

## $\beta$ -Cell Dysfunction under Hyperglycemic Stress: A Molecular Model

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### Abstract

#### **Background:**

Pancreatic  $\beta$  cells respond to chronic hyperglycemia by increasing the synthesis of proinsulin (the precursor molecule of insulin). Prolonged stimulations lead to accumulation of misfolded proinsulin in the secretory track, delayed insulin secretion, and release of unprocessed proinsulin in the blood. The molecular mechanisms connecting the state of endoplasmic reticulum overloading with the efficiency of proinsulin to insulin conversion remain largely unknown.

#### **Method:**

Computer simulations can help us to understand mechanistic features of the  $\beta$ -cell secretory defect and to design experiments that may reveal the molecular basis of this dysfunction. We used molecular crowding concepts and statistical thermodynamics to dissect possible biophysical mechanisms underlying the alteration of the secretory track of  $\beta$  cells and to elucidate the chemistry aspects of the secretory dysfunction. We then used numerical algorithms to relate the degree of biophysical alteration of these secretory compartments with the change of proinsulin to insulin conversion rate.

#### **Results:**

Our computer simulations suggest that overloading the endoplasmic reticulum initiates downstream molecular crowding effects that affect protein translational mechanisms, including proinsulin misfolding, delayed packing of proinsulin in secretory vesicles, and low kinetic coefficient of proinsulin to insulin conversion.

#### **Conclusions:**

Together with previous experimental data, the present study can help us to better understand chemistry aspects related to the secondary translational mechanisms in  $\beta$  cells and how hyperglycemic stress can alter secretory function. This can give a further impetus to the development of novel software to be used in a clinical setup for prediction and assessment of diabetic states in susceptible patients.

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**Abbreviations:** (ER) endoplasmic reticulum, (CP) C-peptide, (GA) Golgi apparatus, (I) insulin, (IAPP) islet amyloid polypeptide, (P) proinsulin, (PC) proinsulin convertase, (PP) preproinsulin, (SV) secretory vesicle, (T1DM) type 1 diabetes mellitus, (T2DM) type 2 diabetes mellitus

**Keywords:** endoplasmic reticulum stress, hyperglycemia, insulin, molecular crowding, proinsulin, type 2 diabetes mellitus

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## Introduction

Insulin is the final product of a sequential biochemical process in the secretory track of the  $\beta$  cell.<sup>1</sup> Initially, the insulin mRNA is translated as a single chain precursor called preproinsulin (PP) that undergoes a series of post-translational modifications, which are required for progression to the active insulin (I). Removal of its signal peptide during insertion into the endoplasmic reticulum (ER) generates the proinsulin (P) molecule. Within the ER, P molecules are folded in their native states, which are then exported to the Golgi apparatus (GA) and packaged in secretory vesicles (SV). The successful conversion of proinsulin to insulin requires the detachment of the C-peptide (CP) chain from the single-stranded polypeptide P. The process is mediated by PC2 and PC3, two proinsulin convertases that are packaged together with proinsulin in SV. If the native structural motif of proinsulin is altered or the accessibility for these convertases to the cleavage sites on the proinsulin surface is obstructed, then the convertases can not liberate the CP, leading to a release of unprocessed proinsulin in the blood.

Various conditions, such as increased metabolic demands and chronic hyperglycemia, raise the synthesis of proinsulin to high limits,<sup>2</sup> overloading the ER. Often, such states lead to dramatic changes of the biophysical environment in the secretory track, such as ER dilation and accumulation of proteinaceous debris in the secretory track.<sup>3-5</sup> Mutations in the insulin gene can also contribute to the alteration of the ER protein folding potential.<sup>3,4,6-10</sup> Data revealed mutant proinsulin species accumulated in the ER, preGolgi intermediates, and GA.<sup>3,4,7,10</sup> Most likely, the presence of mutant proinsulin in the secretory track interferes with the normal translational process, favoring proinsulin misfolding and aggregation.<sup>8</sup> This may explain the 3–5-fold increase of the volume density in the secretory compartments.<sup>7</sup> Normally, the ER and its accessory components undergo adequate adjustments to cope with increasing crowding conditions and ensure optimum proinsulin processing. This adaptation process includes ER dilation and intensified clearing of the environment from toxic residues.<sup>11</sup> Misfolded proinsulin species that escape the “quality control” mechanism may also win the passage competition through the ER and advance on the secretory track. An increased level of misfolded proinsulin in the end part of the secretory track (i.e., vesicles) can favor the entrapment of the proinsulin convertases and restricts the accessibility for

these convertases to the cleavage sites on the surface of the natively folded proinsulin, impeding conversion to insulin and maturation of the SV. Secretion of non-matured vesicles by  $\beta$  cells increases the level of inactive proinsulin in the blood. Elevated blood proinsulin levels were found both at the onset of type 1 diabetes mellitus (T1DM)<sup>12-17</sup> and in type 2 diabetes mellitus (T2DM),<sup>17-36</sup> as well.

Although previous studies were suggestive in revealing consequences of the increased ER overloading in  $\beta$  cells, i.e., accumulation of misfolded proinsulin in the secretory track<sup>4,37-47</sup> and release of unprocessed proinsulin in the blood,<sup>12-36</sup> they do not provide a clear picture of the underlying molecular mechanism connecting the state of ER overloading with the efficiency of proinsulin to insulin conversion. Computer simulations can help us to better understand mechanistic features of this secretory defect and to design experiments that will reveal its molecular basis. In previous studies,<sup>48-50</sup> we predicted peculiar molecular crowding effects in the ER of  $\beta$  cells that may be generated by chronic hyperglycemic conditions. By using basic molecular principles, we have pointed out that prolonged stimulation of the  $\beta$  cells to increase the biosynthesis of insulin [and islet amyloid polypeptide (IAPP)] can lead to a gradual impairment of the processing of their precursor molecules (proinsulin and proamylin) in the ER.<sup>49,50</sup> Moreover, the increase of IAPP production can lead to formation of harmful IAPP preamyloid oligomers, which may accumulate in pancreatic islets<sup>51,52</sup> and/or be secreted in the blood.<sup>52</sup> Increased secretion of amyloidogenic IAPP species in the blood can favor the formation of IAPP amyloids in the heart, causing major cardiac dysfunction.<sup>52</sup>

Here, we extended these molecular crowding concepts to investigate possible biophysical alterations that could manifest on the entire secretory track of the  $\beta$  cells, including the ER, GA, and SV. We then used computer simulations to relate the degree of biophysical alteration of these secretory compartments with the change of the proinsulin to insulin conversion rate. Together with previous experimental data,<sup>3-5,10,51</sup> the present study can help us to better understand secondary translational mechanisms implicated in  $\beta$ -cell dysfunction. This can give a further impetus to the development of novel software to be used in a clinical setup for prediction and assessment of diabetic states in susceptible patients.

## Methods

Molecular crowding concepts<sup>53–55</sup> are derived from the principle that an enhanced volume density of the local environment results in reduced configuration space and distribution of states (less entropy) of the reactant macromolecules in comparison with the macromolecular products. Therefore, the overall entropy loss is lower, which leads to more significant decrease in free energy and higher equilibrium constants for the reaction. This can have a major effect on all processes with a change in excluded volume, such as protein folding, unfolding, and aggregation processes.<sup>48–50,56–62</sup>

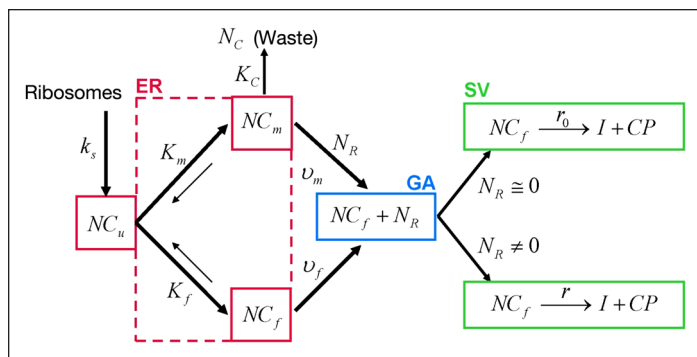
The effect of crowding can be predicted based on the apparent chemical activity coefficient ( $\gamma$ ). For instance, the excess chemical potential of the macromolecules due to the interactions between a newly added macromolecule of type  $j$  in the local environment of volume  $V$  and all the other molecules in the environment can be expressed by

$$\ln \gamma_j = \frac{\Delta F_j}{kT} \cong \ln(1 - f) + \frac{V_j}{V} \frac{1}{1 - f} \left[ \left( \frac{V_k}{V_j} \right)^{2/3} + \left( \frac{V_k}{V_j} \right)^{1/3} + 1 \right] + \left( \frac{V_j}{V} \right)^2 \frac{1}{2(1 - f)^2} \left[ \left( \frac{V_k}{V_j} \right)^{4/3} + 2 \frac{V_k}{V_j} \right] + \left( \frac{V_j}{V} \right)^3 \frac{1}{3(1 - f)^3} \left( \frac{V_k}{V_j} \right)^2 \quad (1)$$

In the above,  $F_j$  is the Helmholtz function,  $kT$  represents the thermal energy,  $V_j$  denotes the average molecular volume of the species  $j$ ,  $V_k$  stands for the average molecular volume of the crowding agent, and  $f$  is the characteristic volume fraction for molecular crowding. **Equation 1** is widely used in studying molecular crowding effects in various biological systems.<sup>48–50,56–62</sup>

**Equation 1** was previously used in computer simulations to predict the change of ER volume density due to molecular overloading.<sup>49,50</sup>

Here, we used molecular crowding concepts to study the biophysical alteration of the secretory track in  $\beta$  cells induced by hyperglycemic stimulations and to estimate the effect of this alteration on the efficiency of proinsulin to insulin conversion. The kinetic scheme for insulin biosynthesis is displayed in **Figure 1**. The insertion of  $N$  unfolded proinsulin in the ER is followed by folding and diffusion to the GA or misfolding and proteolysis by the “quality control” mechanism (waste). Misfolded proinsulin species that escape the “quality control” mechanism in the ER may also be packed in secretory vesicles (SV) ( $N_R$ ) together with natively folded proinsulin ( $NC_f$ ). However,



**Figure 1.** Scheme of the kinetics underlying the synthesis of insulin used in the present computation. The insertion of  $N$  unfolded proinsulin in the ER is followed by folding and diffusion to the GA or misfolding and proteolysis by the “quality control” mechanism (waste). Misfolded proinsulin species that escape the “quality control” mechanism in the ER may also be packed in secretory vesicles (SV) ( $N_R$ ) together with natively folded proinsulin ( $NC_f$ ). Misfolded proinsulins act as a crowding agent, altering the rate ( $r$ ) of proinsulin conversion to insulin.

these misfolded P molecules would not be susceptible to the conversion to active insulin and are secreted in the blood in unprocessed form. Moreover, an increased level of misfolded proinsulin in SVs can act as a crowding agent, restricting the accessibility for convertases to the cleavage sites on the surface of the natively folded proinsulin and impeding conversion to insulin. Consequently, additional unprocessed P molecules can be dispersed into the blood.

### Molecular Crowding Effects in the ER

Proinsulin represents more than 50% of the total protein biosynthesis in the ER during functional stimulation by glucose.<sup>63</sup> Therefore, proinsulin molecular species (i.e., unfolded, folded, and misfolded molecules) are the main contributors to the crowding effects in this secretory compartment. These effects can alter the kinetics of proinsulin synthesis, which can be estimated based on **Equation 1**. To this goal, we first derived the characteristic parameter  $f$  (the volume fraction of the molecular crowding) as a function of the local molecular composition (i.e., unfolded, folded, and misfolded proinsulin species) of the ER. The volume of the ER can be approximated by  $V_{ER} \cong N(V_{P_u}C_u + V_{P_f}C_f + V_{P_m}C_m + \Delta V)$ , where  $V_{P_u}$ ,  $V_{P_f}$ , and  $V_{P_m}$  are the molecular volumes of unfolded ( $u$ ), folded ( $f$ ), and misfolded ( $m$ ) proinsulin species.  $C_u$ ,  $C_f$ , and  $C_m$  denote the population density numbers of these molecules in the local environment,  $C_u + C_f + C_m = 1$ .  $\Delta V$  represents the required “free” space in the ER that allows the P molecule to fold and transfer efficiently to the GA. Under normal glycemic conditions,  $\Delta V \gg V_{P_u}$ , which means that the insertion of additional unfolded P molecules in the ER does not

overwhelm its folding capabilities. Contrarily, β cells under hyperglycemic stimulation, which increases the proinsulin load in the ER, can reach rapidly the limit  $\Delta V \rightarrow 0$ . In this limit, molecular crowding effects are likely to alter the biosynthesis of proinsulin in the ER. The characteristic fraction of molecular crowding ( $f$ ) entering **Equation 1** can be written as  $f = NC_m V_m / V_{ER}$ .

We now can express the kinetic coefficients of proinsulin transition from unfolded ( $u$ ) to natively folded ( $f$ ) and misfolded ( $m$ ) states at equilibrium  $K_f = \frac{C_f}{C_u}$  and  $K_m = \frac{C_m}{C_u}$  in terms of the crowding-free kinetic coefficients,  $K_{0,f}$  and  $K_{0,m}$  and activity coefficients  $\gamma_u$  and  $\gamma_f$  (see **Equation 1**) by

$$K_f = K_{0,f} \exp\left(\frac{\Delta F_u - \Delta F_f}{kT}\right) = K_{0,f} \frac{\gamma_u}{\gamma_f} \quad (2)$$

$$K_m = K_{0,m} \exp\left(\frac{\Delta F_u - \Delta F_m}{kT}\right) = K_{0,m} \frac{\gamma_u}{\gamma_m}$$

$\Delta F_j$ ,  $j = u, f, m$  represents variations of Helmholtz functions accompanying the addition in the local environment of an extra molecule from the unfolded, folded, or misfolded proinsulin species.  $\Delta F_j$  are derived in terms of  $\gamma_j$  as shown in **Equation 1**.

Normally, misfolded proinsulins are targeted for degradation by the “quality control” mechanism in the ER. We assume that the “quality control” mechanism in the ER is characterized by the equilibrium kinetic coefficient of clearance by  $K_c$ . Hence, the amount of cleared misfolded proinsulin is  $N_C = NC_m K_c$ . In susceptible cells, the “quality control” mechanism can be overwhelmed by the increased proinsulin misfolding, which leads to  $K_c < 1$ . In this case, an amount  $N_R = NC_m(1 - K_c)$  of misfolded proinsulins will advance further on the secretory track, together with natively folded proinsulin.

**Equations 1** and **2** reflect the biophysical changes in the ER induced by folding and misfolding of proinsulin. Based on these equations, we can estimate the most likely partition of folded and misfolded P molecules in the ER under molecular crowding conditions.

### Transit of Proinsulin to Golgi Apparatus under Molecular Crowding Conditions

Molecular crowding forces affect the diffusion of various proteins differently, depending on their molecular sizes  $r_j = (3/4\pi V_j)^{1/3}$  and degree of molecular crowding,  $f^{64}$

$$D_j = \left(\frac{1-f}{1+f}\right)^2 D_{0,j} = \left(\frac{1-f}{1+f}\right)^2 \frac{k_B T}{6\pi\eta_0 r_j} \quad (3)$$

Here,  $D_{0,j}$  represents the diffusion coefficient under crowding-free conditions, which depends on the size of protein  $r_j$ , temperature  $T$ , Boltzmann constant  $k_B$ , and viscosity coefficient  $\eta_0$  (the Stokes-Einstein equation). We can use **Equation 3** to estimate the rate of molecular transit from ER to GA under molecular crowding conditions. The transfer rate can be approximated by  $v_j = \frac{1}{\tau_j}$ , where  $\tau_j$  represents the molecular diffusion time  $\tau_j = \frac{a^2}{4D_j} \cdot D_j$  is given by **Equation 3** and  $a$  stands for the characteristic diffusion length. Therefore, the effective transit rates of native and misfolded proinsulins ( $v_f$  and  $v_m$ ) can be expressed in terms of the characteristic value  $v_{0,f} = \frac{4D_{0,f}}{a^2}$  under crowding-free conditions by

$$v_f = \left(\frac{1+f}{1-f}\right)^2 v_{0,f} \text{ and } v_m = \left(\frac{1+f}{1-f}\right)^2 \left(1 \mp \frac{\Delta r}{r_f}\right) v_{0,f} \quad (4)$$

In **Equation 4**,  $\Delta r$  stands for the specific change in size of a misfolded proinsulin,  $r_m = r_f \pm \Delta r$ ,  $\Delta r \ll r_f \cdot v_{0,f}$  can be estimated from measurements of the time required for half of the P molecules to pass to the GA in normal conditions ( $t_{1/2}$ ),  $v_{0,f} \cong \frac{N}{2t_{1/2}}$ ,  $t_{1/2} \cong 15 \text{ min.}^{65}$

**Equation 4** shows that misfolded proinsulin species with large molecular volumes will advance much slower on the secretory path while the transit of smaller ones will be affected to a much lesser extent.

### Molecular Overcrowding Alters Proinsulin to Insulin Conversion in Vesicles

Let  $n$  be the average number of P molecules packed in a secretory vesicle under normal (crowding-free) conditions. We denote the volume of the vesicle by  $V_{SV_0} \cong nV_{P_f} + \Delta v_0$ , where  $\Delta v_0$  represents a characteristic “free space” in the vesicle that is used normally by convertases to reach the cleavage sites on the proinsulin polypeptide chain. Inclusion of misfolded proinsulin species in the secretory vesicle reduces the effective available space in the vesicle by a fraction  $f_{SV} = xnV_{P_m}/V_{SV} \cong xnV_{P_m}/V_{SV_0}$ ; we assume that vesicle volumes do not change significantly ( $V_{SV_0} \cong V_{SV}$ ) under molecular crowding conditions.  $x$  is the fraction of proinsulin in misfolded states in the local environment, which can be estimated based on the amounts of folded ( $NC_f$ ) and misfolded ( $N_R$ ) P molecules exported to the GA under molecular crowding conditions. This reads  $x = N_R/(NC_f + N_R)$ .



Within the SV, the misfolded P molecules act as a crowding agent, inhibiting the conversion of proinsulin to active insulin. To estimate the molecular crowding effects on the kinetics of this chemical process, we can apply similar concepts as those used to derive **Equation 2**. Consequently, the kinetic coefficient ( $r$ ) of the conversion of native proinsulin to insulin under crowding conditions can be written as

$$r = r_0 \exp\left(\frac{\Delta F_P - \Delta F_I - \Delta F_{CP}}{kT}\right) = r_0 \frac{\gamma_P}{\gamma_I \gamma_{CP}}, \quad (5)$$

where  $r_0$  is the crowding-free kinetic coefficient.  $\gamma_P$ ,  $\gamma_I$  and  $\gamma_{CP}$ , the activity coefficients of P, I, and CP, depend explicitly on the volume fraction of the molecular crowding agent  $f_{SV}$  (i.e., misfolded proinsulin).  $\Delta F_P$ ,  $\Delta F_I$ , and  $\Delta F_{CP}$  represent variations of Helmholtz functions accompanying the addition in the local environment of either native proinsulin, insulin, or CP.

**Equations 1–5** provide a selfconsistent mathematical correlation between the degree of biophysical alteration of the secretory track following acute hyperglycemic stimulation and the decreased proinsulin to insulin conversion rate.

### Computational Details

**Equations 1–4** were used in iterative computations<sup>49,50</sup> of  $C_f$  and  $C_m$  to determine the optimal available space in the ER ( $\Delta V/N$ ) leading to maximum folding capabilities at equilibrium, i.e.,  $C_f \gg C_m$  as previously described.<sup>49,50</sup> Then, the value of  $\Delta V/N$  was systematically decreased to determine the molecular crowding conditions that are critical for proinsulin folding. The complete kinetic scheme of the biochemical reactions is shown in **Figure 1**.  $S$  describes the variation in time of the proinsulin load,  $S = N \exp(-k_s t)$ , and  $k_s$  stands for the characteristic kinetic rate. The value of  $k_s$  was set so that the lag time for processing the entire proinsulin load is about two-hours time, under physiological conditions.<sup>66</sup> Backward rates, corresponding to proinsulin transitions from folded and misfolded states to the initial unfolded state, are assumed much slower than forward rates. Numerical simulations were run for two different values of the kinetic coefficient of clearance  $K_c = 10K_m$  and  $K_c = 0.1K_m$ . The initial conditions were set to  $P_j(t = 0) = 0$ , ( $j = u, m, f$ ), and  $S(t = 0) = N$ . We assumed  $V_{P_m} \cong 1.5V_{P_f}$  as the test molecular volume of the misfolded proinsulin species. The volume of the unfolded P molecule was set to  $V_{P_u} \cong 2V_{P_f}$ . A variation of the test molecular sizes  $V_j$  by ~50% does not affect significantly the amount of

processed proinsulin at critical ER dilation.<sup>49,50</sup> However, the relative difference between molecular volumes of folded and misfolded P molecules determines the accumulation of denaturated P molecules in the ER.

Similar line of reasoning was followed in the numerical simulations of the  $P \rightarrow I + CP$  dissociation reaction in SV. The amounts of folded ( $N_{C_f}$ ) and misfolded ( $N_R$ ) P molecules at equilibrium derived earlier were used as initial conditions to predict the output of proinsulin to insulin conversion  $I(t)$  with the kinetic rate  $r$  given by **Equation 5**. The crowding-free kinetic rate  $r_0$  is a free parameter that was set so that the lag time for processing the entire proinsulin load in the vesicle in ideal conditions is about 30 minutes. The overall increase of the exposed surface area to molecular crowding forces following the splitting of proinsulin in insulin and CP conversion was estimated based on scaled particle theory, as described in References 49 and 50. Thus,  $P \rightarrow I + CP$  increases the total surface area by about 40%, which corresponds to a change of the partial volume of the molecules given by  $V_{I+CP} \cong 1.2V_{P_f}$ .

### Results

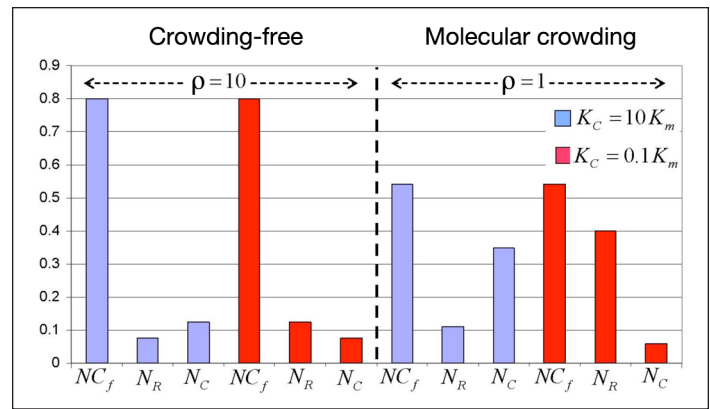
Previous numerical simulations<sup>49</sup> suggested that  $\beta$  cells undergoing increased hyperglycemic stimulations can maintain optimal capabilities of proinsulin biosynthesis in the ER by volume expansion ( $\Delta V \cong 10NV_{P_f}$ ) of the ER and rapid targeting of misfolded proinsulin for degradation ( $K_c \gg K_m$ ). It was also suggested that molecular crowding effects can significantly weaken the ER capabilities to fold and transfer P molecules to the next secretory compartment, the Golgi apparatus.<sup>50</sup>

In **Figure 2**, we display the fraction numbers of folded ( $N_{C_f}$ ) and misfolded ( $N_R$ ) proinsulin passed to the GA, for normal ( $\Delta V \gg NV_{P_f}$ ,  $\rho = \frac{\Delta V}{NV_{P_f}} = 10$ ) and molecular overcrowding ( $\rho = \frac{\Delta V}{NV_{P_f}} \cong 1$ ) conditions. We can see that an increase of the ER volume density to abnormal limits ( $\rho \cong 1$ ) leads to a significant drop of the probability of proinsulin to form native structures and accumulation of misfolded proinsulin in the secretory track. Present estimations are in good agreement with measurements of ER dilation and accumulation of misfolded proinsulin in diabetic  $\beta$  cells.<sup>3–5,7</sup> Data<sup>7</sup> have shown a 1.7-fold average increase in the volume density of the dilated ER in comparison with the normal ER in control  $\beta$  cells. In **Figure 2**, we also display the

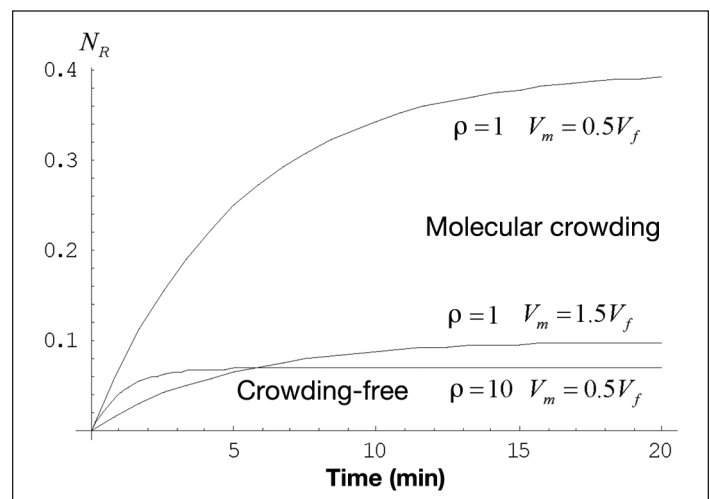
relative amount of misfolded proinsulin cleared by the “quality control” mechanism ( $N_C$ ) for assumed crowding conditions  $\rho = 1$  and  $\rho = 10$ . According to the present numerical simulations, the “quality control” mechanism of the  $\beta$  cell works efficiently, i.e.,  $N_C \gg N_R$ , if the rate of clearance is much larger than that of misfolding,  $K_C \gg K_m$ .

The predicted change in the biosynthesis of proinsulin, which is displayed in **Figure 2**, is actually the result of a superposition of two different but related crowding effects. First, the formation of highly compressed, nonnative structures is favored in an overcrowded environment. We can see in **Figure 2** a significantly increased tendency of proinsulin to misfold under crowding conditions ( $\rho = 1$ ) compared to the fraction of misfolded proinsulin under normal conditions ( $\rho = 10$ ). Second, the passage of proinsulin to the GA under crowding conditions is slower, which increases even further the local molecular crowding and accelerates proinsulin denaturation. We can clearly see this effect by simulating the transport of various misfolded proinsulin species from the ER to GA under time-dependent crowding conditions. In **Figure 3**, we display the predicted amounts of misfolded proinsulins ( $N_R$ ) passed to the GA under normal ( $\rho = 10$ ) and critical crowding ( $\rho = 1$ ) conditions. One can easily notice that misfolded proinsulin species with large molecular volumes (i.e.,  $V_m = 1.5V_f$ ) advance much slower on the secretory path while, the transit of smaller ones (i.e.,  $V_m = 0.5V_f$ ) are affected to a much lesser extent. Based on this prediction, we infer that proteinaceous accumulations in the early part of the secretory path<sup>57</sup> are most likely constituted by misfolded proinsulin species with large molecular volumes and by proinsulin aggregates.

Molecular crowding effects in SV can dramatically alter the proinsulin to insulin conversion. Because the total macromolecular volume ( $V_I + V_{CP}$ ) corresponding to the two product molecules I and CP is normally larger than that of the precursor molecule (P), we obtain from **Equation 1**  $\gamma_P < \gamma_I \gamma_{CP}$ . Hence, an increase of the molecular crowding ( $f_{SV} \rightarrow 1$ ) in SV can favor preservation of proinsulins in intact structures rather than splitting them into insulin molecules and CPs. By using **Equation 5**, we can simulate numerically the increase in time of the amount of insulin  $I(t)$  resulted from the dissociation reaction  $P \rightarrow I + CP$ . The amounts of folded ( $N_C$ ) and misfolded ( $N_R$ ) proinsulin species displayed in **Figure 2** are used here to estimate  $f_{SV}$  and  $r$  (see **Equation 5**). The characteristic time-dependent probability densities in the insulin state corresponding to normal ( $\rho = 10$ ) and molecular crowding ( $\rho = 1$ ) conditions are displayed



**Figure 2.** The amounts of folded ( $N_C$ ) and misfolded ( $N_R$ ) proinsulin passed to the GA relative to the cleared ( $N_C$ ) by the “quality control” mechanism for normal ( $\rho = 10$ ) and molecular crowding ( $\rho = 1$ ) conditions.



**Figure 3.** Predicted amounts of misfolded proinsulins ( $N_R$ ) passed to the GA under normal ( $\rho = 10$ ) and critical crowding ( $\rho = 1$ ) conditions. Small volume, misfolded proinsulin species can advance more easily on the secretory track while large misfolded proinsulin species and proinsulin aggregates accumulate in the early part of the secretory track, increasing the state of molecular crowding.

in **Figure 4**. Our numerical simulations clearly suggest that the presence of misfolded, inactive P molecules in vesicles can impede the conversion of natively folded proinsulin in active insulin.

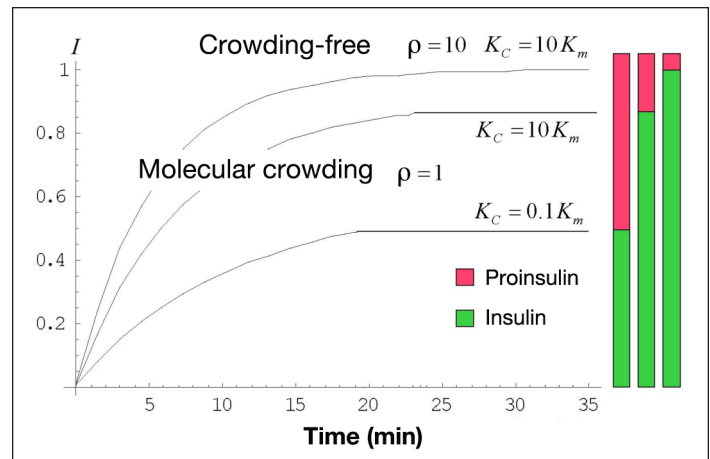
## Conclusions

We used molecular crowding concepts and basic statistical thermodynamics to predict the biophysical alteration of the secretory track in  $\beta$  cells induced by prolonged hyperglycemic stimulations. Our computer simulations suggest that the proinsulin overload in the ER, the main  $\beta$  cell response to hyperglycemic stimulation, generates downstream molecular crowding effects that impair protein translational mechanisms at several different levels.

First, molecular overcrowding in the ER enhances the propensity of P molecules to misfold. The formation of highly compressed, nonnative structures is energetically favored in an overcrowded environment as the entropy gain from this compaction can be significant. Increasing the ER volume density to abnormal limits ( $\rho \approx 1$ ) enhances significantly the probability of proinsulin to form nonnative structures and to accumulate in the secretory track (see **Figure 2**). Present theoretical predictions are in good agreement with measurements of ER dilation and accumulation of misfolded proinsulin in diabetic  $\beta$  cells.<sup>5,7</sup> Our findings provide a mechanistic understanding of previous data showing that supranormal production of nonnative proinsulin may predispose  $\beta$  cells to ER stress and premature loss of  $\beta$  cells.<sup>3,4,67-69</sup>

Second, molecular crowding forces acting in the secretory track can slow down the transport of P molecules from the ER to GA (see **Figure 3**), delaying the packing of proinsulin in SVs and conversion to active insulin. Markedly delayed insulin secretion in response to glucose administration is a common feature of T2DM.<sup>70</sup> From the time-dependent transfer of proinsulin to the GA displayed in **Figure 3**, we can infer that crowding effects in the secretory track represent a possible source of the delayed onset of the insulin response. Moreover, present computer simulations predict that small volume, misfolded proinsulin species can advance more easily on the secretory track while large misfolded proinsulin species and proinsulin aggregates accumulate in the early part of the secretory track, increasing the state of molecular crowding (see **Figure 3**). Misfolded P molecules that are incorporated in SVs are not susceptible to the conversion to active insulin in SVs and are secreted in the blood in unprocessed form.

Finally, crowding effects can play a major role in the maturation process of the SV. Misfolded proinsulin species incorporated in SVs can act as a crowding agent, restricting the accessibility for convertases to the cleavage sites on the surface of the natively folded proinsulin and impeding conversion to insulin. Consequently, unprocessed P molecules can be dispersed into the blood. Elevated levels of proinsulin have been found both at the onset of type T1DM<sup>12-17</sup> and in T2DM<sup>17-36</sup> as well. Two mechanisms are possible reasons why susceptible  $\beta$  cells release unprocessed proinsulin into the blood:<sup>12,32,48</sup> (1) dysfunction of the enzymatic proinsulin processing mechanism and (2) lack of maturation of insulin vesicles due to an intense hyperglycemic stimulation. We now can understand this SV defect based on a physical chemistry basis. The conversion



**Figure 4.** The characteristic time-dependent probability densities in the insulin state  $I(t)$  corresponding to normal ( $\rho = 10$ ) and molecular crowding ( $\rho = 1$ ) conditions. We can notice that the presence of misfolded, inactive P molecules in vesicles can impede the conversion of natively folded proinsulin in active insulin.

of proinsulin in insulin and CP ( $P \rightarrow I + CP$ ) requires an available space ( $\Delta v$ ) in the vesicle that needs to fulfill the condition  $\Delta v > (V_I + V_{CP})I(t) - V_{P_f}[1 - I(t)]$  where  $I(t)$  is the time-dependent probability density in the insulin state (see **Figure 4**). As the composition of the vesicle changes in time due to proinsulin to insulin conversion, the overall available space in the vesicle is shrinking, leading to the upsurge of the crowding effects and delay of proinsulin processing. An increase of the volume fraction of misfolded proinsulin in the vesicle imposes additional space constraints, leading ultimately to  $\Delta v \rightarrow 0$ . This inhibits the proinsulin to insulin conversion and increases the secretion of unprocessed proinsulin in the blood. Secretion of intact proinsulin increases progressively with the accumulation of proteinaceous debris in the secretory track (see **Figures 2 and 4**), i.e., with the development of the disease, which is an agreement with general clinic observations.<sup>34-36</sup>

In conclusion, according to the present computer simulations, overloading the ER in  $\beta$  cells would initiate downstream molecular crowding effects that would affect protein translational mechanisms, including accumulation of misfolded proinsulin in the secretory track, delayed packing of proinsulin in SVs, and low kinetic coefficient of the proinsulin to insulin conversion. The immediate physiological consequence of these cumulative effects would be the increased secretion of unprocessed proinsulin in the blood. Such molecular crowding effects may also affect the synthesis of other cellular components, including the precursor of the IAPP. Our previous molecular simulations<sup>50</sup> suggest that the propensity of IAPP to form preamyloid oligomers, the initial stage of amyloid



deposition in pancreatic islets, is significantly enhanced by molecular crowding effects. Thus, factors that may affect the fine balance between protein synthesis and aggregation may be the key toward understanding many diseases.<sup>71</sup>

The present study provides a molecular basis for the development of novel software that can help to identify defects in translational mechanisms of the β cell, cellular dysfunction, and development of T2DM. The software may prove very informative in a clinical setup for prediction and assessment of diabetic states in susceptible patients.

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