Activation and Processing of Non-anchored Yapsin 1 (Yap3p)*

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A C-terminally truncated form of yapsin 1 (yeast aspartic protease 3), the first member of the novel sub-class of aspartic proteases with specificity for basic residues (designated the Yapsins), was overexpressed and purified to apparent homogeneity, yielding ~1 µg of yapsin 1/g of wet yeast. N-terminal amino acid analysis of the purified protein confirmed that the propeptide was absent and that the mature enzyme began at Ala268. The mature enzyme was shown to be composed of approximately equimolar amounts of two subunits, designated α and β, that were associated to each other by a disulfide bond. C-terminally truncated proyapsin 1 was also expressed in the baculovirus/SF9 insect cell expression system and secreted as azymogen that could be activated upon incubation at an acidic pH with an optimum at ~4.0. When expressed without its pro-region, it was localized intracellularly and lacked activity, indicating that the pro-region was required for the correct folding of the enzyme. The activation of proyapsin 1 in vitro exhibited linear kinetics and generated an intermediate form of yapsin 1 or pseudo-yapsin 1.

Peptide hormones are synthesized as larger precursors that require endoproteolytic cleavage at basic residues in the secretory pathway. In yeast, the Golgi resident, subtilisin-like serine protease, Kex2p (1, 2), is responsible for the processing of pro-α-mating factor and pro-killer toxin at specific basic residue cleavage sites (3–5). However, in Kex2p-deficient mutants, two genes encoding aspartic proteases, YAP3 and MKC7, have been cloned. Yapsin 1 (previously named Yap3p) was able to suppress the cold-sensitive phenotype (7) observed in these mutant cells. Yapsin 2 (now named yapsin 2) was able to suppress the cold-sensitive phenotype (7) and appear to have a common substrate pool with that of Kex2p.

Both enzymes have specificity for basic amino acid residues and are anchored proteins (7–9) and share 53% amino acid identity (7).

The mature enzyme was shown to be composed of approximately equimolar amounts of two subunits, designated α and β, that were associated to each other by a disulfide bond. C-terminally truncated proyapsin 1 was also expressed in the baculovirus/SF9 insect cell expression system and secreted as a yzymogen that could be activated upon incubation at an acidic pH with an optimum at ~4.0. When expressed without its pro-region, it was localized intracellularly and lacked activity, indicating that the pro-region was required for the correct folding of the enzyme. The activation of proyapsin 1 in vitro exhibited linear kinetics and generated an intermediate form of yapsin 1 or pseudo-yapsin 1.

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Our recent work has shown that yapsin 1 is immunologically related to pro-opiomelanocortin converting enzyme, an enzyme with similar backbone size and specificity. Moreover, immunocytochemical studies showed colocalization of immunoreactive yapsin 1-related processing enzymes in neuropeptide-rich regions of mammalian brain and pituitary (13) supporting the existence of mammalian homologues of yapsin 1 involved in pro-neuropeptide processing. We also showed that yapsin 1 can function in vivo to process pro-opiomelanocortin in the mammalian PC12 cell line (17). This indicates a close structural and functional relationship of yapsin 1 with mammalian yapsins. Thus yapsin 1, which has been the most extensively studied member of the yapsin family, represents an ideal model enzyme for learning how the yapsin class of aspartic proteases are synthesized. Studies on the biosynthesis of yapsin 1 will also further define the structure of the enzyme and shed light on the best substrates that can interact with the enzyme. This will ultimately provide an insight into the physiological role that yapsin 1 plays in yeast.

**EXPERIMENTAL PROCEDURES**

**Purification and Analysis of Yapsin 1 Secreted from Overexpressing Yeast**

Carboxyl-terminally truncated soluble yapsin 1 enzymatic activity was found to be secreted into the culture supernatant of a previously described yeast expression system (14) and was used as the starting material for the purification of the enzyme.

**Concentration**—The yeast culture supernatant containing the secreted yapsin 1 enzyme was concentrated by tangential flow filtration at room temperature using a 30-kDa molecular mass cutoff omega membrane in an ultrasette cassette (Filtron, Northborough, MA). A 4-fold diafiltration step to replace the media with 20 mM sodium phosphate buffer, pH 7.0, was also performed. The sample was further concentrated by centrifugation filtration using a Filtron 30-kDa Mac-

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§ The abbreviations used are: GPI, glycosphatidylinositol; YAP3, yeast aspartic protease 3; ACTH, adrenocorticotropin; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; ConA, concanavalin A; HPLC, high pressure liquid chromatography.

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2 N. X. Cawley, V. Olsen, and Y. P. Loh, unpublished observations.
rosey membrane filter. All subsequent procedures were carried out at 4°C.

**Anion Exchange Chromatography (MonoQ1)**—The concentrated medium was applied to a MonoQ HR 5/5 column (Pharmacia Biotech Inc.) connected to a fast protein liquid chromatography system (Pharmacia). The column was allowed to re-equilibrate in buffer A (20 mM sodium phosphate, pH 7.0) before a gradient of 0–25% buffer B (buffer A with 1 M NaCl) was applied. The flow rate was 1 ml/min, and 0.5-ml fractions were collected. The fractions were assayed by the standard ACTH$_{1–15}$ assay, previously described (15). Two fractions on either side of the peak of activity were pooled and re-run on the MonoQ. The peaks of activity were combined and concentrated by centrifugation filtration through a Filtron 30-kDa Microsep omega membrane filter.

**Gel Filtration Chromatography (Superdex G-200)**—The concentrated sample from MonoQ1 was applied to a High Load 16/60 Superdex G-200 superfine column (Pharmacia) that was connected to a Fast Protein Liquid Chromatography (FPLC) system. The column was allowed to re-equilibrate in buffer A (20 mM sodium phosphate, pH 7.0) before a gradient of 0–25% buffer B (buffer A with 1 M NaCl) was applied. The flow rate was 1 ml/min, and 0.5-ml fractions were collected. The fractions were assayed by the standard ACTH$_{1–15}$ assay, previously described (15). Two fractions on either side of the peak of activity were pooled and re-run on the MonoQ. The peaks of activity were combined and concentrated by centrifugation filtration through a Filtron 30-kDa Microsep omega membrane filter.

**Expression and Analysis of Proyapsin 1 from Baculovirus**

Proyapsin 1 and yapsin 1 (Δproyapsin 1) were engineered for expression in the baculovirus expression system. Recombinant YAP3 gene constructs, encoding amino acids 22–532 (proyapsin 1) and 68–532 (Δproyapsin 1), were engineered by PCR using the primer pairs 1/3 and 2/3, respectively (see Table I for primer sequences), and the previously characterized expression vector, pYAP3/LC (8) as template. Both PCR fragments containing a BamHI and a PsI restriction site at their 5’ and 3’ ends, respectively, were subclone into the pAcGP67 baculovirus transfer vector downstream of the strong signal peptide of the acidic glycoprotein, gp67. The pAcGP67 plasmids containing the YAP3 inserts were cotransfected with the BaculoGold viral DNA (Pharmingen, San Diego, CA) into Spodoptera frugiperda (SF9) cells. Recombinant viral particles were identified by plaque assay and harvested for high titer stock generation. For the expression of the recombinant SF9 cells, 10 ml of serum-free medium (SF-900 II SFM, Life Technologies, Inc.) supplemented with penicillin and streptomycin. After reaching 80% confluence, the cells were infected with 18 ml of fresh medium containing 2 ml of purified recombinant baculovirus (1.1×10$^{10}$ pfu/ml). Two days postinfection, the cells and media were collected for analysis by Western blot and by the yapsin 1 activity assay. The media were concentrated and partially purified by concanavalin A (ConA) affinity chromatography (22) prior to analysis, and the soluble cell extracts were analyzed without manipulation except for an aliquot of the Δproyapsin 1 sample which was treated with and without N-glycanase, according to the company’s guidelines (Genzyme, Cambridge, MA) and analyzed by Western blot.

**Other experiments** described below utilized the culture supernatant, from multiple flask of cells expressing proyapsin 1, that were concentrated by centrifugation through a Macrosep 10-kDa omega membrane and frozen at −20°C until analysis. A fresh preparation of a protease inhibitor mixture (Complete$,^\text{TM}$, Boeringer Mannheim, Germany) containing EDTA but not pepstatin A was added prior to the experiments to minimize non-specific degradation during the procedures.

**pH-dependent Activation of Proyapsin 1**—The effect of pH on the activation of proyapsin 1 was investigated. Five μl of the proyapsin 1 sample were incubated in 20 μl of 0.1 M sodium citrate or sodium citrate/sodium phosphate buffers with pH values ranging from pH 2.6 to 7.2 for 20 h at 30°C. Five μl from each incubate were adjusted to pH 4.3 and assayed for yapsin 1 activity by the ACTH$_{1–15}$ assay which was carried out at 30°C for 30 min.
was investigated by a pre-activation experiment. Forty five μl of the proyapsin 1 were incubated in 900 μl of 0.1 M sodium citrate/sodium phosphate buffer, pH 4.3, at 30 °C, in the absence of ACTH1–39. At regular times after the start of the incubation, 90 μl were removed, added to 10 μl of ACTH1–39 (220 μM), and incubated for a further 30 min at 30 °C. ACTH1–15 was then quantitated by HPLC. To investigate if active yapsin 1 itself could activate proyapsin 1, 5 μl of proyapsin 1 were incubated in the presence and absence of 2.2 units of purified yapsin 1 from yeast for 50 min at 30 °C in 0.1 M sodium citrate/sodium phosphate buffer, pH 4.3. ACTH1–39 (220 μM) was then added, and the tubes were incubated for a further 30 min at 30 °C. The presence of ACTH1–15 was then quantitated by HPLC.

To study the molecular changes that occurred after activation, a pre-activation experiment was performed and analyzed by Western blot and assayed for enzymatic activity. Ten ml of the incubates were assayed for the presence of yapsin 1 activity on the Superdex G-200 column resulted in a bimodal activity in the G-200 step. The inclusion of a second MonoQ column resulted in the step with the highest fold purification of yapsin 1 activity (Fig. 1B). Application of the MonoQ1 pool of activity on the Superdex G-200 column resulted in a bimodal distribution of yapsin 1 activity (Fig. 1B) which was attributed to the two hyperglycosylated forms of secreted yapsin 1, a 150–180-kDa and a 90-kDa form that have previously been characterized (8). Fractions 54–63, corresponding to the 150–180-kDa molecular mass form of yapsin 1, were pooled for the next purification step resulting in a 57% recovery of activity in the G-200 step. The inclusion of a second MonoQ column at this time was necessary to remove a contaminating protein, identified by amino acid sequencing as glucan-1,3-β-glucosidase (Fig. 2, lanes 2 and 3), that appeared to be co-purifying with yapsin 1. The shallow NaCl gradient used to elute the protein from the column generated a broad peak of yapsin 1 activity (data not shown); however, only the first half of the peak (fractions 52–57) was pooled, to avoid the glucosidase, and referred to as purified yapsin 1.

Silver stain analysis demonstrated the apparent homogeneity of the yapsin 1 preparation by staining only one protein (Fig. 2, lane 1), and amino-terminal amino acid sequence analysis resulted in two yapsin 1 sequences as follows: sequence 1, A68DGYEEEHTNQQSF; sequence 2, D145INPFGWL(T)GTGS-AI. The internal sequence of yapsin 1, starting at Asp145, has been obtained on four different occasions, each with a different enzyme preparation, rendering this cleavage consistent and specific. When the picomole recovery of amino acids from each cycle of the sequencer was plotted, a least squared linear regression fit allowed an initial concentration of each sequence to be calculated by extrapolation. The average relative ratio of the two sequences was 1.22:1 for sequence 1 relative to sequence 2, demonstrating that ~80% of the yapsin 1 molecules has been processed into two subunits, α and β. To determine if the two yapsin 1 subunits were associated by a disulfide bond, a sample of a partially purified preparation of yapsin 1 was analyzed by SDS-PAGE under nonreducing and reducing conditions followed by Coomassie Blue staining and amino-terminal amino acid sequencing. Coomassie Blue staining of 1.6 μg of unreduced protein from the Superdex G-200 pool of yapsin 1 activity that had been deglycosylated by endoglycosidase H showed a major protein between the 66- and 97-kDa molecular mass standards (Mark12, Novex, San Diego, CA) and a minor protein at ~31 kDa (Fig. 2, lane 2). Amino-terminal amino acid sequencing of the upper band gave a similar relative picomole ratio of the α- and β-subunit sequences of yapsin 1 as the purified preparation described above, whereas the lower band contained only yeast glucan-1,3-β-glucosidase. When a similar aliquot of the protein was run in the presence of β-mercaptoethanol, a reduction in the molecular mass of the yapsin 1 band

### Table II

| Step                  | Total volume (ml) | Protein conc. (μg/ml) | Specific activity (units/μg protein) | Total activity (units) | Recovery (%) | Purification fold |
|-----------------------|-------------------|-----------------------|-------------------------------------|------------------------|--------------|-------------------|
| Media                 | 3900              | 3.4                   | 287.1                               | 3806,946               | 100          | 1                 |
| Concentration         | 20                | 440.9                 | 725.6                               | 6398,341               | 188          | 2.5               |
| MonoQ1                | 1                 | 650.7                 | 6328.7                              | 4118,085               | 108 (64.4)   | 22 (8.7)          |
| Superdex G-200        | 10                | 29.6                  | 7972.9                              | 2359,978               | 62 (57.3)    | 27.8 (1.3)        |
| MonoQ2                | 3                 | 30.1                  | 12,533.3                            | 1131,757               | 29.7 (47.9)  | 43.6 (1.6)        |

*Units are described as the amount of enzyme that generates 0.18 μg of ACTH1–15 from 10 μg of ACTH1–39 in a 100 μl 0.1 M sodium citrate buffer, pH 4.3, at 37 °C for 30 min (15). The numbers in parenthesis reflect the % recovery and fold purification for each step.

### Results

**Purification and Analysis of Yapsin 1 from Yeast**

Yapsin 1 was purified from the culture supernatant of the yeast expression system yielding −1 μg of yapsin 1/g of wet yeast. The purification procedure resulted in an overall recovery of 29.7% of yapsin 1 from the starting media with an overall fold purification of 43.6 (Table II). Anion exchange chromatography of the concentrated culture supernatant on the MonoQ1 column resulted in the step with the highest fold purification of 8.7 and a recovery of −64%. Yapsin 1 eluted from the column in 100–150 mM NaCl (Fig. 1A). Application of the MonoQ1 pool of activity on the Superdex G-200 column resulted in an overall recovery of 29.7% of yapsin 1 from the starting media with an overall fold purification of 43.6 (1.6). The internal sequence of yapsin 1, starting at Asp145, has been obtained on four different occasions, each with a different enzyme preparation, rendering this cleavage consistent and specific. When the picomole recovery of amino acids from each cycle of the sequencer was plotted, a least squared linear regression fit allowed an initial concentration of each sequence to be calculated by extrapolation. The average relative ratio of the two sequences was 1.22:1 for sequence 1 relative to sequence 2, demonstrating that ~80% of the yapsin 1 molecules has been processed into two subunits, α and β. To determine if the two yapsin 1 subunits were associated by a disulfide bond, a sample of a partially purified preparation of yapsin 1 was analyzed by SDS-PAGE under nonreducing and reducing conditions followed by Coomassie Blue staining and amino-terminal amino acid sequencing. Coomassie Blue staining of 1.6 μg of unreduced protein from the Superdex G-200 pool of yapsin 1 activity that had been deglycosylated by endoglycosidase H showed a major protein between the 66- and 97-kDa molecular mass standards (Mark12, Novex, San Diego, CA) and a minor protein at ~31 kDa (Fig. 2, lane 2). Amino-terminal amino acid sequencing of the upper band gave a similar relative picomole ratio of the α- and β-subunit sequences of yapsin 1 as the purified preparation described above, whereas the lower band contained only yeast glucan-1,3-β-glucosidase. When a similar aliquot of the protein was run in the presence of β-mercaptoethanol, a reduction in the molecular mass of the yapsin 1 band.
to ~65 kDa was observed concomitant with the appearance of a diffuse protein at ~34 kDa (Fig. 2, lane 3). Amino-terminal amino acid sequencing of the upper band resulted in the β-subunit sequence of yapsin 1, whereas the lower band resulted in the α-subunit sequence of yapsin 1 in addition to the glucosidase sequence.

Analysis of an Active Site Mutant of Yapsin 1 Expressed in ΔYapsin 1 Yeast

It was demonstrated by Western blot using antiserum MW283 that yapsin 1 and yapsin 1(Asp101 → Glu101) were successfully expressed and secreted at similar levels in the Δyapsin 1 yeast strain (Fig. 3A, lanes 3 and 4). Yapsin 1 was ~5 kDa smaller than the active site mutant. No enzymatic activity was present from the culture supernatant of the mutant yapsin 1, although abundant activity was observed with the normal yapsin 1 (Fig. 3B). Western blot analysis using antiserum VO2377, which is specific for the propeptide of yapsin 1, showed strong immunostaining of yapsin 1(Asp101 → Glu101) (Fig. 3A, lane 1), whereas only trace amounts were evident in the normal yapsin 1 sample (Fig. 3A, lane 2).

Analysis of Baculovirus-expressed Yapsin 1 Recombinants

Western blot analysis of ConA-purified media (1.6 μg of protein) from SF9 cells expressing proyapsin 1 and Δproyapsin 1 demonstrated that proyapsin 1 was secreted into the growth media as a ~60-kDa protein (Fig. 4A, lane 1), whereas the Δproyapsin 1 was not secreted (Fig. 4A, lane 2). Western blot analysis of the corresponding cell extracts (3.8 and 4.3 μg of protein) showed that proyapsin 1 was present as a ~75-kDa protein and Δproyapsin 1 was present as a ~70-kDa protein (Fig. 4A, lanes 3 and 4). Furthermore, Δproyapsin 1 shifted to a lower molecular mass upon treatment by N-glycanase indicating the presence of N-linked sugars on Δproyapsin 1 (data not shown).

The ConA-purified media and the soluble cell extracts (1.3 and ~12 μg of protein, respectively) were further analyzed by the ACTH1–39 activity assay with and without a pre-activation step at pH 4.3 and 37 °C. The ConA-purified media were pre-activated for 12 h, and the soluble cell extracts were pre-activated for 2 h. Yapsin 1 enzymatic activity was found only in the proyapsin 1 samples yielding a ~31-fold increase in yapsin 1 activity from the ConA-purified media and a 5.6-fold increase in yapsin 1 activity from the soluble cell extract as a result of the pre-activations (Table III).

pH-dependent Activation of Proyapsin 1—After incubation of 5 μl of proyapsin 1 for 20 h at various pH values, an aliquot of each incubate was assayed for yapsin 1 activity. A plot of nanogram of ACTH1–15 generated versus preincubation pH demonstrated a pH-dependent generation of yapsin 1 activity (Fig. 5). Activity was generated at acidic pH values between 3.0 and 5.0 with an optimum at pH 4.0. The samples that were incubated below pH 3.0 were unable to be activated by a subsequent incubation at pH 4.3 indicating that the enzyme had
been irreversibly denatured. However, the samples that were incubated above pH 5.0 were able to be activated by a subsequent incubation at pH 4.3 indicating that the enzyme had not been denatured (data not shown).

Time Course Studies on the Activation of Proyapsin 1—When proyapsin 1 was incubated at 30 °C and pH 4.3 in the presence of ACTH$_{1-39}$ for various time points, the activity profile obtained demonstrated an initial lag in the generation of ACTH$_{1-15}$ after which the product was generated in an apparent first-order manner at pH 4.0.

When proyapsin 1 was incubated at 30 °C and pH 4.3 in the absence of ACTH$_{1-39}$ for various time points and then assayed for yapsin 1 activity, it was found that yapsin 1 activity was generated with apparent first-order kinetics (Fig. 6B). When 2.2 units of purified yapsin 1 were incubated with proyapsin 1 at 30 °C and pH 4.3 for 30 min prior to the ACTH$_{1-39}$ assay, the resulting total activity (594 ng of ACTH$_{1-15}$) was approximately equal to the combined activity of the pure yapsin 1 from yeast (406.6 ng ACTH$_{1-15}$) and the activated yapsin 1 from the

**TABLE III**

Summary of the enzymatic activity of proyapsin 1 and Δproyapsin 1 expressed in the baculovirus/Sf9 expression system

| Yapsin 1 molecule | Soluble cell extract | ConA-purified media |
|------------------|---------------------|--------------------|
|                  | No activation       | Activation         |
| Proyapsin 1      | 57.6                | 324                |
| ΔProyapsin 1     | ND                  | 18                 |

**FIG. 5.** pH activation profile of proyapsin 1 expressed in Sf9 cells. Thirteen aliquots of proyapsin 1 (5 μl) were incubated for 20 h at 30 °C with a range of pH values between 2.6 and 7.2. An aliquot of each sample was then adjusted to pH 4.3 and analyzed by the ACTH$_{1-39}$ assay at 30 °C. Activity is plotted as nanograms of ACTH$_{1-15}$ generated/5 μl of proyapsin 1/100-μl reaction as a function of the preincubation pH. Activity was generated in a pH-dependent manner with an optimum at pH 4.0.

**FIG. 6.** Kinetic studies on the activation of proyapsin 1. A, proyapsin 1 was incubated for 7 h with 22 μg ACTH$_{1-39}$, pH 4.3, 30 °C, and at regular time points, the presence of ACTH$_{1-39}$ was quantitated by HPLC. The data presented are a combination of three experiments, and the activity is expressed as nanograms of ACTH$_{1-15}$ generated/5 μl of proyapsin 1/100-μl reaction. Note the lag phase in the profile indicative of the formation of an intermediate. A nonlinear regression fit of the data, calculated by SigmaPlot™, version 4.0, resulted in the following equation, $y = 123.6x + 81.5$, $r^2 = 0.99$. B, proyapsin 1 was preincubated without ACTH$_{1-39}$, pH 4.3, and 30 °C. At different time points, aliquots were removed, added to ACTH$_{1-39}$, and allowed to incubate for a further 30 min at 30 °C. The amount of ACTH$_{1-15}$ generated was then quantitated by HPLC. The data presented are a combination of three experiments, and the activity is expressed as nanograms of ACTH$_{1-15}$ generated/5 μl of proyapsin 1/100-μl reaction. Note the linearity in the generation of yapsin 1 activity demonstrated by its equation, $y = 123.6x + 81.5$, $r^2 = 0.99$. Inset, 5 μl of proyapsin 1 were preincubated with and without 2.2 units of active yapsin 1 from yeast for 30 min, pH 4.3, 30 °C, and then total yapsin 1 activity was determined by the ACTH$_{1-39}$ assay at 30 °C. The results presented are the average of two experiments. Yapsin 1 alone (1) generated 406.6 ng of ACTH$_{1-15}$ and proyapsin 1 alone (2) generated 219.6 ng of ACTH$_{1-15}$. When combined (3) the resulting total activity of 594 ng of ACTH$_{1-15}$ demonstrated that active yapsin 1 did not activate the proyapsin 1.
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V02377 and MW283. In addition to the observed increase in yapsin 1 activity (Fig. 7A), proyapsin 1 was converted from ~65 kDa (Fig. 7B, lanes 1 and 3) to a slightly smaller protein that was immunoreactive with both MW283 and V02377 (Fig. 7B, lanes 2 and 4). The intensity of the immunostained band of proyapsin 1 (unactivated and activated) under nonreducing and reducing conditions remained the same (data not shown) and indicated that baculovirus-expressed proyapsin 1 was not processed into subunits.

**DISCUSSION**

Our previous work demonstrated that recombinant yapsin 1, devoid of its GPI membrane-anchoring domain, was efficiently overexpressed and secreted from a yeast expression system. These forms of yapsin 1 were found to be hyperglycosylated and stable to lyophilization and long term storage under a variety of conditions. In contrast to the yapsin 1 obtained from the cellular extract of the same expression system (14), the stability of the secreted enzyme rendered it useful for kinetic studies since the specific activity remained constant for long periods. In addition, the abundance of yapsin 1 activity in the culture supernatant as compared with the cell extract (8) represented a source of enzyme in quantities potentially sufficient for structural analysis. Yapsin 1 was purified to apparent homogeneity from the culture supernatant yielding ~1 µg of yapsin 1/g of wet yeast. The procedure utilized anion exchange chromatography (Fig. 1A) based on the finding that the isoelectric point (pI) of yapsin 1 was ~4.5 (8), thus rendering this enzyme negatively charged at pH 7.0. Gel filtration chromatography was also used (Fig. 1B), since secreted yapsin 1 was previously determined to be hyperglycosylated, primarily giving a molecular mass of ~150–180 kDa (8, 9). A combination of these procedures was used with an overall recovery of 29.7% and a fold purification of 43.6 (Table II). The unexpected increase in total activity observed in the concentration step (168%, Table II) was initially thought to be from the removal of a low molecular mass inhibitor; however, upon addition of the filtrate back to the retentate, no inhibition of the activity was observed (data not shown). The presence of a small amount of proyapsin 1 in the media (Fig. 3A, lane 2) that became activated during the initial stages of the concentration step may in part account for the increase. The major increase, however, is most likely due to compounded small errors in the pipetting introduced during the many dilutions of up to 1 × 10³ which were required to obtain quantifiable yapsin 1 activity in the highly sensitive ACTH1-15 assay for yapsin 1 (picogram range). This was confirmed when the procedure was repeated on a smaller scale where such large dilutions were not necessary. In that instance the recovery of total yapsin 1 activity in the retentate was ~95% of the starting material.

Purity of the final preparation of enzyme was confirmed by silver stain (Fig. 2, lane 1) and amino-terminal amino acid analysis. As expected, the mature enzyme was shown to start at Ala⁶⁸ indicating that the proregion had been removed, but surprisingly, an additional internal sequence was obtained. Processing in this region resulted in the generation of two subunits designated here as α- and β-subunits each with one of the active site triads (Fig. 8D). The amino-terminal amino acid of the β-subunit, identified as Asp⁶⁵, is situated within a unique loop domain of yapsin 1 arising from a large insertion of ~76 residues in comparison with pepsin, rendering this site susceptible to cleavage by proteases. Whether the loop gets cleaved at this residue only or amino-terminally to this residue followed by aminopeptidase trimming is still unknown. This loop domain is also found in yapsin 2 and may represent an important domain of the yapsins in general. Similar processing of cathepsin D has also been observed in a species- and tissue-specific manner where both active site triads are separated into two subunits by cleavage at a site within a 9-residue insertion (23). Processing into subunits appears to be a rare event for mammalian aspartic proteases, with cathepsin D as one exception, but it has been documented for Aspergillus niger proteinase A (24) and a barley aspartic protease (25). However, until now, association of these subunits was found to be noncovalent.

We have shown here that the yapsin 1 subunits are associated by a disulfide bond (Fig. 2, lanes 2 and 3), and by comparison with the known structure of pepsin (26), the disulfide bond is most likely between Cys¹¹⁷ of the α-subunit and Cys¹⁸⁶ of the β-subunit. The relevance of such a processing is unknown; however, preliminary pulse-chase experiments have indicated that it occurs early in the yeast secretory pathway and may represent a key event in the structure/function relationship of yapsin 1 in vivo.

The amino-terminal amino acid of the α-subunit, identified as Ala⁶⁸, indicated that the proregion was removed at the paired basic residue site, Lys⁶⁶-Arg⁶⁷, a site similar to that in the activation of pro-renin to renin (27, 28). Since this propeptide processing site represents an efficient motif for yapsin 1 cleavage, RXKR (15), it suggested that processing of the propeptide may occur by autoactivation in contrast to renin which relies on a trypsin-like activity, although active renin has been reported to contain some intrinsic activity capable of activating prorenin (29). We investigated this hypothesis by expressing an active site mutant of yapsin 1 and demonstrated that the inactive enzyme was ~5 kDa bigger than the normal active yapsin 1 (Fig. 3, lanes 2 and 4). This expected difference in size corresponded to the theoretical molecular mass of the propeptide, the presence of which was confirmed by Western blot (Fig. 3, lane 1) and is consistent with the inability of the active site mutated yapsin 1 to remove its own propeptide. Also,

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| Sample | ng ACTH1-15 generated |
|--------|----------------------|
| Unactivated | 72.0 |
| Activated* | 2505.6 |

**FIG. 7.** Proyapsin 1 was incubated for 22 h at pH 7.2 and pH 4.3. A, an aliquot of each was assayed for yapsin 1 activity, and the results were expressed as nanogram of ACTH1-15 generated/µl of proyapsin 1/100-µl reaction. B, additional aliquots were analyzed by Western blot under reducing conditions on a 12% Trisglycine SDS-PAGE gel. Lanes 1 and 2 were probed with antiserum MW283, and lanes 3 and 4 were probed with antiserum V02377. Note the size difference between the immunoreactive bands in the unactivated (lanes 1 and 3) and the activated samples (lanes 2 and 4) stained with both antisera.

\[ \text{V. Olsen, N. X. Cawley, and Y. P. Loh, manuscript in preparation.} \]
Fig. 8. Schematic diagram of the structural components of yapsin 1. A, the molecule contains a typical signal sequence (ss, amino acids 1–21), a proregion (pro, amino acids 22–67), and the mature enzyme (amino acids 68–569). The GPI membrane anchor is situated in the extreme carboxyl-terminal domain of the molecule. B, from the studies of the baculovirus-expressed proyapsin 1, an intermediate or pseudo-yapsin 1 has been identified presumably as a result of a self-cleavage at Lys$^{56}$ (i.e. Lys$^{18}$ of the propeptide). C, the remainder of the proregion is then likely to be removed at the Lys$^{56}$-Arg$^{57}$ cleavage site by an intra-molecular mechanism. D, from the studies on yapsin 1 expressed in yeast, it was verified that the proregion had been completely removed at Lys$^{56}$-Arg$^{57}$. The mature enzyme is also processed into an α- and β-subunit resulting in Aasp$^{145}$ as the amino-terminal amino acid of the β-subunit. The subunits are associated by a disulfide bond predicted to be between Cys$^{137}$ and Cys$^{150}$. The carboxyl terminus is removed during the GPI-anchoring process which is predicted to occur at Asn$^{145}$. The two active site aspartic acid residues are indicated by an asterisk.

since both constructs were expressed in yapsin 1-deficient yeast strains, we can also conclude that the two other known endogenous processing enzymes with specificity for this type of cleavage site, Kex2p and Mke7p (yapsin 2), were not able to process the mutant proyapsin 1 either. It appears, therefore, that removal of the propeptide is dependent upon active yapsin 1 molecules.

To study further the activation of yapsin 1 in vitro, yapsin 1 was expressed in the baculovirus/Sf9 system, with and without its proregion (proyapsin 1 and Δproyapsin 1, respectively), and analyzed with respect to its properties. Both recombinant proteins were expressed in this system; however, only proyapsin 1 was secreted (Fig. 4, lane 1). Absence of the proregion resulted in the expression of an intracellularly localized protein (Fig. 4, lane 4) that contained no apparent activity even when pre-activated (Table III). These observations are consistent with a misfolded protein that was transport-incompetent. However, that it had entered the secretory pathway is supported by the result that it was N-glycanase-sensitive (data not shown). In contrast to Δproyapsin 1, proyapsin 1 was secreted from the cells and possessed significant levels of activatable yapsin 1 activity (Table III), showing that the proregion was required for the correct folding and secretion of yapsin 1, and only as a correctly folded enzyme was it able to be activated. This activation process was characterized further and shown to occur optimally at pH 4.0 with <10% activated in the pH range of 5.5–6.0 and no apparent activation at the more neutral pH values (Fig. 5). In addition, the enzyme was irreversibly denatured below pH 3.0 (data not shown), a result that had been observed previously (30). The observed progressions of product formation in both the activity profile experiment (Fig. 6A) and the pre-activation experiment (Fig. 6B) indicated that the mechanism of activation was intramolecular, a result that was further supported by the result that purified yapsin 1 from yeast was unable to activate the proyapsin 1 (Fig. 6B, inset).

Previous reports by us (8) and others (9) have deduced that yapsin 1 is located to the extracellular side of the plasma membrane via its GPI membrane anchor and is presumed to be its site of action. Since the pH of yeast growth media is acidic (pH 5.0–6.0) and becomes more acidic with growth, the periplasmic space may represent the primary compartment where autoactivation occurs. However, yapsin 1 was first cloned based on its ability to process the yeast prohormone, pro-α-mating factor (6), in the secretory pathway of KEX2-deficient yeast. Since the synthesis of biologically active α-mating factor requires the subsequent action of two other Golgi resident enzymes, Kex1p (31), a carboxypeptidase B-like enzyme and a dipeptidyl-aminopeptidase (32), it is assumed that a sufficient amount of proyapsin 1 must become activated in the late Golgi in order for mature α-factor to be generated. This is supported by the fact that proyapsin 1 was expressed and functionally active in mammalian PC12 cells (17) where the pH ranges from pH 6.2 in the trans-Golgi network (33) to pH 5.0–5.5 in mature secretory vesicles (34).

Investigation of the biosynthesis and maturation of aspartic proteases such as endothiапепсин (35), cathepsin D (36), pepsin (37), Rhizopus niveus aspartic proteinase-I (38), and yeast proteinase A (39, 40) have all demonstrated the importance of their proregion in the folding of the enzyme and the regulation of their activity. For pepsin, the model aspartic protease, its proregion, which contains 11 basic residues, is partially stabilized by ionic interactions with the negatively charged aspartic acid residues of the active site (41). Upon acidification, the aspartic acid residues become protonated and less charged (pKa = 4.3) causing destabilization and an intramolecular cleavage at Leu$^{16}$-Ile$^{17}$ within the propeptide resulting in the generation of an intermediate form of the enzyme or pseudo-pepsin. The intermediate form undergoes a further intermolecular or intramolecular cleavage of the remainder of the proregion to generate the mature active enzyme. It is interesting to note the similarity to yapsin 1 in this respect in that a potential cleavage site exists at Lys$^{16}$-Phe$^{17}$ within the propeptide. Because of this structural similarity and that cleavage of a mono-lysine residue by yapsin 1 has previously been documented (15), it is predicted that a yapsin 1 intermediate, formed from an intramolecular cleavage presumably at this site, exists. Our activation studies support this prediction, since during the time course experiment, an initial lag phase (within 3 h) in the generation of yapsin 1 activity was observed (Fig. 6A). This observation is consistent with a molecular event that involves the generation of an intermediate, a process that has been documented for other aspartic proteases (36, 37, 42). Further
support came from the pre-activation experiment where the immunoreactive proyapsin 1 was converted to a slightly smaller protein upon activation (Fig. 7A). Since this band still contained the propeptide antigenic site for antiserum VO2377, i.e. Tyr57-Glu61 (Fig. 7A, lane 4), the size shift is consistent with removal of an amino-terminal portion of the proregion and the assignment of this band as an intermediate, i.e. pseudo-yapsin 1. Although trimming at the carboxyl terminus cannot be completely ruled out, the use of serum-free medium, a 2-day incubation to limit cell lysis and the addition of protease inhibitors, renders the presence of potential proteases specific for the carboxyl terminus of proyapsin 1 highly unlikely. Furthermore, the inability of 6 μM pepstatin A to inhibit formation of pseudo-yapsin 1 (data not shown) indicates that another aspartic protease was not responsible for the processing of proyapsin 1 and provides further evidence that the process was intra-molecular since the active site, already tightly bound to the proregion, was inaccessible to the inhibitor.

Although the generation of pseudo-yapsin 1 appears to be fairly rapid, within 3 h as evidenced by the lag in Fig. 6A and a time course study (data not shown), the generation of yapsin 1 from pseudo-yapsin 1 appeared to be extremely slow. The concentration of proyapsin 1 in the starting material was estimated by Western blot from a standard curve made with purified yapsin 1 from yeast (data not shown) and based on 100% conversion of this amount, the amount of enzymatic activity was inaccessible to the inhibitor. Attempts at increasing the efficiency with either calcium or protease was not responsible for the processing of proyapsin 1 and provides further evidence that the process was intra-molecular.

In conclusion, our studies with yeast have demonstrated that yapsin 1 is a heterodimer stabilized by an intramolecular disulfide bond, and the final proregion cleavage site, which was yapsin 1-dependent, was confirmed to occur at the Lys56-Arg67 bond and may be the reason why pseudo-yapsin 1 appears to get stuck in that form. Other pseudo-aspartic proteases, such as pseudo-chymosin and pseudo-cathepsin D (42, 43) under certain conditions, have also been found to be poorly converted.

In conclusion, our studies with yeast have demonstrated that mature yapsin 1 is a heterodimer stabilized by an intramolecular disulfide bond, and the final proregion cleavage site, which was yapsin 1-dependent, was confirmed to occur at the Lys56-Arg67 bond and may be the reason why pseudo-yapsin 1 appears to get stuck in that form. Other pseudo-aspartic proteases, such as pseudo-chymosin and pseudo-cathepsin D (42, 43) under certain conditions, have also been found to be poorly converted.

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