Influence of A7-B7 Disulfide Bond Deletion on the Refolding and Structure of Proinsulin

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Abstract To probe the role of [A7-B7] disulfide bond in the structure and folding of proinsulin, [A7, B7Ser]-proinsulin was prepared. The differences in the *in vitro* refolding, oxidation of free thiol groups, circular dichroism (CD) spectra, antibody and receptor binding assays, and sensitivity to tryptic digestion between the mutant and the wild type proteins were studied. The deletion of [A7-B7] disulfide bond in proinsulin resulted in a significant decrease of α-helix content of the molecule and a great increase in sensitivity to tryptic digestion. The [A7-B7] disulfide bond deleted proinsulin showed a great loss of its receptor binding activity. The *in vitro* refolding study indicated that the rate of the oxidation of free thiol groups in the mutant was a little slower at the later stage as compared to the native molecule, but the deletion of [A7-B7] disulfide bond had little effect on the refolding yield. A possible proinsulin folding pathway way was proposed. During the folding, the intra-A chain disulfide bond forms first and very fast. The formation of the [A20-B19] disulfide bond is more crucial than [A7-B7] disulfide bond and forms very likely earlier than the latter.

Key words disulfide bond; *in vitro* refolding; proinsulin; secondary structure

Disulfide bond in proteins usually serves as a major element for structural stabilization. Formation of the disulfide bond also has important impact on protein folding pathway. The folding pathways of several proteins containing disulfide bonds, such as bovine pancreatic trypsin inhibitor (BPTI)[1, 2], RNase A[3-5] and insulin-like growth factor I (IGF-I)[6-8], have been widely studied. Insulin is a protein containing three disulfide bonds, i.e., one intra-A chain [A6-A11], two inter-chain [A7-B7] and [A20-B19]. In the biosynthesis of insulin, the three disulfide bonds are formed in an intramolecular manner. Much progress in the study of the folding pathway of insulin precursor has been made recently. It was demonstrated that the intra-A chain disulfide bond forms first in the folding pathway of insulin precursor[9] and a putative folding pathway of insulin precursor has been proposed[10]. Des-B30-[A7, B7Ser]-insulin and DKP-[A7, B7Ser]-insulin were also studied[11, 12]. The data revealed that the [A7-B7] inter-chain disulfide bond is crucial for maintaining the native conformation and biological activity of insulin, and two inter-chain disulfide bonds are important for efficient folding/secretion of insulin precursor in yeast[11]. Yet, the detailed role of [A7-B7] disulfide bond in the structure and folding of insulin precursor has to be fully elucidated.

In this paper, [A7, B7Ser]-human proinsulin ([A7, B7Ser]-HPI) was prepared by replacing cysteine residues with serine at A7 and B7 positions in the HPI, and its physico-chemical properties, biological activities as well as the *in vitro* refolding behaviors were studied.

1 Materials and Methods

1. 1 Materials

Escherichia coli strain DH5α was used as host cell. The expression vector pBV220 was a gift from Dr. HOU Yun-De of the Institute of Virology, Chinese Academic of Preventive Medicine. Plasmid pJG103 containing human proinsulin gene was previously constructed in our laboratory (derived from pBV220)[13]. PCR primers were synthesized by Sangon. T_4 DNA ligase, *Taq* DNA polymerase, *Eco*RI, *Bam*HI were purchased from Promega. Trypsin with an activity of 10 400 u/mg was a Sigma product. DTT was from BBI. 5,5'-dithio-bis-(2-nitrobenzonic acid) (DTNB) and BSA were from SABC. Other chemicals of analytical or chromatographic grade were all local products. ^{125}I-insulin radioimmunoassay kit was kindly provided by Navy Radioimmunoassay Technique Center (Beijing, China).

1. 2 Construction of mutant gene and preparation of protein

The [A7, B7Ser]-*HPI* gene was obtained by site-directed mutagenesis using PCR with pJG103 as the template. After cleavage with *Eco*RI and *Bam*HI, the mutant gene was inserted into pBV220 and the recombinant expression plasmid was transformed *E. coli* strain DH5α. The positive colony was identified by restriction mapping analysis and further confirmed by DNA sequencing. Both *HPI* and [A7, B7Ser]-*HPI* were expressed and proteins purified following procedure described previously[13]. The crude protein was then proceeded a final purification by reversed-phase fast protein liquid chromatography (RP-FPLC) on a Resource™ (3 mL) column and then analyzed by 15% native PAGE. The

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purified proteins with about 95% purity were lyophilized and stored at –20 °C for later testing.

1. 3 *In vitro* refolding assay of fully reduced proteins

1 mg HPI or [A7, B7Ser]-HPI was dissolved in 1 mL 0.1 mol/L Tris-HCl buffer (pH 8.0), containing 8 mol/L urea, 1 mmol/L EDTA and 20-fold (in terms of thiol group) excess of DTT. After incubation for 2 h at 37 °C, the reaction mixture was dialyzed against 2000 volumes of refolding buffer (pH 10.8), containing 0.05 mol/L glycine-NaOH, at 4 °C for 24 h to remove reducing agent and denaturant. 10 μL of refolding product was then analyzed by both 15% native PAGE and 15% non-reductive SDS-PAGE. After staining the gel with Coomassie brilliant blue, the proinsulin bands were quantified by densitometry using software Glyko Bandscan (Glyko, Inc., USA).

1. 4 Analysis of tryptic digestion

2 mg HPI or [A7, B7Ser]-HPI was dissolved in 1mL 0.05 mol/L Tris-HCl buffer (pH 7.2). Trypsin was dissolved in 1 mmol/L HCl to different concentrations. 2 μL trypsin was then added to 20 μL HPI or [A7, B7Ser]-HPI at defined enzyme /substrate (*W/W*) ratios. After incubation at 37 °C for 0.5 h, the reaction was stopped by addition of equal volume of 2× native PAGE loading buffer. 10 μL of reaction mixture was analyzed by 15% native PAGE. The corresponding des-B30Thr-insulin bands were quantified by software Glyko Bandscan.

1. 5 Analysis of thiol groups oxidation

2 mg HPI or [A7, B7Ser]-HPI was dissolved in 1 mL of 0.05 mol/L Tris-HCl buffer (pH 8.0), containing 8 mol/L urea, 1 mmol/L EDTA, and 20-fold (in terms of thiol group) excess of DTT. After 2 h incubation at 37 °C, the pH of the reaction mixture was adjusted to 2~3 and then the reaction mixture was loaded onto a desalting FPLC HiTrap™ column (5 mL). The column was eluted with 0.1% TFA to remove DTT in excess. The reduced protein fraction was then collected. 20 μL sample was mixed with 0.5 mL DTNB solution to determine numbers of thiol group at 0 h. The rest sample was mixed with 1/10 volume 0.5 mol/L glycine-NaOH (pH 10.8) and incubated at 4 °C for refolding. The numbers of thiol group at various time periods during refolding were determined with DTNB according to the Ellman's method[14].

1. 6 Analysis of receptor and antibody binding activities

Crude insulin receptor preparation was obtained from human placenta for insulin receptor binding assay[15] and immuno assay was performed as described in the manual of ^{125}I-insulin radio immunoassay kits. Two measurements were calculated as mean±SD.

1. 7 Circular dichroism (CD) studies

The HPI or [A7, B7Ser]-HPI was dissolved in double distilled water (pH 7.0). Protein concentration was determined by UV absorbency, and adjusted to 0.2. CD spectra were recorded by a Jasco-715 circular dichroism spectropolarimeter at 25 °C and from 190 nm to 240 nm. The cell path length was 1 mm. The relative secondary structure contents were calculated according to the method of Chen *et al.*[16].

2 Results

2. 1 Comparison of *in vitro* refolding of HPI and [A7, B7Ser]-HPI

HPI and [A7, B7Ser]-HPI were fully reduced by DTT and then dialyzed against the refolding buffer (pH 10. 8) for renaturation. In native PAGE gel, HPI and [A7, B7Ser]-HPI purified by RP-FPLC are shown to be a major single band with similar mobility rate (lanes 1 and 2 in Fig.1). Both crude refolded products show many bands on the gel (lanes 3 and 4 in Fig.1). The relative percentage of the band of the correctly refolded product was quantified by densitometry. The refolding yield for [A7, B7Ser]-HPI is 88%, almost equals the yield for HPI of 82%. The same result can be seen by non-reductive SDS-PAGE analysis (Fig.2). Most refolded products are in monomeric form, and only small amounts are dimers and polymers (lanes 3 and 4 in Figs.1 and 2).

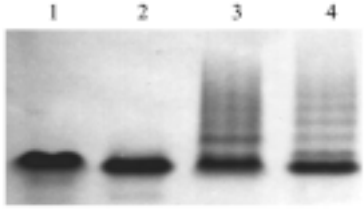

Fig.1 15% native PAGE analysis of HPI and [A7, B7Ser]-HPI before and after the *in vitro* refolding

1, 2, HPI and [A7, B7Ser]-HPI purified by RP-FPLC, respectively; 3, 4, crude *in vitro* refolding products of HPI and [A7, B7Ser]-HPI, respectively.

2. 2 Sensitivity of HPI and [A7, B7Ser]-HPI to tryptic digestion

Both HPI and [A7, B7Ser]-HPI were digested with trypsin, and analyzed by native PAGE (Fig. 3). Des-B30Thr-insulin as well as some intermediates during tryptic digestion of HPI can be observed [15]. Des-B30Thr-insulin shows similar mobility rate on native PAGE gel to that of porcine insulin [Fig. 3(A)]. The tryptic peptide mapping pattern of [A7, B7Ser]-HPI is similar to that of HPI as both proteins share the same tryptic cleaving sites. The mobility rate of des-

B30Thr-[A7, B7Ser]-insulin is a little slower than that of porcine insulin [Fig. 3(B)]. With the increase of trypsin concentration, the acquired des-B30Thr-[A7, B7Ser]-insulin was partially further converted into des-octapeptide-[A7, B7Ser]-insulin at the enzyme/substrate ratio of 1/400. The insulin analogue band disappeared completely at the enzyme/substrate ratio of 1/25. Meanwhile, HPI could only be digested to des-B30Thr-insulin even at the enzyme/substrate ratio of 1/25. This suggests that [A7, B7Ser]-HPI is much more sensitive to tryptic digestion than HPI. The yields of des-B30Thr-insulin or des-B30Thr-[A7, B7Ser]-insulin at different enzyme/substrate ratios were quantified and plotted in Figure 3(C).

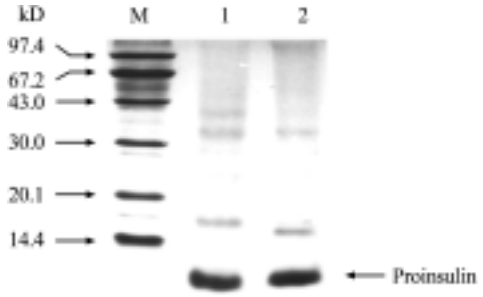

Fig.2 15% non-reductive SDS-PAGE analysis of crude *in vitro* refolding products of HPI and [A7, B7Ser]-HPI

M, molecular weight marker; 1, crude refolded HPI; 2, crude refolded [A7, B7Ser]-HPI.

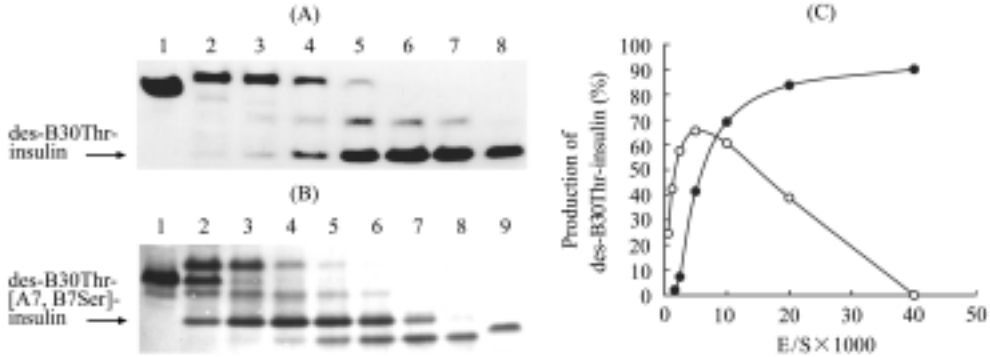

Fig.3 Tryptic digestion assay of HPI and [A7, B7Ser]-HPI by 15% native PAGE analyses in (A) and (B), respectively

(A) 1, HPI. 2 to 7, digestion products at enzyme/substrate (*W/W*) ratios of 1/600, 1/400, 1/200, 1/100, 1/50, 1/25, respectively; 8, porcine insulin. (B) 1, [A7, B7Ser]-HPI; 2 to 8, digestion products at enzyme/substrate (*W/W*) ratios of 1/1600, 1/800, 1/400, 1/200, 1/100, 1/50, 1/25, respectively; 9, porcine insulin. The lanes for des-B30Thr-insulin and des-B30Thr-[A7, B7Ser]-insulin were indicated in (A) and (B), respectively. (C), a plot indicating the digestic production rate at different E/S ratios. ●, des-B30Thr-insulin; ○, des-B30Thr-[A7, B7Ser]-insulin.

2. 3 *In vitro* oxidation of the thiol groups of reduced proteins

The time courses of the *in vitro* oxidation of thiol groups of reduced HPI and [A7, B7Ser]-HPI are shown in Figure 4. In the first half hour, the oxidation rate of reduced [A7, B7Ser]-HPI was very fast, similar to that of HPI. After one hour, although oxidation of reduced HPI was continuously at a relatively high speed, the oxidation rate of reduced [A7, B7Ser]-HPI decreased significantly.

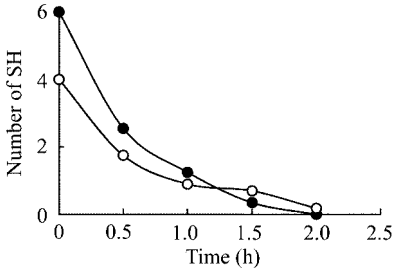

Fig. 4 Time course of oxidation of reduced HPI (●) and [A7, B7Ser]-HPI (○)

2. 4 Antibody and receptor binding assays

The results of antibody and receptor binding assays of HPI and [A7, B7Ser]-HPI are shown in Figure 5(A) and 5(B), respectively. Compared the dosages used for 50% inhibition of ^{125}I-insulin binding between HPI and [A7, B7Ser]-HPI, the antibody binding activity of [A7, B7Ser]-HPI remains at 20.6%. However, the receptor binding activity of [A7, B7Ser]-HPI is only 1.6% of that of HPI.

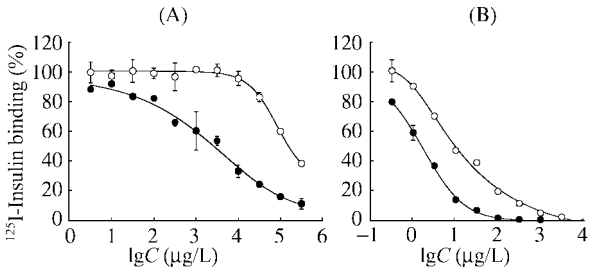

Fig. 5 Biological activity assays of HPI (●) and [A7, B7Ser]-HPI(○)

(A) antibody binding activity assay; (B) receptor binding activity assay. Data are shown as mean±SD of two measurements.

2. 5 Comparison of CD spectra

There are significant differences between the CD spectra of HPI and [A7, B7Ser]-HPI (Fig. 6). The relative secondary structure contents were calculated. The α-helix of [A7, B7Ser]-HPI is about 24%, much lower than that of HPI, which is 39%.

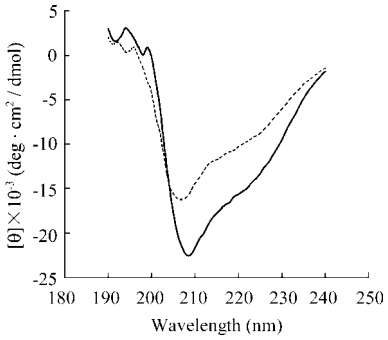

Fig. 6 CD spectra of HPI and [A7, B7Ser]-HPI

The block line represents HPI, and the dotted line represents [A7, B7Ser]-HPI.

3 Discussion

The three disulfide bonds are absolutely conserved in evolution of insulin family members. Deletion of the intra-A chain disulfide bond resulted in an unfolding of the N-terminal α-helix of insulin A chain (A1-A8) and great decrease of biological activity[15,17,18]. It was shown that the deletion of [A7-B7] inter-chain disulfide bond yielded even more serious effect on the structure and the biological activity of insulin[11,12]. In our study, significant changes exist in CD spectrum between [A7, B7Ser]-HPI and HPI, and the α-helix content in the mutant is much lower than in the native molecule. This agrees well with the results reported previously[11,12]. The tryptic digestion shows that [A7, B7Ser]-HPI is much more sensitive to proteolytic action than HPI, which indicates that the removal of the [A7-B7] disulfide bond results in a great structural change in proinsulin molecule. This also agrees well with the CD data. The receptor binding activity of [A7, B7Ser]-HPI is much lower than that of HPI, which is also similar to the data reported previously[11,12]. However, its immunoactivity is partially retained. This indicates that the conformation for antibody binding sites may have not been changed greatly, although great changes have taken place in its receptor binding site as elucidated[12].

Guo *et al.*[11] found that removal of the [A7-B7] disulfide bond resulted in a great decrease in the yield of recombinant insulin precursor secreted from yeast, and the mutant without the inter-chain disulfide bond [A20-B19] even failed to secrete from the yeast cell. Based on these observations, they concluded that the two inter-chain disulfide bonds were important for efficient folding/secretion *in vivo*, and they also inferred that the [A20-B19] disulfide bond formed earlier than [A7-B7]. While in our study, the *in vitro* refolding yield of [A7, B7Ser]-HPI is even a little higher than that of the wild type, indicating that the deletion of [A7-B7] disulfide bond has little effect on the refolding yield of proinsulin. With two less thiol groups than reduced HPI, the reduced [A7, B7Ser]-HPI may have less possibility to form dimers or polymers during refolding. It was suggested that there is a complex quality control system in the *in vivo* secretory pathway, and only proteins that pass a stringent selection can be secreted at last[19]. Based on our study, the decrease of the α-helix content and the increase of sensitivity to tryptic digestion indicate a much looser conformation of [A7, B7Ser]-HPI than the wild type. Even if the [A7, B7Ser]-insulin precursor might have folded correctly in yeast cell, its structure is unstable and easier to be attacked by protease *in vivo*, so that the secretion yield of [A7, B7Ser]-insulin precursor decreased greatly[11].

The time course of oxidation of thiol groups in reduced protein may provide some information to illustrate the folding pathway. In our earlier work, the differences in the time courses of oxidation of reduced insulin A chain, reduced A and B chains, and reduced proinsulin between the [A6-A11] disulfide bond deleted mutant and the wild type were studied. The intra-A chain disulfide bond forms first in the folding pathway of insulin precursor was proposed[9]. This

was also verified by the investigation of the oxidation of recombinant porcine insulin precursor as a major folding pathway[10]. Herein we compared the time course of oxidation of reduced [A7, B7Ser]-HPI with that of HPI and the final refolding yields of both proteins. The result indicates that more than two thiol groups in the reduced [A7, B7Ser]-HPI disappeared in the first half hour and the speed of oxidation was fast. This suggests that, similar to HPI, the [A6-A11] intra-A chain disulfide bond in [A7, B7Ser]-HPI has formed first. The oxidation rate of [A7, B7Ser]-HPI was then slowed down, indicating a possibly slow formation of the [A20-B19] inter-chain disulfide bond, which means that deletion of the [A7-B7] inter-chain disulfide bond does have influence on the formation of [A20-B19] disulfide bond. Even though, as reduced [A7, B7Ser]-HPI has two less thiol groups as compared with reduced HPI, the former protein gives an equal, if not higher, yield to the latter (Figs. 1 and 2). In another study of our laboratory on [A20-B19] disulfide bond deleted HPI, it was demonstrated that the deletion of [A20-B19] disulfide bond did not affect the oxidation of the other four thiol groups significantly, but the refolding yield was decreased to about 45% as compared to 88% for [A7, B7Ser]-HPI at similar conditions (unpublished data). This indicates that formation of the [A20-B19] disulfide bond is more important than that of [A7-B7] disulfide bond in the proinsulin folding pathway and the former disulfide bond is very likely to form earlier than the latter. Based on all the above results, we propose a very possible proinsulin folding pathway: During the folding, the intra-A chain disulfide bond forms first and very fast, which can stabilize partially folded A chain secondary structure[9]. The C-peptide may serve as an intra-molecular chaperone-like function to prevent B chain from aggregation and help A chain with partially native like structure to pair with B chain to form the two inter-chain disulfide bonds[20]. The formation of the [A20-B19] disulfide bond is more crucial than [A7-B7] disulfide bond for the correct folding of proinsulin and forms very likely earlier than the latter.

References

1. Darby NJ, Morin PE, Talbo G, Creighton TE. Refolding of bovine pancreatic trypsin inhibitor via non-native disulphide intermediates. *J Mol Biol*. 1995, **249** (2): 463 – 477
2. Dadlez M, Kim PS. Rapid formation of the native 14-38 disulfide bond in the early stages of BPTI folding. *Biochemistry*. 1996, **35** (50): 16153 – 16164
3. Xu X, Scheraga HA. Kinetic folding pathway of a three-disulfide mutant of bovine pancreatic ribonuclease A missing the [40-95] disulfide bond. *Biochemistry*. 1998, **37** (20): 7561 – 7571
4. Iwaoka M, Juminaga D, Scheraga HA. Regeneration of three-disulfide mutants of bovine pancreatic ribonuclease A missing the 65-72 disulfide bond: Characterization of a minor folding pathway of ribonuclease A and kinetic roles of Cys65 and Cys72. *Biochemistry*. 1998, **37** (13): 4490 – 4501
5. Volles MJ, Xu X, Scheraga HA. Distribution of disulfide bonds in the two-disulfide intermediates in the regeneration of bovine pancreatic ribonuclease A: Further insights into the folding process. *Biochemistry*. 1999, **38** (22): 7284 – 7293
6. Hober S, Forsberg G, Palm G, Hartmanis M, Nilsson B. Disulfide exchange folding of insulin-like growth factor I. *Biochemistry*. 1992, **31** (6): 1749 – 1756
7. Narhi LO, Hua QX, Arakawa T, Fox GM, Tsai L, Rosenfeld R, Holst P *et al.* Role of native disulfide bonds in the structure and activity of insulin-like growth factor 1: Genetic models of protein-folding intermediates. *Biochemistry*. 1993, **32** (19): 5214 – 5221
8. Hober S, Uhlén M, Nilsson B. Disulfide exchange folding of disulfide mutants of insulin-like growth factor I *in vitro*. *Biochemistry*. 1997, **36** (15): 4616 – 4622
9. Yuan Y, Wang ZH, Tang JG. Intra-A chain disulphide bond forms first during insulin precursor folding. *Biochem J*. 1999, **343** (Pt 1): 139 – 144
10. Qiao ZS, Guo ZY, Feng YM. Putative disulfide-forming pathway of porcine insulin precursor during its refolding *in vitro*. *Biochemistry*. 2001, **40** (9): 2662 – 2668
11. Guo ZY, Feng YM. Effects of cysteine to serine substitutions in the two inter-chain disulfide bonds of insulin. *Biol Chem.* 2001, **382** (3): 443 – 448
12. Hua QX, Nakagawa SH, Jia W, Hu SQ, Chu YC, Katsoyannis PG, Weiss MA. Hierarchical protein folding: Asymmetric unfolding of an insulin analogue lacking the A7-B7 interchain disulfide bridge. *Biochemistry*. 2001, **40** (41): 12299 – 12311
13. Tang JG and Hu MH. Production of human proinsulin in *E. Coli* in a non-fusion form. *Biotechnol Lett*. 1993, **15** (7): 661 – 666
14. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys.* 1959, **82**: 70 – 77
15. Dai Y, Tang JG. Intra-A chain disulfide bond (A6-11) of insulin is essential for displaying its activity. *Biochem Mol Biol Int.* 1994, **33** (6): 1049 – 1053
16. Chen YH, Yang JT, Martinez HM. Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion. *Biochemistry*. 1972, **11**: 4120 – 4131
17. Hua QX, Hu SQ, Frank BH, Jia W, Chu YC, Wang SH, Burke GT *et al*. Mapping the functional surface of insulin by design: Structure and function of a novel A-chain analogue. *J Mol Biol*. 1996, **264** (2): 390 – 403
18. Dai Y, Tang JG. Characteristic, activity and conformational studies of [A6-Ser, A11-Ser]-insulin. *Biochim Biophys Acta*. 1996, **1296** (1): 63 – 68
19. Ellgaard L, Molinari M, Helenius A. Setting the standards: Quality control in the secretory pathway. *Science*. 1999, **286** (5446): 1882 – 1888
20. Chen LM, Yang XW, Tang JG. Acidic residues on the N-terminus of proinsulin C-Peptide are important for the folding of insulin precursor. *J Biochem.* 2002, **131** (6): 855 – 859