The Prosequence of *Rhizopus niveus* Aspartic Proteinase-I Supports Correct Folding and Secretion of Its Mature Part in *Saccharomyces cerevisiae*

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Ryouichi Fukuda, Hiroyuki Horiuchi, Akinori Ohta, and Masamichi Takagi

*From the Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan*

Extracellular *Rhizopus niveus* aspartic proteinase-I (RNAP-I) was secreted effectively by *Saccharomyces cerevisiae* when RNAP-I with its preprosequence was synthesized in this organism (Horiuchi, H., Ashikari, T., Amachi, T., Yoshizumi, H., Takagi, M., and Yano, K. (1990) *Agric. Biol. Chem.* 54, 1771–1779). Certain deletions (Δpro, Δ1, Δ2), and amino acid substitutions (M1) in the prosequence blocked secretion of RNAP-I, although the pro tease protection assay revealed that even Δpro could be translocated across the membrane of the endoplasmic reticulum. When Δpro or M1 was synthesized simultaneously with the wild-type preprosequence in *S. cerevisiae*, secretion of RNAP-I was recovered. Therefore, the physical linkage of the prosequence to the mature region is not a prerequisite for secretion of active RNAP-I. Purified RNAP-I with the prosequence once denatured in 6 M guanidine HCl could be renatured and activated to have its enzymatic activity by removing guanidine HCl in *vitro*, but RNAP-I without the prosequence could not. Furthermore, the wild-type prosequence helped the recovery of the activity of the denatured RNAP-I in *trans*, but the prosequence of M1 with which secretion of RNAP-I was not observed in *vitro*, did not. From these results we concluded that the prosequence of RNAP-I supports correct folding of RNAP-I in the endoplasmic reticulum lumen and its subsequent secretion in *S. cerevisiae*. The functional role of the prosequence of an aspartic proteinase was elucidated.

Most secretory proteins have polypeptides consisting of 20–30 amino acid residues called signal sequences at the N termini of the mature parts. Signal sequences play important roles in membrane translocation in both prokaryotes and eukaryotes (for review see Larriba, 1993). Besides signal sequences, some of hydrolases, hormones, and growth factors have additional polypeptides called prosequences. Prosequences have been shown to serve a variety of functions.

It is reported that the prosequence of bovine pancreatic trypsin inhibitor has information necessary for correct disulfide bond formation (Weissman and Kim, 1992). It is also indicated that the prosequence of human parathyroid hormone facilitates accurate and efficient function of its signal sequence (Wiren et al., 1988). In addition, the prosequence of mouse nerve growth factor is necessary for the secretion of correctly processed and biologically active nerve growth factor (Suter et al., 1991). Furthermore, it is reported that the prosequence of preprosomatostatin can protect α-globin from degradation in the ER and enables intracellular transport and secretion when it is fused to the N terminus of α-globin and expressed in mammalian cells (Stoller and Shields, 1989).

Most of proteases have prosequences at the N termini, C termini, or both of mature enzymes. Prosequences of proteases are thought to be involved in the inactivation of mature enzymes after synthesis until localization in the destined places either in or out of the cells (Neurath, 1989). However some of the prosequences seem to have other functions. The prosequences of some serine proteases such as subtilisin E of *Bacillus subtilis* (Zhu et al., 1989), α-lytic protease of *Lysobacter enzymogenes* (Baker et al., 1992), and carboxypeptidase Y of *Saccharomyces cerevisiae* (Winther and Sorensen, 1991) are shown to have the function to help refolding of the denatured mature parts in *vitro*. In addition, the vascular sorting signal of carboxypeptidase Y is within its prosequence (Valls et al., 1990). Moreover, it is proposed that the prosequence at the C terminus of serine protease of *Serratia marcescens* and that of IgA protease of *Neisseria gonorrhoeae* form pores in the outer membrane through which the mature parts are translocated (Wandersman, 1992). In comparison with these cases, little is known about the functional role of prosequences of aspartic proteases. It is presumed that the prosequence of proteinase A of *S. cerevisiae* promote folding of the mature part (van den Hazel et al., 1993), but clear evidence is not obtained.

*Rhizopus niveus*, a filamentous fungus, secretes large amounts of glucoamylases, aspartic proteases, etc. extracellularly. We have cloned and sequenced genes encoding aspartic proteinase-I (RNAP-I) (Horiuchi et al., 1988a), aspartic proteinase-II, -III, -IV, and -V (RNAP-II to -V) (Sakaguchi et al., 1992), and ribonuclease Rh (Horiuchi et al., 1988b). RNAP-I is synthesized as a precursor form with a prosequence (21 amino acid residues) and a prosequence (45 amino acid residues) at the N terminus of the mature part (323 amino acid residues). RNAP-I is also secreted extracellularly with high efficiency in *S. cerevisiae* when the prepro-RNAP-I gene is expressed under the control of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene promoter (Horiuchi et al., 1990) or GALL gene promoter of *S. cerevisiae* (about 150 mg/liter under the optimum condition). This situation opens a possibility of analyzing the functional role of the prosequence of RNAP-I taking advantage of the refined *S. cerevisiae* gene-manipulating system.

In this paper, we analyzed the function of the prosequence of RNAP-I and found that the prosequence of RNAP-I was essential for both secretion of mature part in *S. cerevisiae* in *vitro* and
refolding of denatured mature RNAP-I in vitro. We therefore propose that the prosequence of RNAP-I guides correct folding of RNAP-I in vivo probably in the ER lumen, thereby making it possible for the enzyme to go through the secretory pathway.

MATERIALS AND METHODS

Strains and Media—Escherichia coli JA221 (recA1 leuB6 tryE6 hsdR30 hsdM16 lacIq lacZM15) was used as a host for plasmid construction and strain CJ236 (dai1 ungI thi-1 relA1, cPlO5[F'cam]) as a host for site-directed mutagenesis. Strain MV1190 [Δ(lac-proAB) thiA proA150] was used for selection of transformants and E. coli ER2733 (F− trpD6 proA3 lacI2ΔM15) was used as a host for production of GST, GST-pro, and GST-M1.

S. cerevisiae strain R27-7C-1C (MATa his3 leu2 ura3 trp1) and EH13-15 (MATa topl) were used as hosts for secretion and purification of mature RNAP-I and pro-RNAP-I. Yeast cells were cultured aerobically at 30 °C. For selection of yeast transformants, YNB medium (0.67% yeast nitrogen base without amino acids, 2% glucose) was used with appropriate supplements. RNAP-I secretion in liquid medium was tested in YNBDC medium (YNB containing 2% casamino acids and 0.05% uracil).

Enzymes and Reagents—Nucleic acid modification enzymes were purchased from Takara Shuzo Co. (Kyoto, Japan) and under conditions suggested by the manufacturer. Oligonucleotides for site-directed mutagenesis were synthesized with DNA Synthesizer 391 (Applied Biosystems).

Plasmid Construction—Recombinant DNA manipulations were done by the standard methods (Maniatis et al., 1982). Yeast transformation was carried out using the lithium acetate procedure described by Ito et al. (1983).

Plasmid pYP2841, in which the whole prosequence of RNAP-I gene was deleted, was constructed as follows. A 0.5-kb EcoRI-SalI fragment containing the coding sequence for the prosequence and part of the mature part of RNAP-I was isolated from pYPR2831 (Horiuchi et al., 1990), and it was cloned between EcoRI and SalI sites of M13mp19. Then an XhoI site and a BamHI site were introduced before and after the prosequence of RNAP-I gene, respectively, by site-directed mutagenesis. Strain MV1190 (Δ(lac-proAB) thiA proA150) was transformed with pYP2841.

Plasmids pYP2842 and pYP2843 encode RNAP-I derivative ΔI and ΔII, in which the region from lys to Pro+ and from Ala6 to Thr+ was deleted, respectively. Plasmid pYP2881S encodes an RNA polymerase derivative in which the whole prosequence of RNAP-I gene was deleted. These plasmids were used as a template for site-directed mutagenesis by the method previously described by Horiuchi et al. (1990). Yeast transformation was done as follows. Purified mature RNAP-I (125 μl, 0.114 mg/ml) and 4 μg of plasmid DNA was used as a template for PCR amplification. The amplified DNA was treated with exonuclease I and Shrimp alkaline phosphatase. The treated DNA was used as a template for PCR amplification.

Plasmid pYP2844 encodes an RNA polymerase derivative in which the whole prosequence of RNAP-I gene was deleted. This plasmid was used as a template for site-directed mutagenesis by the method described by Horiuchi et al. (1990).

Pro-RNAP-I was isolated from pYP2841. Those cells were cultivated in YEP medium (2% peptone, 1% yeast extract, 2% galactose) at 30 °C for 24 h. The pH of the culture supernatant was adjusted to 6.2, and the solution was concentrated by ultrafiltration and dialyzed against 20 mM sodium phosphate (pH 6.2). The solution was applied on a CM-Sepharose fast protein liquid chromatography column equilibrated with 20 mM sodium phosphate (pH 6.2) by Sephadex G-25 column (NAP column, Pharmacia), and these proteins were used for in vitro renaturation assay.

Part of mature RNAP-I thus purified was used to prepare rabbit polyclonal antiserum. Production and Purification of the Prosequences of RNAP-I—The prosequences of RNAP-I were produced and purified as a fusion proteins with glutathione S-transferase.

E. coli MV1190 cells harboring the plasmid pGEX-31 or pGEX-44 were cultivated in 10 ml of LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl containing 50 μg/ml of ampicillin at 37 °C overnight. The culture was inoculated to 300 ml of the same medium, and it was cultivated at 37 °C for 6 h. Then isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM into the culture, and it was further cultivated for 3–5 h. Cells were collected, resuspended in purification buffer (20 mM sodium phosphate (pH 7.3), 150 m NaCl, 100 mM EDTA, 10 mM EGTA, 100 μg/ml of phenylmethylsulfonyl fluoride, 0.5 mg/ml leupeptin) and homogenized with Braun homogenizer for 1 min. Triton X-100 was added to a final concentration of 1%, and the solution was left on ice for 30 min. Then it was centrifuged at 100,000 × g for 10 min at 4 °C. The supernatant was loaded on a glutathione Sepharose 4B column (1.6 × 10 cm), which had been equilibrated with the purification buffer plus 1% Triton X-100. After being washed with the purification buffer, the column was eluted with 5 mM glutathione in the same buffer. The eluted fraction was collected, and the buffer was exchanged to 20 mM sodium phosphate (pH 6.2) by Sephadex G-25 column (NAP column, Pharmacia), and these proteins were used for in vitro renaturation assay.

In Vitro Renaturation Assay—In vitro cis renaturation assay was done as follows. Purified mature RNAP-I (125 μl, 0.114 mg/ml) and...
mature RNAP-I (1 ml, 0.281 mg/ml) was prepared as described above. Aliquots (20 µl each) were mixed with equal molar of GST, GST-pro, or cerevisiae pro-RNAP-I (125 µl, 0.239 mg/ml), both in 20 m~ sodium phosphate (pH 6.2), were mixed with 375 µl of 8 M guanidine HCl in the same buffer. These plasmids were transferred to S. cerevisiae strain R27–7C-1C and extracellular secretion of mature RNAP-I in the transformants was examined. Any transformants that were expected to produce Δ1, Δ2, or M1 did not show a halo due to the extracellular RNAP-I activity on casein-containing plates (data not shown). The extracellular proteinase activity in each of these transformants expressing Apro, Δ1, Δ2, and M1 was less than 1% of that of RNAP-I expressed with the wild-type prosequence and not different from that of the transformant harboring the control vector pYE209 (Table I). The culture supernatants were also subjected to SDS-PAGE, and the bands corresponding to the pro- and mature RNAP-I were not found except for the case of production of RNAP-I with the wild-type prosequence (data not shown). Thus the complete or partial deletion of the prosequence and the replacement of 2 amino acid residues in it blocked secretion of RNAP-I. In Western blotting analysis of the cell extracts of these transformants using anti-RNAP-I antisera, the bands corresponding to Apro, Δ1, Δ2, and M1 were observed in the cell extracts (data not shown).

To test whether Apro was present in the cytoplasm or translocated across the ER membrane, the microsomal fraction was prepared from the cells expressing Apro, and then, the existence of trypsin-resistant Apro was investigated by protease protection assay. Fig. 2 shows that Apro in intact microsomal fraction was resistant to proteolysis (Fig. 2, lane 2), whereas it was sensitive when microsomal membrane was solubilized with 1% Triton X-100 (Fig. 2, lane 3).

Thus, even Apro could most likely be translocated across the ER membrane into the ER lumen.

Secretion of RNAP-I with Mutated Prosequences by trans-Complementation of the Wild-Type Prosequence in Vivo—We examined whether the prosequence of RNAP-I that was synthesized as a peptide could recover the secretion of Apro or M1. The plasmid pYGpro encoding the wild-type prosequence of RNAP-I under the control of GAL1 promoter was constructed. This plasmid was introduced with pYPR2841 (encoding Apro) or pYPR2844 (encoding M1) into S. cerevisiae R27–7C-1C. These plasmids were designed to produce only Apro or M1 in the glucose medium but the prosequence of RNAP-I, too, in the galactose medium.

These transformants were cultured first in YNBDc (glucose-containing) liquid medium for 24 h at 30°C and then in YNBC or YNBBG (galactose-containing) liquid medium for additional 24 h. The extracellular proteinase activities of the culture supernatants were determined. In the presence of galactose, the activity of the transformants that synthesized Apro or M1 together with the prosequence was 19 or 30% of the wild type (Fig. 3). On the contrary, little activity was detected with these transformants in glucose medium. Furthermore, in SDS-PAGE analysis of the supernatant having proteinase activity, the band corresponding to the authentic RNAP-I was detected (data not shown).

Renaturation of Denatured RNAP-I in the Presence of the
Prosequence in Vitro—The function of the prosequence of RNAP-I was analyzed in vitro. Mature RNAP-I and pro-RNAP-I of which the prosequence was not processed were purified from the culture supernatant of the cells harboring the plasmids pYE209 and pYGpro (a), pYPR2831 and YEp24 (b), pYPR2841 and pYGpro (c), and pYPR2844 and pYGpro (d) were measured. Proteinase activity in each of the supernatants is shown as percent of that in the culture supernatant of the cells harboring the plasmids (b) in the same medium.

Next, we examined whether the mutated prosequence of M1, with which secretion of the mature part was not observed in vivo, could renature the denatured mature RNAP-I in vitro. Prosequence of M1 was also supplied as a in-frame fusion with glutathione S-transferase (GST-M1) and used in in vitro experiment as described above. The result is shown in Fig. 5. Denatured RNAP-I did not regain activity in the presence of GST-M1, even after 48-h incubation (data not shown).

**DISCUSSION**

We analyzed the effect of the prosequence of RNAP-I on secretion of its mature part in the expression system of S. cerevisiae as a host. Secretion of RNAP-I was blocked by the complete or partial deletion of the prosequence or by the substitution of 2 amino acid residues in it (Table I). Even without the prosequence, Δpro was proved by protease protection assay to be translocated across the ER membrane in the presence of the prosequence (Fig. 2).

The prosequence synthesized as a peptide could complement in trans the defective secretion of Δpro. In addition, it rescued in trans the secretion of M1 having a mutated prosequence (Fig. 3). Therefore, the prosequence of RNAP-I is essential for secretion of the mature part and probably works after translocation of RNAP-I protein across the ER membrane into the lumen.

At the same time, the prosequence of RNAP-I had a function to activate, probably by helping correct folding, once denatured mature part in vitro. That is, pro-RNAP-I could be renatured and activated with high efficiency after denaturation in vitro, whereas mature RNAP-I itself could not (Fig. 4). Furthermore, the wild-type prosequence synthesized as a peptide in and purified from E. coli helped renaturation of the denatured mature RNAP-I in trans, but the prosequence of M1 did not (Fig. 5). These results suggest that the prosequence of RNAP-I is necessary for correct folding of RNAP-I protein, thereby supporting secretion of the mature part in vivo.

The prosesquences of some serine proteases are shown to help the renaturation of the denatured mature parts in vitro (Zhu et
mature part extracellularly, because it cannot be folded correctly in the cells. Furthermore, it is also suggested that the wild-type prosequence thereby to promote secretion of the mature RNAP-I.

The prosequence of aspartic proteinases have similar functions. These results are quite interesting, because the prosequences of the two structurally and probably also evolutionarily different proteases, serine protease and aspartic proteinase have similar functions.

The process of M1 could neither support secretion of the mature part in vivo nor reнатure the denatured mature RNAP-I in vitro. These results suggest that M1 is not secreted extracellularly, because it cannot be folded correctly in the cells. Furthermore, it is also suggested that the wild-type prosequence serves to fold the mature part correctly in vivo and thereby to promote secretion of the mature RNAP-I.

Analysis of crystal structure of porcine pepsinogen revealed that Lys51 in the prosequence lies medially between the two active site aspartic acid residues Asp31 and Asp74 and that adjacent Tyr25 occupies the P1-binding pocket forming a hydrogen bond to Asp74 (pepsin numbering) (James and Sielecki, 1986). In rhizopuspepsinogen, these residues, Lys53 and Tyr25, are structurally conserved and expected to interact with the mature part directly (Chen et al., 1991). They are also conserved in prosequences of many aspartic proteinases, including RNAP-I (Horiiuchi et al., 1988a; Sogawa et al., 1983; Tonouchi et al., 1988; Hayano et al., 1987; Razanamampany et al., 1992), and expected to play important roles in the interaction between the prosequence and the mature part of RNAP-I.

The mutated position in the prosequence of M1, in which Asp-Pro was substituted for Ala-Leu, is very close to the conserved Lys31-Tyr55 and may influence its function. Furthermore, prosequences of aspartic proteinases are rich in basic amino acid residues. In the prosequence of M1, one negative charge is introduced by replacing Ala49 with acidic amino acid residue, Asp, and structural flexibility is expected to decrease as a result of replacement of Leu with Pro. From the analysis of other RNAP-IIs with mutated prosequences in which single amino acid residues are substituted, it is also observed that positive charges of amino acid residues and structural flexibility in the prosequence play important roles in secretion of RNAP-I by S. cerevisiae. The substitution of those amino acid residues may prevent the correct interaction between the prosequence and the mature part and thereby the prosequence of M1 cannot help correct folding of the mature part fold correctly.

In general, secretory proteins which are not folded correctly are retained in the ER lumen (Helenius et al., 1992). From subcellular fractionation analysis and indirect immunofluorescence observation using anti-RNAP-I antisera, most Δpro and M1 seem to localize in the ER. Therefore, it is presumed that both Δpro and M1 cannot be folded correctly and that they are retained in the ER lumen.

Based on the results presented in this paper, we propose a model on the behavior of RNAP-I in the ER lumen of S. cerevisiae (Fig. 6). RNAP-I with the wild-type prosequence is folded correctly by the function of the prosequence in the ER lumen and secreted extracellularly. The prosequence is processed by its own activity autocatalytically or by intracellular or extracellular proteases of the host S. cerevisiae. On the other hand, certain deletions and amino acid substitutions in the prosequences block correct folding of the mature parts, so that these RNAP-IIs with mutated prosequences are retained in the ER lumen. When the wild-type prosequence is supplied in trans, they can take correct structures and are secreted extracellularly. In case of RNAP-I that has an amino acids-substituted prosequence, it is folded correctly in the presence of the wild-type prosequence supplied in trans and the mutated prosequence is removed, thus the mature part with proteinase activity is detected in the culture supernatants.

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