Biophysical Chemistry 140 (2009) 115–121

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Dilation of the endoplasmic reticulum in beta cells due to molecular overcrowding? Kinetic simulations of extension limits and consequences on proinsulin synthesis

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ARTICLE INFO

Article history:
Received 10 October 2008
Received in revised form 10 December 2008
Accepted 11 December 2008
Available online 24 December 2008

Keywords:
Beta cell dysfunction mechanism
Molecular crowding effect
Proinsulin denaturation
Diabetes
ER dilation
ER stress condition

ABSTRACT

Insulin regulates the energy homeostasis of the human body. This is synthesized in the endoplasmic reticulum (ER) of pancreatic beta cells from proinsulin. Chronic hyperglycemia increases considerably the proinsulin secretion, overcrowding the ER. Recent experimental evidence demonstrates that such states favor the proinsulin denaturation. The biophysical mechanism of this cellular dysfunction remains largely unknown. We use basic molecular principles and numerical simulations of time-dependent crowding conditions in the ER to show that crowding effects enhance the propensity of proinsulin molecules to (mis)fold in compressed, non-native structures. Present results suggest: *i*) misfolding events and toxic accumulations increase dramatically if the proinsulin load exceeds 50% of the available space and *ii*) insufficient lag time for the relaxation of the ER between consecutive proinsulin uploads can cause irreversible alterations of folding capabilities. Present study may prove useful in generating new testable statements on circumstances leading to the development of diabetes.

1. Introduction

Insulin (*I*) is the final product of a sequential biochemical process $PP \rightarrow P \rightarrow I$ (see, for review, the paper by Howell and Bird [1]). Initially, the insulin mRNA is translated as a single chain precursor called pre-proinsulin (*PP*). *PP* undergoes a series of post-translational modifications which are required for progression to the active insulin. Removal of the signal peptide from the *PP* molecular chain during insertion into the endoplasmic reticulum (ER) generates the proinsulin (*P*) molecule. Within the ER, the proinsulin molecules are folded in their native states, which are then exported to the Golgi apparatus and packaged in secretory vesicles. The successful conversion of proinsulin to insulin requires the detachment of the *C* peptide chain from the single-stranded polypeptide *P*. The process is mediated by specific endoproteases which are packaged together with proinsulin molecules in secretory vesicles.

Increasing amounts of evidence demonstrate that toxic accumulations within secretory compartments of beta cells represent a major cause of impaired insulin synthesis leading to diabetes [2–20]. While mutations in the insulin gene and transient replication errors are major contributors to the cellular proteotoxicity increase, secondary translational mechanisms could also have significant implications in this process. Compelling experimental data reveal that *i*) misfolded proinsulin species accumulate in the ER [9,13,17], *ii*) the volume density in this compartment increases considerably [9,19], usually by 3–5 times [9] and *iii*) misfolded proinsulin molecules are prone to aggregation [12]. All these results [9,12,13,17,19] clearly suggest the existence of an accelerated misfolding of proinsulin within the ER which exacerbates the proteotoxicity within the secretory track. Deciphering chemistry circumstances and molecular mechanisms leading to an increase of proinsulin misfolding and accumulation in the ER is the main focus of the present report.

More than 50% of the proteins synthesized in the ER during beta cell stimulation by glucose are proinsulin molecules [21]. Generally, chronic hyperglycemia leads to an increased rate of proinsulin synthesis, overcrowding the ER [19,22–24]. Inherently, placing an enormous burden on the ER enhances the probability of protein misfolding. To maintain optimum level conditions for proinsulin processing, the ER undergoes adequate volume adjustments [25,26] and increases the rate of clearance of the local environment from toxic residues [2,4,11,25]. However, the ER can be pushed too far in susceptible cells. Eventually, the ER volume fails to dilate and stress conditions ensue [19]. This causes a wide range of beta-cell dysfunctions leading eventually to the development of diabetes [6,7,11,19,27,28].

The implication of the molecular crowding effects on the insulin synthesis in beta cells was not addressed previously although experimental data suggest that these effects may be a source of various stresses culminating in cellular dysfunction [3,6,7,10,11,14–17,19,22–24]. Studies of the effects induced by molecular crowding forces on proinsulin folding and transport in the ER can improve our understanding on molecular mechanisms that elicit an impaired insulin synthesis. Crowding effects may not be limited solely to the ER, similar forces could also act in the next secretory compartments (Golgi and vesicles) leading to altered packaging of proinsulin in vesicles, entrapment of proinsulin convertases and/or restricted accessibility for these convertases to the cleavage sites on the surface of the proinsulin and a general depressed kinetic rate of the transformation of the *native* proinsulin in active insulin and *C*-

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doi:10.1016/j.bpc.2008.12.003

peptide, as suggested recently [20]. The main aim of the present work is the analysis of proinsulin folding dynamics under crowding conditions generated by the insertion of large amounts of precursor *P* molecules in the ER. We discuss critical conditions that can lead to long-lasting crowding effects and identify prerequisites that may favor the return to normal, physiological states. Our study may prove useful in generating new testable statements on circumstances leading to impaired insulin production and development of diabetes.

1.1. Molecular crowding effects

Under normal, physiological crowding in the ER, i.e. 100 mg/ml [25], proteins are folded in native, three-dimensional structures. Each protein type has a particular molecular volume V_f in this state. Typically, folding pathways lead also to the formation of species with smaller ($V_m \leq V_f$) or larger ($V_M \geq V_f$) volumes. This common phenomenon in protein folding reflects the opposing effects of the fundamental tendency of a system to seek out the lowest energy levels and, at the same time, populate as many levels as possible given the available thermal energy [29,30]. When the local volume density increases to asymptotic values, less volume is left for molecules to survey states with extended geometries from the configuration space, resulting in reduced configuration space and distribution of states (less entropy). The entropies of molecular states corresponding to small molecular volumes do not decrease as much. Accordingly, the increase of local crowding results in less overall entropy loss, which leads to more significant decrease in free energy and higher equilibrium constants for transitions to compact molecular structures. According with recent studies [31–33], this can have a major effect on all processes with a change in excluded volume, such as protein folding and aggregation processes. Increase of crowding in cellular compartments can slow down the local movement of macromolecules by up to two orders of magnitude, depending on the size and shape of the molecules [31–33]. It can also shift to much higher values the equilibrium constants of specific biochemical reactions [20,31–39].

It is becoming evident from the above considerations that the degree of volume exclusion has a key role in the factors that define which state proinsulin molecules will adopt inside the ER. Exceeding the local molecular crowding much over the physiological limit enhances the propensity of proinsulin molecules to (mis)fold in highly compressed, nonnative structures and/or to form proinsulin aggregates, as the entropy gain from compaction of proinsulin molecules can be significant. This fact raises the following questions. Let $N^{(0)}$ be the average load of proinsulin in the ER under normal translational regulation conditions of proinsulin synthesis. The corresponding partial volume of proinsulin can be written as

$$V \cong N^{(0)} V_u P_u + N^{(0)} V_f P_f + N^{(0)} V_m P_m + N^{(0)} V_M P_M + \Delta V. \quad (1)$$

V_u, V_f, V_m and V_M are characteristic volumes occupied by proinsulin molecules in unfolded, folded and misfolded states while, P_u, P_f, P_m and P_M stand for the probability densities in these states, so that at any time t, $\Sigma_i P_i(t) = 1$. ΔV stands for the required extra-space in the ER that allows proinsulin molecules to fold and transfer properly to the Golgi compartment. How much does the ER need to expand to minimize the crowding effects induced by the increase of the proinsulin load ($N \gg N^{(0)}$)? Assuming that the ER reaches the critical limit of dilation, which leads to an escalation of the crowding conditions, what fraction of the proinsulin precursor molecules (unfolded proinsulin) will be able to fold in native states and move on the secretory track?

2. Methods

To answer these questions in an effective manner, we adapted a classical numerical simulation routine [40] to study proinsulin folding and transport to the Golgi apparatus (GA) in a crowded environment (see the Appendix). Within the model, we assume that crowding conditions change in time as a result of insertion of additional proinsulin precursor molecules in the ER, folding and misfolding events, degradation of denaturated species and exit of native molecules from the ER.

2.1. Time-dependent ansatz to describe molecular crowding

Basic molecular principles assure that, in a highly volume-occupied environment, the reactivity of a particular solute species is determined by the number of molecules of that solute per unit of available volume, which is an effective concentration called thermodynamic activity. The available volume is the part of total volume that may be occupied by the center of mass of a particular solute species at a particular instant. Thus, because molecules are mutually impenetrable, the presence of a significant volume fraction of macromolecules in the medium is a source of constraints on the placement of an additional macromolecule. These constraints depend upon the relative sizes, shapes, and concentrations of all macromolecules in that environment [32,33]. The effect of crowding can be assessed in terms of an apparent chemical activity coefficient (γ). γ measures 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. γ_j is derived as a function of the average molecular volume of the species j (V_j), the average molecular volume of the crowding agent (V_k) and a characteristic fraction (f) of molecular crowding

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

In the equation above, F_j is the Helmholtz function and k_T represents the thermal energy. Eq. (2) indicates that the composition of a crowded system can change to minimize the total free energy of that system. Thus, molecular crowding can shift equilibria towards a state of the system in which excluded volume is minimized. The extent to which a particular macromolecular species excludes volume to its neighbors generally increases with the ratio of surface to volume of that species. Eq. (2) was derived by Boublìk [41] for a binary system of hard particles, based on the scaled particle theory [42]. This equation has been used under various mathematical formulations to describe a large variety of molecular crowding effects in biological systems [31–39,43–45]. Based on Eq. (2), it is not too difficult to understand that, in an overcrowded environment, folding pathways that involve equilibrium states as well as transition states [46] having small volumes will prevail over those requiring larger volumes. Thus, if the transition state leading to native proinsulin has a larger volume than that of the misfolded proinsulin, the proper precursor of the folded proinsulin (i.e. the unfolded proinsulin) tends to misfold under crowding conditions.

Within such a multicomponent system, the additional molecule j may undergo interactions with molecules of various types (k) and volumes (V_k). A straightforward adaptation of Eq. (2) to describing the energy change induced by steric repulsions between constituent molecules of a multicomponent system can be obtained by assuming that V_k represents an effective volume (V_k^{eff}). V_k^{eff} accounts for the molecular species more likely to contribute to the local crowding at the time of introducing the additional molecule j in the local environment. V_k^{eff} can be written as

$$V_k^{\text{eff}} \cong V_u P_u + V_f P_f + V_m P_m + V_M P_M. \quad (3)$$

As P_i, $i = u,f,m,M$, depends on the time t, Eq. (3) reflects chemical changes in time of the local environment. It describes in terms of

probability densities the type of molecules that are more likely to constitute the surrounding environment of the additional molecule j, at a given time t. In a similar manner, we define the characteristic fraction of molecular crowding by

$$f \cong \left(1 + \frac{\Delta V}{V_u P_u + V_f P_f + V_m P_m + V_M P_M}\right)^{-1}. \quad (4)$$

The present approach neglects entropic contributions due to the heterogeneity of the system. The addition of different molecular species in the system introduces extra terms in Eq. (2) that depend on concentrations and geometrical characteristics of the constituent molecules. Therefore, Eqs. (1)–(4) remain a good approximation of the energy change induced by steric repulsions in a multicomponent system, as long as the number of interacting species is kept small.

The net outcome of the steric effects on individual proinsulin molecules can be modified by diffusional motion of the molecules [47]. This is because the driving force in the diffusion process of particles requires the existence of a gradient of concentration, which makes the crowd to disperse in time. Inherent local fluctuations in the particle density may also alter the steric effects. Nevertheless, the overall increase of crowding in the ER will slow down the local movement of proinsulin molecules [33]. The effective rate of transit of proinsulin molecules through the ER towards the Golgi compartment can be approximated by

$$k_t = \frac{1}{\tau}\frac{v^{(0)}}{v}, \quad (5)$$

where τ represents the time of translation through the ER under physiological conditions and $\frac{v^{(0)}}{v}$ is a correction due to the restriction on the movement of the molecules in a crowded environment. $v^{(0)} = \frac{V^{(0)}}{N^{(0)}}$ and $v = \frac{V}{N}$ are derived from Eq. (1) and represent the partial volumes of proinsulin under physiological, crowding free conditions ($\Delta V \gg NV_u$) and increased crowding conditions ($\Delta V \to 0$), respectively.

A relaxation of local crowding conditions is also to be expected from the "quality control" system which clears denaturated proinsulin species from the ER [25]. In calculations, we assumed that the rate of clearance (k_c) is in the range of values similar to the transit rate k_t, $k_c \cong k_t$.

The complete scheme of the biochemical reactions involving different proinsulin species in the ER is shown in Fig. 1. Computation details are given in the Appendix.

3. Results

Our numerical simulations show that crowding effects are negligible if the average extra-space ΔV in the ER is about 10 times larger than the volume of loaded proinsulin $\Delta V \cong 10NV_f$, where N is the amount of proinsulin precursor molecules inserted in the ER. Under such conditions, folding and transport of proinsulin to the Golgi apparatus are optimal. In contrast, a fully loaded ER (i.e. $V \cong NV_f$) does not provide an appropriate environment for the synthesis of proinsulin. This leads to a dramatic increase of proinsulin misfolding, slower traffic and accumulation of misfolded species. Actually, crowding conditions become visible in simulations when the extra-space in the ER decreases below $10NV_f$ $\left(\frac{\Delta V}{NV_f} < 10\right)$ and become critical in the limit $\Delta V \to NV_f$. This can be inferred from Fig. 2a, where we display the evolution in time of the characteristic probability densities P_m and P_M for various values of the parameter $v = \frac{\Delta V}{NV_f}$. In Fig. 2b, we show the evolution in time of the amount of folded proinsulin passed to the Golgi apparatus (N_G) relative to the initial value of the proinsulin load (N), $n_G = \frac{N_G}{N}$. By looking at Fig. 2b, we can notice the progressive decrease of n_G with decreasing v. $v \cong 2$ corresponds to a sudden drop of n_G. A further decrease of v ($v \to 1$) is critical for folding and transport of proinsulin to the Golgi apparatus. Apparently, the ER cannot efficiently process additional proinsulin molecules at the critical volume density $v^{(c)} \cong 1.75$ (not shown). Present estimations are in good agreement with experimental data reported recently [9] from measurements of ER dilation and accumulation of misfolded proinsulin in diabetic beta cells. These data [9] have shown a 1.7 times average increase in the volume density of the dilated ER in comparison with the normal ER in control beta cells.

In overcrowded environments ($v = 2$), the propensity of proinsulin molecules to misfold and accumulate in the ER increases dramatically so that, the protein "quality control" system of the ER must increase the clearance rate more than 10÷20 times to cope with the volume exclusion effects. The accumulation of misfolded proinsulin increases the local crowding, accelerating the proinsulin misfolding process. This becomes apparent from Fig. 3a, where we compare the evolution

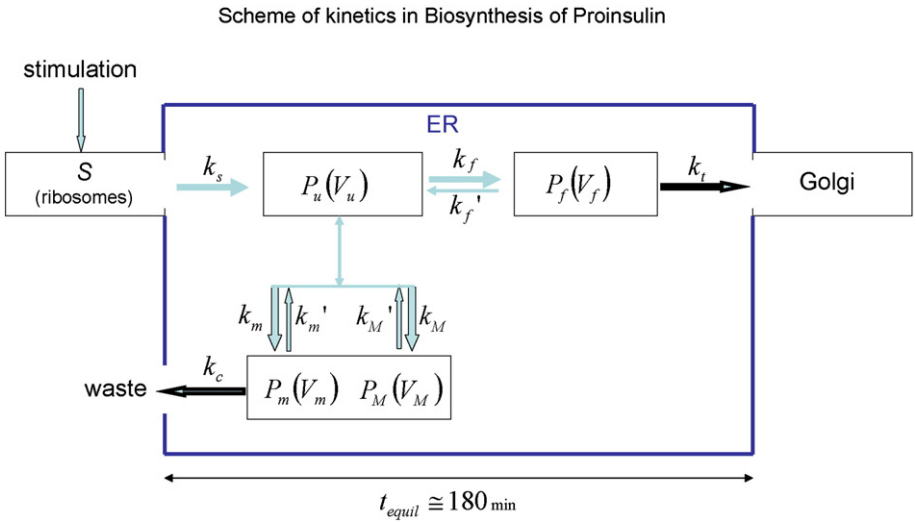

Fig. 1. Scheme of the proinsulin kinetics used in present computation. Under normal, physiological crowding in the ER, proteins are folded in native, three-dimensional structures. Each protein type has a particular molecular volume V_f in this state. (V_u denotes the molecular volume of the unfolded state.) Crowding conditions favor the formation of species with smaller ($V_m \leq V_f$) volumes. The probability of forming proinsulin species with larger ($V_M \geq V_f$) volumes is small in this case. Crowding conditions change in time as a result of insertion of additional proinsulin precursor molecules in the ER ($S \xrightarrow{k_s} P_u$), folding ($P_u \xrightarrow{k_f} P_f, P_f \xrightarrow{k_f'} P_u$) and misfolding events ($P_u \xrightarrow{k_{m,M}} P_{m,M}$, $P_{m,M} \xrightarrow{k_{m,M}'} P_u$), degradation ($P_{m,M} \xrightarrow{k_c} waste$) of denaturated species and exit of native molecules from the ER ($P_f \xrightarrow{k_t} GA$).

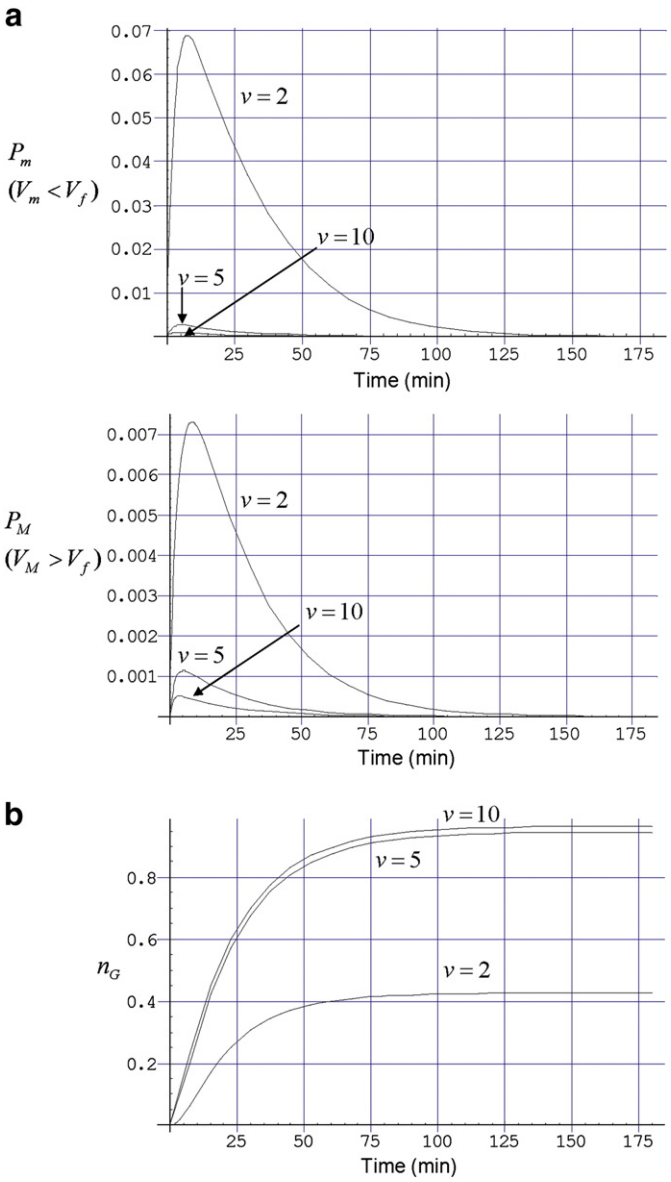

Fig. 2. (a) The evolution in time of the characteristic probability densities P_m and P_M for various values of the free parameter v. v represents the ratio between the dilation of the ER (ΔV) and the size of the proinsulin load (N). (b) The evolution in time of the amount of folded proinsulin passed to the Golgi apparatus (N_G) relative to the initial value of the proinsulin load (N), $n_G = \frac{N_G}{N}$.

in time of the probability distribution in misfolded states (P_m) for $k_c \cong k_t$ and $k_c \cong 10k_t$. From Fig. 3b, we can observe that the deficit in the output of native proinsulin is larger than 50% even for $k_c \cong 10k_t$. A relaxation of the crowding conditions requires both ER dilation ($\Delta V \rightarrow 10NV_f$) and rapid degradation of denaturated species ($k_c \gg 10k_t$).

In normal beta cells, molecular crowding effects may occur temporarily, such as those induced by successive upsurges of the translational regulation of proinsulin. For instance, three consecutive uploads ($3 \times N^{(0)}$) of proinsulin in the ER will induce different dynamics of proinsulin molecules depending on the time interval between loads. In Fig. 4, we compare time evolutions of population densities (P_m) in misfolded states corresponding to successive proinsulin uploads in the ER separated by 2 h time intervals with those in which the proinsulin upload takes place at 1 h time interval. From Fig. 4, we can see that an insufficient time for a complete relaxation of the ER, i.e. less than an usual 2 h lag time [1], may lead to an accumulation of the misfolded proinsulin species even if the initial molecular crowding effects were negligible ($\Delta V \cong 10NV_f$).

4. Discussion

Starting from basic molecular principles, we derived time-dependent correlations between the increase of molecular crowding in the ER and decrease of the amount of native proinsulin transported to the Golgi apparatus. Our study suggests that, generally, volume exclusion effects generated by overcrowding can reduce the efficiency of the ER to process proinsulin molecules. In order to minimize the volume exclusion in the ER, the precursor (unfolded) proinsulin molecules tend to misfold, forming structures with small molecular volumes ($V_m \leq V_f$), as the entropy gain from compaction of proinsulin molecules can be significant. This explains the accumulation of misfolded proinsulin species in the ER of diabetic beta cells [9,13,17].

In the extreme case of chronic hyperglycemia, overloading of the ER may occur frequently. The volume available in the ER for adding new proinsulin molecules drops to low values, which may lead to intense crowding effects. Therefore, increased proinsulin synthesis in these cells requires that both the size of the ER and its accessory components undergo continuous adjustment [26]. This size adaptation is achieved through signaling pathways from the ER to the nucleus whose components include the ER-associated transmembrane proteins Ire1, PERK (PKR-like ER kinase), and potentially other signaling receptors [48,49]. The pathways trigger the synthesis of a variety of resident ER and cytosolic proteins, which are required for proper protein folding, regulation of translation, membrane lipid synthesis, and transport. Failure of the ER to dilate may lead to stress and development of the unfolded protein response [25]. The presence of considerable amounts of denaturated protein in apoptotic cells [50,51] suggests that the decrease of the beta-cell mass is a consequence of the failure of the protein regulation mechanism [52]. Augmenting the capacity of the ER "quality control" system can,

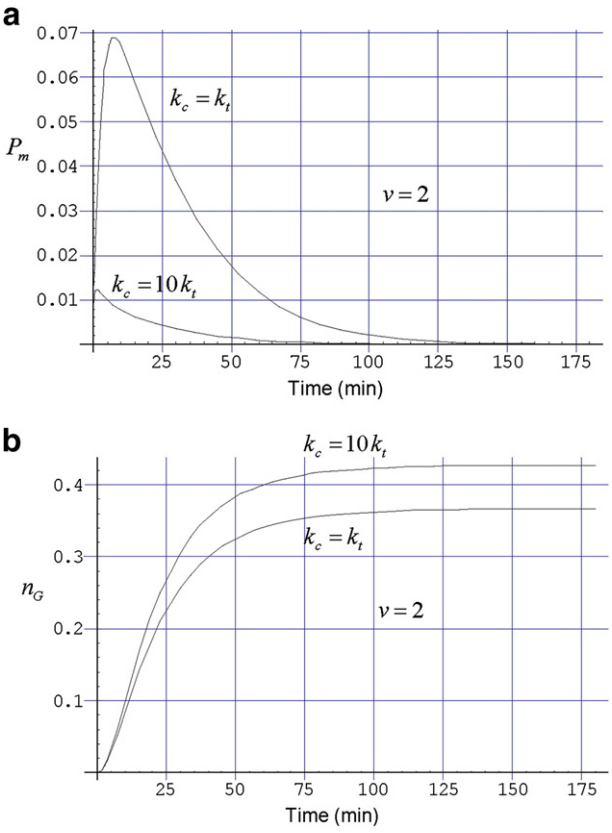

Fig. 3. (a) The evolution in time of P_m for $k_c \cong k_t$ and $k_c \cong 10k_t$ under critical crowding conditions (v=2). (b) The evolution in time of the amount of native proinsulin passed to the Golgi apparatus (N_G) relative to the size of the initial proinsulin load (N) under intense molecular crowding conditions (v=2).

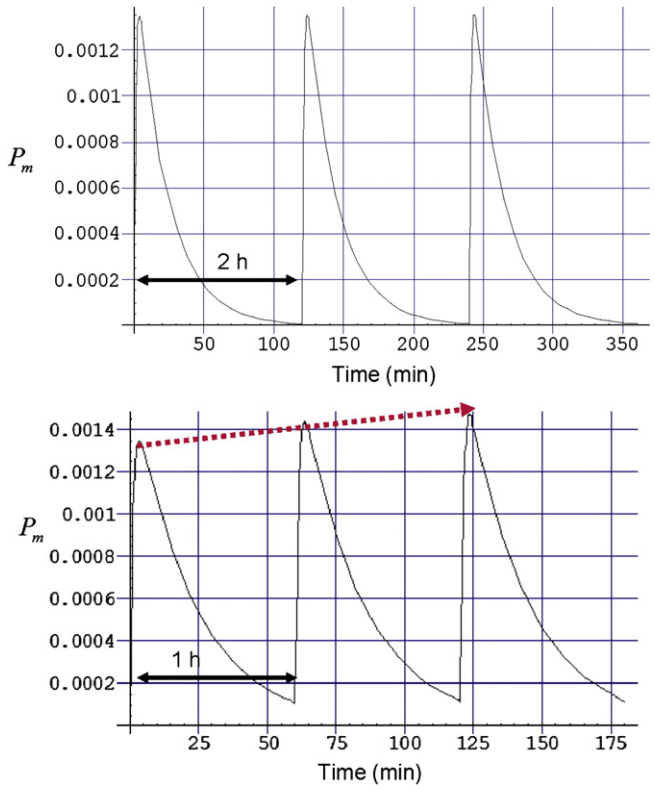

Fig. 4. We compare the evolution in time of the population densities (P_m) of the misfolded proinsulin states corresponding to successive proinsulin loads separated by 2 h time intervals (upper panel) with that in which the proinsulin upload in the ER takes place at 1 h time interval (lower panel). The red arrow shows the tendency of accumulation in time of misfolded proinsulin in the ER due to insufficient time of relaxation of the ER. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in principle, reduce crowding effects [25]. However, the rate of clearing the ER from misfolded proinsulin must increase considerably under acute crowding conditions, i.e. to reach values 10–20 times higher than that corresponding to normal physiological conditions, as we have shown above. Present results suggest that crowding effects and, subsequently, the propensity of proinsulin to misfold become negligible if the ER dilation reaches the limit of 10-fold the volume of the proinsulin load, i.e. $\Delta V \cong 10NV_f$. While reaching this limit of extension could be rather difficult, experiments have shown that ER dilates significantly under chronic hyperglycemia [9]. Our numerical simulations agree relatively well with experimental data showing that a 1.7 times increase of the volume density in the ER leads to an accelerated proinsulin denaturation [9,19].

Present kinetic simulations indicate the time interval between uploads of proinsulin in the ER as the main source leading to a transformation of the inherent temporary crowding effects into long-lasting ones. Insufficient lag time for a complete relaxation of the ER may cause an accumulation of misfolded proinsulin, thus contributing to a progressive cellular dysfunction. This aspect deserves special attention in the context of type 2 diabetes. It is possible that metabolic perturbations cause an exacerbation of the ER stress condition in diabetic cells via crowding effects. Consequently, temporary hyperglycaemia may accelerate the stress condition and development of apoptosis in such cells.

From an experimental perspective, a possible accumulation of toxic proinsulin species in the ER can be assessed by quantitative immunogold labeling for the *C*-peptide [9] along the secretory pathway. Based on the known proinsulin to insulin conversion in immature secretory granules, immunogold labeling for *C*-peptide detectable in the ER and up to the Golgi apparatus reflects proinsulin immunoreactivity, whereas that over immature and mature secretory granules represent free *C*-peptide. In time, the distribution of immunolabeled *C*-peptide will change showing an accumulation in the ER and a shortage of the insulin secreted from the beta cell.

5. Conclusions

The present model predicts that the amount of natively folded proinsulin molecules in the ER (proinsulin molecules which can eventually be transformed in active insulin after additional processing in the next secretory compartments) decreases dramatically in an overloaded ER. This implies that the efficiency of the drugs used to stimulate diabetic beta cells declines in time. Rather, therapeutic strategies should focus on removing toxic beta-cell residues and reestablishing the insulin synthesis to normal.

One more thing needs to be emphasized here. It is recognized that, under supraphysiological conditions related to the chronic hyperglycemia, the secretion of amylin is abnormally increased [53]. Amylin is a small peptide which is secreted by similar stimuli to those that secrete insulin. Usually, amylin can precipitate out in these cells. Secretion of large amounts of amylin into the extracellular environment can lead to the formation of amyloid deposits [54]. Inherently, amyloid deposits constitute a permanent source of crowding in islets, because the extracellular environment is devoid of molecular chaperones. Although extracellular aggregates can be degraded by immune-system pathways including macrophages, this process is often slowed down by disease and aging. Amyloid deposits disturb normal cellular function and represent a common feature in the pathogenesis of several type 2 diabetes [50,54–57].

In addition, the present study suggests that even a temporary increase of the frequency of mutations in the insulin gene, or other transient replication errors, may have actually long term consequences on the beta cell function. Thus, if the increase of mutant proinsulin synthesis cannot be handled efficiently by the intrinsic cellular repair mechanism [9,12,17,18] then, the accumulated toxic residues in the secretory pathway can decrease the chemical activity of the native proinsulin and interfere with the conversion to insulin.

Present study may prove useful in generating new testable statements on circumstances leading to impaired insulin production and development of diabetes.

Acknowledgements

The author would like to thank the John von Neumann Institute for Computing, Research Centre Jülich, where much of this work was done, for its hospitality. The author also thanks Professors C.J. Rhodes and C. Ionescu-Tirgoviste for useful discussions on the physiology of the beta cell.

Appendix A. Computational details

We used iterative computations [40] of kinetic rates and probability densities corresponding to molecular states involved in the biosynthesis of proinsulin to determine the optimal value of ΔV leading to maximum folding capabilities ($Pf \gg P_{m,M}$). Then we systematically decreased the value of ΔV to determine critical crowding conditions. The complete kinetic scheme of these biochemical reactions is shown in Fig. 1. The corresponding system of kinetic equations reads...

$$\begin{aligned}
\frac{dP_u}{dt} &= k_s S - (k_f + k_m + k_M)P_u + k_f{}'P_f + k_m{}'P_m + k_M{}'P_M \\
\frac{dP_f}{dt} &= k_f P_u - k_f{}'P_f - k_t P_f \\
\frac{dP_m}{dt} &= k_m P_u - k_m{}'P_m - k_c P_m \\
\frac{dP_M}{dt} &= k_M P_u - k_M{}'P_M - k_c P_M .
\end{aligned} \tag{6}$$

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 2 h time, under physiological conditions [1]. k_c denotes the rate of clearing misfolded structures by the "quality control" system in the ER and k_t denotes the rate of transport of folded proinsulin from the ER to the Golgi apparatus (see, Eq. (5)). k_f, k_m and k_M represent the kinetic coefficients characterizing transitions of proinsulin molecules from unfolded to folded and misfolded states, while k_f', k_m' and k_M' are the kinetic coefficients of backward reactions. Molecular principles discussed in above indicate that crowding conditions increase the propensity of macromolecules to form structural states characterized by small volumes. Therefore, backward rates, corresponding to proinsulin transitions from folded and misfolded states to the initial unfolded state, are much slower than forward rates ($k_f' \ll k_f$, $k_m' \ll k_m$, $k_M' \ll k_f$). Misfolding from the folded state would require the access of a partly unfolded state. Such a transition state would therefore have a larger volume than the folded state and it may be inhibited by molecular crowding. Thus, the reaction pathway $P_f \rightarrow P_{m,M}$ can be neglected within the present approach.

Kinetic coefficients characterizing transitions of proinsulin molecules from unfolded to folded and misfolded states under crowding conditions are derived in terms of the crowding-free kinetic coefficients ($k_{0,f}$, $k_{0,m}$ and $k_{0,M}$) and activity coefficients (γ_u, γ_f, γ_m and γ_M),

$$\begin{aligned} k_f &= k_{0,f}\exp\left(\frac{\Delta F_u - \Delta F_f}{kT}\right) = k_{0,f}\frac{\gamma_u}{\gamma_f} \\ k_m &= k_{0,m}\exp\left(\frac{\Delta F_u - \Delta F_m}{kT}\right) = k_{0,m}\frac{\gamma_u}{\gamma_m} \\ k_M &= k_{0,M}\exp\left(\frac{\Delta F_u - \Delta F_M}{kT}\right) = k_{0,M}\frac{\gamma_u}{\gamma_M}. \end{aligned} \tag{7}$$

ΔF_i, $i=u,f,m,M$, represents variations of Helmholtz functions accompanying the addition in the local environment of an extra molecule from the unfolded, folded or misfolded proinsulin species. ΔF_i are derived in terms of γ_i, as shown in Eq. (2). The characteristic values for $k_{0,m}$ and $k_{0,M}$, characterizing proinsulin misfolding under crowding-free conditions, are assumed to be much smaller than the rate of folding ($k_{0,f}$), $k_{0,m}=k_{0,M}\leq 0.01k_{0,f}$. All other coefficients are extracted from published experimental data, as follows. $k_{0,f}$ is essentially the inverse of the time interval (t) in which a proinsulin molecule forms its characteristic disulfide bridges under physiological, crowding-free conditions, $k_{0,f}\cong\frac{1}{t}$, where $t\cong 1$ min [58]. The translation time τ entering the kinetic rate k_t of proinsulin transport from the ER is adjusted in the present computation so that half of proinsulin molecules passed to the Golgi apparatus in $t_{1/2}\cong 15$ min [58,59]. In calculations, we assumed that the rate at which the "quality control" system is clearing the ER from toxic residues resulted from misfolded proinsulin is in the range of values similar to the transit rate k_t, $k_c\cong k_t$. The backward rates k_f', k_m' and k_M' were assumed to be small, e.g., ~1% of the forward rates, from reasons discussed above.

The initial conditions in the present stochastic numerical simulations are set to $Pi(t=0)=0$, ($i=u,m,M,f$), and $S(t=0)=N$. The extent of dilation of the ER (ΔV) is given in terms of the molecular volume (V_f) of the native proinsulin species. In the present computation, we considered $V_m\cong 0.5V_f$ and $V_M\cong 1.5V_f$ as test values for the molecular volumes of the two misfolded proinsulin species. The volume of the unfolded proinsulin molecule is set to $V_u\cong 2V_f$. A variation of the test molecular sizes V_i by ~50% does not affect significantly the amount of processed proinsulin (n_G) at the limit value $v\cong 2$ of the ER dilation (not shown). However, it is not difficult to understand that the relative difference between the molecular volumes of the folded and various misfolded proinsulin molecules is crucial for the accumulation of denaturated proinsulin molecules in the ER.

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