PROINSULIN DISULFIDE MATURATION AND MISFOLDING IN THE ENDOPLASMIC RETICULUM

Ming Liu*, Yulin Li§, Douglas Cavener§, and Peter Arvan*¶

Running Title: Disulfide Mispairing in Proinsulin

Upon nonreducing tris-tricine-urea-SDS-PAGE, newly-synthesized proinsulin from pancreatic islets of normal rodents forms a predominant band of fast mobility representing the native disulfide isomer, which is efficiently secreted. In addition are recovered at least two slower-migrating "isomer #1 and #2" bands, not discernible under reducing conditions, which represent a minor proportion of molecules that exhibit less efficient secretion. Although rats and mice have two proinsulin genes, three distinct-migrating species are also produced upon proinsulin expression from a single wild-type human proinsulin cDNA. The 'Akita-type' proinsulin mutation, which causes dominant-negative diabetes mellitus due to point mutation C(A7)Y that leaves B7-cysteine without its disulfide pairing partner, is recovered as a form that near quantitatively co-migrates with the aberrant "isomer #1 band" of proinsulin. Anomalous migration is also demonstrated for several other proinsulin mutants lacking a single cysteine. In islets from PERK-/- mice, which exhibit premature loss of pancreatic beta cells, the hypersynthesis of proinsulin increases the amount of nonnative proinsulin isomers. Such findings appear consistent with an hypothesis that supranormal production of nonnative proinsulin forms may predispose to pancreatic beta cell toxicity.

Proinsulin is comprised sequentially of three regions, the amino-terminal B-chain, the proteolytically-removable C-peptide linker region, and the carboxyl-terminal A-chain. Proinsulin contains six cysteine residues that form the three evolutionarily-conserved disulfide bonds of the insulin/IGF-1 superfamily, including B7-A7 and B19-A20 interchain pairs and the A6-A11 intrachain pair. At 4.5 amino acids translated per second (1), it can be estimated to require 25 s or less to synthesize nascent preproinsulin in the endoplasmic reticulum, and the three disulfide bonds are expected to form either cotranslationally or shortly thereafter (2).

Recent evidence suggests that B-chain mutants have increased disulfide mispairing in the secretory pathway (3; 4), even for certain mutants that exert a generally favorable effect on the ultimate stability of the native state (5). Certainly, related members of the insulin/IGF-1 family are predisposed to forming nonnative disulfide bridges during progression through the protein folding pathway (6-8). Of the 15 potential disulfide isomers that might be formed, a few specific disulfide mispaired combinations, called swap I and swap II isoforms, may be preferred (9; 10), although these conclusions are based primarily on folding studies performed in vitro.
Recent hypotheses suggest that a subpopulation of wild-type proinsulin might also misfold in pancreatic beta cells (11). Proinsulin misfolding would appear to be linked to the dominant negative pathogenesis of diabetes mellitus in the 'Akita' diabetic mouse, in which one allele of mouse proinsulin-II has the C(A7)Y mutation, which leaves B7-cysteine without its natural disulfide pairing partner (12; 13). However, recent studies have thus far been unable to discern any qualitative or quantitative differences in proinsulin folding either in the islets of Akita mice (versus normal mice) or in CHO cells expressing the Akita-type mutant proinsulin (11).

We have been recently engaged in the analysis of disulfide maturation of newly-synthesized insulin precursor by nonreducing tris-tricine-urea-SDS-PAGE, as the method has excellent sensitivity to disulfide mispairing (5; 14). We now report distinct monomeric proinsulin structures produced in normal pancreatic islets or from the wild-type proinsulin cDNA, as well as a proinsulin cDNA expressing the Akita-type mutation. The results for the first time identify qualitative and quantitative differences in folding between wild-type and mutant proinsulin and also indicate a subpopulation of wild-type proinsulin molecules engaged in intramolecular disulfide mispairing.

Materials And Methods

Materials — Guinea pig anti-insulin was from Linco (St. Charles, MO). Polyclonal anti-BiP was from ABR. Zysorbin was from Zymed Laboratories. 35S-methionine/cysteine mixture was from ICN. Methionine/cysteine-deficient DMEM, dithiothreitol, and Ficoll 400 were from Sigma. MG115 was from Calbiochem. Isopropyl-beta-D-thiogalactopyranoside (IPTG) and proteinase inhibitor cocktail were from Roche Molecular Biochemicals.

Isolation and Labeling of Rat and Mouse Pancreatic Islets — Islets were isolated from male Sprague-Dawley 200 - 250 g rats. Briefly, the pancreas was retro-perfused with collagenase, excised and digested in a sterile tube, filtered through a stainless steel mesh, and islets enriched by discontinuous Ficoll gradient centrifugation. Isolated islets were washed and recovered overnight in RPMI 1640 with 11.1 mM glucose containing 10% fetal bovine serum plus 1% penicillin-streptomycin before experiments. Islets from 18 day old PERK-/- and PERK+/+ mice (15) were also isolated by pancreatic collagenase digestion, harvesting by gradient centrifugation, and finally recovered overnight in serum-containing medium.

Islets were washed twice in methionine- and cysteine-deficient medium plus 1% RIA-grade BSA, 50 ug/ml soybean inhibitor, and 10 mM HEPES pH 7.35. The islets were then metabolically labeled with 35S-amino acids in the same medium, and when a chase was employed (Fig. 1), it included complete medium plus 10% fetal bovine serum.

Proinsulin mutagenesis — A human proinsulin cDNA was subcloned into the pcDNA3 vector. With this as template, mutant proinsulin cDNAs were created by three PCR reactions. Reaction #1 used forward and reverse primers to amplify a cDNA stretch encoding the signal peptide and the downstream site of the introduced mutation. Reaction #2 used another set of primers to amplify a cDNA encoding the mutation point as well as the
remaining carboxyl-terminal region of proinsulin. The products from reactions #1 and #2 were designed to share ~20 bp overlap in the region of the introduced mutation. Finally, a third PCR reaction used the primers at the 5' and 3' ends of the proinsulin coding sequence along with both gel purified products from the first two PCR reactions as template, thereby generating the full-length mutant proinsulin cDNA. These were gel purified and ligated into the pGEM T-vector (Promega). Mutations were confirmed by direct DNA sequencing, and the mutants subcloned into pcDNA3 using KpnI and EcoRI restriction sites.

SDS-PAGE — In this paper, unless expressly stated, all media and subsequently-employed buffers were devoid of reducing reagents. In selected experiments, pre-treatment of live cells before lysis with 10 mM N-ethyl maleimide to block free thiols had no effect on the fractional recovery of newly-synthesized nonnative proinsulin isomers. A resolving gel (11.2% acrylamide, 0.35% bis-acrylamide, 10% Prosieve-50, 6.0 M urea, 0.1% SDS in 1.0 M Tris, pH 8.45) was overlaid with 0.4 cm height of spacer gel containing 9.3% acrylamide, 0.6% bis-acrylamide, 0.1% SDS in 1.0 M Tris, pH 8.45, without Prosieve or urea. The stacking and running the tris-tricine-urea-SDS-PAGE was as previously described (5). ¹²⁵I-proinsulin gel standard was from Linco (catalog #9015).

RESULTS and DISCUSSION

Proinsulin Synthesis in Rat Pancreatic Islets— We examined by nonreducing tris-tricine-urea-SDS-PAGE (14) the immunoprecipitable proinsulin synthesized in rat pancreatic islets during a 5 min ³⁵S-amino acid labeling. The newly-synthesized proinsulin existed as a major faster-migrating species as well as two faintly-detected slower-migrating species (arrows highlighted by asterisk, Fig. 1). While proinsulin conversion intermediates also migrate more slowly than proinsulin under these gel conditions (16), a 5 min period is too short an interval for nascent proinsulin to have initiated proteolytic processing in immature secretory granules (17). After a 30 min chase following the 5 min labeling, more slowly-migrating bands appeared, consistent with conversion intermediates (bracket, Fig. 1). Under such short labeling conditions, none of the proinsulin forms were co-precipitated from cell lysates during immunoprecipitation with a

Immunoprecipitation — At the end each experiment, cell lysates (and chase media if any) were treated with a proteinase inhibitor cocktail, pre-cleared with Zysorbin and subjected to immunoprecipitation with guinea pig anti-insulin as previously described (14). Immunoprecipitates were boiled for 5 min in gel sample at a final concentration of 1% SDS, 12% glycerol, and 0.0025% serva blue in 50 mM Tris, pH 6.8.
polyclonal antibody against the ER molecular chaperone, BiP.

When radiolabeling was extended to 30 min, more $^{35}$S-proinsulin was recovered. In conjunction with this was the increased biosynthesis of the two slower-migrating species (marked with arrows). During the 30 min labeling period, conversion intermediates (marked with bracket) were already apparent. These bands increased in abundance during a subsequent 30 min chase in conjunction with appearance of the labeled insulin band, but this was not observed for the anomalously slow-migrating species of proinsulin. Rather, the slow-migrating proinsulin was co-precipitated from cell lysates during immunoprecipitation with anti-BiP, despite that this subpopulation is minor by comparison to the faster-migrating native proinsulin. Thus, proinsulin exists as a large native-folded subpopulation plus minor isomeric subpopulations that have achieved a different, nonnative conformation.

Recombinant Proinsulin Synthesis in Heterologous Cells— Rat pancreatic beta cells synthesize both proinsulin-I and proinsulin-II gene products. To determine whether the minor, slower-migrating proinsulin species were unique to rat islets, we examined heterologous cells transfected to express the polypeptide encoded by a human proinsulin cDNA, which has a slightly different sequence than that of rat proinsulins. Fig. 2A shows full-length gels derived from insulin immunoprecipitation of untransfected and transfected CLA14 cells and their bathing media; these cells were IPTG-induced to overexpress ATF6 and ER molecular chaperones, which tends to increase recombinant secretory protein expression (14). The first two lanes of Fig. 2A indicate the spectrum of nonspecific radiolabeled bands recovered from these cells, and medium. After transfection with a proinsulin cDNA, nonreducing tris-tricine-urea-SDS-PAGE analysis revealed three newly-synthesized proinsulin species (although the band spacing is slightly different than that from rat islets), with the fastest band predominating and already beginning to be secreted during a 50 min chase. These bands collapsed into a single species of slower mobility under reducing conditions, strongly indicating that the minor, slower-migrating proinsulin bands are derived from a common translation product representing isomers that differ from native proinsulin by the pattern of their disulfide bonds. To confirm this point we performed a two-dimensional gel analysis (Fig. 2B). 293T cells were transfected with the proinsulin cDNA and three specific insulin-immunoprecipitable bands were recovered by nonreducing SDS-PAGE (lanes 2, 3) over and above background from cells transfected with empty vector (lane 1). Individual species were excised from the nonreducing gel. Original samples from mock-transfected or transfected cells (that had not previously been subjected to eletrophoresis) were compared under reducing conditions (lanes 6, 7) against each of the nonreduced excised bands that were now re-run in a second, reduced, dimension. A standard of chemically-purified iodinated proinsulin was run as a control under nonreduced (lane 5) or reduced (lane 11) conditions. The data establish that each of the nonreduced proinsulin species co-migrate with reduced proinsulin in the second dimension (Fig. 2B).

When exploring the fate of heterologously expressed proinsulin in 293T cells, we found that these distinct, heterologously-expressed proinsulin forms do not appear to be handled identically in
the secretory pathway. Specifically by 4 h of chase, essentially all of the major, faster-migrating proinsulin band went on to be secreted (Fig. 2C) while the slower-migrating species that were recovered intracellularly at 1 h were not fully recovered at the 4 h chase time, being only partially secreted (more about this, below).

Two exposures of the same gel are shown in Fig. 2D, to enhance visibility of more poorly recovered proinsulin forms. Wild-type proinsulin, as before, was recovered primarily as the well-secreted native isomer and two more slowly-migrating bands that are arbitrarily named isomer #1 and isomer #2 bands, respectively. It is clear from the darker exposure that during the 2.5 h chase the native proinsulin band was secreted with high efficiency (~90%) while the isomer #1 band was also secreted, but with ~50% efficiency, and the isomer #2 band was secreted with still lower efficiency. The different percentages of these isoforms recovered from cells vs. media and their identity under reducing conditions indicates that the isoforms differ by disulfide pairing and are not created artifactualy during sample preparation for electrophoresis.

Expression in islet beta cells of proinsulin lacking one of the 6 critical Cys residues (Cys-A7) is responsible for dominant-negative pathogenesis in the Akita diabetic mouse (11-13; 18-20). It was therefore of interest to examine proinsulin mutants lacking individual cysteine residues in relation to the electrophoretic mobility of wild-type proinsulin isomers. We began by creating point mutants in the least important and most important proinsulin disulfide bonds (Fig. 2D). It has already been reported that a mutant lacking both A6 and A11 cysteines (which normally produce the intrachain disulfide bond of insulin) does not prevent formation of a band with normal gel mobility nor the ability of the protein to be secreted (14; 21). Expression of the C(A11)S proinsulin mutant yielded two bands: the faster (minor) form essentially co-migrated with native proinsulin and was secreted with high efficiency, and a band running similarly to the isomer #1 band that was secreted with ~50% efficiency. This is consistent with a portion of the C(A11)S single mutant to have a simple unpaired A6-cysteine yet form the critical B7-A7 and B19-A20 disulfide bonds, resulting in a band of normal mobility and secretion behavior. By contrast, most C(A11)S mutant co-migrating with the isomer #1 band is likely to have disturbed formation of either the B7-A7 or B19-A20 disulfide bond, probably because of intramolecular attack by the unpaired A6-cysteine residue. Interestingly, expression of the C(A20)A mutant yielded only a single band: a form of the slowest mobility, nearly co-migrating with the isomer #2 band of proinsulin, unable to be secreted.

It has previously been reported that the misfolding of the Akita mutant of mouse proinsulin-II, C(A7)Y, cannot be distinguished from the folding of normal mouse proinsulin-II, when expressed either in pancreatic islets or in CHO cells (11). We therefore introduced the C(A7)Y mutation into the human proinsulin cDNA and examined the recombinant product in 293T cells; a representative experiment (in duplicate) is shown in Fig. 3A. A decreased amount of the radiolabeled hAkita mutant was recovered by nonreducing tris-tricine-urea-SDS-PAGE. Moreover, the recovered hAkita mutant proinsulin was quite obviously distinct in appearance from that of wild-type proinsulin, exhibiting no band comparable to the native proinsulin species, with most of the population co-migrating with the
isomer #1 band of proinsulin. Virtually none of the hAkita mutant was secreted at any chase time (data not shown), unlike the (albeit impaired) secretion of the isomer #1 band of wild-type proinsulin.

*En route* to the manufacture of all six single Cys mutants of proinsulin, we have examined the C(B19)A point mutant, which leaves A20-cysteine unpaired. Once again, the mutant proinsulin nearly quantitatively failed to form a band comparable to the native proinsulin isomer, and was not secreted. Instead, the C(B19)A proinsulin largely disappeared intracellularly after a 5 h chase in a manner that was partially inhibited by the presence of the proteasome inhibitor MG115 (Fig. 3B). Such a finding suggests that at least some of the nonnative proinsulin isomers undergo disposal by ER-association degradation via the ubiquitin-proteasome system.

Taken together, the data in Figs. 2 and 3 prove that multiple proinsulin isoforms can be produced from a single proinsulin translation product, and that distinct disulfide pairings lead to discrete proinsulin bands that have different fates within the secretory pathway.

*Proinsulin Isomers in Islet beta Cells of Mice Deficient for PERK*—Mice with knockout of the PERK gene product develop severely decreased beta cell mass leading to overt diabetes mellitus in the fourth postnatal week (15; 22). Prior to the onset of diabetes, supranormal biosynthesis of proinsulin in the pancreatic islets has been observed. (22). The dysregulation of proinsulin biosynthesis is probably the result of a failure of the normal PERK phosphorylation of the eukaryotic translational initiation factor eIF2alpha on residue Ser51, because homozygous mice with knock-in of eIF2alpha-S51A also display deficiency of pancreatic beta cells (23). We therefore chose to examine proinsulin biosynthesis from the islets of wild-type and PERK−/− mice by nonreducing tris-tricine-urea-SDS-PAGE, in relation to total protein synthesis followed by conventional Laemmli SDS-PAGE (24). As shown in Fig. 4 in islets from wild-type mice, proinsulin synthesis was greater at a prevailing glucose concentration of 17 mM than at 5 mM, and this was accompanied by an increase in total islet protein synthesis. When comparing islets of PERK−/− mice to wild-type mice, proinsulin biosynthesis was increased relative to total protein synthesis, especially at high glucose. In conjunction with increased proinsulin biosynthesis was an increase in slower-migrating proinsulin isomers (marked with downward arrows). Once again, these bands were distinct from mouse proinsulin conversion intermediates (standards from betaTC3 cells shown at right). The slower-migrating proinsulin isomers were significantly increased in absolute amounts but not significantly increased in relative amounts (~17%) of proinsulin, yet these findings confirm the hypothesis that islet beta cells of PERK−/− mice exhibit supranormal production of nonnative proinsulin (25). In conjunction with the discovery of dominant-negative diabetes in the Akita mouse, the current results appear consistent with a view that increased abundance of misfolded proinsulin may predispose to pancreatic beta cell toxicity (19).
REFERENCES

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FIGURE LEGENDS

Fig. 1. Newly-synthesized proinsulin in rat pancreatic islets analyzed by nonreducing tris-tricine-urea-SDS-PAGE. Isolated rat islets were divided into distinct samples that were radiolabeled with $^{35}$S-amino acid mixture for the times indicated and then lysed immediately or after a 30 min chase period. Fifty islets were used for each sample. The cell lysates and 30 min chase medium were immunoprecipitated with a polyclonal antibody to insulin that recognizes all insulin forms. The immunoprecipitates were analyzed by nonreducing tris-tricine-urea-SDS-PAGE. In addition to the established positions of proinsulin (Pro), insulin (Ins), and conversion intermediates (bracket), two proinsulin bands migrating more slowly than native proinsulin were identified (double arrows marked with asterisk) and one of these forms appears to be co-precipitated with anti-BiP.

Fig. 2. Recombinant proinsulin synthesized from the human proinsulin cDNA in heterologous cells. (A) Forty-eight hours after transfection (Proins) or mock-transfection (Con) of the CLA14 subclone of CHO cells (14), the cells were pulse-labeled with $^{35}$S-amino acids for 1 h and chased for 50 min. The cells ("C") were lysed, chase media ("M") collected, and both were analyzed by tris-tricine-urea-SDS-PAGE under nonreduced or reduced conditions. Nonreduced proinsulin appeared as a predominant faster migrating species and two slower migrating forms (contained within bracket area) and these forms collapsed into a single band as noted under reduced conditions. The full-length resolving gel does not recover obvious evidence of higher molecular mass proinsulin complexes although labeled proinsulin recovery near the top of the resolving gel (11) cannot be excluded. (B) 293T cells transfected either with empty vector (lanes 1, 6) or proinsulin cDNA (lanes 2, 3, 7) were pulse labeled as in (A) but without chase. After immunoprecipitation with anti-insulin, the samples were analyzed by nonreducing tris-tricine-urea-SDS-PAGE in comparison to a chemically-purified proinsulin standard (lane 5, marked "$^{125}$I"). The nonreduced gel was dried without fixation and after autoradiography, the slower-migrating proinsulin forms from lane 3 were individually cut from the dried nonreducing gel, separately from the major proinsulin species (which had co-migrated with chemically-purified $^{125}$I-proinsulin). Each excised band was re-swollen and boiled in fresh gel sample buffer plus 100 mM DTT, and analyzed in a second gel in which the original samples were also run under reducing conditions (lanes 6, 7), along with a reduced, purified proinsulin standard ("$^{125}$I", lane 11). (C) Transfected 293T cells were pulse labeled as in (A) and chased for the times indicated. A small quantity of the nonnative proinsulin isomers is secreted but this secretion clearly does not go as efficiently to completion as is the case for the faster-migrating native proinsulin band. (D) Two exposures of pulse-labeled 293T cells transfected with an empty vector (Control) or cDNAs encoding the wild-type human Proinsulin (Proins.) or proinsulin bearing the point
mutations indicated. The cells were pulse-labeled for 90 min and chased for 2.5 h, and both cell lysates ("C") and chase media ("M") were analyzed by immunoprecipitation with anti-insulin. In addition to the native proinsulin isomer that was very efficiently secreted over 2.5 h, two slower migrating forms of proinsulin were arbitrarily designated isomer #1 and isomer #2 bands, and these were secreted with decreased efficiency. The C(A11)S mutant was comprised of forms that co-migrated primarily with native proinsulin and isomer #1, while the C(A20)A mutant yielded a nonsecreted product that co-migrated with the isomer #2 band of proinsulin.

Fig. 3. Additional single cysteine point mutants of proinsulin analyzed by nonreducing tris-tricine-urea-SDS-PAGE. 293T cells were mock-transfected (Control) or transfected with a cDNA encoding wild-type human proinsulin or that containing (A) the Akita mutation C(A7)Y or (B) C(B19)A. In (A), the cells were radiolabeled for 1 h, in duplicate, and lysed without chase. The hAkita mutant was recovered at decreased levels and the band co-migrated largely with the proinsulin isomer #1 band. In (B), the cells were radiolabeled for 90 min and chased for the times indicated, in the absence or presence of 20 μM MG115, a proteasome inhibitor. The C(B19)A mutant also exhibited a mobility consistent with nonnative proinsulin, was not secreted, and was degraded over 5 h in a manner partially protected by the proteasome inhibitor.

Fig. 4. Newly-synthesized proinsulin in the isolated pancreatic islets from wild-type and PERK−/− mice. Fifty islets were analyzed in each sample. All islets were preincubated for 40 min at the respective glucose concentrations of either 5.5 mM or 17 mM. The islets were washed twice in methionine-free, cysteine-free medium at the glucose concentrations indicated and then radiolabeled for 20 min with 35S-amino acid mixture in the same media. The islets were then lysed and immunoprecipitated with anti-insulin, and analyzed by nonreducing tris-tricine-urea-SDS-PAGE (shown in upper panel). The supernates of the insulin immunoprecipitations were then analyzed by conventional Laemmli SDS-PAGE (shown in lower panel). [As a standard in the tris-tricine-urea-SDS-PAGE above, beta-TC3 cells were pulse-labeled for 30 min with 35S-amino acid mixture and chased for 30 min before cell lysis and insulin immunoprecipitation.] Note that the islets of PERK−/− mice hypersynthesize native proinsulin as well as the slower-migrating species of proinsulin (downward arrows) that do not co-migrate with proinsulin conversion intermediates (bracket).
Liu et al., Figure 1
Liu et al., Figure 3
Liu et al., Figure 4
Glucose (mM):

Newly-Synthesized Immunoprecipitated Proinsulin