subjected to ultrasonic treatment before being transferred into sample cuvettes.

2.3 Surface morphology

The morphologies of insulin aggregates and SACI were observed by transmission electron microscope (TEM) (Hitachi H-600 transmission electron microscope, JOEL Ltd., Japan) with an accelerating voltage of 75 kV. To prepare the samples for TEM measurements, a drop of sample solution (1 mg/mL) was deposited on a carbon-coated copper grid (200 meshes), then excess of sample solution was removed with filter paper. After being negatively stained with 1 wt/v% phosphotungstic acid for 60 s and a thorough air-drying, the samples were observed in the electron microscope.

2.4 Atomic force microscopy (AFM)

Samples were prepared as described above and incubated up to 50 h. After that, samples were diluted 20-fold with water and then immobilized on freshly cleaved mica for 10 min. The mica surface was washed with pure water, dried gently with a nitrogen flow and then left to dry overnight. All AFM images were captured in tapping mode with spring constant of 40 N/m and resonant frequency of 300 kHz, using a Nanoscope Multimode (Vecco Instruments, USA).

2.5 Circular dichroism (CD)

Conformational changes of insulin in the SACI assembly were evaluated by circular dichroism. All CD measurements were conducted by scanning from 190 to 310 nm on a JASCO CD spectropolarimeter (J-1500-150, USA) at room temperature. All measurements were performed in 0.1 M phosphate buffer solution (pH 7.4) and the concentration was 1 mg/mL. Each test was recorded in a quartz cuvette having a path length of 1 mm. Each spectrum is the average of three acquisitions.

2.6 SEC-HPLC measurement

Samples were detected by using size-exclusion high-performance liquid chromatography (SEC-HPLC): LC-15C Liquid Chromatograph, SPD-15C UV/Vis detector (Shimadzu, Japan), Shodex KW-802.5 column (300×8.0 mm, 5.0 µm particles) with a mobile phase containing 5 mM phosphate buffer solution (pH 7.4), with a flow rate of 1 mL/min and UV detection at 220 nm. Data processing was performed with the LC-15C solution software Version 1.26 (Shimadzu).

2.7 Isothermal titration calorimetry (ITC)

ITC experiments were performed on a MicroCal VP-ITC at 25°C in Tris–HCl buffer (10 mM, pH 7.4). In a typical titration experiment, insulin solution (0.50 mM) was placed in the 1.334 mL sample cell of the calorimeter, while Cp1-11 solution (1.50 mM) was loaded into the 250 µL injection syringe. All solutions were degassed under vacuum for 20 min before the titration experiment to avoid forming air bubble. The Cp1-11 solution was titrated into the sample cell at a sequence of 21 injections of 12 µL aliquots, respectively. The contents of the sample cell were stirred at 400 rpm throughout the measurements to ensure thorough mixing. The heat of Cp1-11 dilution in the buffer alone was subtracted from the titration data (both normalized to 0) for each experiment. Calorimetric data were analysed using origin © 7.5 software.

2.8 Computer simulation

The in silico analysis and prediction were performed with a series of softwares. The initial structure of insulin was obtained from protein data bank (PDB ID: 4INS), which is an insulin dimer. One insulin molecule was selected from the dimer, and then relaxed with molecular dynamics simulations through GROMACS52-54 for 100 ns. The insulin molecule was explicitly solvated by water using a TIP3P model,55 and ions were added to neutralize the system. The ff99SBildn force field56 was used to determine the interaction energies between different atoms. Simulations were performed in a cubic box with periodic boundary conditions, and the size of water box was chosen to have a minimal distance of 10 Å between solute and boundaries of the box. NVT ensemble and V-rescale thermostat57 were used for temperature coupling with a coupling constant of 0.1ps, and the temperature was set as 310 K. Non-bonded van der Waals interactions were truncated at a cutoff distance of 1 nm, and the particle-mesh Ewald summation58 was used for Coulomb interactions with a switching distance of 1 nm. Neighborlists were utilized and updated every 10 integration steps. The LINCS algorithm59 was used to constrain all bonds.

During relaxation, C- tail of insulin, i.e., C-terminal segment of B-chain as defined in this paper, showed high flexibility. Different conformations were selected from the simulation trajectory, and then used for later prediction. Pepsite2 was employed to predict the probable binding sites between insulin and Cp1-11. As a result, one insulin conformation with the lowest statistical significance (p-value<0.03) was found, which means that this conformation should be the most probable one that can closely bind Cp1-11. Then PEP-SiteFinder was used to predict the 3-dimensional structure of the complex. Later, the complex conformation was relaxed again by GROMACS for 500 ns.

2.9 Cytotoxicity test

The cytotoxicity of SACI was evaluated via MTT assay by
using L929 cells. The cells were cultured in Dulbecco’s modified eagle medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL of penicillin and 100 µg/mL of streptomycin at 37 °C, under an atmosphere of 5% CO2 and 95% relative humidity. The cells were seeded in a 96-well microtiter plate (Baoxin Co. Ltd, Chengdu, China) at a density of 10^4 cells/well and incubated in 100 µL of DMEM/well for 24 h. The culture media were replaced with fresh culture media containing serial dilutions of SACI, and the cells were incubated for 24 h. Then, 10 µL MTT solution (5 mg/mL) was added to each well and incubated for 5 h at 37 °C. At the end of the assay, the blue formazan reaction product was dissolved by adding 0.3 mL DMSO. Finally, the absorbance was measured at a wavelength of 492 nm using a microplate reader. The cell viability (%), relative to that of the control cells cultured in a medium without SACI, was calculated from \[\frac{[A]\text{test}}{[A]\text{control}} \times 100\%\], where \([A]\text{test}\) and \([A]\text{control}\) are the absorbance values of the wells (with SACI) and control wells (without SACI), respectively. For each sample, the final absorbance was the average of those measured from six wells in parallel.

### 2.10 Animal experiments

The *in vivo* experiments were carried out with male Sprague-Dawley (SD) rats (8 weeks old, 210–240 g) and permitted by the Animal Research Committee of the University. Rats were induced diabetic by STZ according to previous works.60, 61 The diabetic rats were divided three experimental groups, each containing six rats. Diabetic rats were subcutaneously injected with insulin, SACI-1/1 and SACI-2/1 solution, at a dose of 4 U/kg (0.1 mg/mL), separately. Blood glucose levels (BGLs) were measured using the blood taken from the tail vein by a one-touch blood glucose monitoring system (OneTouch®UltraEasy™, LifeScan, Inc. Milpitas, CA). Plasma for insulin measurements was obtained by collecting 1.5 mL blood samples (taken at 0, 10, 30, 60, 90, 120, 180, 240 and 360 min) from the tail vein in to a polypropylene tube containing heparin sodium, which was then centrifuged at 5000 rpm for 20 min. Insulin concentration in plasma samples was measured by porcine insulin ELISA Kit according to the protocol. All the samples were stored at −10 °C before analysis. Data processing was performed with statistical method (n=5). Data are presented as mean ± SD. Analysis of variance was used for statistical comparison. A *p* value <0.05 was considered significant.

### 3. RESULTS AND DISCUSSION

We first investigated the effect of Cp1-11 on insulin aggregation by dynamic light scattering (DLS, Figure 1 and Figure S1) and TEM/AFM (Figure 2, Figure S2 and Figure 3) to monitor the size variations. Insulin aggregates were intentionally prepared at high concentrations and low pH, with a size of 132.4 nm as detected by DLS, which corresponds to that of insulin oligomers, as the sizes of monomeric (5.7 kDa) or dimeric (11.4 kDa) insulin are smaller than 5 nm.51, 62 On the other hand, Cp1-11 was added with insulin at molar ratios of 1:1 and 2:1, which were named as SACI-1/1 and SACI-2/1, respectively. It is interesting to find that although SACI-2/1 still exhibited the peak of insulin oligomers, the newly appeared peak at 4-5 nm indicated that there were some monomeric insulin molecules resulting from their interaction with Cp1-11. This phenomenon demonstrated that the addition of Cp1-11 helps the disaggregation of insulin oligomers, which is consistent with previous reports about the function of C-peptide and its fragments.47, 49 However, we also noted that there was no new peak for SACI-1/1 sample, indicating that a 1:1 ratio may not enough to obviously disaggregate the insulin oligomers. TEM images also showed the morphological variations after complexing with Cp1-11: the spherical particles became loose and some small particles appeared, which demonstrated that the SACI has different morphology compared with insulin aggregates and it is liable to release insulin. Moreover, AFM images showed that insulin had formed mature fibers while SACI was still nanoparticles.

![Fig. 1. Hydrodynamic diameter distributions of insulin aggregates and SACI-2/1 by dynamic light scattering.](image1)

![Fig. 2. TEM images of insulin aggregates and SACI-2/1.](image2)

![Fig. 3. AFM images of insulin (a), SACI-1/1(b) and SACI-2/1 (c) after incubation for 50 h (1 mg/mL, pH=7.0, 25°C).](image3)
after incubation for 50 h (Figure 3). The SACI solution retained clear without visible aggregation even after 20 days (Figure S3). The above results have collectively demonstrated that Cp1-11 can interact with insulin to disaggregate insulin oligomers and inhibit the further fibrillation of insulin.

From a theoretical point of view, the binding of Cp1-11 peptide to insulin is considered to promote the disaggregation of insulin oligomers into monomers, which correlated well with the results of size exclusion high performance liquid chromatography (SEC-HPLC). SEC-HPLC confirmed that Cp1-11 peptide can interact with insulin oligomers resulting in the formation of Cp1-11/insulin monomers, as the new eluting peak at 9.1 min appeared after adding Cp1-11 peptide (Figure 4). In addition, the aggregation of insulin over a series of concentrations was investigated by comparing the eluting peaks in SEC-HPLC (Figure S4). A high concentration of insulin had an earlier eluting peak than that of low concentration, which indicates that insulin aggregates much more easily with an increase in insulin concentration. SEC-HPLC results suggest that Cp1-11 peptide can transform insulin oligomers to monomers through interaction with insulin.

Circular dichroism (CD) is a powerful technique to investigate the secondary structural change of proteins. The three major far-UV CD signals are noted as a large positive peak at 201 nm, and two well-defined negative peaks at 208 nm and 222 nm. CD results indicated that the secondary structures of insulin were retained after interacting with Cp1-11 peptide as there was no significant difference between the overall secondary structural composition of insulin and SACI (Figure 5).

It is also noted that the magnitudes of the two negative minima, i.e., 208 nm (α-helix) and 222 nm (β-sheet), can be analyzed as the ratio of [θ]_{208}/[θ]_{222} to indicate the self-association profiles of insulin. As shown, SACI-2/1 has higher [θ]_{208}/[θ]_{222} ratio (1.23) than that of insulin with aggregates (1.17), indicating less self-association of insulin while it is in the form of SACI.

The interaction between Cp1-11 and insulin was further investigated using isothermal titration calorimetry (ITC). As the binding stoichiometry, binding affinity, binding enthalpy and entropy change can be determined, ITC is normally utilized to measure the heat change resulting from the direct interaction between different molecules. In this work, we could not characterize the exact heat change caused by interactions between Cp1-11 and insulin because Cp1-11 showed an obvious endothermic effect during titration into the buffer (Figure 6a). The endothermic effect was due to the disaggregation of Cp1-11 upon dilution, which neutralized the heat response upon the titration of Cp1-11 to insulin (Figure 6b). By subtracting the endothermic effect of the titrants, a clear exothermic effect was obtained between Cp1-11 and insulin (Figure 6c). Although this result suggests that thermodynamic parameters cannot be defined by ITC analysis, the exothermic effect indicates that Cp1-11 and insulin have a direct molecular interaction.

To present a further insight into the effect of Cp1-11 peptide on an atomic level, computer simulation has been performed. According to our knowledge, the spatial structure of SACI has not yet been determined experimentally. In this work, Pepsite266,67 was first employed to predict the probable binding sites between insulin and Cp1-11, then PEP-SiteFinder68 was used to predict the 3-dimensional structure of SACI (Figure S5). The most probable conformation was further refined by molecular dynamics simulation with GROMACS.52 As shown in Figure 7a, residues of insulin with high probability to bind Cp1-11 are mainly distributed at the C-terminal tail of insulin’s B-chain (named as C-tail in this paper), which are very similar to the binding interface of the insulin dimer (Figure S6). The most popular conformation of SACI (Figure 7b) is very similar to the prediction (Figure S5). In the complex, Cp1-11 binds with insulin as an insert between the C-terminal tail and the N-terminal helix of B chain, shaping a short beta-sheet in combination with C-tail. Subsequently, three conformations...
of insulin were compared in Figure 7c, which are SACI, insulin/receptor, and the insulin dimer, respectively. The C-tail of insulin was found to display three distinct orientations in those complexes. In the case of the insulin dimer, the C-tail is very close to the helical segment of the B-chain and also the N-terminus of A-chain (called “closed state” in this paper), leading to a compact complex with another insulin molecule (Figure S6). On the other hand, the C-tail undergoes a hinge-like rotation away from the insulin core (called “open state” in this paper), so as to enable insulin receptor engagement. Interestingly, the orientation of the C-tail situates between the closed and open states, thus it is named as the intermediate state.

As an alternative, we focus on the atomic binding sites of Cp1-11 when complexed with insulin, thus a system with Cp1-11 : insulin = 1:1 is suitable. Our simulation showed that the insulin/Cp1-11 complex share very similar binding sites with insulin/insulin dimer, and the binding affinity is enough to destroy the insulin dimer. In this viewpoint, Cp1-11 : insulin = 1:1 is enough for the ideal case. While in real experimental conditions, the peptides in aggregation state and/or unbound state will also attribute to the higher ratio (2:1) between Cp1-11 and insulin.

Fig. 6. The heat response during Cp1-11 was titrated into the insulin solution at 25°C. (a) Blank heat response upon the injection of 1.5 mM Cp1-11 solution into 10 mM Tris–HCl buffer (pH 7.4). (b) Raw heat response data during the titration of Cp1-11 (1.5 mM) into insulin (0.5 mM). (c) The calibrated isothermal titration data of Cp1-11 (1.5 mM) into insulin (0.5 mM) by subtracting the blank heat response of Cp1-11 into the buffer.

Computer simulation showed that Cp1-11 can insert into the space between C-terminal tail and N-terminal helix of insulin’s B-chain, resulting in the dissociation of the insulin dimer. In addition, the appearance of the intermediate state (in SACI) can also decrease the energy barrier for the transition from the closed state (in insulin dimer) to the open state (in insulin/receptor), thus accelerating the kinetics for the formation of insulin/receptor, which will be proved later as indicated by the rapid decrease of blood glucose level in animal experiments.

It is also noted that the computer simulation suggested a 1:1 binding of Cp1-11 with insulin, but all of the experimental results suggested that the 2:1 complex performed better than 1:1 complex. For the simulation with Cp1-11 : insulin = 2:1, the aggregation of peptides should be considered and the interactions would be more complicated.
The effective disaggregation of insulin oligomers by Cp1-11 peptide suggests that it may have potential in diabetes therapy. To test its biocompatibility, the cytotoxicity of SACI at a series of concentrations were evaluated by MTT assay using L929 cells. As shown in Figure 8, both SACI-1/1 and SACI-2/1 display good cell viability in the range of 105-130%, with a concentration of up to 100 µg/mL, indicating the safety of SACI for biomedical applications.

All of the in vitro experiments indicate that SACI may help to enhance the therapeutic effect of insulin by promoting the disaggregation of its oligomers, thus animal experiments were carried out in vivo to examine this possibility. Streptocozotin (STZ)-induced type 1 diabetic rats were used in this work as their pancreatic islet function was completely destroyed by STZ.13, 60, 61 The solution of insulin, SACI-1/1 and SACI-2/1 were subcutaneously injected into rats at the same dosage of insulin (4 U/kg). As shown in Figure 9a, both SACI-1/1 and SACI-2/1 led to a significant reduction in blood glucose level (BGL) within 30 min, which clearly indicated the rapid-acting character of SACI. Additionally, the pharmacodynamic activities of SACI and insulin were compared by the area under the curve (AUC) (Figure 9b). The AUC of SACI-2/1 is significantly lower than that of insulin and SACI-1/1, indicating the highest bioavailability of SACI-2/1.70

The effective decrease of BGL of diabetic rats after the injection of SACI should be attributed to the immediate release of insulin in vivo. The reason is that Cp1-11 peptide can interact with insulin to promote the disaggregation of insulin oligomers into monomers, which can interact with insulin receptors more easily after subcutaneous administration and improve the therapeutic effect.

As shown in Figure 9b, the serum insulin levels in diabetic rats reached the highest concentration at 60 min. The results of insulin release correlated well with the results of the BGL changes (Figure 9a), which indicate that SACI-2/1 could most effectively promote rapid insulin release at the beginning and control postprandial hyperglycemia in diabetic rats. It’s noted that higher serum insulin concentration, can be due to many different reasons like better absorption, enhanced stability, reduced metabolism, or inhibited renal clearance and so on, however, faster release is one of many reasons that have been proved in vitro data, other reasons need to be testified next.

4. CONCLUSIONS

In summary, effective inhibition of insulin aggregation has been achieved by applying Cp1-11 peptide, which can interact with oligomeric insulin and facilitate its disaggregation into the physiologically active monomeric form. Computer simulation indicates that Cp1-11 can insert into the space between the C-terminal tail and the N-terminal helix of insulin’s B-chain, leading to the dissociation of insulin aggregates. By this mechanism, the supramolecular assembly of Cp1-11 and insulin, i.e. SACI, can improve the bioavailability and therapeutic effect of insulin. The animal experimental data shows that subcutaneous injection of SACI leads to fast release of insulin in the circulation and enhances its effect on the control of blood glucose levels. We therefore conclude that SACI has potential applications in the treatment of diabetes. Moreover, the strategy of using peptide to modulate the release profiles can be widely explored for various protein/peptide drugs, such as calcitonin in the treatment of bone-related diseases.71
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