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Secretion and processing of insulin precursors in yeast

(peptide hormone/dibasic sequences/spacer peptides/proinsulin)

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ABSTRACT **A series of dibasic insulin precursors including proinsulin was expressed and secreted from *Saccharomyces cerevisiae*. Recombinant plasmids were constructed to encode fusion proteins consisting of a modified mating factor α1 leader sequence and an insulin precursor. The leader sequence serves to direct the fusion protein into the secretory pathway of the cell and to expose it to the Lys-Arg processing enzyme system. The secreted peptides were purified from the fermentation broth and characterized by sequencing and amino acid analysis. Processing at one or both dibasic sequences was shown in proinsulin and in other insulin precursors containing a short spacer peptide in place of the C peptide. In contrast, no processing was observed in the absence of a spacer peptide in the insulin precursor molecule, e.g., B-Lys-Arg-A (where A and B are the A and B chain of human proinsulin, respectively). This type of single-chain insulin precursors isolated from such constructions could be enzymatically converted into insulin by treatment with trypsin and carboxypeptidase B. The above results suggest that the C-peptide region of proinsulin serves to direct the trypsin-like converting enzyme to process at the two dibasic sequences. We propose that in hormone precursors in general the spacer peptides serve to expose dibasic sequences for processing.**

Human preproinsulin consists of a prepeptide of 24 amino acid residues followed by proinsulin containing 86 amino acid residues in the configuration: prepeptide-B-Arg-Arg-C-Lys-Arg-A in which C is the C peptide of 31 amino acid residues (1), and A and B are the A and B chain of human proinsulin, respectively. The prepeptide is removed during transport of the nascent polypeptide into the endoplasmic reticulum. By the time it reaches the Golgi, the disulfide bridges of proinsulin (2) have been established (3–5). Proinsulin is further processed at the two dibasic sequences by a trypsin/carboxypeptidase B-like enzyme system to insulin (for review see Tager and Steiner, ref. 2, and Steiner *et al.*, ref. 6). Processing at dibasic amino acid sequences seems to be a general property of a series of hormone precursors, e.g., proforms of glucagon (7–9), gastrin (10, 11), somatostatin (12), and pancreatic polypeptide (13).

In the yeast, *Saccharomyces cerevisiae*, a similar system within the secretory compartments (14, 15) processes the primary yeast mating factor α1 (MFα1) gene product into mature α-factor (αF), a peptide of 13 amino acid residues (16). The MFα1 gene encodes the following precursor: prepeptide-Lys-Arg-$(Glu\text{-}Ala)_2$-αF-Lys-Arg-$(Glu\text{-}Ala)_3$-αF-$(Lys\text{-}Arg\text{-}Glu\text{-}Ala\text{-}Asp\text{-}Ala\text{-}Glu\text{-}Ala\text{-}\alpha F)_2$ (17). Processing of the precursor peptide includes a trypsin-like cleavage at the Lys-Arg sequences and a dipeptidyl aminopeptidase-like cleavage that removes the Glu-Ala and Asp-Ala sequences (14).

To study the enzyme processing system in yeast and to evaluate the hormone precursor concept, we have constructed yeast strains with recombinant plasmids coding for a series of dibasic insulin precursors fused to a modified MFα1 leader sequence. The expression levels in the supernatant after fermentation were measured by RIAs for insulin and C peptide. The expression products have been purified from yeast culture supernatants and characterized by amino acid sequence analysis to evaluate the pattern of proteolytic processing.

MATERIALS AND METHODS

Plasmid Constructions. Preproinsulin cDNA was obtained in the following way: total RNA purified (18) from human pancreas was reverse transcribed (11) with avian myeloblastosis virus reverse transcriptase and d(GCTTTATTC-CATCTCTC) as first-strand primer. This heptadecamer is complementary to a sequence in the 3′-untranslated region of the human preproinsulin mRNA (1). After preparative urea/polyacrylamide gel electrophoresis of the cDNA, the second strand was synthesized on this template with large fragment of DNA polymerase I and a tridecamer d(CAGA-TCACTGTCC) complementary to the cDNA in the 5′-untranslated region as second-strand primer. After S1-nuclease digestion the human preproinsulin double-stranded cDNA was purified by PAGE, tailed with terminal transferase, and cloned in the *Pst* I site on pBR327 (19) in *Escherichia coli*. A fragment of the cDNA clone starting at the codon for the first amino acid of proinsulin and ending in the 3′-nontranslated region was then generated: the desired flush-end start of this gene was obtained by primer repair synthesis (20) using the primer TTTGTGAACCAAC, while the end of the gene was generated by filling-in of an *Hga* I site and addition of an *Xba* I linker. This fragment was ligated to a subclone of MFα1 inserted into pUC13 (21) so that the filled-in *Hin*dIII site of MFα1 [position 263 (17)] was joined to the sequence encoding proinsulin. The Glu-Ala-Glu-Ala-encoding sequence immediately prior to the proinsulin sequence was then removed by *in vitro* loop-out mutagenesis (22). Genes encoding different insulin precursors were made by further *in vitro* loop-out mutagenesis using chemically synthesized 30-mer oligonucleotides to delete the original C-peptide-encoding sequences. In yeast expression plasmids subsequently constructed, the modified MFα1-insulin precursor sequences were inserted between the promoter and transcription termination regions of the triose phosphate isomerase gene of *S. cerevisiae*. The triose phosphate isomerase promoter was isolated as a 900-base-pair fragment extending from an upstream *Bgl* II site to an *Eco*RI site at position −10 of the triose phosphate isomerase sequence (23) generated by BAL-31 digestion and linker insertion. The

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Abbreviations: A, B, and C, A chain, B chain, and C peptide of human proinsulin, respectively; MFα1, yeast mating factor α1; αF, α-factor; IRI, immunoreactive insulin; IRC, immunoreactive C peptide.

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*Eco*RI site of the MFα1 presequence was generated by linker addition to the filled-in *Hin*fI site at position −70 of the MFα1 sequence (17). The terminator sequence was inserted as a 700-base-pair fragment starting at the *Xba* I site at position 736 of the triose phosphate isomerase sequence and ending at the *Bam*HI site of a linker inserted at the downstream *Eco*RI site. Together these fragments provide sequences that ensure a high rate of transcription for the insulin precursor-encoding genes and also provide presequences that direct the insulin precursors into the yeast secretory pathway and lead to the secretion of the expression products into the growth medium. The entire expression unit was inserted as an ≈2.3-kilobase partial *Sph* I to *Bam*HI fragment into the unique *Sph* I–*Bam*HI site of plasmid CPOT (24). This high copy number plasmid contains the *Schizosaccharomyces pombe* triose phosphate isomerase gene (*POT*) and the *S. cerevisiae LEU2* gene as well as pUC13 sequences and the entire 2μ plasmid of *S. cerevisiae*. CPOT-plasmid derivatives coding for the various dibasic insulin precursor molecules were transformed into *S. cerevisiae* strains carrying deletions in the triose phosphate isomerase gene by selecting for growth on glucose. Such mutants are normally unable to grow on glucose as the sole carbon source, and they grow very slowly on a galactose/lactate medium. Due to this growth deficiency, there is a strong selection for plasmids containing the triose phosphate isomerase gene, even when growing on rich, glucose-containing medium. The plasmid construction is shown in Fig. 1.

Expression Levels. The yeast strains containing plasmids encoding the different insulin precursors (see Table 1) were grown on YPD medium (25). Immunoreactive insulin (IRI) was measured by RIA (26) using semisynthetic human insulin (Novo Industri A/S, Denmark) as standard. Only insulin-like molecules with correct disulfide bridges would be measured in this assay. For the insulin precursor, B-Arg-Arg-C--peptide-Lys-Arg-A (human proinsulin), the expression level of immunoreactive C peptide (IRC) was measured by use of a human C-peptide RIA (27). In this assay human ^{125}I-labeled Tyr-C peptide was used as tracer, and a guinea pig anti-human C-peptide serum, M1228 (28), which reacts equally well with human C peptide and human proinsulin, was used as antibody. Human proinsulin was used as standard (29).

Peptide Purification and Characterization. IRC material was purified from yeast strain MT-509 supernatant by use of the following steps: LiChroprep RP-18 column (Merck), gel filtration on a Sephadex G-75 column, HPLC on a 10-μm Waters μBondapak C_{18} column. IRI material was purified by chromatography on a LiChroprep RP-18 column, immunoaffinity chromatography on an anti-insulin Sepharose column (MCA OXI 001, Novo Industri A/S), and HPLC on a Waters μBondapak C_{18} column.

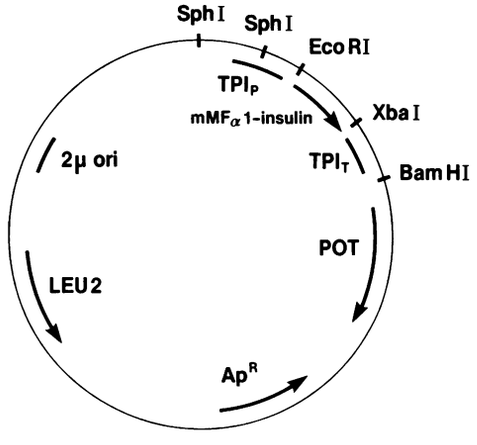

FIG. 1. Yeast expression plasmids for dibasic insulin precursors. mMFα1, modified MFα1 leader sequence (17) in which the C-terminal Glu-Ala-Glu-Ala sequence has been removed. Insulin, insulin-precursor encoding sequences (see Table 1). TPI$_P$ and TPI$_T$ triose phosphate isomerase promoter and terminator sequences, respectively. *Bam*HI, *Eco*RI, *Sph* I, and *Xba* I: restriction enzyme sites. Only restriction sites relevant to the construction of the expression plasmids have been indicated.

Conversion of Disulfide Bridge-Linked B-Lys-Arg-A to Human Insulin. The conversion was carried out as a trypsin digestion of B-Lys-Arg-A at basic pH to hydrolyze the peptide bond Arg_{B32}-Gly_{A1}. The product, B-Lys-Arg A, was purified and digested with carboxypeptidase B to insulin (Fig. 2). B-Lys-Arg-A (471 mg) was suspended in 26.4 ml of H_2O. The mixture was chilled to 4°C, and 3.0 ml of 0.25 M NaOH was added to dissolve the insulin precursor. Six milligrams of trypsin (Novo Industri A/S) in 200 μl of water was added. The reaction was carried out at 4°C for 5 hr and stopped by the addition of 600 μl of 4 M HCl. The yield as determined by HPLC (method given below) was 91.5% of B-Lys-Arg A, and the main part of the remaining 8.5% was undigested precursor. The product was purified by preparative HPLC on a column (5 × 25 cm) of octadecyl-dimethylsilyl substituted silica (average particle size, 15 μm; pore size, 100 Å). The column was equilibrated and eluted (isocratically) with 0.185 M KCl/0.6 mM HCl, pH 3.15, containing 37% (vol/vol) ethanol at a flow rate of 2 liters/hr. B-Lys-Arg A eluted at 4.3 column volumes and could be isolated from the alcoholic pool by crystallization (33). The purified material was dissolved to a concentration of 5 mg/ml in 50 mM Tris·HCl buffer, pH 9.3, and digested with carboxypeptidase B (5 μg/ml) (Boehringer Mannheim) at 37°C. Aliquots were removed from the digest mixture at 0 min, 2 min, 5 min, and 30 min, acidified to pH 1.5 with 4 M HCl and analyzed by HPLC on a 5-μm Nucleosil RP C_{18} column (4 × 200 mm) equilibrated and isocratically eluted with 33 mM $(NH_4)_2SO_4$/1.5 mM H_2SO_4 containing 29.4% (vol/vol) acetonitrile at 30°C at a flow rate of 1 ml/min (Fig. 2). Peptides were detected by measurement of absorption at 214 nm. The conversion was completed after 30 min (Fig. 2), and ethanol was added to the digest mixture to 60% (vol/vol). The human insulin was purified by anionic exchange chromatography on a QAE-Sephadex column (34). The overall yield of human insulin was 76%.

RESULTS

The entire MFα1 leader sequence, encoding 89 amino acid residues (17), was used in the initial studies for the plasmid constructions in the following general configuration: MFα1 leader(1–83)-Lys-Arg-Glu-Ala-Glu-Ala-insulin precursor. The expression products purified from supernatants of yeast cultures containing such constructions showed that the major part of the precursor molecules was in the form of either a Glu-Ala-Glu-Ala-insulin precursor (in the particular case, 89%), or a Glu-Ala-insulin precursor (2%). Only a minor part (9%) was in the form of a "correctly" N-terminal processed insulin precursor (results not shown). Similar results have been reported by Brake *et al.* (35), who used the entire MFα1 leader sequence fused to human epidermal growth factor. We interpreted these findings as a result of low activity of the dipeptidyl aminopeptidase-like enzyme system that normally is responsible for the last step in the correct processing of the yeast αF (14). To overcome the problem of heterogeneously

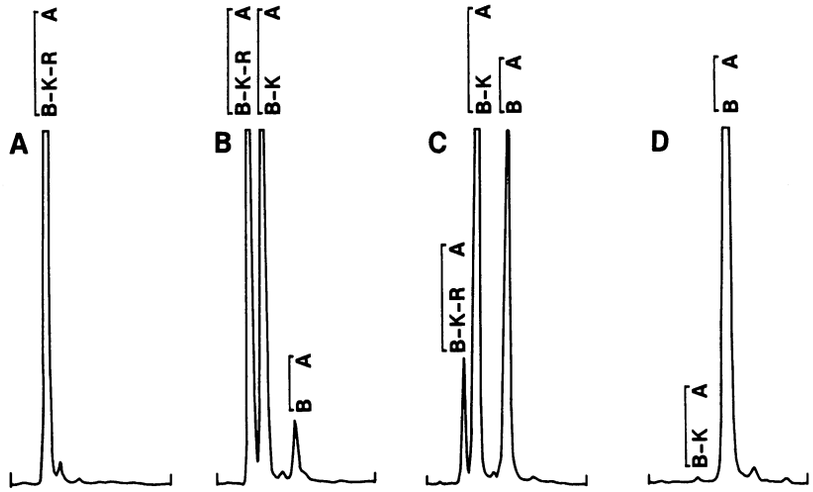

FIG. 2. HPLC traces illustrating the last step in the conversion of B-Lys-Arg-A to B A (human insulin). B-Lys-Arg-A was digested at basic pH to B-Lys-Arg A, which was purified and further digested with carboxypeptidase B. Aliquots were removed from the latter digest mixture at 0 min (*A*), 2 min (*B*), 5 min (*C*), and 30 min (*D*) and analyzed by HPLC. K, lysine; R, arginine.

processed N-terminal insulin precursor sequences, the Glu-Ala-Glu-Ala-coding sequence of the MFα1 leader was removed by site directed mutagenesis (22), and the rest of the present study was carried out by use of this modified MFα1 leader.

Expression Level. The 10 insulin precursors of the present study are listed in Table 1, which also shows the expression levels as measured by RIA for insulin and C peptide. In several of the RIAs performed on crude yeast culture supernatants a dilution effect was observed, i.e., the calculated amount tends to increase with increasing dilution of the sample. However, when the samples were diluted $\approx$1:500 or more, the value tended to stabilize around a constant level given in Table 1. The dilution effect observed is a result of the assay technique used in which a well-defined peptide (insulin or proinsulin) is used as standard for measuring insulin-like and C peptide-like peptides. Although the absolute level of expression in the various strains may vary by $\approx$30% from day to day, the ratio between different constructions was essentially constant when grown in parallel.

The insulin precursors can be divided into two main groups, one containing two dibasic sequences separated by a spacer peptide (Table 1, constructions **1–6**) and another without spacer peptide containing only a single dibasic sequence (constructions **7–10**). In the first group, the length of the spacer peptide has been varied from 2 amino acid residues to full-length human C peptide containing 31 amino acid residues. Changing the length of the C-peptide region in the interval from 31 to 4 amino acid residues did not affect the expression level of insulin-like peptide, whereas a further decrease from 4 to 2 amino acid residues increased the amount of insulin-like peptides as measured by RIA. The nature of the first dibasic sequence being either Arg-Arg or Lys-Arg did not seem to affect the expression level.

In the second group of insulin precursors containing one dibasic sequence and no spacer peptide (constructions **7–10**) the expression level of insulin-like peptides was significantly higher than in the first group, and the nature of the dibasic sequence seemed to influence the amount of insulin-like peptide produced. The amount of IRC material produced from the proinsulin precursor was within the same range as insulin-like peptide produced from the group without spacer peptide. This might indicate that the primary gene product is produced equally efficiently for all the precursors studied.

Characterization of Secreted Peptides. The expression products from the proinsulin precursor construction were purified from the fermentation broth by a combination of HPLC and immunoaffinity chromatography. Both IRC and IRI were purified, and the characterization was carried out by microsequence analysis (Table 2). The dominating IRC was found to be intact C peptide, although it was not possible to judge from the sequence analysis whether or not a minor part (less than 5%) was in the form of C peptide-Lys and/or C peptide-Lys-Arg. As can be seen from Table 2, no intact proinsulin could be isolated from the fermentation broth. At least seven different HPLC peaks were found to contain insulin immunoreactive material. Peptide material from the four dominating peaks was analyzed by microsequencing and shown to be intermediate forms of proinsulin/insulin (Table 2). One of the minor insulin-like peptides was found to

Table 1. Expression levels of insulin precursors as determined by radioimmunoassays

Construction	Insulin precursor encoding sequence	Yeast strain	Immunoreactive insulin, μM	Immunoreactive C peptide, μM
1	B-Arg-Arg-C-peptide-Lys-Arg-A*	MT-509	0.18	1.71
2	B-Arg-Arg-Glu-Ala-Glu-Asp-Leu-Gln-Lys-Arg-A	ZA-300	0.14	—
3	B-Arg-Arg-Glu-Ala-Pro-Leu-Gln-Lys-Arg-A	MT-642	0.16	—
4	B-Arg-Arg-Glu-Ala-Leu-Gln-Lys-Arg-A	MT-641	0.17	—
5	B-Lys-Arg-Glu-Ala-Leu-Gln-Lys-Arg-A	MT-632	0.18	—
6	B-Arg-Arg-Leu-Gln-Lys-Arg-A	MT-631	0.31	—
7	B-Arg-Arg-A	MT-616	1.00	—
8	B-Arg-Lys-A	MT-660	1.60	—
9	B-Lys-Lys-A	MT-655	2.00	—
10	B-Lys-Arg-A	MT-593	1.70	—

*Identical to human proinsulin encoding sequence.

Table 2. Expression products from the proinsulin precursor construction

Peptide	Approximate amount, μM
C-peptide	1.30
B-Arg-Arg C peptide-Lys-Arg-A	0.02
B-Arg-Arg-C peptide-(Lys-Arg) A	0.05
Diarginyl insulin (B-Arg-Arg A)	0.10
Monoarginyl insulin (B-Arg A)	0.02
Insulin- and proinsulin-like peptides (minor peaks)	0.05

cochromatograph with human insulin in the HPLC system, but the amount was too small for a complete sequence analysis.

The expression products from the other precursor constructions containing two dibasic sequences (constructions **2–6**) were isolated by use of immunoaffinity chromatography on an anti-insulin column, followed by HPLC as described for the proinsulin construction. For these constructions, it was found that 95% of the IRI material present in the yeast supernatant could be bound to and subsequently eluted from the anti-insulin column. When analyzed by HPLC the IRI material was found to be heterogeneous in all the constructions in that five to seven peaks containing IRI could be isolated. From the sequencing of IRI-containing fractions it could be concluded that processing had occurred at one or both dibasic sites in all the constructions containing a spacer peptide and two dibasic sequences, and no single-chained, unprocessed precursor molecules could be isolated. Furthermore, neither the length of the spacer peptide in the range from 2 to 31 residues nor the nature of the first dibasic sequence (Arg-Arg or Lys-Arg) did significantly influence the processing pattern of these insulin precursors.

The purification and characterization of secreted peptides from plasmid constructions encoding precursors without a spacer peptide (constructions **7–10**) are exemplified by the B-Lys-Arg-A precursor. IRI material from the culture supernatant of strain MT-593 was purified essentially as described above. The material eluted from the HPLC column contained only one peak with IRI. The overall purification yield of IRI was 79%. The peptide material from this peak was sequenced and found to be a single-chained peptide of 53 amino acid residues (average repetitive yield, 92.1%) with the sequence B-Lys-Arg-A, i.e, no processing at the dibasic sequence had occurred. Amino acid analysis showed the composition expected for the precursor. Furthermore, digestion of the peptide with trypsin [3% (wt/wt), pH = 8.6, 37°C, 18 hr] resulted in a peptide that coeluted with a standard of desoctapeptide (B_{23}–B_{30}) insulin in the HPLC system and that had the amino acid composition and amino acid sequence of desoctapeptide (B_{23}–B_{30}) insulin. Changing the dibasic sequence to either Arg-Arg, Arg-Lys, or Lys-Lys (constructions **7–9**) did not lead to any processing; i.e., only the single-chained unprocessed molecule could be isolated. Expression levels as measured by RIA differed significantly between the four dibasic constructions (Table 1). We cannot exclude the possibility that the precursor molecules are actually produced at the same rate, but that establishment of correct disulfide bridges proceeds, for some steric reason, with a faster rate for the Lys-Lys precursor than, e.g., for the Arg-Arg precursor.

The assay techniques and purification procedures used should be kept in mind when evaluating the above results. Only insulin-like molecules with correct disulfide bridges would contribute to the IRI level, and only these molecules would be absorbed on the anti-insulin column. Thus, free A and B chains, molecules with incorrect disulfide bridges, dimers, polymers, etc. would not be detected in the RIA and would not be absorbed by the anti-insulin column.

***In Vitro* Conversion into Human Insulin.** The four purified insulin precursors without spacer peptide, B-X-Y-A, could be converted into B A (human insulin) by *in vitro* digestion with trypsin and carboxypeptidase B by the method used by Kemmler *et al.* (36) for the conversion of proinsulin to insulin. The yield of such conversions was ≈50% due to an initial trypsin cleavage on the C-terminal side of both the X and Y residues, resulting in equal amounts of B-X-Y A and B-X Y-A, which were further digested with carboxypeptidase B to give equal amounts of B A and B Y-A. When the ratio of trypsin to insulin precursors was kept low [0.1–0.01% (wt/wt)], only trace amounts of desoctapeptide (B_{23}–B_{30}) insulin and des(B_{30})-insulin were generated.

Digestion of B-Lys-Arg-A with trypsin at a high pH value decreased the reactivity of trypsin with lysine residues (37) and resulted in a high yield (more than 90%) of B-Lys-Arg A, which could be nearly quantitatively (more than 99%) converted into B A by digestion with carboxypeptidase B (Fig. 2). The biosynthetic human insulin generated from B-Lys-Arg-A showed identity with human insulin in a series of comparative studies including crystal structure, amino acid sequence analysis, amino acid composition, peptide mapping, and basic disc electrophoresis (results not shown). The biological potency of the biosynthetic human insulin in the mouse blood glucose assay (38) was 28.1 units/mg ($P <$ 0.05, confidence limit; 25.7–30.7 units/mg). The potency of the fourth international insulin standard is 24.0 units/mg (38).

DISCUSSION

The enzyme system in yeast responsible for the maturation of yeast pheromone precursors has been the subject of several investigations (17, 39, 40). The proteolytic processing of the yeast αF precursor is initiated by a selective cleavage at the Lys-Arg sequence (14, 41). The structural gene encoding the Lys-Arg-specific processing enzyme system (*KEX2* gene) has been isolated from *S. cerevisiae* (39). The yeast endopeptidase specific for dibasic sequences isolated by Mizuno and Matsuo (40) is different from the *KEX2* gene product and does apparently not participate in normal αF processing (42). *In vitro* studies have shown that the *KEX2* gene product cleaves also at Arg-Arg and Lys-Lys sequences (39) and processing at Lys-Arg, Arg-Arg, and Arg-Lys sequences probably occurs during the maturation of the yeast killer toxin precursor (43, 44). The present study shows that processing at the first Lys-Arg sequence occurs in hybrid genes encoding chimeric proteins in which insulin precursors are fused to the MFα1 leader sequence. Removal of the gene segment encoding the last four residues (Glu-Ala-Glu-Ala) of the leader sequence did not seem to affect the processing at the first Lys-Arg sequence. Similar results have been reported for the MFα1 leader sequence fused to invertase (45) and other foreign proteins (35). In these instances the amino acid sequence and the tertiary protein structure close to the first Lys-Arg do not seem to influence the processing significantly.

In contrast, processing at the dibasic sequences located between the B and A chains seems to be strongly influenced by the accessibility of the cleavage site. In all the insulin precursors containing spacer peptides and two dibasic sequences (constructions **1–6**) processing at one or both sites was observed, whereas no processing was observed in the insulin precursors without spacer peptides (constructions **7–10**). These results, however, are complex to interpret in terms of susceptibility to processing of the dibasic sequences

in precursors with established disulfide bridges. Thus the efficient establishment of correct disulfide bridges could be an important parameter since the tertiary protein structure close to the dibasic sites differs between linear and folded insulin precursor molecules. Furthermore, it seems reasonable to assume that the ability of the different insulin precursor molecules to establish the disulfide bridges depends on the spacer peptide chain between residues B_{30} and A_1, the C-peptide region.

It is generally believed that the C-peptide region of proinsulin mediates the formation of correct disulfide bridges (46, 47). This is in contrast with the present findings in which the C-peptide region has been removed (constructions **7–10**), and still correct disulfide bridges are established in the insulin precursor molecules. Steiner *et al.* (48) have suggested that in the pancreatic β cell the C-peptide region serves as a spacer to elongate the peptide to a minimum length for efficient membrane transport into the endoplasmic reticulum. In the present study in yeast this minimum length has apparently been achieved, even in the case of insulin precursors with no spacer peptides (53 residues) by the fusion of the precursor to the 85-residue modified MFα1 leader. Given the great similarity between the specificity of the Lys-Arg processing enzyme system in yeast and the proinsulin converting enzyme system in the pancreatic β cell, it is reasonable to suggest that the function of the C peptide of proinsulin is to expose the two dibasic sequences so that they are available for processing. This hypothesis might be further extended to include other spacer peptides of hormone precursors. In the case of proglucagon processing in the pancreatic α cell, the glucagon sequence is flanked by two dibasic sequences and two spacer peptides (9) with the length of 30 amino acid residues (7, 49) and 6 amino acid residues (7), respectively. Processing of the glucagon precursor in the pancreatic α cell occurs at the dibasic sequences at the N-terminal end (49, 50) and at the C-terminal end of the glucagon, but not at the Arg-Arg sequence in position 17 or 18 of the glucagon molecule (7, 9). Thus the function of the spacer peptides might be to expose for processing the two dibasic sequences flanking the glucagon sequence.

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