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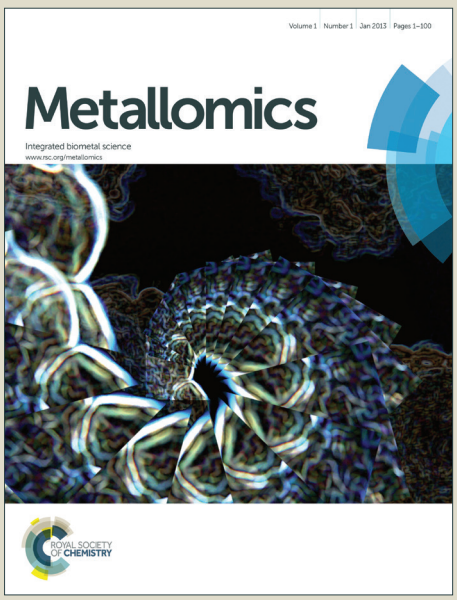

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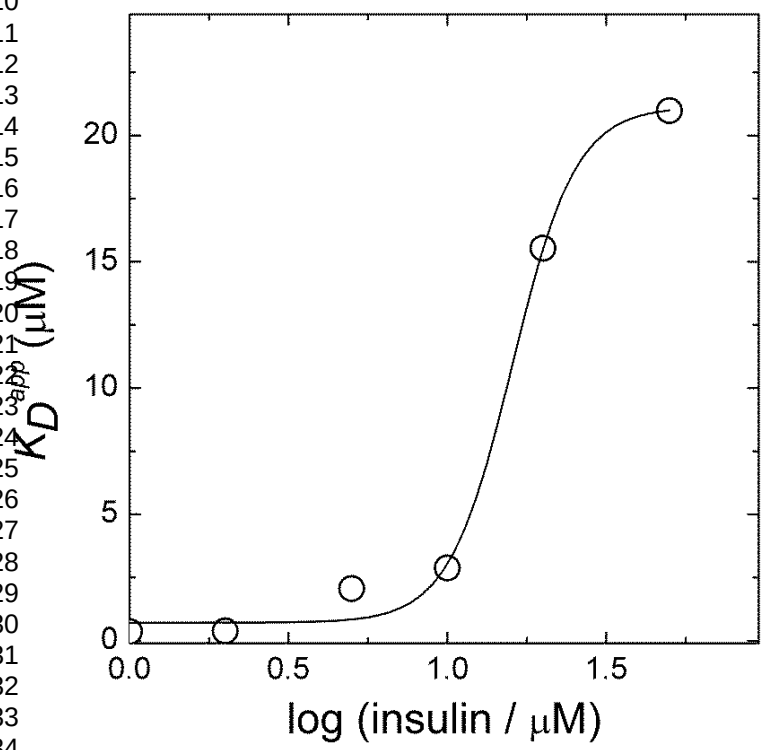

$$Zn^{2+} + \text{insulin} \rightleftharpoons \text{Zn-insulin}$$

$$K_D = 0.40\ \mu M$$

Affinity of zinc and copper ions to insulin monomers

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Zinc is an essential trace element involved in the correct packing and storage of insulin. Total zinc content in the pancreatic β-cells is among the highest in the body and changes in the Zn^{2+} levels have been found to be associated with diabetes. The most common form of Zn-insulin complex is a hexamer containing two zinc ions. However, zinc can also form other complexes with insulin, whereas dissociation constants of these complexes are not known. We have determined that the dissociation constant value for the monomeric 1:1 Zn-insulin complex is equal to 0.40 μM. The apparent binding affinity decreases drastically at higher insulin concentrations where the peptide forms dimers. Cu^{2+} ions also bind to monomeric insulin, whereas the apparent Cu^{2+}-binding affinity depends on HEPES concentration. The conditional dissociation constant for Cu^{2+}-insulin complex is equal to 0.025 μM. The analysis demonstrates that insulin cannot form complexes with zinc ions in circulation due to the low concentration of free Zn^{2+} in this environment.

Introduction

Insulin is the main peptide hormone involved in glucose metabolism and universally used in the treatment of type I diabetes. Insulin is produced by β-cells of the pancreas and its secretion into circulation is influenced by different stimuli, including increased blood glucose level. The insulin molecule consists of a 21-residue A chain and a 30-residue B chain connected by two interchain disulfide bonds. At micromolar concentrations insulin self-associates into dimers[1] and at millimolar concentrations it readily forms globular hexamers that normally contain two zinc ions.[2-3]

Insulin has inherent properties for interaction with zinc ions and this transient metal plays an essential role in all stages of insulin metabolism, from production and storage to secretion and utilization.[4] The zinc content of pancreatic β-cells is among the highest in the body. Zinc ions are actively transported into secretory vesicles inside the β-cells by the help of pancreas-specific zinc

transporter ZnT8.[5] Inside the secretory granules insulin forms crystalline arrays of hexamers that contain two Zn^{2+} ions per hexameric unit. Thus, a substantial amount of Zn^{2+} is co-secreted with insulin. Additionally, the secretory granules of β-cells may contain Zn^{2+} in excess to what is necessary to form Zn-insulin hexamers, which results in an even greater yield of free Zn^{2+} during exocytosis.[6] Co-secreted zinc can act as an autocrine inhibitory modulator of insulin secretion,[7] however, it is not known whether zinc can modulate the action of insulin.

Once in the extracellular microenvironment, the Zn^{2+}/ insulin complex dissociates. It is mentioned that timely removal of secreted free Zn^{2+} from the extracellular space is critical in order to maintain the normal function of insulin[6] because monomeric insulin is the active form recognized by receptors. During transfer from vesicles to the portal vein, insulin is diluted approximately 10^7-fold; at this dilution the Zn^{2+}/insulin complexes dissociate within seconds,[8] however, the final level of dissociation depends on the concentration of free zinc ions in the serum and the affinity constant of the Zn^{2+}/insulin complex.

The dissociation constant for the complex formed, K_D, is one of the most essential parameters characterizing any biochemical interaction. Both the amount of the complex formed under physiological conditions as well as the putative effects of the dyshomeostasis of interacting partners on the balance of the complex formation depend directly on the K_D value. Despite the obvious importance of insulin interaction with Zn^{2+} ions, the affinity of the formation of zinc-insulin complexes has not been thoroughly studied. We could find only one estimate for the dissociation constant of the Zn^{2+}-insulin complex in the range of 2 μM,[9] which, unfortunately, is determined in the presence of Tris buffer that has very complex interactions with transition metal ions. Insulin hexamer can exist in three different allosteric forms: T6 (tense), R3T3, and R6 (relaxed). R6 is the most stable form of hexameric insulin (For a review on the allosteric properties of insulin see Ref [4]). It is known that the rate of 2,2′,2″-terpyridine assisted Zn^{2+} release from the R6 conformation of hexameric insulin is 70 000 fold slower than that from T6 state which most probably also means higher affinity of zinc to R6 state.[10]

In the present study, we determined the dissociation constants for reversible insulin-zinc and insulin-copper complex formation using the changes in the intrinsic fluorescence of insulin. Copper does not associate with insulin under physiological conditions and we studied its interactions with insulin only because of its similarity to Zn^{2+}. We show that the metal-binding center of insulin is not selective for Zn^{2+} and it can bind Cu^{2+} with increased affinity. Some peptide aggregation occurred during the zinc-binding experiments at higher insulin concentrations which resulted in drastically decreased metal-binding affinity. In the case of copper we demonstrated that the concentration of buffer has an influence on the apparent affinity

of insulin towards Cu^{2+} ions and derived the value for the conditional (buffer independent) dissociation constant for Cu^{2+}-insulin complex.

Experimental

Materials. Lyophilized bovine insulin, $CuCl_2$ and Thioflavin T (ThT) were from Sigma-Aldrich (St. Louis, MO, USA); 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane-1-sulfonic acid (HEPES) Ultrapure from USB Corporation (Cleveland, OH, USA). $ZnCl_2$ and NaCl were extra pure from Scharlau (Barcelona, Spain). All solutions were prepared in fresh Millipore Q water.

Sample preparation. Stock solutions of insulin (50 μM) were prepared by dissolving an appropriate amount of the lyophilized peptide in 0.02 M HEPES and 0.1 M NaCl, pH 7.3. The insulin solution contained traces of zinc in the molar ratio less than 1:10 as determined by AAS. After 30 minutes incubation the stock solution was diluted with buffer to the appropriate concentration and used for experiments.

Fluorescence spectroscopy. Fluorescence spectra were collected on a Perkin–Elmer LS-55 fluorescence spectrophotometer (Perkin Elmer, Waltham, MA, USA). For the detection of intrinsic tyrosine fluorescence of insulin, excitation at 270 nm was used and the emission was recorded at 305 nm. The titration of insulin with metal ions was carried out in a 0.5 cm path length quartz cuvette by adding of 1–10 μL aliquots of the stock solutions of the respective metal salt to the insulin solution. After each addition the solution was stirred for 10 seconds and the average fluorescence intensity was measured over a 30 s period. In the control experiment without salts added the intrinsic fluorescence of insulin was constant for at least 1 hour confirming the instability of the peptide solution. The fluorescence of the amyloid dye used, ThT, was measured at 480 nm using excitation at 440 nm.

Size-exclusion chromatography. The oligomeric composition of insulin samples was determined by SEC on a Superdex 75 10/300 column (GE Healthcare, Giles, United Kingdom) connected to an Äkta Purifier system (GE Healthcare, Giles, United Kingdom). 0.02 M HEPES pH 7.3 containing 0.1 M NaCl was used as an elution buffer.

Calculation of dissociation constants. The values of the dissociation constants and the stoichiometries of the Zn^{2+}-insulin and Cu^{2+}-insulin complexes were calculated by fitting the titration data to the following quadric equation:

$$I = \frac{I_\infty + (I_0 - I_\infty)}{[L]} \times \left(\frac{[L] + [Me^{2+}] + K_D app) - \sqrt{(K_D + [L] + [Me^{2+}])^2 - 4 \times [Me^{2+}] \times [L]}}{2} \right) \quad (1),$$

where $[Me^{2+}]$ is the total concentrations of metal ions, [L] is the concentration of metal binding sites and K_D is the dissociation constant of metal-insulin complex. I_0, I and I_∞ are the fluorescence intensities of the peptide sample in the absence, the presence, and the saturation of metal ions.

Non-linear least-square fitting and statistical analysis of the data were performed using Origin 6.1 program (OriginLab Corporation, USA).

Results and Discussion

Binding of Zn^{2+} ions to insulin.

The intrinsic fluorescence of the tyrosine residues in the insulin molecule was sensitive to the binding of zinc (and also copper) ions to the peptide. Fig. 1 shows that the fluorescence intensity of insulin decreases during titration with Zn^{2+} ions. The effect of Zn^{2+} was reversible: addition of 10 mM of EDTA restored 90% of the initial fluorescence intensity (See Supplement Fig. 1). The binding curves can be quantitatively described by Eqn.1 and the K_D^{app} values and the number of binding centers can be determined. The superscript "app" denotes the dependence of the dissociation constant on the composition of the solution; in the case of Zn^{2+} ions the K_D values were independent of the buffer concentration and depended on the insulin concentration (Supplement Fig. 2). The binding stoichiometry was shown to be equal to 1:1 at low insulin concentrations, where the L values found in the result of fitting were close to the insulin concentration. Due to the increase in K_D values the L could not be treated as a variable at high insulin concentrations and we used insulin concentration as L in data fitting suggesting the same stoichiometry at all insulin concentrations.

The K_D^{app} values increased with increasing insulin concentration, indicating better binding at low insulin concentrations. The semilogarithmic plot of K_D versus insulin concentration (Fig. 2) shows a sharp threshold in the apparent affinity at concentrations between 10 and 20 μM. We suggest that the observed decrease in the Zn^{2+} binding affinity at higher insulin concentrations may be associated with insulin dimerization, since the K_D value for dimerization is in the same concentration range.[11-12] Size-exclusion chromatography (SEC) analysis showed that the injection of 2 μM insulin exposed one peak with elution volume of 15.04 ml, which corresponds to the monomeric state of protein. At the same time, injection of 50 μM insulin decreased the

elution volume to 13.79 ml indicative of peptide oligomerization (Supplement Fig. 3). Elution volume of 50 μM insulin corresponds to the molecular mass of 10 kDa, which is consistent with the dimeric state of insulin. The presence of 200 μM Zn^{2+} in the probe did not cause any shift of insulin peak in SEC confirming that Zn^{2+} keeps insulin in a soluble state and does not induce oligomerization.

The dissociation constant value at low insulin concentrations (≤ 2 μM), K_D = 0.40 μM corresponds to the binding of an equimolar amount of Zn^{2+} ions to monomeric insulin. However, we cannot draw a conclusion that the K_D value at high insulin concentrations corresponds to low affinity of insulin dimers towards zinc ions. First, the intensity of the intrinsic fluorescence of the tyrosine residues in insulin showed slow equilibration after adding each new portion of Zn^{2+} into the solution. Aggregation of the peptide was apparent due to an increase in the turbidity of the solution, thus the process of Zn^{2+} binding may be more complex at high insulin concentrations. In principle Zn^{2+}-insulin complexes with different stoichiometry can form at higher insulin concentrations and as long as we do not know their fluorescent characteristics or cannot determine their presence using some other method we cannot exclude the possibility that the decrease in affinity is not due to precipitation. The nature of aggregates formed at high insulin concentrations was tested with the well-known amyloid dye ThT. Insulin can form amyloid aggregates also at neutral pH,[12-13] however, amyloid formation was suppressed by Zn^{2+} ions.[12] ThT fluorescence did not increase in the presence of insulin aggregates, which indicated that no insulin fibrils were formed during metal ion titration experiments. The K_D^{app} value for the monomeric Zn^{2+}-insulin complex was considerably smaller than the IC50 value estimated from the inhibition curves of insulin fibrillization by Zn^{2+}.[12]

As the oligomeric insulin binds calcium ions besides Zn^{2+}, we also examined the effects of calcium ions on the insulin intrinsic fluorescence and zinc binding properties. Ca^{2+} had no effect on either the intrinsic fluorescence of insulin or on its Zn^{2+} binding affinity.

Affinity of insulin towards Cu^{2+} ions.

Addition of Cu^{2+} leads to the decrease in the intrinsic fluorescence of the molecule (Fig. 3). The K_D^{app} values were calculated from the respective titration curves according to Eqn. (1). The K_D values show that insulin is not selective to zinc, suggesting that most likely the molecular basis for the interaction of insulin with zinc and copper ions is similar.

In contrast to the Zn^{2+}-insulin complex, the fluorescence intensity of insulin in the presence of Cu^{2+} ions stabilized quickly also at high peptide concentrations. It is known that HEPES buffer used in this study forms a complex with Cu^{2+} ions and affects the corresponding apparent

dissociation constant.[14] We found that K_D^{app} values vary when different concentrations of HEPES buffer are used (Table 1 and Fig 4). In the same experiment with Zn^{2+}ions no significant difference between binding constants was observed (Supplementary Fig. 2), indicating that HEPES does not form detectable complexes with Zn^{2+}.

Since HEPES does not form ternary complexes with metal ions,[14] it is still recommended for copper binding studies; however, its influence on the binding equilibrium is taken into account by calculating the dissociation constants at zero buffer concentration (conditional dissociation constant).

Conditional constants K_D in HEPES were calculated from the apparent dissociation constants using the appropriate correction functions:[14]

$$\log K_D = \log K_D^{app} - C \qquad (2)$$

$$C = \log\left(1 + \beta_{MeL} \times \frac{C_L}{1 + 10^{-pH + pK_a}}\right) \qquad (3)$$

where C_L is the buffer concentration, K_a is the deprotonation constant of the buffer, and β_{MeL} is the dissociation constant for the metal^{2+}-HEPES complex. The conditional dissociation constants for Cu^{2+}-insulin complexes (Table 1) and the K_D value 0.025 μM were obtained. Thus, the metal-binding site of insulin is not selective for Zn^{2+} and it can bind Cu^{2+} with even better affinity. The semilogarithmic plot of the dependence of insulin affinity on the insulin concentration, shown in Fig.5, was also similar to the one obtained with zinc ions.

Biological relevance.

The biological significance of zinc-insulin interaction relies clearly on the biological availability of zinc in the pancreatic cells due to ZnT8 transporter activity. Zinc ions are related to several aspects of insulin metabolism. First, zinc co-crystallizes with insulin into dense secretory granules, second, zinc-ions co-secreted with insulin are involved in the regulation of the response to glucose levels.[7,15-16] In principle, the monomeric insulin can interact with zinc ions in pancreatic β-cells as well as in circulation. In the secretory vesicles of pancreatic β-cells the concentration of zinc and insulin are 11 and 21 mM, respectively.[17] The K_D value for the monomeric Zn^{2+}-insulin complex equal to 0.40 μM was calculated from our data. In secretory vesicles at high concentrations insulin ends up in crystalline arrays of hexamers containing two zinc ions, but the primary function of zinc ions is not to catalyze the hexamer formation since similar hexameric structures are also formed in the absence of zinc ions.[15] Considering the

affinity and zinc content in pancreatic cells, monomeric insulin can be present in the form of a zinc complex.

The total concentration of zinc in the plasma is around 11 μM, however, the level of labile or exchangeable zinc is considerably lower. Approximately 28% of the zinc pool in plasma is tightly bound to α_2-macroglobulin.[18] The rest of zinc is bound to serum albumin, which is present at extremely high 0.5 – 0.75 mM concentrations and has two Zn^{2+}-binding sites characterized with dissociation constant values 100 nM and ~1 μM.[19] Thus, albumin can bind all labile zinc in serum to its high-affinity sites which are not saturated with zinc ions and the concentration of free zinc ions is too low for binding to insulin that is present at subnanomolar (57 - 280 pM) concentrations.[20] Accordingly, the prevalent form of insulin in circulation is free monomeric insulin that interacts with the insulin receptor.

Conclusions

Insulin monomers bind zinc ions with dissociation constant of 0.40 μM and copper(II) ions with conditional dissociation constant 0.025 μM. In secretory granules insulin is in complex with zinc ions, whereas in circulation it exists as a free monomer.

Acknowledgements

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Table 1. Effect of HEPES on the Cu^{2+} binding to insulin.

The dependence of apparent dissociation constants K_D^{app} of Cu-insulin complexes on the HEPES concentrations and the conditional dissociation constant (K_D) values calculated according to Eqn 2 and 3.

Concentration of HEPES (mM)	C [a)]	K_D^{app} (μM) SD	K_D (μM)
5	0.67	0.10 0.03	0.021
20	1.19	0.40 0.08	0.026
50	1.57	1.00 0.20	0.027

[a)] correction function values calculated according to Eqn. 3

Figures

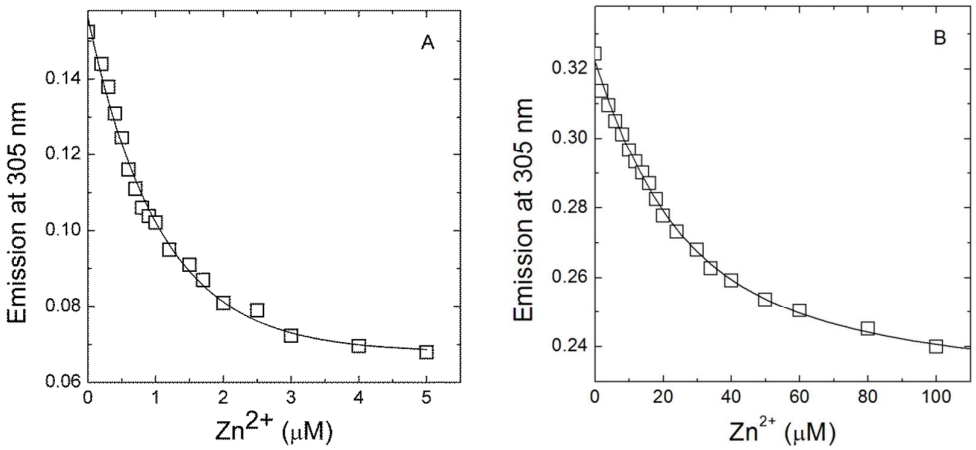

Figure 1. Binding of Zn^{2+} ions to insulin. Decrease of the intrinsic tyrosine fluorescence of insulin with increasing concentration of Zn^{2+}. A 1 μM insulin, solid line correspond to Eqn. 1, K_D^{app} = 0.40 0.11 μM and binding stoichiometry 0.93 ± 0.17; B 20 μM insulin,solid line corresponds to K_D^{app} = 12.8 3.8 μM and binding stoichiometry 0.97 ± 0.29. Titration was carried out at pH 7.4 in 0.02 M HEPES buffer containing 0.1 M NaCl.

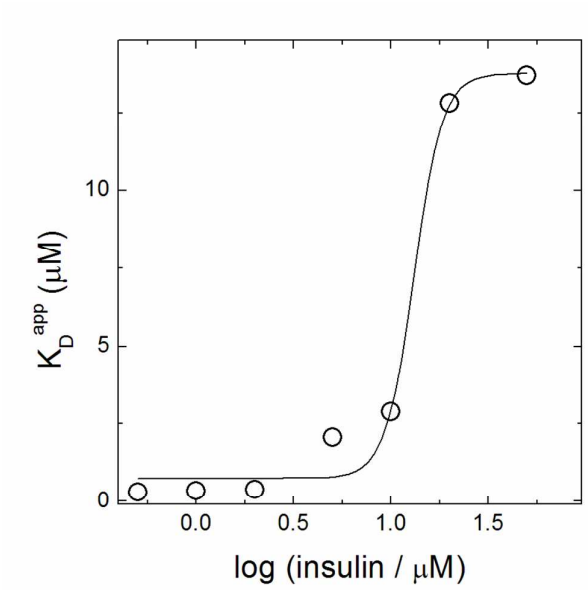

Figure 2. Dependence of the apparent dissociation constant values of Zn^{2+}-insulin complex on the insulin concentration.

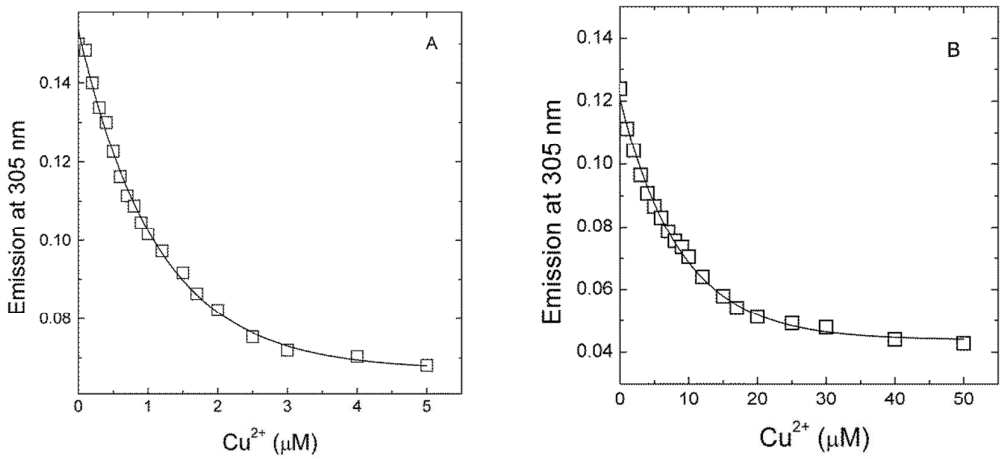

Figure 3. Binding of Cu^{2+} ions to insulin. Decrease of intrinsic tyrosine fluorescence with increasing concentration of Cu^{2+}. A 1 μM insulin, K_D = 0.40± 0.09 μM and stoichiometry 0.97 ± 0.14; B 10 μM insulin, K_D = 2.21± 0.38 μM. Titration was carried out at, pH 7.4 in 20 mM HEPES buffer, 0.1 M NaCl; Excitation at 270 nm.

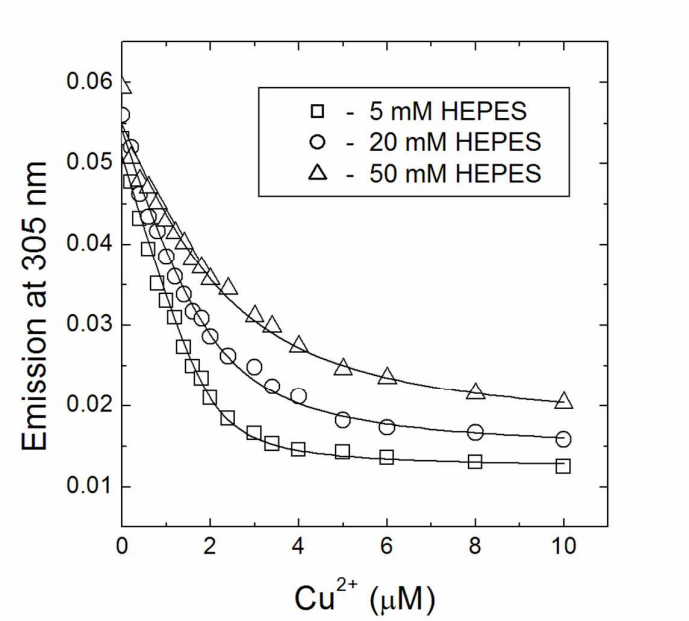

Figure 4. The effect of HEPES buffer concentration on the binding of Cu^{2+} ions to insulin. pH 7.4, 0.1 M NaCl, buffer concentrations are shown in Legend

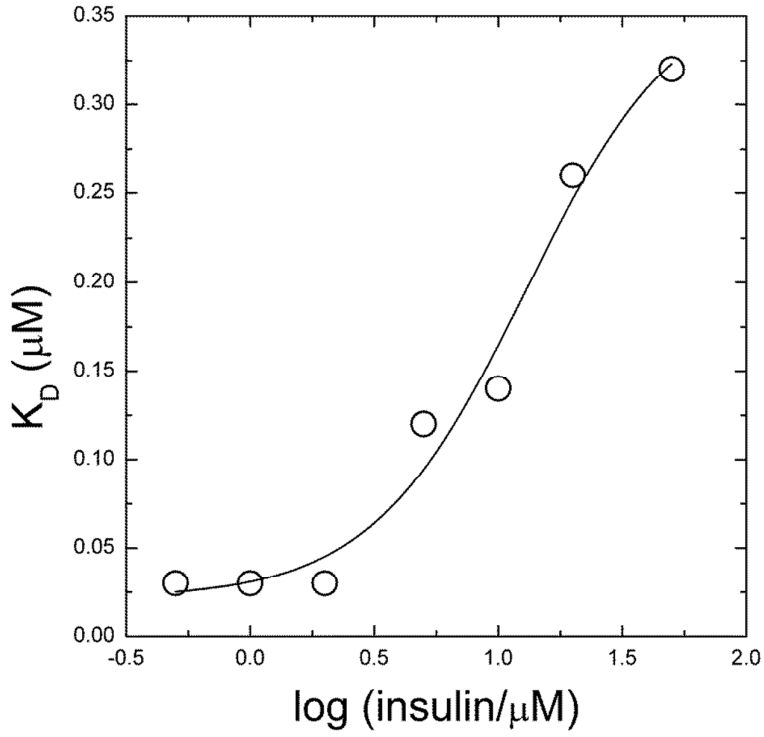

Figure 5. Dependence of the apparent dissociation constant values of Cu^{2+}-insulin complex on the insulin concentration