Aminopeptidase Y, a New Aminopeptidase from Saccharomyces cerevisiae

PURIFICATION, PROPERTIES, LOCALIZATION, AND PROCESSING BY PROTEASE B*

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A novel aminopeptidase, termed aminopeptidase Y, was purified from yeast, Saccharomyces cerevisiae, as two molecular forms of 70 and 75 kDa, which were identical immunologically, catalytically and in N-terminal sequence up to 14 residues. They contained 0.81 and 0.84 mol of zinc atom/mol of protein, respectively. N-Glycocanase generated a single 54-kDa species from both forms, and endoglycosidase H gave a 54-kDa species. Immunoblotting after subcellular fractionation showed that aminopeptidase Y is localized in the vacuole/lysosome-soluble compartment as the 70-kDa form. Aminopeptidase Y hydrolyzed all amino acid-4-methylcoumaryl-7-amides (MCA) examined, especially Lys- and Arg-MCA. Dipeptidyl-MCAS were far more rapidly hydrolyzed (Lys-Ala-MCAlys-MCA was markedly enhanced by Goa+, Arg-MCA/Arg"CA ArgMCk Dipeptidyl-MCAS were far more rapidly hydrolyzed (Lys-Ala-MCAlys-MCA was markedly enhanced by Goa+, Arg-MCA/Arg"CA ArgMCk Dipeptidyl-MCAS were far more rapidly hydrolyzed. Rabbit anti-aminopeptidase Y antiserum adsorbed over half the aminopeptidase activities in vacuolar extract from wild-type yeast cells. Aminopeptidase Y antisera inhibited aminopeptidase-generated a single 53-kDa species from both mutant cells from which the genes of four yeast vacuoles had been deleted contained aminopeptidase Y as a 74-kDa form. This was converted to the mature form (70 kDa) by vacuolar extract of wild-type cells and by purified yeast vacuolar protease B.

Yeast vacuoles, which are equivalent to lysosomes found in higher eukaryotic organisms (1), contain a protein degradation system composed of several kinds of proteases, proteases A and B (PrA, PrB), carboxypeptidases Y and S (CPY, CPS), and aminopeptidases I and Co (API, APCo) (for review, see Ref. 2). The vacuole/lysosome protein degradation system plays a role in protein turnover, changing the proteolytic activities to adapt to the nutritional conditions (3). In the absence of the vacuolar proteases, yeast cells cannot survive under poor nutritional conditions (4). Recent results show that both vacuoles and lysosomes are responsible for proteolysis of not only endocytosed or phagocytosed proteins but also cytosolic proteins (Ref. 5 and for a review, see Ref. 6). The vacuoles also function for storage of amino acids (7), especially basic amino acids (8). For the complete degradation of proteins to amino acids, exopeptidases such as carboxypeptidases and aminopeptidases are needed.

PrA, PrB, and CPY are imported as proforms and processed on the way to or after reaching the vacuoles; they function as the mature proteases (9). Of these proteases, PrA seems primarily responsible for the processing of a number of vacuolarzymogens, since the pep4 mutant lacking the activity of PrA also lacks the activities of PrB, CPY, and alkaline phosphatase (10), and at least CPY and PrB were accumulated as larger precursors (11, 12). Conversion of pro-CPY to mature CPY is catalyzed by PrB, which might be activated both by PrA and autocatalytically (13, 14), or by an alternate activation cascade in which active intermediates are formed by the direct action of PrA (15).

Aminopeptidases exist ubiquitously in organisms (16) and play a role in catabolizing peptides and processing bioactive peptides, as well as functioning as leukotriene A4 hydrolase (17). Despite many studies on aminopeptidases, nothing is known about their processing for activation or sorting. Several yeast aminopeptidases have been reported as vacuolar enzymes, but many of them correspond to API (9). We describe here a new and highly potent vacuolar aminopeptidase, termed aminopeptidase Y (APY). This paper deals with the purification, enzymological properties, localization, and processing of this major yeast vacuolar aminopeptidase. The accompanying paper (36) describes the molecular cloning of the APY gene and the construction of an APY-deleted mutant.

MATERIALS AND METHODS

Chemicals

Bestatin, pepstatin, amastatin, phosphoramidon, E64, and the follow-
ing 4-methylcoumaryl-7-amide (MCA) substrates were purchased from
Pepitide Institute, Inc.: Lys-, Arg-, Leu-, Met-, Ala-, Phe-, Lys-Ala-,
and Pro-Phe-Arg-MCA. Ser-, Tyr-, Gly-, Glu-, Pro-, and Arg-Arg-MCA and
peptides were from Bachem Feinchemikalien. Molecular mass markers
were from Life Technologies, Inc. PMSF and Ficoll 400 were from
Sigma. Zymolyase-100 T was from Seikagaku Kogyo. 125I-Labeled pro-
tein A was from Amersham. Endoglycosidase H (endo-β-N-acetylglu-
cosaminidase) and N-glycanase (peptide-N(4-N-acetyl-β-glucosaminyl-
asparginase) amide) were from Genzyme. Fixed Staphylococcus cells
and N(2-furylacryloyl)-Phe-Phe were from Calbiochem.

General Methods

Protein concentration was determined with the BCA protein assay
(reagent (Pierce Chemical Co.). SDS-polyacrylamide gel electrophoresis
was run according to Laemmli (18). The anti-APY IgG fraction was
prepared from antisera raised in rabbit against APY with protein
agarose (Calbiochem). Atomic absorption analysis was carried out by
using a polarized Zeeman atomic absorption spectrophotometer (Hita-
chi Z-7000).

Yeast Cells and Growth

Yeast strains used were D273-1B (ATCC25657) as wild-type and
ABYS1 (MATa, pra1, prb1, pre1, cpr1, ade) lacking vacuolar proteases

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†The abbreviations used are: PrA, protease A; PrB, protease B; CPY,
carboxypeptidase Y; CPS, carboxypeptidase S; API, aminopeptidase I;
APCo, aminopeptidase Co; APY, aminopeptidase Y; APY1, 70-kDa form
of APY; API, 75-kDa form of APY; MCA, 4-methylcoumaryl-7-amide;
PMSF, phenylmethanesulfonyl fluoride; MOPS, 3-(N-morpholino)pro-
panesulfonic acid; HPLC, high-performance liquid chromatography;
dansyl, 5-dimethylaminonaphthalene-1-sulfonil; PNA, p-nitroanilide.
**Yeast Aminopeptidase Y**

Yeast aminopeptidase was isolated from S. cerevisiae grown on a dialyzed amino acid-MCA substrate. The activity was determined by measuring the decrease in absorbance at 280 nm or the increase in fluorescence at 325 nm and 390 nm. The enzyme was purified by hydroxylapatite chromatography and Sephadex G-200 column chromatography. The enzyme was further purified by affinity chromatography on N-terminal 4-hydroxynaphthylcarbamoyl-Sepharose and Protamine-Sepharose affinity columns. The purified enzyme was homogeneous and had a molecular mass of approximately 26 kDa.

**Subfractionation of Yeast Cells**

The yeast cells were grown to the late logarithmic stage and centrifuged at 10,000 x g for 10 min. The supernatant was collected and used as the crude cell extract. The cell extract was then subjected to density gradient centrifugation in a sucrose gradient (50% to 80%) to separate the plasma membrane fraction. The plasma membrane fraction was then subjected to centrifugation at 100,000 x g for 10 min. The supernatant was collected and used as the crude mitochondrial fraction. The crude mitochondrial fraction was then applied to a Sephadex G-200 column (2 x 27 cm) equilibrated with 0.1 M NaCl, 10% glycerol, 0.3 M tris-HCl (pH 7.5). The active fractions were collected and concentrated to 10 ml.

**Enzyme Assay**

Enzyme assays for aminopeptidase were carried out by the following three methods.

1. Method A—With amino acid-MCA as the substrate, fluorescence due to the 7-amino-4-methylcoumarin produced was measured at $\lambda_{ex}$ 380 nm and $\lambda_{em}$ 460 nm. During the purification steps, 20 µM ARG-MCA was used as the substrate in 0.5 ml of 50 mM MOPS (pH 7.5). After the reaction was stopped by addition of 0.5 ml of 50% ethanol, 10 µM EDTA, 0.1 M Tris-HCl (pH 7), each fraction was processed as described (21).

2. Method B—Hydrolysis of dipeptidyl-MCA to amino acid and amino acid-MCA was estimated at $\lambda_{ex}$ 270 nm and $\lambda_{em}$ 350 nm. Hydrolysis of dipeptidyl-MCA to amino acid and amino acid-MCA was estimated by the following three methods. In method B-1, dipeptidyl-MCAs (0.1 mM) were hydrolyzed by the enzyme in 0.1 ml of 50 mM MOPS (pH 7.5) at 30 °C. After the reaction was stopped by addition of 0.5 ml of 50% ethanol, 10 µM EDTA, 0.1 M Tris-HCl (pH 7.5), hydrolyzed dipeptidyl-MCA was quantified by the increase of fluorescence at $\lambda_{em}$ 325 nm and $\lambda_{em}$ 390 nm. The fluorescence ratios of Lyu-Ala-MCA/Lyu-MCA and Arg-MCA/Arg-MCA were taken to be 1.8, respectively. Further hydrolysis of dipeptidyl-MCA, via amino acid-MCA, to 7-amino-4-methylcoumarin could be estimated at $\lambda_{em}$ 380 nm and $\lambda_{em}$ 460 nm as in method A. In method B-2, assay solutions prepared as in method B-1 were subjected to high-performance liquid chromatography (HPLC) with a reversed-phase column (Supersphere RP-8, Merck Co.), using 11% acetonitrile, 0.1% trifluoroacetic acid for Lyu-Ala-MCA and Arg-MCA/Arg-MCA or 14% acetonitrile, 0.1% trifluoroacetic acid for Pro-Pho-Arg-MCA. Hydrolysis of each substrate was estimated from the diminution of peak area of dansylated substrate peptide or from the new peak of dansylated N-terminal amino acid. One unit was defined as the enzyme required to hydrolyze 1 µmol of substrate/min in each assay system.

**Immunoblotting**

Proteins in the electrophoresed SDS-polyacrylamide gel were electroblotted onto a polyvinylidene difluoride membrane (Millipore Co.). The antigen-antibody complex was visualized with goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad) using 4-chloro-1-naphthol. To quantify the antigen-antibody complex, 125I-protein (Amersham) was added, and autoradiography was conducted with a Bio-image analyzer BAS 2000 (Fuji Film Co.).

**Enzyme Purification**

**Purification of Aminopeptidase Y**—Three kg of commercial baker's yeast cells (Saccharomyces cerevisiae, Oriental Yeast Co., Japan) was homogenized in a Dynomill in isotonic buffer, 0.6 M sorbitol, 10 mM Tris-HCl (pH 7.5), and a crude membrane-rich fraction was prepared by differential centrifugation (28). The fraction was sonicated in 10 mM Tris-HCl (pH 7.5), and the extract was obtained by centrifugation at 15,000 x g for 45 min followed by 150,000 x g for 45 min. The extract (1850 ml) was treated with 1 ml PMSF, adjusted to pH 7.5 with NaOH, and applied to a DEAE-cellulose column (6 x 17 cm) equilibrated with 10 mM Tris-HCl (pH 7.5) (1.4 liters total in 65 fractions). Fraction numbers 20–44 were collected, and NaCl was added to give a concentration of 0.5 M. Next, zinc-chelating Sepha-rose column chromatography was carried out in 0.5 M NaCl, 10% glycerol, 20 mM Tris-HCl (pH 7.5). After application of the active fractions from DEAE-cellulose chromatography, the column (1.6 x 25 cm) was washed with 1 liter of the buffer. Absorbed materials were eluted with a linear pH gradient from the above buffer to 0.5 M NaCl, 10% glycerol, 10 mM NaHPO4, (pH 3.3) (400 ml total in 60 fractions). The active fractions eluted at around pH 6.5 (fraction numbers 36–42, 165 ml) were collected. After the dialysis against 10% glycerol, 20 mM Tris-HCl (pH 7.5), the active fractions were applied to a DEAE-cellulose column (1 x 44 cm) equilibrated with the buffer. The absorbed materials were eluted with a 0–0.5 M linear gradient of NaCl in the buffer. The active fractions eluted around 0.1 M NaCl were collected and concentrated to 1.6 ml by ultrafiltration with a PM-10 membrane (Amicon). The concentrate was applied to a Sephadex G-200 column (2 x 92 cm) and developed with 0.2 M NaCl, 10% glycerol, 10 mM MOPS (pH 7.5). The combined active fractions (29 ml) were dialyzed against 1 liter of 10% glycerol, 20 mM HEPEs (pH 7.5) and applied to a protamine-Sepharose column (1 x 27 cm) equilibrated with the same buffer. Elution was carried out with a linear gradient to 4 M NaCl, 10% glycerol, 10 mM NaHPO4, (pH 3.3) (150 ml total in 45 fractions). The column was chilled with ice, and the flow rate was 1 ml/min. The active fractions which eluted around 2 M NaCl (fraction numbers 37–48) were collected and applied to a hydroxyapatite column (1 x 27 cm) equilibrated with the starting buffer, 10% glycerol, 20 mM HEPEs (pH 7.5). The adsorbed enzymes were eluted with a linear gradient to 10% glycerol, 0.2 M potassium phosphate (pH 7.5) (400 ml total in 100 fractions). Two active...
peaks appeared. The first part of the first peak, which eluted around 0.1 m potassium phosphate (fraction numbers 40–55), was collected as APY. The latter half of the second peak, which eluted around 0.13 m potassium phosphate (fraction numbers 56–69) was rechromatographed on the hydroxyapatite column under the same conditions as before. The active fractions were collected as before and combined with those previously obtained.

**Purification of Protease B—Preparation of crude extract of bakers’ yeast (1 kg) and protein precipitation with ammonium sulfate were carried out as described (27).** The purification was carried out with five steps of column chromatography: DEAE-cellulose, hydroxyapatite, DEAE-cellulose (second), Sephadex G-200, and HPLC with hydroxylapatite column (HCA column, Mitsui Toatsu Chemicals). Activity of protease B was measured according to Saheki and Holzer (28) with Azocoll as the substrate. The purified protease B (72 mg) showed a specific activity of 16.8 A\textsubscript{280} nm min\textsuperscript{-1} mg\textsuperscript{-1}.

**RESULTS**

**Purification of Yeast Aminopeptidase**—The final purification step on a hydroxyapatite column is shown in Fig. 1. The two peaks of activity coincided with the peaks of absorbance at 280 nm, and each of the two purified components showed a single band on SDS-polyacrylamide gel electrophoresis (Fig. 2). The enzyme from the first peak fractions showed a molecular mass value of 70 kDa and the other a value of 75 kDa. These values were the same under nonreducing conditions (data not shown). Thus, aminopeptidase Y was distinguished as APY\textsubscript{70} and APY\textsubscript{75}, respectively. Estimation of molecular mass by gel filtration gave values of 70 kDa for APY\textsubscript{70} and 80 kDa for APY\textsubscript{75} (data not shown). APY\textsubscript{70} and APY\textsubscript{75} were obtained in amounts of 3.8 and 3.5 mg, respectively, from 3 kg of cells (Table I).

**Molecular Difference between APY\textsubscript{70} and APY\textsubscript{75}—To establish whether APY\textsubscript{70} and APY\textsubscript{75} are the same enzyme or not, partial activity of APY toward MCA was progressively inhibited. There was no essential difference between APY\textsubscript{70} and APY\textsubscript{75}.

**Table I**

| Step          | Protein | Total activity* | Specific activity |
|---------------|---------|----------------|------------------|
| Extract       | 18,700  |                |                  |
| DEAE-cellulose| 5230    |                |                  |
| Zinc-chelating Sepharose | 901 | 194 | 0.215 |
| DEAE-cellulose| 103     | 129            | 1.25             |
| Sephadex G-200| 46.4    | 324            | 6.97             |
| Protamin-Sepharose | 12.6 | 249           | 19.8             |
| Hydroxyapatite| APY\textsubscript{70} | 3.8 | 144 | 38.0 |
|               | APY\textsubscript{75} | 3.5 | 98  | 28.0 |

* One unit was defined as amount of the enzyme to release 1 pmol of AMC/min under the assay condition.

**Endoglycosidase H or N-glycanase treatment of aminopeptidase Y**. One μg of APY\textsubscript{70} or APY\textsubscript{75} was treated with endoglycosidase H (0.25 unit) for 6 h at 37 °C and electrophoresed on SDS-polyacrylamide gel (7.5%). a–f, APY\textsubscript{70}; g–i, APY\textsubscript{70} and APY\textsubscript{75}; a, d, and g, before treatment; b, e, and h, treatment with endoglycosidase H; c, f, and i, treatment with N-glycanase.

**Fig. 3**. Endoglycosidase H or N-glycanase treatment of aminopeptidase Y. One μg of APY\textsubscript{70} or APY\textsubscript{75} was treated with endoglycosidase H (0.25 unit) for 6 h at 37 °C and electrophoresed on SDS-polyacrylamide gel (7.5%). a–f, APY\textsubscript{70} and APY\textsubscript{75}; a, d, and g, before treatment; b, e, and h, treatment with endoglycosidase H; c, f, and i, treatment with N-glycanase.

**Summary of purification of aminopeptidase Y**

The enzyme activity was measured by method A using Arg-MCA as the substrate. The activity of APY could not be accurately determined because of hydrolysis of the substrate by other enzymes in the initial crude fractions.

**Atomic Absorption Analysis**—Many aminopeptidases contain a zinc atom in the active site, and atomic absorption analysis showed the presence of 0.84 and 0.81 zinc atom/molecule in APY\textsubscript{70} and APY\textsubscript{75}, respectively.

**Effects of Additives on the Activity of APY**—The activities of APY\textsubscript{70} and APY\textsubscript{75} with Arg-MCA as the substrate were strongly inhibited by metal chelators, EDTA, EGTA, and o-phenanthroline (Table II). These results, together with the inhibition by the aminopeptidase-specific inhibitors bestatin and amastatin and the presence of a zinc atom, show that APY is a typical aminopeptidase.

In regard to metal ions, 0.5 mM Co\textsuperscript{2+} enhanced the activities 8–9-fold, whereas Ca\textsuperscript{2+} and Mg\textsuperscript{2+} had little effect, and Mn\textsuperscript{2+}, Cu\textsuperscript{2+} and Zn\textsuperscript{2+} were inhibitory. The inhibition of APY by o-phenanthroline was restored only by Co\textsuperscript{2+} (data not shown).

**Substrate Specificity of APY and Effects of Co\textsuperscript{2+} on the Activity**—The substrate specificity of APY toward MCA substrates was examined in the presence of 0 and 0.25 mM Co\textsuperscript{2+} (Table III). Peptidyl-MCAs were far more rapidly hydrolyzed than amino acid-MCAs, and ratios of the release rates of same N-terminal amino acids from these substrates in the absence of cobalt were as follows; Lys-Ala-MCA/Lys-MCA = 350, Pro-Phe-Arg-MCA/Pro-MCA = 750. Co\textsuperscript{2+} had different effects, depending on the substrate used. Hydrolysis of amino acid-MCAs was enhanced by Co\textsuperscript{2+}. In contrast, hydrolysis of peptidyl-MCAs was inhibited.

The concentration dependence of the Co\textsuperscript{2+} effect on the activity of APY was examined (Fig. 4). With Arg-MCA as the substrate, Co\textsuperscript{2+} enhanced the activity of APY concentration-dependently. On the other hand, the activity toward Arg-Arg-MCA was progressively inhibited. There was no essential difference between APY\textsubscript{70} and APY\textsubscript{75}.

Various peptides were hydrolyzed by APY, and the release rates of the N-terminal amino acids were measured (Table IV). APY hydrolyzed the peptides, except Gly-Gly-Gly, at various

**Fig. 2**. SDS-polyacrylamide gel electrophoresis of aminopeptidase Y. Two μg of each aminopeptidase were boiled with SDS and 2-mercaptoethanol and electrophoresed on SDS-polyacrylamide gel (7.5% acrylamide). The proteins were visualized with Coomassie Blue. a, APY\textsubscript{70}; b, mixture of the purified APY\textsubscript{70} and APY\textsubscript{75}; c, APY\textsubscript{75}.
TABLE II
Effects of various reagents on the activity of aminopeptidase Y

| Reagent                         | Concentration m M | Remaining activity % | Activity Ratio |
|---------------------------------|-------------------|----------------------|----------------|
| None                            | 100               | 100                  | 1              |
| EDTA                            | 1                 | 0.1                  | 0.1            |
| EGTA                            | 0.1               | 0.1                  | 0.1            |
| o-Phenanthroline                | 0.1               | 0.1                  | 0.1            |
| p-Chlormercurobenzoic acid      | 0.1               | 0.1                  | 0.1            |
| MTrH                            | 0.1               | 0.1                  | 0.1            |
| N-Ethylmaleimide                | 0.1               | 0.1                  | 0.1            |
| Dithiothreitol                  | 0.1               | 0.1                  | 0.1            |
| o-Phenanthroline                | 0.1               | 0.1                  | 0.1            |
| Diisopropyl fluorophosphate     | 0.1               | 0.1                  | 0.1            |
| Bestatin                        | 0.02              | 0.02                 | 0.1            |
| Aminstatin                      | 0.02              | 0.02                 | 0.1            |
| Phosphoramidon                  | 0.02              | 0.02                 | 0.1            |
| E64                             | 0.02              | 0.02                 | 0.1            |
| CaCl2                           | 0.9               | 94                   | 103            |
| MgCl2                           | 0.9               | 104                  | 90             |
| MnCl2                           | 0.3               | 33                   | 32             |
| CoCl2                           | 0.5               | 770                  | 940            |
| CuSO4                           | 0.5               | 10                   | 13             |
| ZnSO4                           | 0.5               | 28                   | 21             |
| Triphosphate                    | 1                 | 0.1                  | 0.1            |
| Phosphate                       | 1                 | 7                    | 7              |
| Phosphate                       | 1                 | 56                   | 62             |

TABLE III
Hydrolysis of MCA substrates by aminopeptidase Y

| Substrate        | Activity | Ratio | Activity Ratio |
|------------------|----------|-------|----------------|
| Lys-Ala-MCA      | 6.13     | 14.6  | 0.42           |
| Arg-Arg-MCA      | 1.94     | 5.32  | 0.36           |
| Pro-Phe-Arg-MCA  | 1.06     | 1.12  | 0.95           |
| Lys-MCA          | 1.06     | 0.042 | 25.2           |
| Arg-MCA          | 0.918    | 0.035 | 26.2           |
| Leu-MCA          | 0.355    | 0.023 | 15.4           |
| Met-MCA          | 0.346    | 0.021 | 16.5           |
| Ala-MCA          | 0.264    | 0.020 | 13.2           |
| Ser-MCA          | 0.110    | 0.0084| 13.1           |
| Phe-MCA          | 0.070    | 0.0050| 14.0           |
| Tyr-MCA          | 0.056    | 0.0045| 12.4           |
| Pro-MCA          | 0.040    | 0.0015| 2.7            |
| Gly-MCA          | 0.016    | 0.0048| 3.3            |
| Gla-MCA          | 0.010    | 0.0007| 14.3           |

rates with different effects of Co2+ on the activity. Hydrolysis of Leu-Leu and Arg-Val was activated 3.7-fold by Co2+, respectively. Esterification of both dipeptides resulted in greater susceptibility to APY, although the effect of Co2+ then became inhibitory.

Localization of Aminopeptidase Y—Localization of APY was examined by subtraction of the yeast cells followed by immunoblot analysis (Fig. 5). Enrichment of individual fractions was ascertained in terms of the enhanced specific activities of marker enzymes: carboxypeptidase Y for vacuoles, cytochrome c oxidase for mitochondria, and NADPH-oxidase for microsomes. Antiseras raised against APY2 and APY3 cross-reacted completely, and here we used antisera against APY3. The similar distribution profile of APY to that of carboxypeptidase Y confirmed the vacuolar localization of this enzyme. The enzyme was localized in the soluble fraction of vacuoles in the molecular form of APY2. By comparing the intensity of the band in the vacuole fraction with that of purified APY, we estimated that APY accounts for about 1% of vacuolar proteins.

Absorption of Vacuolar Aminopeptidase Activity by Anti-APY IgG—The contribution of APY to total aminopeptidase activities in the vacuoles was estimated by immunoblot analysis. As shown in Fig. 6, absorption with 31 µg of anti-APY IgG left only 12% of Lys-Ala-MCA-hydrolyzing activity, 32% of Lys-MCA-hydrolyzing activity (18% when assayed in the presence of Co2+), and 47% of Leu-MCA-hydrolyzing activity. Western blot analysis showed that a little APY still remained after the absorption. These results show that APY accounts for the major part of the aminopeptidase activities at least for these examined substrates in this organelle.

Conversion of Aminopeptidase Y Precursor to Mature Form—ABY1 mutant cells are mutants from which the genes of vacuolar proteases (proteases A and B, carboxypeptidases Y and S) have been deleted. In the mutant cells, it was expected that APY might exist as a precursor form, since protease A and protease B are responsible for processing or activation of vacuolarzymogens (10). We therefore attempted to detect a precursor form of APY in vacuolar extract of ABY1 mutant cells and examined its conversion to the mature form (Fig. 7). Since the soluble fraction of wild-type cell vacuoles was used in small amount, 1/10 of the protein of ABY1, the Lys-Ala-MCA-hydro-
lyzing activity of the wild-type extract was as low as that of ABYS1. Neither soluble fraction showed much change in aminopeptidase activities during incubation. However, when the two vacuolar extracts were mixed, the hydrolytic activity toward Lys-Ala-MCA gradually increased during incubation (Fig. 7A). This increase was inhibited by PMSF but not by pepstatin. Immunoblot analysis revealed a 74-kDa precursor form of APY (pro-APY) in the vacuoles of ABYS1 cells. During incubation for 30 min, about half the APY precursor was converted to mature APY, and conversion was complete at 3 h (Fig. 7B).

Inhibition of the conversion of 74-kDa pro-APY to mature 70-kDa APY by PMSF strongly suggested that vacuolar serine protease, protease B, is responsible for processing of the pro-APY. To confirm this, we prepared 31-kDa protease B of high purity, as shown by SDS-polyacrylamide gel electrophoresis (Fig. 8A). The Azocoll-hydrolyzing activity of protease B was completely inhibited by PMSF (not shown). Incubation of the vacuolar soluble fraction of ABYS1 cells with protease B caused a gradual increase of Lys-Ala-MCA-hydrolyzing activity (Fig. 8B) concomitant with conversion of the 74-kDa precursor form to the 70-kDa mature form as ascertained by immune blot analysis (Fig. 8C).

About half of the pro-APY was converted to 70-kDa APY in 8 min, although at this point, the Lys-Ala-MCA-hydrolyzing activity was still only 35% of that at 30 min. This apparent disagreement between the conversion of the mature form and activity was also seen when pro-APY was activated by extract of wild-type cell vacuoles; about half of pro-APY was converted to 70-kDa form at 30 min, although the increase of activity was only about 20% of that at 3 h (Fig. 7).

**DISCUSSION**

A number of yeast aminopeptidases have been described (for review, see Ref. 9), but the best characterized are methionine aminopeptidase and API. The methionine aminopeptidase is a cytosolic aminopeptidase which removes the N-terminal methionine of newly synthesized proteins (29). API is a vacuolar aminopeptidase with a molecular mass of 640 kDa, composed of 12 subunits. API is typical leucine-aminopeptidase which is highly active toward Leu-p-nitroanilide (PNA), but weakly toward Lys-PNA (30). Aminopeptidase II preferentially hydrolyzes Lys-PNA and Leu-PNA, but its periplasmic location (31) distinguishes it from API. The other known aminopeptidases also differ from API in location, substrate specificities, or molecular weight, except APCo. APCo has not been purified yet, but the activating effect of Co²⁺ on its hydrolysis of amino acid-PNA (32) is similar to that in the case of API. Since the
source strain of APCo was the ABYS1 mutant (32), there is a possibility that the reported activity of APCo may be derived from the 74-kDa precursor of APY, although the reported molecular mass of APCo is 100 kDa.

The immunoblot experiment with anti-APY IgG indicated that APY is responsible for most of the hydrolyzing activity to release N-terminal basic amino acids and over half the molecular mass of APCo is 100 kDa.

The activating effect of Co²⁺ on the APY activity was observed in the case of APY, however, the key factor is not the nature of the N-terminal amino acid but seems to be the length of the peptides. Esterification of the dipeptides Leu-Leu and Arg-Val altered the effect of Co²⁺ from activator to inhibitor. Further studies are needed on the action of Co²⁺, but binding site occupancies on APY could be an important factor.

Aminopeptidases have been assayed conveniently by using amino acid-conjugated chromophores, such as p-nitroaniline, β-naphthylamide, and MCA. However, such dipeptide-like chromogenic or fluorogenic substrates occupy only the P₁ site (subsite where the N-terminal amino acid of the substrate is bound), whereas the P’₁ site (subsite where the penultimate amino acid of the substrate is bound) of methionine aminopeptidase is known to function critically in the removal of N-terminal methionine (34). We therefore developed a convenient method which can cover the P’₁ site as well as the P₁ site of the enzyme. The method utilizes the lower fluorescence of amino acid-MCA than dipeptidyl-MCA at λₘₐₓ 325 nm, λₑₐₓ 390 nm (Arg-Arg-MCA/Arg-MCA = 1.8, Lys-Ala-MCA/Ala-MCA = 1.7). Estimation of the activity of APY by this method showed that Lys-Ala-MCA and Arg-Arg-MCA were far more rapidly hydrolyzed than Lys-MCA and Arg-MCA (350- and 150-fold, respec-
Yeast Aminopeptidase Y

In yeast vacuoles, the PEP4 gene product PrA is primarily responsible for the processing of vacuolar protein precursors to their mature forms. PrA either processes those precursors directly or is essential for the activation of processing proteases. Pr-A or PrB is one of the precursors processed by PrA, and after subsequent autocatalysis, activated PrB successively processes pro-CPY (15) and pro-CPS (35) to the mature forms. The present study showed that maturation of pro-APY is also involved in the vacuolar proteolytic processing system. Although the precise mechanism remains to be elucidated, aminopeptidase Y is the first aminopeptidase known to be activated by proteolytic processing.

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