Endoplasmic Reticulum Overcrowding as a Mechanism of β-Cell Dysfunction in Diabetes

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ABSTRACT This study suggests a molecular mechanism that explains the accumulation of denaturated proinsulin in the endoplasmic reticulum (ER) of β-cells. Such states were frequently observed in β-cells experiencing increased demand for insulin production and were shown to lead to secretory dysfunction and diabetes. Here, a self-consistent kinetic model is used to investigate changes in protein translation due to ER overloading. The model is based on a molecular theory that relates the molecular composition and level of molecular crowding in the ER to the kinetic rates of protein folding/misfolding and transit to the Golgi apparatus (GA). This study suggests that molecular crowding forces can increase protein misfolding and impair the transport to the GA, thus overwhelming the quality control mechanism in the ER. A continual accumulation of toxic residues in the ER enhances even further the molecular crowding, accelerating protein denaturation. This article shows that molecular crowding affects differently the transit of various proteins through the ER. Apparently, the molecular crowding level that can inhibit the transport of native proinsulin to the GA influences to a lesser extent the transit of proamylin, a much smaller peptide cosynthesized with proinsulin in the ER. Smaller-volume misfolded proinsulin species may also win the passage competition through the ER and move on the secretory track. However, misfolded proinsulin fails the conversion to active insulin. This study can help us to decipher circumstances leading to the alteration of the secretory function in susceptible β-cells and the onset of diabetes.

INTRODUCTION

The endoplasmic reticulum (ER) serves several important functions, including posttranslational modification, folding, and assembly of newly synthesized secretory proteins, and its proper function is essential to cell survival. Under normal physiological conditions, the volume density of all proteins in this organelle can reach 100 mg/ml (1), of which >50% is proinsulin molecules (2). Another important component of the ER is proamylin, a much smaller peptide cosynthesized with proinsulin at a ratio of ~1:10 (3). Various conditions, such as increased metabolic demand and chronic hyperglycemia, raise the synthesis of proinsulin (and proamylin!) to high limits (4), overcrowding the endoplasmic reticulum (ER). Genetic mutations can also constitute a source of crowding for the secretory pathway, as indicated by recent data (5–10). The experiments revealed that mutant proinsulin species accumulate in the ER, pre-Golgi intermediates, and Golgi apparatus (6,8,9), leading to a three- to fivefold increase in the volume density in these compartments (6). Under normal conditions, 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 (1). In susceptible cells, the adaptation process fails, leading to stress and cellular dysfunction (1,11). All these data (4–9,11–22) suggest that molecular crowding may possibly represent a general feature of the diabetic β-cells. Previous computational simulations suggest that crowding effects become negligible if the ER dilation reaches a limit of 10-fold the volume of the protein load (23). Although experiments have demonstrated that the ER dilates significantly under chronic hyperglycemia (6), reaching this limit of extension could be rather difficult in susceptible β-cells. Therefore, a dramatic alteration of the secretory function of β-cells can ensue.

Increased molecular crowding in the ER can impair the secretory function of β-cells via two possible main mechanisms. First, protein transport from the ER to the Golgi apparatus (GA) is inhibited. Second, the continual increase of molecular crowding in the ER enhances the propensity of proteins to misfold. The formation of highly compressed nonnative structures is favored in an overcrowded environment, as the entropy gain from this compaction can be significant. Probably, smaller-volume protein species, such as proamylin and compacted misfolded proinsulin molecules, can win the passage competition through the ER and advance on the secretory track. Proamylin can eventually be converted to active amylin at the end of the secretory track, whereas misfolded proinsulin is likely to fail the conversion to insulin. Parallel changes of proinsulin and amylin blood levels are features of type-2 diabetes (24,25). The aim of this study is to predict effects of the increased protein loads in the ER on reaction rates that control the biosynthesis of proinsulin and proamylin. The approach can help us to identify secondary translational mechanisms that could have significant implications in the dysfunction of β-cells and onset of diabetes.

The basics of the molecular crowding theory can be found in works by Ellis (26), Ellis and Minton (27), and Hall and

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Minton (28). Crowding effects in biological processes rely on 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 a 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, misfolding, and aggregation processes (23,29–35). The net outcome of the steric effects on individual proteins can be modified by their translational motion (36). This is because the driving force involved in the transit of the molecules requires the existence of a gradient of concentration, which makes the crowd disperse in time. Inherent local fluctuations in the particle density may also alter the steric effects. Overall, the increase of crowding in the ER will slow down the local movement of proteins (28) and can foster other competing reactions, such as specific binding to other molecular species (26–28) and aggregation (30,32,33,35), making the processes even more complicated.

A mathematical model to simulate time-dependent crowding conditions in the ER was developed recently (23). The ansatz relates the molecular composition and level of molecular crowding in the ER to the kinetic rates of protein folding and misfolding. Here, the approach is extended to describe the effect of molecular crowding forces on the ability of proinsulin and proamylin to fold and transit the ER, thus advancing on the secretory track. From numerical simulations, the critical level of molecular crowding leading to the accelerated denaturation of proinsulin molecules will be predicted. In this limit, folding pathways that involve structural states with small volumes prevail over the native state, a fact that will become apparent from numerical simulations presented in this article.

MATHERIALS AND METHODS

The effect of molecular crowding, as described by the general theory (26–28), can be assessed by using an apparent chemical activity coefficient, γ. 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 species j, V_k stands for the average molecular volume of the crowding agent, and f is the characteristic volume fraction for molecular crowding.

Changes of the molecular composition in the ER under crowding conditions

The concept of molecular crowding can readily be adapted to assess changes of the secretory function in β-cells subject to increased protein loads in the ER. It is assumed that the typical size distribution of protein species in the ER consists of unfolded, folded, and misfolded states (Fig. 1). The misfolded states include species with molecular volumes (V_m) smaller than the characteristic volume, V_f, of the native states ($V_m \leq V_f$), as well as species with molecular volumes, V_M, larger than that of the native state ($V_M \geq V_f$). Therefore, the protein system consists of macromolecules that can be distinguished based on size. 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. 1 to describe the energy change induced by steric repulsion between constituent molecules of a multicomponent system can be obtained by assuming that V_k represents an effective volume (V_k^{eff}), as described previously (23). V_k^{eff} accounts for the molecular species more likely to contribute to the local crowding at the time of introducing the additional molecule into the local environment. Each protein system composing the local environment, i.e., proinsulin ($i = 1$) and proamylin ($i = 2$), contributes separately to V_k^{eff}. Therefore, V_k^{eff} can be approximated by $V_k^{\text{eff}} \cong \sum_{i,j} V_{ij}P_{ij}$, where $j = u, f, m$, and M, and $i = 1$ and 2. The index u represents the molecular volume of the unfolded protein i (the precursor of the folded/misfolded protein i) whereas m, M, and f stand for misfolded and natively folded states, as described above. The valueP_{ij} characterizes the probability densities in unfolded, misfolded, and natively folded states, so that $\sum_j P_{ij}(t) = 1$, and $j = u, f, m$, and M. As $P_{ij}(t)$ depends on time t, V_k^{eff} 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 ij at a given time t. The corresponding partial volume of each protein system is $V_i \cong N_i \sum_j V_{ij}P_{ij} + \Delta V$, and the characteristic fraction of molecular crowding can be approximated by $f \cong (1 + \Delta V(\sum_{i,j} V_{ij}P_{ij})^{-1})^{-1}$ (23). N_i is the average load of protein of type i in the ER, i.e., $i = 1$ is proinsulin and $i = 2$ is proamylin under normal translational regulation conditions. ΔV

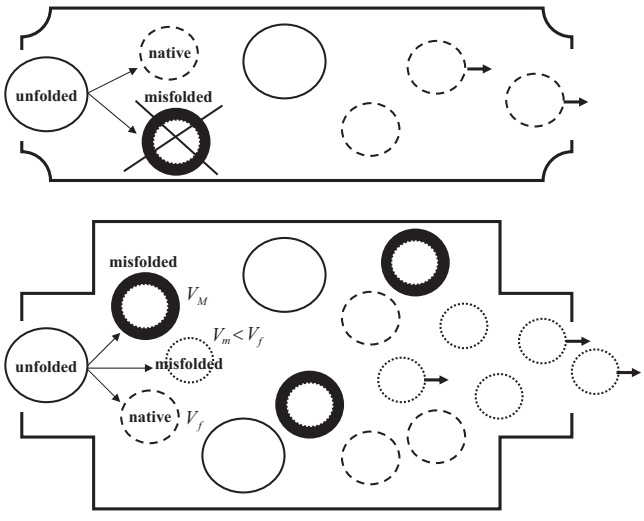

FIGURE 1 Pictorial representation of molecular crowding effects on the protein biosynthesis. Under normal physiological conditions (*upper*), proteins inserted in the ER are folded and transited to the GA. Misfolded species are degraded by the quality control mechanism in the ER. Molecular overcrowding in the ER can increase protein misfolding, overwhelming the quality control mechanism in the ER (*lower*). Small-volume misfolded protein species ($V_m < V_f$) are favored by crowding conditions and they can transit much easier to the GA. Accumulation of misfolded species (V_M) in the ER leads to a significant dilation of the ER.

represents the maximum possible volume extension in the ER that allows the protein to explore configuration states along their folding pathway (37,38), fold properly, and transfer easily to the GA. This approach remains 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.

For each protein system, the effective kinetic coefficients characterizing the transitions from unfolded to folded and misfolded states are derived in terms of the crowding-free kinetic coefficients ($k^0_{if}, k^0_{im},$ and k^0_{iM}) and activity coefficients (γ_{iu}, γ_{if}, γ_{im}, and γ_{iM}),

$$\begin{aligned} k_{if} &= k^0_{if}\exp\left(\frac{\Delta F_{iu}-\Delta F_{if}}{kT}\right) = k^0_{if}\frac{\gamma_{iu}}{\gamma_{if}} \\ k_{im} &= k^0_{im}\exp\left(\frac{\Delta F_{iu}-\Delta F_{im}}{kT}\right) = k^0_{im}\frac{\gamma_{iu}}{\gamma_{im}} \\ k_{iM} &= k^0_{iM}\exp\left(\frac{\Delta F_{iu}-\Delta F_{iM}}{kT}\right) = k^0_{iM}\frac{\gamma_{iu}}{\gamma_{iM}}. \end{aligned} \tag{2}$$

ΔF_{ij} ($j = u, f, m, M$; $i = 1, 2$) 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_{ij} values are derived in terms of γ_{ij}, as shown in Eq. 1, whereas k^0_{ij} can be extracted from experimental data (see Appendix 1).

The overall increase of crowding in the ER will slow down the local movement of proinsulin molecules (28). The effective rate of transit of proinsulin molecules through the ER toward the Golgi compartment can be approximated by (23)

$$g_{ij} = g^0_{ij}\frac{v^0}{v}, \tag{3}$$

where g^0_{ij} is the rate of transit of proinsulin (i = 1) and proamylin (i = 2) species ($j = f, m, M$) under physiological, noncrowding conditions and v^0/v is a correction due to the restriction on the movement of the molecules in a crowded environment (Appendix 2).

The complete scheme of the biochemical reactions involving each protein system i in the ER is shown in Fig. 2, and the corresponding system of kinetic equations reads

$$\begin{aligned} \frac{dP_{iu}}{dt} &= k_{is}S_i - \left(k_{if}+k_{im}+k_{iM}\right)P_{iu} + k'_{if}P_{if} + k'_{im}P_{im} + k'_{iM}P_{iM} \\ \frac{dP_{if}}{dt} &= k_{if}P_{iu} - k'_{if}P_{if} - g_{if}P_{if} \\ \frac{dP_{im}}{dt} &= k_{im}P_{iu} - k'_{im}P_{im} - g_{im}P_{im} - c_{im}P_{im} \\ \frac{dP_{iM}}{dt} &= k_{iM}P_{iu} - g_{iM}P_{iM} - c_{iM}P_{iM} \\ n_{Gi} &= g_{if}P_{if} + g_{im}P_{im} + g_{iM}P_{iM} \\ n_{Ci} &= c_{im}P_{im} + c_{iM}P_{iM} \end{aligned} \tag{4}$$

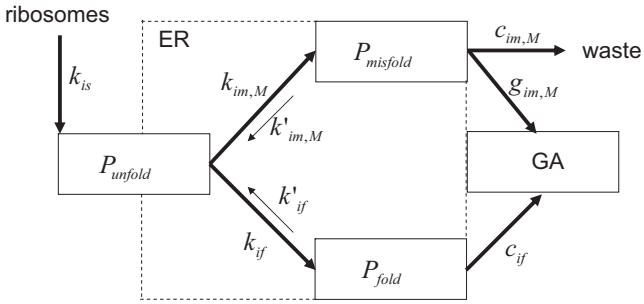

FIGURE 2 Scheme of the proinsulin kinetics used in this computation.

S_i is a function describing the variation in time of proinsulin ($i = 1$) and proamylin ($i = 2$) loads, $S_i = N_i \exp(-k_{is}\, t)$, and k_{is} stands for the characteristic kinetic rate. The value of k_{is} was set so that the lag time for processing the entire protein load (N_i) is ~2 h under physiological conditions (39). g_{ij} ($j = f, m, M$) denotes the rate of transport of proteins from the ER to the GA, and c_{ij} ($j = m, M$) is the rate of clearing misfolded structures from the ER. In calculations, it is assumed that the rate at which the quality control system clears the ER of toxic residues resulting from misfolded proinsulin (c_{ij}) is in the range of values similar to the transit rate (g_{ij}), $c_{ij} \cong g_{ij}$. k_{if}, k_{im}, and k_{iM} represent kinetic coefficients characterizing transitions of proinsulin molecules from unfolded to folded and misfolded states, whereas k'_{if}, k'_{im}, and k'_{iM} are kinetic coefficients of backward reactions. Molecular principles indicate that crowding conditions increase the propensity of macromolecules to form structural states characterized by small volumes (26–28). Therefore, backward rates, corresponding to proinsulin transitions from folded and misfolded states to the initial unfolded state, are much slower than forward rates ($k'_{if} << k_{if}$, $k'_{im} << k_{im}$, $k'_{iM} << k_{iM}$). 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 might be inhibited by molecular crowding. Thus, the reaction pathway $P_{if} \rightarrow P_{im,M}$ can be neglected within the approach described here.

Numerical computations

This study used an iterative computation scheme of the kinetic rates and probability densities corresponding to molecular states involved in the biosynthesis of proinsulin and proamylin to determine the optimal value of ΔV leading to maximum folding capabilities ($P_{if} >> P_{im,M}$).The value of ΔV was then systematically decreased to determine critical crowding conditions. The initial conditions in the stochastic numerical simulations presented here are set to $P_{ij}(t = 0) = 0$, ($i = u, m, M, f$; $i = 1, 2$), and $S_i(t = 0) = N_i$, $N_1/N_2 \cong 10$. The extent of dilation of the ER (ΔV) is given in terms of the molecular volume ($\sum_{i=1,2} N_i V_{if}$) of the native protein species. Here, this study considered $V_{2f}/V_{1f} \cong 0.6$ (the ratio of the molecular weight cubic roots of the two peptides) and $V_{im} \cong 0.5\, V_{if}$ and $V_{iM} \cong 1.5\, V_{if}$ as test values for the molecular volumes of the two misfolded protein species. The volume of the unfolded protein molecule is set to $V_{iu} \cong 2\, V_{if}$.

One more thing needs to be discussed at this point. The kinetic rates of proinsulin misfolding under crowding-free conditions, $k_{0,\,m}$ and $k_{0,\,M}$ here, are assumed to be much smaller than the rate of folding, $k_{0,\,f}$. This reflects the natural tendency of proteins to form native structures, as well as the ability of specialized components of the ER to target misfolded proteins and degrade them. Fig. 3 observes generic temporal evolutions of misfolded proteins ($n_C = n_{C1} + n_{C2}$) cleared by the quality control mechanism in the ER for $k^0_{im} = k^0_{iM} = 0.001\, k^0_{if}$, $k^0_{im} = k^0_{iM} = 0.01\, k^0_{if}$, and $k^0_{im} = k^0_{iM} = 0.1\, k^0_{if}$. As expected, k^0_{im} and k^0_{iM} are critical for the probability distributions

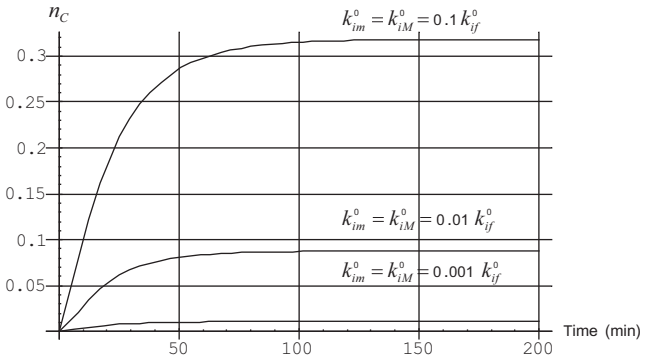

FIGURE 3 Generic temporal evolutions of the amount of misfolded proinsulin cleared by the quality control mechanism in the ER for $k^0_{im} = k^0_{iM} = 0.001\, k^0_{if}$, $k^0_{im} = k^0_{iM} = 0.01\, k^0_{if}$, and$k^0_{im} = k^0_{iM} = 0.1\, k^0_{if}$.

of various protein species in the ER. However, k^0_{im} and k^0_{iM} enter as scaling factors in computations and should not affect the conclusions pertaining to the crowding effects in the ER. All results reported in the next section correspond to folding conditions characterized by $k^0_{im} = k^0_{iM} = 0.01\ k^0_{if}$.

RESULTS

The approach presented here provides the mathematical correlation between molecular crowding, protein folding, and translation within the secretory track of a cell. The approach can be used to estimate the alteration of the biochemical processes involved in the biosynthesis of proinsulin and proamylin in β-cells experiencing increased protein loads in the ER (hyperglycemic conditions). Stochastic numerical simulations carried out in this study suggest that volume exclusion effects due to overcrowding the early part of the secretory pathway are critical to the amount of processed proinsulin. Under conditions in which the volume available in the ER for adding newly synthesized proinsulin molecules drops to low values, e.g., the ratio $v = \Delta V / \sum_i N_i V_{if}$ between the dilation of the ER (ΔV) and the mean volume of proinsulin and proamylin ($\sum_i N_i V_{if}$) is in the range $v < 2$, molecular crowding effects can decrease dramatically the amount of proinsulin passing to the GA. This can be inferred from Fig. 4, which displays the evolution in time of the total amount of proinsulin (both folded and misfolded species) passed to the GA (N_{G1}) relative to the initial value of the proinsulin load (N_1), $n_{G1} = N_{G1}/N_1$. Note the progressive decrease of n_{G1} with decreasing v. $v \cong 1$ corresponds to a sudden drop of n_{G1}. A further decrease of v is critical for folding and transport of proinsulin to the GA. Under conditions assumed by the numerical simulations presented here, the amount of proinsulin passing through the ER to reach the GA for $v < 1$ is mainly constituted by misfolded proinsulin species that escaped the degradation process.

Based on Eq. 1, it is not too difficult to understand that in an overcrowded environment, folding pathways that involve equilibrium states as well as transition states (40) with 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 (Fig. 5 *a*). Fig. 5 *b* compares the probability densities in the native state (P_{1f}) and misfolded states (P_{1m}) of proinsulin molecules characterized by small molecular volumes ($V_{1m} < V_{1f}$), at critical crowding conditions corresponding to $v = 2$ and $v = 1$. This study can observe that an upsurge in the local molecular crowding much over the physiological level becomes a source of accelerated denaturation of proinsulin molecules. In an overcrowded environment, folding pathways that involve structural states with small volumes

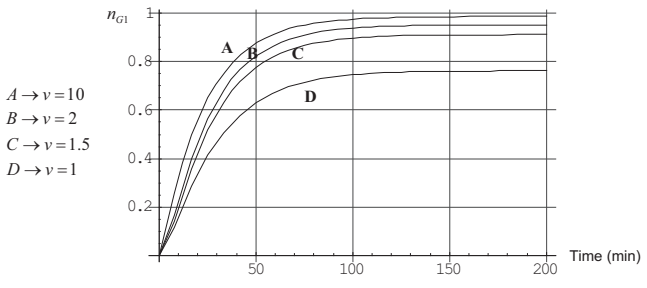

FIGURE 4 The evolution in time of the amount of folded proinsulin passed to the Golgi apparatus (N_{G1}) relative to the initial value of the proinsulin load (N_1), $n_{G1} = N_{G1}/N_1$.

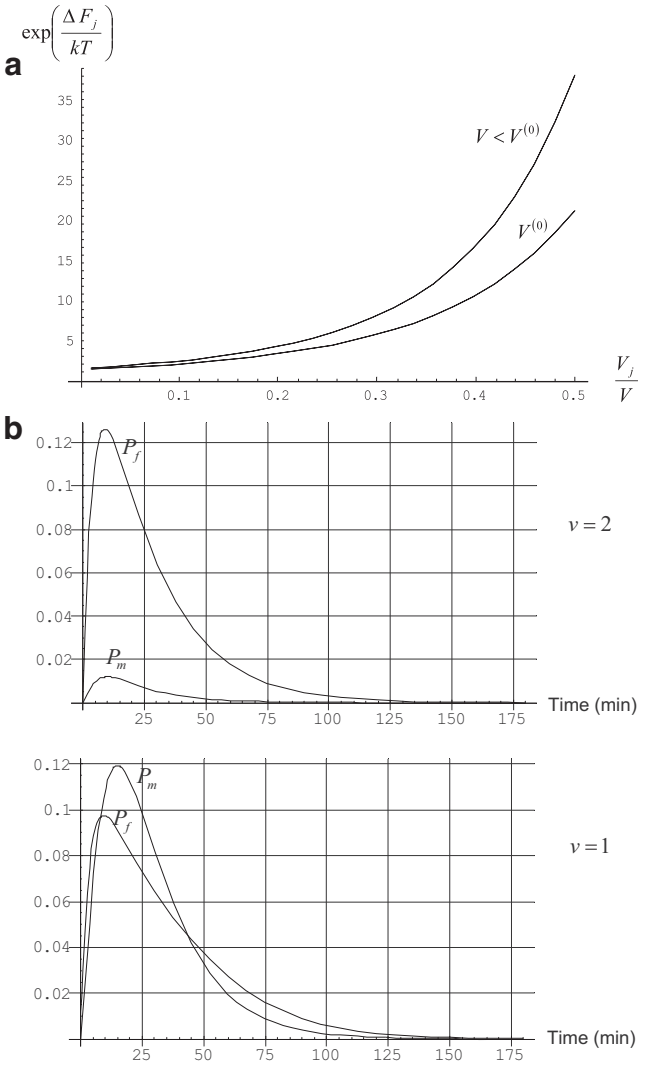

FIGURE 5 (*a*) Variation of the Helmholtz function as a function of the volume (V_f) of the molecule to be added in a crowded environment for $f = 0.3$ (see Eq. 1). Calculations were done for two different volumes of the local environment, V and $V^{(0)}$ ($V < V^{(0)}$). For simplicity, it was assumed that $V_j = V_k$. From the energy change, it was inferred that folding pathways that involve equilibrium states, as well as transition states having small volumes, will prevail over those requiring larger volumes. (*b*) Probability densities P_f and P_m corresponding to the natively folded and misfolded states ($V_m < V_f$) for increased crowding conditions $v = 2$ and $v = 1$.

(V_{1m}) will prevail over the native state of a volume (V_{1f}) and states requiring larger volumes (V_{1M}).

The effect of crowding on the amount of processed proinsulin depends strongly on the initial load of proinsulin and proamylin ($N_1 + N_2$) and the degree of dilation (ΔV) of the ER and, to a lesser extent, molecular volumes (V_{ij}, $j = u,f,m,M$; $i = 1,2$). A variation of the test molecular sizes, V_{ij}, by ~50% does not affect significantly the amount of processed proinsulin (n_{G1}) at the limit value $v \cong 2$ of the ER dilation (not shown). However, the relative difference between molecular volumes of possible misfolded proinsulin molecules is crucial for controlling the predominant misfolded species, as shown in Fig. 3. Not surprisingly, molecular crowding forces leave the biosynthesis of proamylin ($i = 2$) practically unaltered. For $v = 2$, there is almost no molecular crowding effect on the transit of proamylin to GA. Under critical molecular crowding conditions, i.e., $v = 1$, <1% of proamylin molecules are restrained from passing to the GA. Fig. 6 compares the total amount of proinsulin and proamylin passed to the GA, $n_G = N_{G1} + N_{G2}/N_1 + N_2$, under crowding free ($v = 10$) and critical crowding ($v < 1$) conditions. I observe that for $v = 0.75$, the fraction of proamylin ($i = 2$) transited to the GA is almost similar to that corresponding to misfolded proinsulin ($i = 1$).

DISCUSSION

Starting from basic molecular principles, this study derived simple mathematical correlations between the increase of molecular crowding in the ER of a β-cell and the decrease of the amount of native proinsulin processed in the ER and transported to the GA. This study suggests that volume exclusion effects generated by molecular crowding induced by an intense upsurge of the proinsulin load in the ER and/or an accumulation of misfolded proinsulin can reduce the efficiency of the ER to process proinsulin molecules. Moreover, the proper precursor of the natively folded proinsulin (i.e., the unfolded proinsulin) may also tend to misfold under crowding conditions. From Figs. 4 and 5, I can infer that the accumulation of misfolded proinsulin enhances even further the propensity for misfolding and restricts the transfer of native proinsulin molecules to the GA. Smaller-volume, misfolded proinsulin species that escaped from the quality control mechanism in the ER may win the transit competition and advance on the secretory track. As shown in Fig. 5 *b*, within a highly crowded ER, misfolded proinsulin represents the majority of the molecular species advancing on the secretory track. However, these misfolded proinsulin molecules would not be susceptible to conversion to active insulin in secretory vesicles and may be secreted unprocessed in the blood (42) (reviewed by Porte and Kahn (41)). This is the main result of the report presented here. It suggests that secondary translational mechanisms can have significant implications in the accumulation of misfolded proinsulin in the ER.

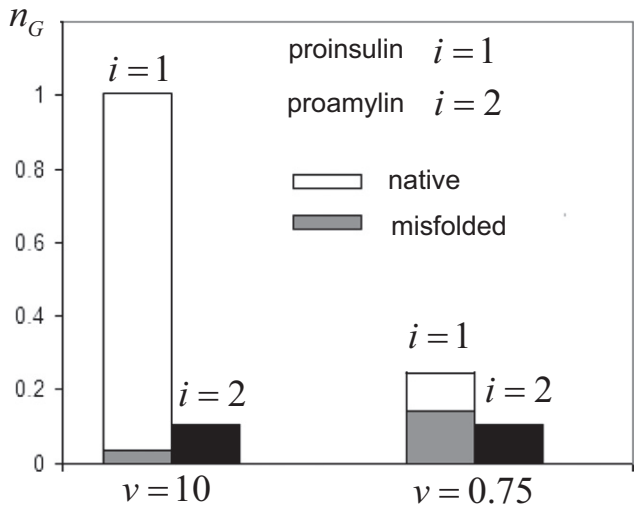

FIGURE 6 Molecular crowding forces leave the biosynthesis of proamylin ($i = 2$) practically unaltered. Under critical molecular crowding conditions, i.e., $v < 1$, <1% of proamylin molecules are restrained from passing to the GA. For $v = 0.75$, the fraction of proamylin transiting to the GA is almost similar to that corresponding to misfolded proinsulin ($i = 1$).

A similar computational approach was used here to assess the biosynthesis of proamylin. Not surprisingly, the effects of molecular crowding on proamylin synthesis are minimal, according to the numerical simulations described here. This is because proamylin has a much smaller molecular volume than proinsulin, so that the level of molecular crowding inhibiting the synthesis of proinsulin leaves the proamylin translation unaltered. Therefore, the net effect of molecular crowding in the ER is an imbalance of the two main secretory lines of the β-cell, proinsulin $\rightarrow$ insulin and proamylin $\rightarrow$ amylin (as displayed in Fig. 6). Normally, the synthesis of amylin, also called islet amyloid polypeptide (IAPP), is coordinated with that of insulin, but in many patients with diabetes, especially type-2 diabetes, the synthesis of IAPP increases as that of insulin decreases (25). Human IAPP is amyloidogenic and responsible for the formation of toxic preamyloid oligomers in pancreatic islets, β-cell death, and development of type-2 diabetes mellitus (43,44). Recently, I discovered accumulation of IAPPs in failing hearts of diabetic patients (45), suggesting that toxic IAPP oligomers can circulate in the blood. Understanding conditions favoring the increased secretion of IAPPs in the blood and IAPP oligomer formation is therefore crucial for linking type-2 diabetes to heart failure (45). According to results presented here, the change of the insulin/IAPP ratio in diabetes could reflect molecular overcrowding of the ER and subsequent secretory dysfunction of β-cells.

In the extreme case of chronic hyperglycemia, overloading of the ER may occur frequently. The volume available in the ER for adding new unfolded proteins for processing drops to low values, which may lead to intense crowding

effects. Therefore, increased protein synthesis in these cells requires that both the size of the ER and its accessory components undergo continuous adjustment (4). 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 (46,47). 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 (1). Augmenting the capacity of the ER quality control system can, in principle, reduce crowding effects (1). However, the rate of clearing the ER from misfolded proinsulin must increase considerably under acute crowding conditions.

Numerous studies have indicated that stress on the ER is the main cause of β-cell dysfunction leading to the development of diabetes (15–17,19–22). It has been demonstrated (16,17,19–22) that pancreatic β-cells respond to ER stress by activating the unfolding protein response. This is part of a complex mechanism by which cells limit or repair the molecular damage. The mechanism involves an increased synthesis of molecular chaperones (48), which can protect unfolded proteins to aggregate or target misfolded proteins for degradation. If ER stress is prolonged, or the adaptive response fails, apoptosis is triggered (21,43,44). The presence of considerable amounts of denaturated protein in apoptotic cells (43,44) is clear evidence that the decrease of the β-cell mass is a consequence of the failure of the protein regulation mechanism (49). The analysis presented here reveals that a continual accumulation of toxic residues enhances molecular crowding even further, thus accelerating proinsulin denaturation and dysfunction of the β-cell. These findings support recent experimental results showing that supranormal production of nonnative proinsulin may predispose the ER to cell toxicity and premature loss of pancreatic β-cells (8,9,50–52).

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 (6) along the secretory pathway. Based on the known proinsulin-to-insulin conversion in immature secretory granules, immunogold labeling for C-peptides detectable in the ER and up to the Golgi apparatus reflects proinsulin immunoreactivity, whereas that over immature and mature secretory granules represents free C-peptides. 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 β-cell. However, a direct proof of the concept of crowding-induced misfolding requires the use of engineered mutant proinsulin molecules that, when translocated into the ER, form molecular aggregates that cannot be exported. The insertion of a critical amount of such proteins will alter the ability of the ER to fold endogenous proinsulin, which will be reflected in a shortage of the insulin secreted from the β-cell. Recently, Engel et al. (35) published an experimental approach to evaluate the effects of crowding on the folding of a model protein in vitro (35). Their work shows that molecular crowding compacts the unfolded test protein and favors the aggregation of intermediate folding products, which is in line with the results of the numerical simulations presented here.

APPENDIX 1

The kinetic coefficient k^0_{1f} can be estimated from published experimental data. For proinsulin ($i = 1$), this is essentially the inverse of the measured folding time (t) under physiological, crowding-free conditions, $k^0_{1f} \cong \frac{1}{t}$, where $t \cong 1$ min (53). As proinsulin and proamylin are cosynthesized in the ER and undergo similar structural transformations along the secretory track, it is assumed that $k^0_{1f} \cong k^0_{2f}$. The kinetic rates of protein misfolding under crowding-free conditions, k^0_{im} and k^0_{iM}, are assumed to be much smaller than the rate of folding, k^0_{if}. This reflects the natural tendency of proteins to form native structures, as well as the ability of specialized components of the ER to target misfolded proteins and degrade them. Within the preliminary computations, various test values for k^0_{im} and k^0_{iM}, such as $k^0_{im} = k^0_{iM} = 0.001\ k^0_{if}$, $k^0_{im} = k^0_{iM} = 0.01\ k^0_{if}$, and $k^0_{im} = k^0_{iM} = 0.1\ k^0_{if}$ were used. Generic temporal evolutions of the amounts of misfolded proinsulin cleared by the quality control mechanism in the ER are displayed in Fig. 3.

APPENDIX 2

Equation 3 reads $g_{ij} = g^0_{ij}\ v^0/v$, where g^0_{ij} is the rate of transit of proinsulin ($i = 1$) and proamylin ($i = 2$) species ($j = f,\ m,\ M$) under physiological, noncrowding conditions and v^0/v is a correction due to the restriction on the movement of the molecules in a crowded environment (23). g^0_{ij} is a function of the average time of transit (τ) of proteins through the ER and the relative change in size of the molecular species during this transit, $C_{ij} \cong V_{if}/V_{ij}$, $j = f, m, M$, $i = 1, 2$. Therefore, g^0_{ij} can be written as $g^0_{ij} \cong C_{ij}/\tau$. The translation time, τ, will be adjusted in computations so that half of the protein molecules will pass to the Golgi apparatus in $t_{1/2} \cong 15$ min (53,54). $v^0 = V^0_1 + V^0_2/N^0_1 + N^0_2$ and $v = V_1 + V_2/N_1 + N_2$ are specific volume densities in the ER, which can be derived as described in the Computation section. The former represents the volume density under physiological, crowding-free conditions, i.e., $N^0_1\ V^0_{1u} + N^0_2\ V^0_{2u} << \Delta V$, where V^0_i is the partial volume of each protein system and N^0_i stands for its load in the ER, under crowding-free conditions. The latter is the volume density under increased crowding conditions, for which it was assumed $N_1 + N_2 >> N^0_1 + N^0_2$, so that the ER reaches the limit of dilation ($\Delta V \rightarrow 0$) (23).

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