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Zn(II) IONS CO-SECRETED WITH INSULIN SUPPRESS INHERENT AMYLOIDOGENIC PROPERTIES OF MONOMERIC INSULIN

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Running head: Zn(II) ions inhibit insulin fibrillation

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Insulin, a 51-residue peptide hormone is an intrinsically amyloidogenic peptide, forming amyloid fibrils *in vitro*. In the secretory granules insulin is densely packed together with Zn(II) into crystals of $Zn_2Insulin_6$ hexamer, which assures osmotic stability of vesicles and prevents fibrillization of the peptide. However, after release from the pancreatic β-cells insulin dissociates into active monomers, which tend to fibrillize not only at acidic but also at physiological pH values. The effect of co-secreted Zn(II) ions on the fibrillization of monomeric insulin is unknown, however, it might prevent insulin fibrillization. We showed that Zn(II) inhibits fibrillization of monomeric insulin at physiological pH values by forming a soluble Zn(II)Insulin complex. Inhibitory effect of Zn(II) ions is very strong at pH 7.3 (IC_{50}=3.5 μM), whereas at pH 5.5 it progressively weakens, pointing towards participation of the His residue(s) in complex formation. Obtained results indicate that Zn(II) ions might suppress fibrillization of insulin at its release sites and in circulation. It is hypothesized that misfolded oligomeric intermediates occurring in insulin fibrillization pathway, especially in zinc-deficient conditions, might induce autoantibodies against insulin, which leads to β-cell damage and autoimmune type I diabetes.

Keywords: zinc, insulin, fibrillization, temperature dependence, fluorescence spectroscopy, Thioflavin T

INTRODUCTION

Insulin, a peptide hormone crucial for glucose metabolism, is produced in the islets of Langerhans by pancreatic β-cells. The peptide is synthesized in the endoplasmatic reticulum and concentrated into secretory granules in the Golgi apparatus [1]. After processing by prohormone convertases PC1/3 and PC2 [2] insulin forms water-insoluble crystals of zinc-hexamer ($Zn_2Insulin_6$) in the slightly acidic environment (pH 5.5) of secretory granules [3-4]. Zn(II) content in the pancreatic beta cells is among the highest in the body reaching ten millimoles per liter [5], whereas one third of it is localized in secretory granules together with insulin [6]. Zn(II) is uploaded to the granules with assistance of a pancreas-specific zinc transporter ZnT8 localized on the membranes of the granules [7]. ZnT8 knockout mice, where secretory granules of insulin are zinc-depleted, show normal insulin biosynthesis, processing, and release, which indicates that Zn(II) ions are not ultimately required in the processes upstream from insulin release [8].

Amyloidogenic properties of insulin are known since 1940ties [9-10]. Fibrillation of insulin has been intensively studied at low pH values and high peptide concentration mostly as a suitable model of protein fibrillation [11]. However, insulin can also fibrillize at physiologically relevant neutral pH values [12-13]. It is noteworthy that insulin does not form fibrils in the secretory granules, where its concentration is extremely high reaching 21 mM [14]. Zn(II) ions, concentration of which is around 11 mM in secretory granules [14], stabilize insulin at high peptide concentrations by forming crystals of $Zn_2Insulin_6$ hexamers [15-16]. However, insulin does not fibrillize in the Zn(II)-depleted secretory

granules of transgenic ZnT8 knockout mice [8], indicating that Zn(II) is not the only factor preventing insulin fibrillization in secretory granules. In the absence of Zn(II) insulin still forms oligomeric structures, mainly hexamers, which may prevent insulin fibrillization at high peptide concentrations. Indeed, insulin fibrillization at high peptide concentrations is slower than that at low concentrations indicating that oligomerization inhibits insulin fibrillization [17]. Insulin is a rare exception within the amyloidogenetic peptides, since the propensity for fibrillization of other peptides increases at elevated concentration. It has also been demonstrated that insulin oligomers must dissociate to monomers before fibrillization [18]. Dissociation of insulin crystals into monomers occurs immediately after secretion of the insulin and also after its injection. Thus, a question may arise what factors prevent the fibrillization of considerably amyloidogenic insulin monomers in pancreatic extracellular space, sites of the injection and also in circulation.

It is noteworthy that despite widespread use of insulin for treatment of type I diabetes the incidence for formation of insulin fibrillar deposits at the site of repeated injections is relatively rare. Such a condition, categorized as injection amyloidosis, has been observed at the sites of repeated injection of neutral porcine insulin [19-20]. As a rule injection solutions of insulin are supplemented with approximately 0.3 molar equivalents of Zn(II) ions [12, 21]. It can be suggested that co-injected Zn(II) ions might also suppress the fibrillization of monomeric insulin and formation of insulin fibril deposits at the sites of repeated injection as well as in circulation. Influence of Zn(II) on insulin at very low concentration of the peptide would therefore be of great interest with regards to its action and metabolism.

It is known that metal ions like Zn(II) and Cu(II) have pronounced effects on the fibrillation of a variety of amyloidogenic peptides like Alzheimer's amyloid peptide [22], synuclein [23-24], tau protein and prion protein, whereas metal ions can enhance [23-24] or inhibit fibrillation [25]. In principle, the fibrillization of monomeric insulin can also be suppressed by metal ions, and its suppression by Zn(II) that is co-secreted with insulin, can be physiologically relevant. Moreover, fibrillization studies of monomeric insulin are also necessary for getting better understanding about the mechanism of insulin fibrillization as the critical amyloidogenic intermediate in fibrillization of insulin as well as other amyloidogenic peptides/proteins is a partially unfolded monomer [13, 26-27]. Secretory granules of insulin contain besides Zn(II) ions also C-peptide [28] and amylin that are present in equimolar and approximately 10-fold lower amounts than insulin respectively [29]. Insulin is shown to interfere amylin fibrillation [30] whereas C-peptide affects insulin oligomerization pattern [31-32] and suppress amylin fibrillation[29], however the effects of the co-released peptides on the fibrillization of insulin are not determined.

In the current work we studied systematically the effects Zn(II) ions, C-peptide, amylin and environmental conditions on the fibrillization of monomeric insulin at physiological pH and demonstrate that Zn(II) ions suppress fibrillation of monomeric insulin through differential stabilization of monomeric ground state over partially open transition state leading to amyloidogenesis. It is proposed that Zn(II) that are co-secreted together with insulin may prevent fibrillation of insulin at its release sites and in circulation. C-peptide and amylin did not influence the insulin fibrillization.

EXPERIMENTAL

Materials – Lyophilized insulin was purchased from Sigma-Aldrich (St. Louis, USA), amylin was from rPeptide (Bogart, GA, USA) and C-peptide from Nordic Biosite (Helsinki, Finland). Thioflavin T (ThT) and $CuCl_2x2H_2O$ were from Sigma-Aldrich (St. Louis, USA); HEPES Ultrapure, MB Grade was from USB Corporation (Cleveland, USA). $ZnCl_2$ and NaCl were extra pure from Scharlau (Barcelona, Spain). All solutions were prepared in fresh MilliQ water.

Sample preparation - Stock solution of insulin was prepared as follows: Insulin was dissolved in 20 mM HEPES and 100 mM NaCl, pH 7.3 at a concentration of 50 μM. After 30 minutes incubation the insulin stock solution was diluted with buffer and used for experiments. Oligomeric composition of insulin samples was determined by SEC on Superdex 75 10/300 column (GE Healthcare, Giles, United Kingdom) connected to a Äkta Purifier system (GE Healthcare, Giles, United Kingdom) by using 20 mM HEPES and 100 mM NaCl, pH 7.3 as elution buffer.

Monitoring insulin fibrillization by ThT fluorescence – in a standard experiment freshly prepared stock solution of insulin was diluted to a final concentration of 2.5 μM in 20 mM HEPES and 100 mM NaCl, pH 7.3 containing 2.5 μM of ThT and appropriate amount of Zn_2Cl_2. 450 μl of each sample was incubated in a 0.5 cm path length quartz cell, equilibrated at 50°C and agitated with a magnetic stirrer at 250 rpm. Increase in ThT fluorescence was measured at 480 nm using excitation at 440 nm on a Perkin-Elmer LS-45 fluorescence spectrophotometer (Perkin-Elmer,Waltham, MA, USA) equipped with a magnetic stirrer. When insulin was incubated without agitation at similar conditions the fibrillization process was extremely slow and only a slight increase in ThT fluorescence was observed during prolonged incubation (5 days). Agitation at 250 rpm was found optimal for monitoring the insulin fibrillization and the effect of agitation was similar to that observed for Alzheimer's amyloid peptide fibrillation [25]. In temperature dependence studies temperature was varied from 35°C to 50°C. In the second series of experiments concentration of insulin was varied from 2.5 to 30 μM.

The kinetic curves of fibrillation were fitted to several sigmoidal functions including Avrami, Gompertz and Boltzmann equations using program Origin (OriginLab, Northhampton, MA, USA) and it was found that the Boltzmann equations (equation. 1) provides the best fit of the experimental data and allows calculation of the kinetic parameters of fibrillization.

$$y = \frac{A_2 - A_1}{1 + e^{-[\,(t - t_0)\,]\,/\,\tau}} + A_2\,, \quad (1)$$

where A_1 is the initial fluorescence level, A_2 is the maximum fluorescence, t_0 is the time when fluorescence has reached half maximum; $1/\tau = k$ is the apparent rate constant of the fibril growth and lag time is approximated by t_0-2τ as suggested earlier (33). IC_{50} values were calculated according to hyperbolic dose-response curves as described in [25].

Transmission Electron Microscopy - The grids were placed on an adhesive solid surface and 3 μl of previously centrifuged (30 min, 12000 g) peptide solution was pipetted on each grid and let air-dry. Then drops of 2% uranylacetate were spotted on a parafilm plate and grids were placed on them with the upper side down (to bring the probe and the contrasting solution in contact). Probes were kept in uranylacetate for 10 min, then removed and washed with milliQ water. After that, excess water was removed with a filter paper and the grids were placed into a special carrier. TEM images from the samples were created on SELMI-SUMY EM-125 instrument at 75 kV accelerating voltage and recorded onto high resolution, 60x90 mm negative film.

ESI-MS measurements. Samples of 1 to 50 μM of insulin in 100 mM ammonium acetate pH 7.3, incubated for 10 min within the gastight class syringe at 50°C were injected into the electrospray ion source of QSTAR Elite ESI-Q-TOF MS instrument (Applied Biosystems, Foster City, USA) by a syringe pump at 10 μl/min and ESI-MS spectra were recorded during up to 5 min in m/Z region from 500 – 3000 Da at following instrument parameters: ion spray voltage 5500 V; source gas 45 l/min; curtain gas 20 l/min; declustering potential 60V; focusing potential 320 V; detector voltage 2300V. ESI MS spectra were analyzed with the program Bioanalyst (Applied Biosystems, Foster City, USA).

Equilibrium dialysis. Dialysis experiment was performed with cellulose ester Spectra/Por dialysis membrane tubing with a molecular weight cut-off of 1000 Da (Spectrum Laboratories, USA) containing 1.5 ml of 10 μM insulin dissolved in 20 mM HEPES and 100 mM NaCl, pH 7.3. Dialysis tubing was equilibrated with 500 ml of dialysate solution containing 20 mM HEPES, 100 mM NaCl, 5μM $ZnCl_2$, pH 7.3 under agitation for 24 hours at 4°C until equilibrium was achieved. At the end of the experiment, samples were taken from both the inside and outside solutions, acidified with 1 M HCl and analyzed for zinc content by atomic absorption spectroscopy on PerkinElmer 3100 instrument (Perkin Elmer, Waltham, MA, USA).

RESULTS

Monitoring the in vitro formation of insulin fibrils

Insulin fibrillization experiments were performed with freshly solubilized peptide under continuous agitation by monitoring an increase in the fluorescence of the fibril-reactive dye ThT *in situ*. In these conditions a relatively fast increase in ThT fluorescence was observed in accordance with a typical two-phase growth curve (Fig.1). The kinetic curves of fibrillization fitted well to Boltzmann equation (Eqn.1) and the midpoint of fibril formation (t_0) and the rate constants for the fibrillization process were calculated. Fibrillization was monitored in the presence of 2.5 μM or 10 μM ThT. In a separate experiment we demonstrated that ThT inhibits insulin fibrillization at concentrations higher than 10 μM . Experiments conducted with 2.5 μM insulin showed that the fibrillization rate constant increased and duration of lag phase decreased with increasing temperature (Fig. 1). The activation energy E_a = -84 kJ/mol was found from the slope in Arrhenius plot (Fig. 1 inset). In further experiments insulin fibrillization was carried out at 50°C for practical reasons. Fibrillation curves at 2.5 and 5 μM concentration of insulin exposed similar kinetics and lag phase, whereas at concentrations above 10 μM lag phase increased and the values of the rate constant for fibril growth decreased (Fig. 2).

Monomer-dimer equilibrium of insulin studied by ESI MS. ESI MS spectrum of 2 μM insulin exposed +4 and +5 peaks of monomeric insulin (Fig. 3a), whereas at higher peptide concentrations +6 and +7 peaks of dimeric insulin started increase in the spectra (Fig. 3b,c). Monomeric insulin was also major peak in the spectra at 50 μM insulin (Fig. 3c), where substantial part of insulin is supposed to be dimeric. Such a behavior is indicative for partial dissociation of insulin dimers to monomers during electrospray ionization. Moreover, in calculating the dissociation constant for insulin dimers it should also be considered that ionization efficiency of monomers and dimers is not equal in ESI MS. To get binding isotherm the fractional content of insulin dimer peaks was calculated by dividing summarized area of all insulin dimer peaks to the summarized areas of all insulin peaks in ESI MS spectra and latter parameter was plotted against insulin concentration (Fig. 3d). Obtained curve was fitted to equation of hyperbola and maximal fractional content F_{max} = 0.18±0.03 indicates that 80% of insulin dissociate to monomers during electrospray ionization, which could also be expected as insulin dimer dissociation constant is relatively high. Obtained K_D = 18.9 ±0.6 μM describes equilibrium of insulin dimer dissociation. Our ESI MS results at pH 7.3 are similar with earlier nanospray ESI MS studies, however, slightly different charge states for different species were observed at pH 3.3 [21]. Based on SEC and ESI MS studies we can conclude that at the concentration 2.5 μM insulin is prevalently monomeric and the effects of insulin oligomerization on the fibrillation kinetics are negligible.

Effects of Zn(II) on the fibrillization of insulin. Effect of Zn(II) on the fibrillization of 2.5 μM insulin was studied at pH 7.3 and 5.5. At pH 7.3 Zn(II) ions decreased the fibrillization rate constant and increased the lag phase of the process in a concentration dependent manner, whilst the maximal level of ThT fluorescence was not affected (Fig. 4a). Inhibitory effect of Zn(II) characterized by IC_{50} (defined as Zn(II) concentration where a twofold decrease of the fibrillization rate constant was observed) was equal to 3.5±0.9 μM (Fig. 4a). At pH 5.5 the effect of Zn(II) was much weaker and only an insignificant (10 %) decrease in fibrillation rate was observed in the presence of 20 μM Zn(II) (Fig. 4b).

Effects of C-peptide and amylin on the fibrillization of insulin. Fibrillization of 2.5 μM insulin at pH 7.3 and 50° C was not affected by 2.5 and 5 μM C-peptide as well as by 0.5 μM amylin (Fig. 5), indicating that micromolar concentrations of these peptides does not affect fibrillization of monomeric insulin.

Size-exclusion chromatography of insulin. SEC analysis showed that injection of 10 μM insulin exposes one peak with elution volume of 14.5 ml (Fig. 6a), while increasing the insulin concentration to 100 μM decreased the elution volume (Fig. 6b) indicative for peptide oligomerization at higher concentrations in agreement with literature data [21, 33]. SEC of the aliquots of fibrillation mixture showed that fibrillization is accompanied with the loss of the insulin from the solution (Fig. 6c). Fibrillization of 10 μM insulin is completely suppressed in the presence of 5 μM Zn(II) (Fig. 6a) and SEC analysis of fibrillization mixture in these conditions indicates that the peptide exposes similar SEC peak as

initial solution of 10 μM insulin (Fig. 6d). Moreover, the presence of 5 μM Zn(II) in elution buffer did not cause shift of insulin peak in SEC confirming that 5 μM Zn(II) keeps insulin in a soluble state and does not induce changes in oligomers of insulin in our experimental conditions.

Equilibrium dialysis. Equilibrium dialysis of 10 μM insulin solution against 5μM of Zn(II) showed that in these conditions insulin binds 0.4 mole Zn(II) per mole, which corresponds to the value of the dissociation constant K_{Zn} = 7.5 μM.

Characterization of insulin fibrils by transmission electron microscopy. Insulin samples showing high ThT fluorescence (agitated for more than 30 min) showed the presence of fibrils in TEM, confirming that the increase of ThT fluorescence reflects peptide fibrillization in our assay. In the samples of 10 μM insulin with added 5 μM Zn(II), almost no aggregates were detected at pH 7.3 after 30 min agitation, however, at pH 5.5 insulin fibrils were observed (Fig. 3e), which confirms that 5 μM Zn(II) protects insulin from fibrillation at pH 7.3 but not at acidic pH values.

DISCUSSION

Fibrillization of insulin has been thoroughly studied for more than sixty years, however, majority of studies have been performed with millimolar concentrations of Zn(II)-insulin that fibrillizes at elevated temperatures and in acidic conditions [12]. Low pH is necessary for fibrillization since at high millimolar concentrations and neutral pH Zn(II)-insulin is dominantly in the form of hexamers resistant towards fibrillization [18]. Addition of Zn(II) ions that stabilize hexameric form is commonly used in injection solutions of insulin [12, 21].

Zinc-free apo-insulin can fibrillize not only at acidic but also at physiologically relevant pH values especially in the presence of chemical compounds such as ethanol, urea and guanidinium that are able to dissociate insulin to monomers [12-13].Insulin is a rare exception within the amyloidogenic peptides since its dilution increases the propensity for fibrillization, whereas the fibrillization of other peptides is favored at high peptide concentrations. Such a behavior indicates that oligomerization inhibits insulin fibrillization [17]. Thus, it follows that the fibrillization of the monomeric biologically active form of insulin, which is the most aggregation prone form of the peptide, is insufficiently studied.

Behavior of monomeric insulin can be studied at low peptide concentrations. According to the literature insulin dimers begin to dissociate when diluted to concentrations below 100 μM [34] [35] and 10 μM insulin is assumed to be essentially monomeric [21]. The dissociation constant, K_D value for insulin dimers determined by static and dynamic laser light scattering is equal to 12.5 μM at pH 7.3 and 25°C [36]. Our ESI MS studies at 50°C and pH 7.3 yielded K_D= 18.9 μM, which is in good agreement with the literature. Accordingly, at 2.5 μM concentration insulin is prevalently monomeric and the equilibrium concentration of dimers is approximately 0.22 μM.

Our results demonstrate that fibrillization of insulin depends only slightly on the insulin concentration in the range from 2.5 to 10 μM, whereas at higher concentrations the fibrillization lag time increased and the fibrillization rate slowed down. Inhibition of fibril formation at higher insulin concentrations indicates that formation of insulin oligomers is off-pathway process for the fibrillization. Secondly, as the rate constant for fibril growth at concentrations below 10 μM is constant, we can conclude that the rate limiting step of fibril growth is most probably connected with some intramolecular event like conformational change. Indeed, the fibrillization of monomeric insulin is characterized by relative large enthalpy of activation E_a = -84 kJ/mol indicating that formation of fibrillization-competent structure is accompanied by substantial conformational changes. The native conformation of insulin in $Zn(II)_2Insulin_6$ hexamers [37] as well as its monomers is essentially α-helical [38]. Multiple techniques including FT IR and CD demonstrate that insulin fibril formation is accompanied by extensive unfolding of the molecule to allow conversion from α-helical to β-sheet conformation [11, 21], necessary to build up the cross-β framework of insulin fibrils [39] Apparently, such a conformational change in disulphide-linked native insulin is causing the observed large enthalpy of activation.

From the compounds co-released with insulin, only Zn(II) exposed a pronounced inhibitory effect upon the fibrillization of monomeric insulin at pH 7.4. IC_{50} equal to 3.5 μM indicates that Zn(II) ions

inhibit fibrillization already at low micromolar concentration and addition of four-fold excess of Zn(II) almost completely suppressed the formation of insulin fibrils. Equilibrium dialysis of insulin against 5μM of Zn(II) confirmed that the inhibitory effect is in the same range with the Zn(II) binding affinity of insulin K_{Zn} = 7.5 μM at low concentrations. At pH 5.5 the inhibitory effect of Zn(II) was much weaker as 20 μM Zn(II) did not affect the fibrillization rate constant and caused only a slight increase of the lag phase. The pH dependence points towards participation of His residue(s) in the Zn(II)-induced inhibition of insulin fibrillization. It is known that His residue at position B10 that is located in the vicinity of the six residue B chain segment (B12-B17) contributing to the formation of cross-beta structure [40] participates in the binding of Zn(II) ions. It could be suggested that binding of Zn(II) to His10 may hinder the formation of β-sheet rich conformation compatible for fibrillization nearby. Thus, the interaction of Zn(II) with the monomeric insulin inhibits its fibrillization most probably through differential stabilization of monomeric ground state over partially open conformation that is necessary for fibrillization. A schematic model describing the effect of Zn(II) ions on the fibrillation of insulin is presented in Fig. 7.

Biological context Insulin is present in the secretory granules at extremely high concentration reaching 20 mM [14]. The main factor preventing insulin fibrillization in secretory granules is the formation of stabile $Zn(II)_2insulin_6$ hexamers (in zinc-enriched granules) or insulin oligomers (in zinc-depleted granules). Nevertheless, the fibrillization may also occur at the sites of its release where insulin dissociates to monomers that are the most fibrillization prone forms of the peptider. Based on the current results we suggest that Zn(II) ions that are co-secreted with insulin from pancreatic β-cells might help insulin to avoid fibrillization at its release sites after dissociation of hexamers. Insulin fibrillization is physiologically undesirable process for many reasons. First, fibrillization removes monomeric insulin out of secretion and prevents its interaction with insulin receptors. Second, insulin fibrillization occurs over intermediate misfolded oligomers [41-42] and prefibrillar aggregates, composed from 500 or more monomers and which dimensions might reach 14 Å [43]. These intermediates might be cytotoxic as demonstrated in case of Alzheimer's amyloid peptides [44] and also in the case of many other peptides and proteins. Besides being cytotoxic, these large misfolded oligomeric intermediates can also be immunogenic if they occur in circulation. Such a scenario is feasible in the case of insulin.

Diabetes type 1 is an autoimmune disease, which is characterized by presence of autoantibodies against insulin and some other pancreatic proteins. These diabetes-related autoantibodies are present already in preclinical asymptomatic latent period of the disease and several studies have shown that insulin autoantibodies are the first to appear in young children, implying that insulin may be the primary autoantigen in most cases of childhood type 1 diabetes [45]. The reason why autoantibodies that attack and destroy pancreatic β-cells are generated is currently largely unknown. It cannot be ruled out that the pathology is related to generation of antibodies against misfolded insulin oligomers occurring on-pathway to insulin fibrillization. Based on current results it can be hypothesized that Zn(II) ions co-secreted from pancreatic β-cells protect organism from insulin fibrillization and formation of intermediary nonnative insulin oligomers/aggregates. It can also be hypothesized that disturbances in zinc metabolism and especially zinc deficiency might enhance insulin fibrillization with intermediary formation of misfolded oligomers, which are immunogenic and might induce generation of insulin autoantibodies. Currently there is no direct evidence confirming the suggested hypothesis, however, epidemiological studies associate diabetes with zinc deficiencies that is in good agreement with the suggested hypothesis.

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FIGURE LEGENDS

Figure 1. Temperature dependence of insulin fibrillization as followed by ThT fluorescence. Conditions: 2.5 μM of insulin in 20 mM HEPES, 100 mM NaCl, pH 7.3. Insulin was incubated at 50°C -■-; 45°C; -□- ; 40°C - ●- ; 35°C -○- in a quartz cell with continuous agitation in the presence of 2.5 μM ThT. Solid lines correspond to fits of the data to Boltzmann equation (Eq.1). Inset – temperature dependence in Arrhenius coordinates.

Figure 2. Effect of insulin concentration on its fibrillization. Conditions: 2.5 μM -■-; 5 μM -□- ; 10 μM -●-; 20 μM -○-; 30 μM -▲- insulin in 20 mM HEPES, 100 mM NaC, pH 7.3, was incubated at 50°C in a quartz cell with continuous agitation in the presence of 2.5 μmol/L ThT. Solid lines correspond to fits of the data to Boltzmann equation (Eq.1). Inset – dependence of fibrillization rate constant from the insulin concentration.

Figure 3. Determination of the apparent dissociation constant for insulin dimers by ESI MS. ESI-MS spectra of (a) - 2 μM; (b) - 10 μM; (c) - 50 μM insulin in 100 mM ammonium acetate, pH 7.3; T = 50ºC. Monomeric insulin exposes charge states +4 and +5 and dimeric insulin +6 and +7. (d) - Dependence of the relative intensity of insulin dimer peaks from the concentration of insulin. Solid line shows the fitting curve with K_{Cu} = 18.3 μM.

Figure 4. Effect of Zn(II) on fibrillization of insulin. Conditions: 2.5 μM of insulin in 20 mM HEPES, 100 mM NaCl at pH 7.3 (a) and 5.5 (b) was incubated at 50°C in a quartz cell with continuous agitation in the presence of 2.5 μM ThT and various concentrations of Zn(II) as shown in the figure. Solid lines correspond to fits of the data to Boltzmann equation (Eq.1). Inset – dependence of fibrillization rate constant from the concentration of Zn(II).

Figure 5. Effects of C-peptide and amylin on the fibrillation of insulin Conditions: 2.5 μM of insulin in 20 mM HEPES, 100 mM NaCl, 2.5 μM ThT at pH 7.3 was incubated at 50°C in a quartz cell with continuous agitation in the absence of other peptides (-○-) and in the presence of 2.5 μM C-peptide (-■-) or 1 μM amylin (-●-).

Figure 6. Size exclusion chromatography of insulin samples and TEM image from insulin fibrils. (a) 10 μM insulin; (b) - 100 μM insulin; (c) - 10 μM insulin agitated at 50°C for 60 min; (d) 10 μM insulin agitated in the presence of 5 μM Zn(II) for 60 min; Superdex 75 column, incubation and elution buffer 20 mM HEPES and 100 mM NaCl, at pH 7.3, 25°C. (e) TEM image from fibrils formed by agitation of 10 μM insulin in the presence of 5 μM Zn(II) for 60 min at pH 5.5.

Figure 7. Mechanism for assembly and fibrillogenesis of insulin in the presence of Zn(II) ions. Insulin is hexameric at high peptide concentration both in the presence and absence of Zn(II). Insulin hexamers dissociate to dimers and monomers at low peptide concentration whereas peptide monomers can also bind Zn(II) ions. Monomeric insulin tends to fibrillize via a partially unfolded intermediate that is also assumed to be important for receptor binding. Zn(II)-insulin monomer formation is off-pathway for fibrillization as it prevents opening of the conformation of monomeric insulin.

Figure 1

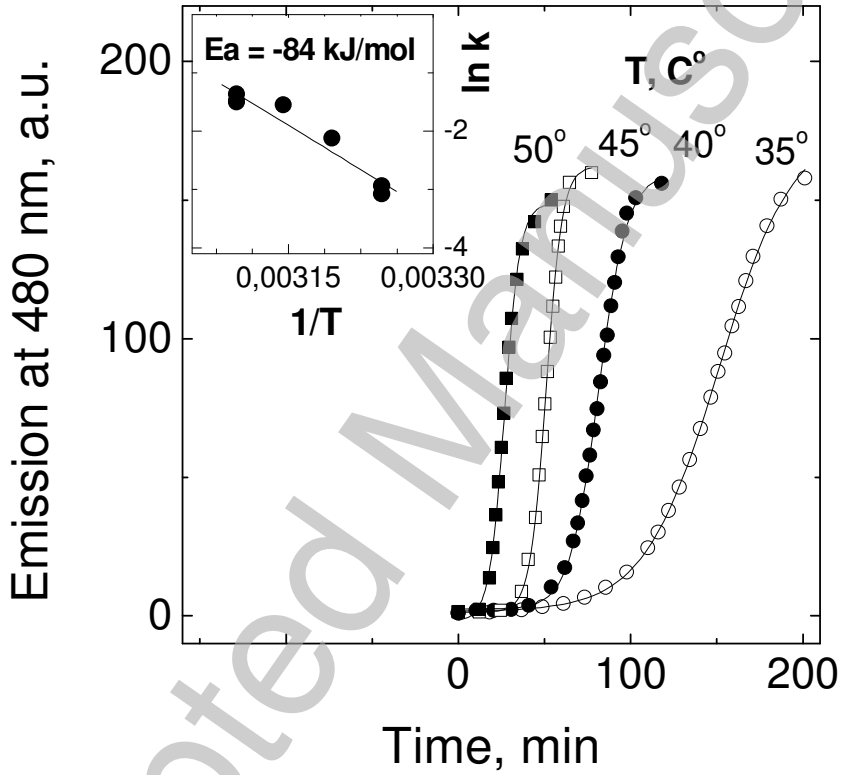

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Figure 2

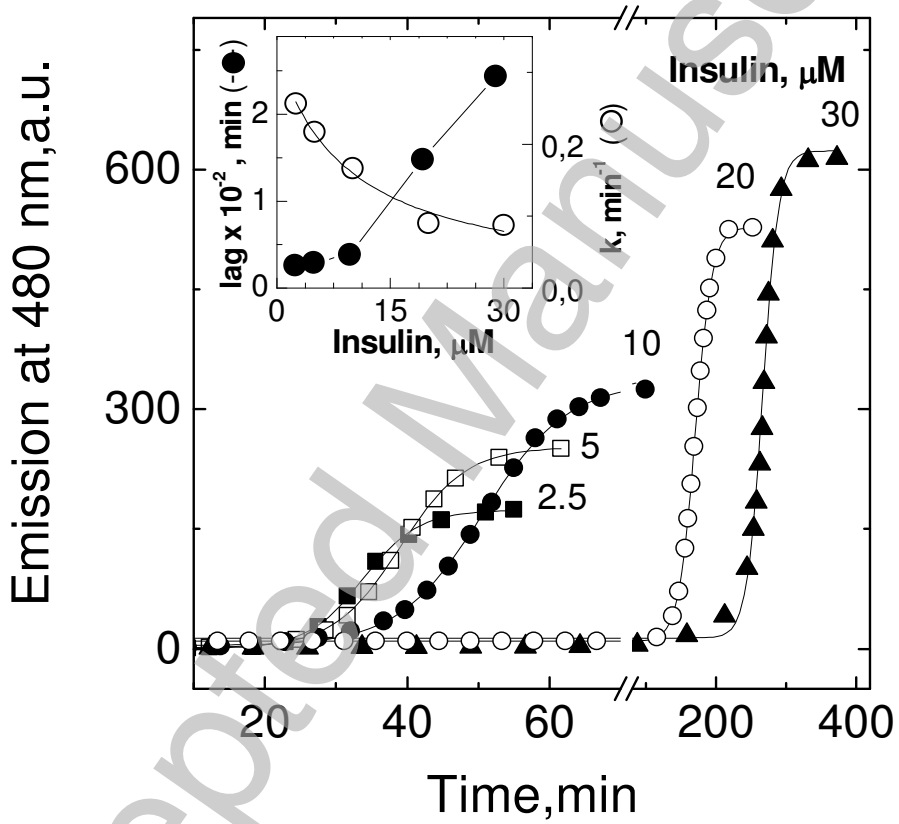

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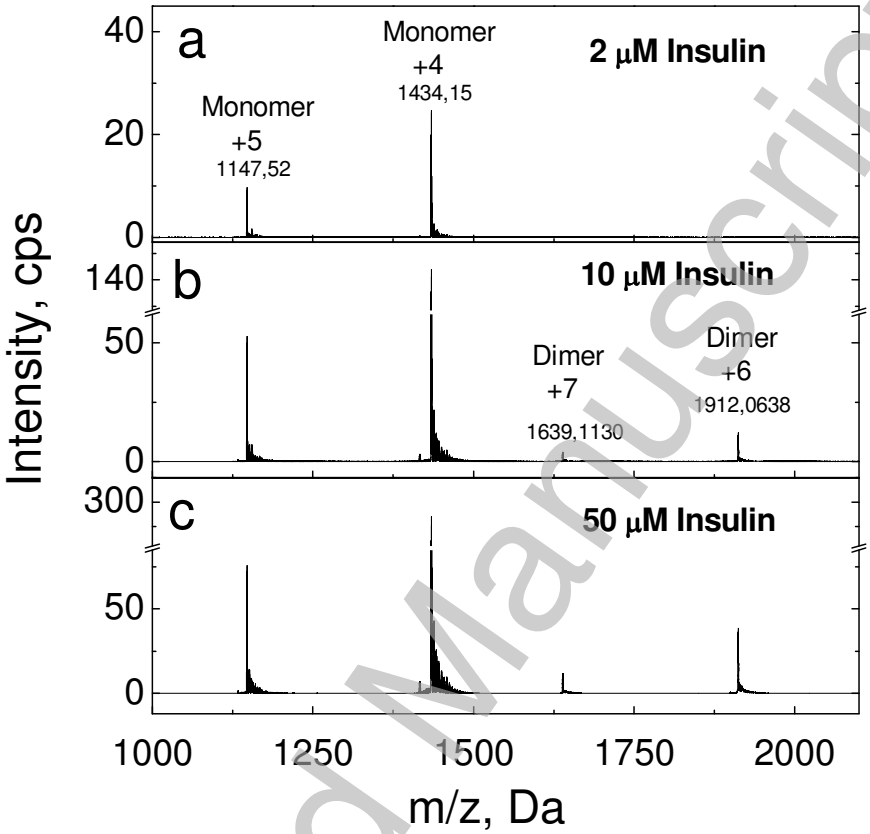
Figure 3



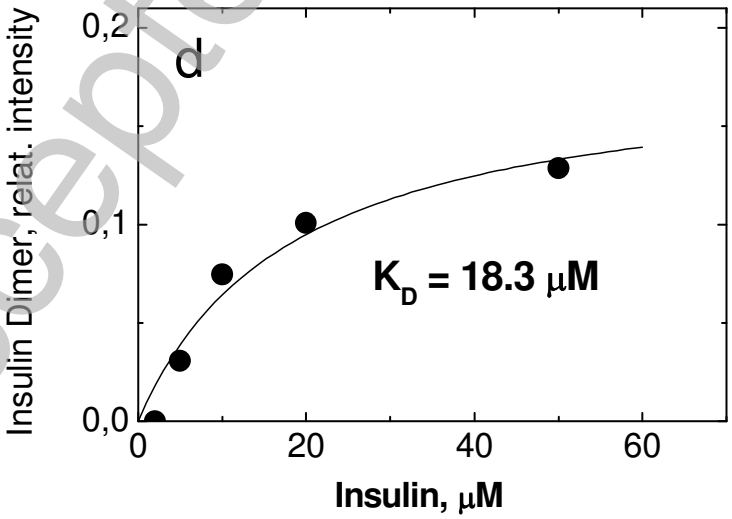


Figure 4

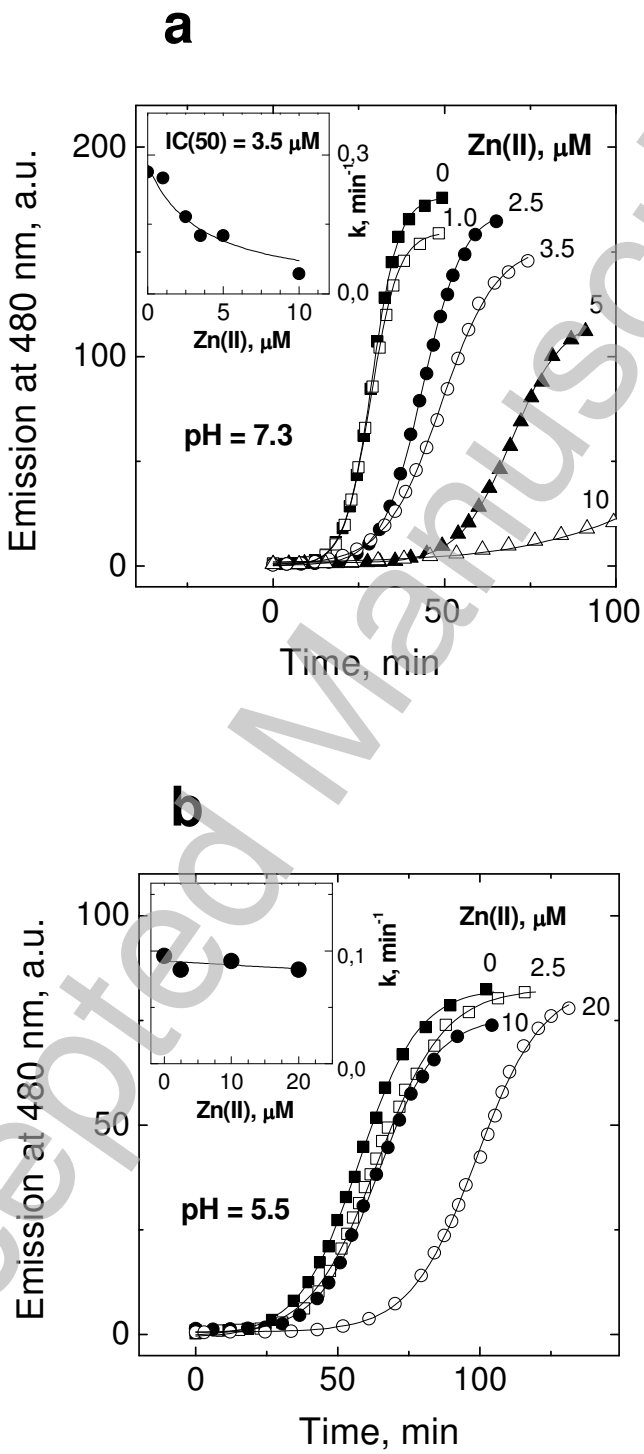


Figure 5

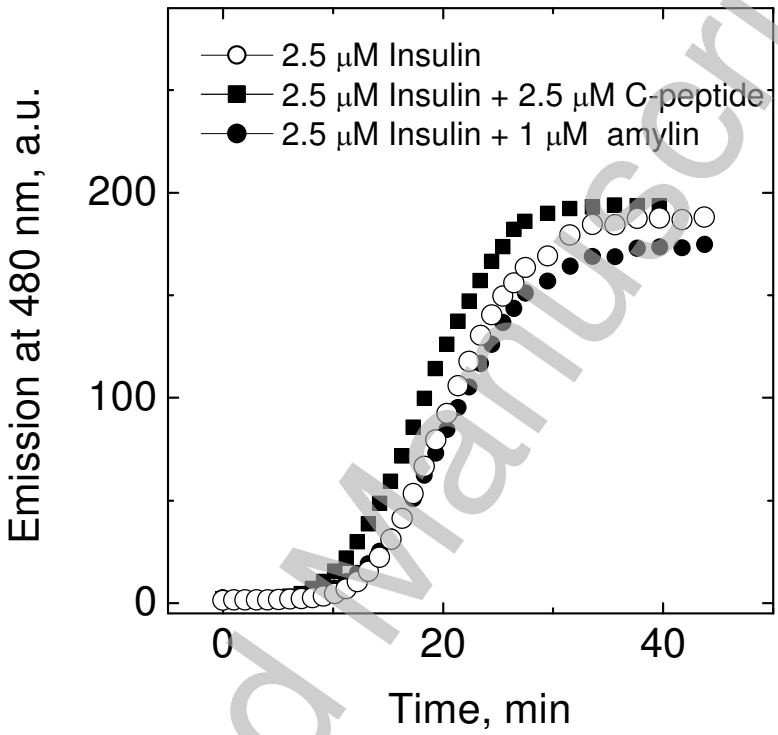


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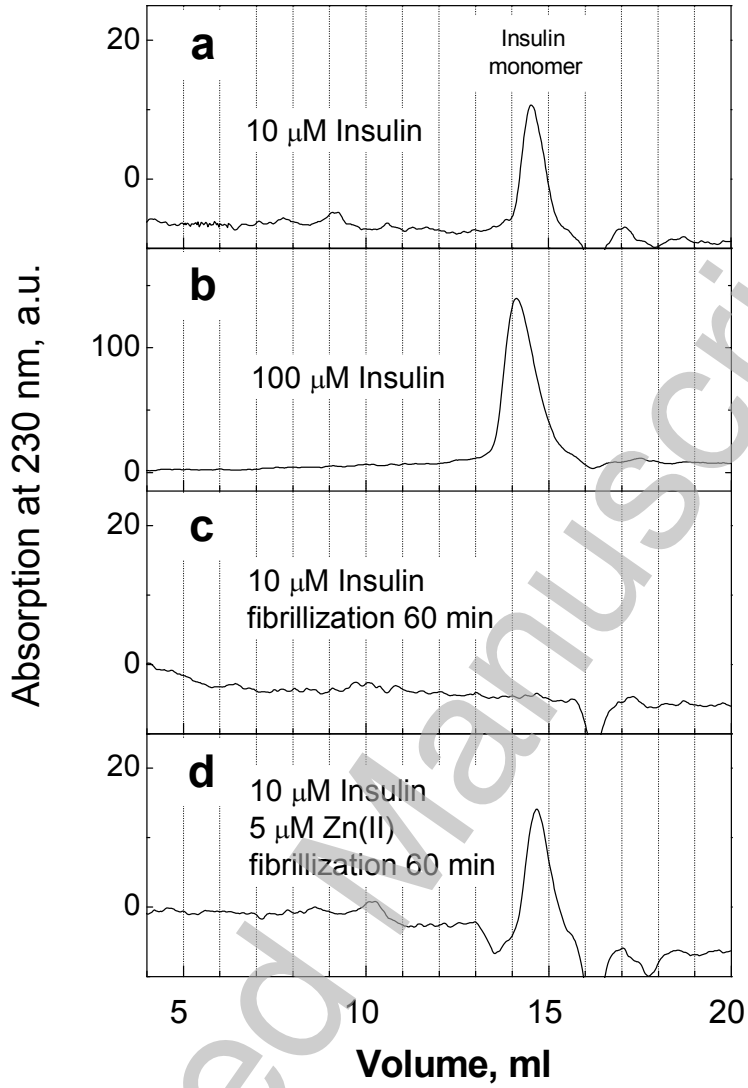
Figure 6



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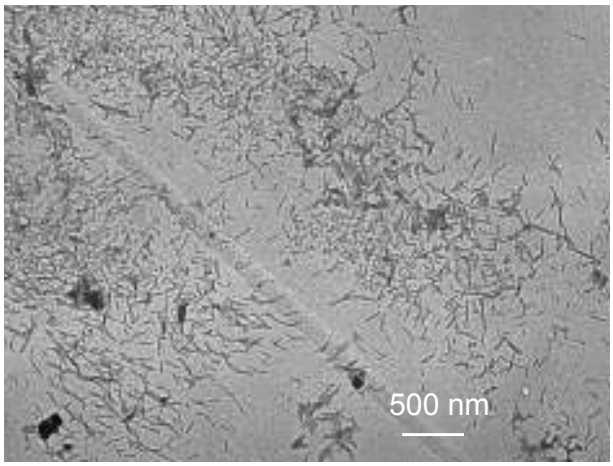

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Figure 7