Proteolysis in Eukaryotic Cells

IDENTIFICATION OF MULTIPLE PROTEOLYTIC ENZYMES IN YEAST*

(Received for publication, February 24, 1984)

Tilman Achstetter, Othnel Emter, Claudia Ehmann, and Dieter H. Wolf‡

From the Biochemisches Institut der Universität Freiburg, Hermann-Herder-Str. 7, D-7800 Freiburg i. Br., Federal Republic of Germany

A previous study led to the discovery of new proteinases in yeast (Achstetter, T., Ehmann, C., and Wolf, D. H. (1981) Arch. Biochem. Biophys. 207, 445-454). The search for proteolytic enzymes active in the neutral pH range has been extended. Studies were done on a mutant lacking four well-known proteinases involved in protein degradation, the two endoproteinases A and B and the two carboxypeptidases Y and S. Twenty-nine chromogenic peptides (amino terminally blocked peptidyl-4-nitroanilides) as well as [3H]methyl-L-casein were used as substrates in this search. For the detection of endoproteolytic activity using chromogenic peptide substrates two versions of the assay were used. In one system the direct cleavage of the 4-nitroanilide bond was measured. In the second, the cleavage of the chromogenic peptide at some site other than the 4-nitroanilide bond was measured. Both variations led to the discovery of multiple proteinase activities. Regulation of these proteolytic activities under different growth conditions of cells was observed. Proteolytic activity on [3H]methylcasein was also found. Ion-exchange chromatography and gel filtration were used for the reproducible separation of the multiple proteolytic activities.

The fundamental role proteolysis plays in intracellular regulation at the post-translational level has become evident in recent years (1-4). Understanding of this regulation depends on our knowledge of the intracellular peptidases and on the cellular processes which they catalyze. As has been shown, the unambiguous assignment of a certain peptidase to its respective intracellular target protein requires in many cases both biochemical and genetic studies (5). The unicellular eukaryote yeast is an ideal candidate to extensively study peptidase bond hydrolysis as this organism is easily accessible to such investigations. Eight peptidases, two endoproteinases (6-8), two carboxypeptidases (9-12), three aminopeptidases (13-15), and one dipeptidase (14) have been characterized in Saccharomyces cerevisiae (for review, see Ref. 3), and a variety of processes have been found which are proteolytically regulated (for review, see Ref. 5). From in vitro studies, endoproteinases A and B were considered the central regulatory enzymes in yeast responsible for a variety of regulatory proteolytic phenomena (16-19). However, genetic studies have ruled out this possibility (20-24) and have uncovered the role of these enzymes in protein turnover (20, 23, 24; for review, see Ref. 5). This led us to the assumption that more specific proteinases must exist which are responsible for known and probably many as yet unknown regulatory processes.

To avoid overlapping activities, mutants deficient in proteinase B, carboxypeptidase Y, and carboxypeptidase S were used and led to the detection of a variety of peptidase activities (25, 26). Endoproteolytic activities were explored in early stationary phase cells and cells grown on ethanol using 14 chromogenic substrates designed for testing relatively specific serum endoproteinases (25). Many questions remained unanswered and some of them will be approached in the present study. The basis of the endoproteinase assay used (25) is the release of 4-nitroaniline from peptides of the structure R-(AA)n-(AA)n-(AA)n-(AA)n-NA (1) (Fig. 1, 1). If, however, cleavage of some other peptide bond occurs, a peptide still containing the 4-nitroanilide residue remains and is not detected (Fig. 1, 2A). In this case, proteolysis can be detected by further digestion of this 4-nitroanilide by an aminopeptidase (Fig. 1, 2B).

The questions to be answered are: 1) can additional peptidase activities be found in yeast cell extracts by such a modification of the peptidase assay; 2) can chromogenic peptides with amino acid sequences different from the previously tested ones (25) uncover new proteolytic activities in yeast; 3) despite the fact that the major unspecific peptidases are absent in the strain used, do other undetected proteolytic activities exist, which are able to cleave unspecific substrates such as casein; and 4) do newly detectable proteolytic activities undergo regulation under different growth conditions?

For studies done under vegetative growth conditions, the quadruple mutant ABYS1 lacking the four vacuolar peptidases proteinase A, proteinase B, carboxypeptidase Y, and carboxypeptidase S was used. Experiments under sporulation conditions were done using the diploid triple mutant BYS-D2 homozygous for the absence of proteinase B, carboxypeptidase Y, and carboxypeptidase S.

This study presents a catalogue of new peptidase activities in yeast. Subsequent purification and characterization should give insight into their actual number and allow further re-
search on this class of enzymes in the regulation of cell metabolism.

MATERIALS AND METHODS

RESULTS AND DISCUSSION

We investigated the endoproteolytic cleavage of 29 chromogenic substrates in our mutant strain lacking four proteolytic enzymes, proteinases A and B and carboxypeptidases Y and S. In addition to the 14 substrates previously tested which contained 4-nitroaniline bound to arginine or lysine and to the hydrophobic amino acids leucine or phenylalanine (25), 15 substrates in which 4-nitroaniline is bound to valine, alanine, methionine, cystine, or proline were used. All substrates were originally designed for a variety of different mammalian proteinases (see Table I, Miniprint Supplement). Activities found against these 29 substrates in the soluble cell extract of yeast were determined in cells grown under different conditions. The different growth phases included logarithmic and stationary phase in complete medium containing glucose as carbon source, logarithmic phase in complete medium containing ethanol as carbon source, logarithmetic phase in glucose-mineral medium containing leucine as sole nitrogen source and the nitrogen starvation conditions needed to induce sporulation in diploid cells. To ensure the detection of peptide cleavage at a site other than the 4-nitroanilide bond, tests were done in the presence and absence of aminopeptidase M.

Activities Measurable in the Absence of Aminopeptidase M in Extracts of Cells Grown Logarithmically in Complete Medium—Activities were found against 10 of the chromogenic substrates without addition of aminopeptidase M in cell extracts of logaritmically growing cells in complete medium (Table II). These substrates were Suc-Ala-Ala-Pro-Leu-NA, Cbz-Ala-Ala-Leu-NA, Cbz-Gly-Gly-Leu-NA, MeO-Suc-Ala-Ala-Ala-NA, Suc-Ala-Ala-Pro-Met-NA, Suc-Ala-Ala-Pro-Phe-NA, Suc-Phe-Leu-Phe-NA, Bz-Ile-Glu-Gly-Arg-NA, Bz-Phe-Val-Arg-NA, Bz-Pro-Phe-Arg-NA, and Bzl-Cys-NA. Of the substrates tested, all the peptides containing leucine and methionine as the AA, residue were cleaved, whereas all peptides containing valine, alanine, proline, and lysine at this position were not cleaved at a measurable rate. Of the peptides containing phenylalanine or arginine as the AA, residue only some were split to a considerable extent.

Activities Measurable in the Presence of Aminopeptidase M in Extracts of Cells Grown Logarithmically in Complete Medium—When aminopeptidase M was present in the assays, substantial increase in activity was measurable against all substrates sensitive in the absence of aminopeptidase M except Bz-Phe-Val-Arg-NA (Table II). Furthermore, activity became apparent against 12 substrates on which cleavage was undetectable without aminopeptidase M (Table II). These substrates include: Ac-Ala-Ala-Pro-Ala-NA, Ac-Ala-Ala-Ala-NA, MeO-Suc-Ala-Ala-Pro-Val-NA, Suc-Ala-Ala-Val-NA, Suc-Tyr-Leu-Val-NA, Suc-Gly-Gly-Phe-NA, Suc-Phe-Pro-Phe-NA, Cbz-Gly-Pro-NA, H-D-Pro-Phe-Arg-NA, Tos-Gly-Pro-Arg-NA, H-D-Val-Leu-Lys-NA, and Tos-Gly-Pro-Lys-NA.

From these observations several general conclusions can be drawn: first they show that the intrinsic aminopeptidase activity in yeast is generally not sufficient to liberate amino acids from the peptides generated by endoproteolytic cleavage of the substrates at sites other than the 4-nitroanilide bond; second, they point to the fact that the substrates, which were designed for the detection of enzymes acting on 4-nitroanilides can serve as powerful tools in uncovering additional enzymes of different specificity; and third, they show that yeast contains enzymes which exhibit specificities different from those for which these substrates were originally designed.

However, these findings complicate the interpretation of data concerning the amount and the regulation of proteinase activity in crude preparations. A clear statement about proteinase specificity and regulation can be made only in those cases where activity is measurable with externally added aminopeptidase M, in which event the assay measures endoproteolytic cleavage at sites other than the 4-nitroanilide bond. The variation of aminopeptidase activities of different specificity during growth (27) additionally complicates the picture. However, inclusion of aminopeptidase M in the assay will result in the sum of both types of activities and will give at least a rough estimate of activity changes in different growth stages.

Activities Measurable in the Presence of Aminopeptidase M in Extracts of Stationary Phase Cells Grown in Complete Medium—When measured in the presence of aminopeptidase M, a considerable increase in activity was visible in stationary phase cells when compared to logarithmically growing cells with Ac-Ala-Ala-Pro-Ala-NA, Cbz-Gly-Pro-NA, Bz-Ile-Glu-Gly-Arg-NA, Bz-Phe-Val-Arg-NA, Bz-Pro-Phe-Arg-NA, H-D-Pro-Phe-Arg-NA, and H-D-Phe-Pip-Arg-NA. A decrease in activity as compared to logarithmically growing cells was detectable with the four substrates Suc-Ala-Ala-Pro-Leu-NA, Suc-Tyr-Leu-Val-NA, Suc-Gly-Gly-Phe-NA, and H-D-Val-Leu-Lys-NA (Table II). This is the first report of proteinases that are specifically present in logarithmically growing yeast cells.
Table II

Proteinase activity in the soluble extract of mutant cells of different growth phases measured with 29 different chromogenic peptide substrates.

For enzyme determination in vegetatively growing cells, strain ABYS1 was used. For enzyme determination in cells under sporulation conditions, strain BYS-D2 was used. Cell extracts were prepared and tests were done as outlined under "Materials and Methods." A, test without aminopeptidase M; B, test with aminopeptidase M added. 20 to 400 μg of protein were included in the test. One unit is defined as the amount of enzyme that catalyzes the release of 1 μmol of product/min under test conditions. (ND, not determined.)

| Substrate | Logarithmic on YPD | Stationary on YPD | Logarithmic on YPD, complete medium | Logarithmic on YPD, mineral medium | Sporulation medium |
|-----------|--------------------|------------------|-------------------------------------|-----------------------------------|-------------------|
|           | A                  | B                | A                                  | B                                | A                 |
| Suc-Ala-Ala-Pro-Leu-Met | -0.1              | -0.2             | 0.1                                | 0.2                              | 0.0               |
| Ac-Ala-Ala-Pro-Leu-Met | 0.1               | 0.0              | 0.1                                | 0.1                              | 0.0               |
| Cbz-Ala-Ala-Pro-Leu-Met | -0.1              | -0.2             | 0.1                                | 0.2                              | 0.0               |
| H-D-Phe-Phe-Pro-Leu-Met | 0.2               | 0.5              | 0.2                                | 0.5                              | 0.2               |
| Cbz-Ala-Ala-Pro-Leu-Met | 0.3               | 1.0              | 0.4                                | 1.4                              | 0.7               |
| H-D-Val-Leu-Lys-NA | 0.2               | 0.5              | 0.3                                | 0.5                              | 0.4               |
| MeO-Suc-Ala-Ala-Pro-Met | 0.4               | 2.4              | 0.3                                | 2.7                              | 1.0               |
| Cbz-Ala-Ala-Pro-Met | 0.1               | 2.1              | 0.2                                | 2.2                              | 1.8               |
| Cbz-Gly-Pro-NA | 0.1               | 0.2              | 0.1                                | 0.1                              | 0.1               |
| Cbz-Arg-Val-NA | 0.1               | 0.1              | 0.1                                | 0.1                              | 0.1               |
| Cbz-Met-Pro-Leu-NA | 0.1               | 0.1              | 0.1                                | 0.1                              | 0.1               |
| Cbz-Val-Leu-Val-NA | 0.1               | 0.1              | 0.1                                | 0.1                              | 0.1               |
| Cbz-Asp-Trp-Arg-NA | 0.1               | 0.1              | 0.1                                | 0.1                              | 0.1               |
| Cbz-Asp-Trp-Arg-NA | 0.1               | 0.1              | 0.1                                | 0.1                              | 0.1               |

Activities Measurable in the Presence of Aminopeptidase M in Extracts of Cells Grown Logarithmically on Ethanol—When ethanol was used as the carbon source, activities in cell extracts in most cases were very similar to those found in stationary phase cells grown on glucose (Table II). Some increase in activity over stationary phase conditions was observed with the substrates Cbz-Ala-Ala-Leu-NA, Cbz-Gly-Gly-Leu-NA, Suc-Tyr-Leu-Val-NA, Bz-Pro-Phe-Arg-NA, H-D-Phe-Pip-Arg-NA, H-D-Val-Leu-Lys-NA, and Tos-Gly-Pro-Lys-NA in the presence of aminopeptidase M. A considerable increase was observed with Suc-Ala-Ala-Pro-Leu-NA. In contrast, activities measured under the same conditions against Suc-Phe-Pro-Phe-NA and Cbz-Gly-Pro-Pro-NA decreased as compared to stationary phase cells grown in glucose (Table II).

Activities Measurable in the Presence of Aminopeptidase M in Extracts of Cells Grown Logarithmically in Mineral Medium Containing Leucine as Sole Nitrogen Source—Activities in the presence of added aminopeptidase M in extracts of cells grown logarithmically in mineral medium containing leucine as sole nitrogen source are characterized by a pattern similar to cells growing logartihmically on glucose complete medium. They differ in that activities against Ac-Ala-Ala-Pro-Ala-NA, Bz-Phe-Val-Arg-NA, and H-D-Pro-Phe-Arg-NA are increased under those conditions and reach values found in glucose-grown stationary phase cells or cells grown logartihmically on ethanol. In addition, the activity against Bz-Asp-Trp-Arg-NA appears rather selectively and the activity against MeO-Suc-Ala-Ala-Pro-Met-NA is increased when compared to all the above-mentioned growth conditions. A selective disappearance of the activity against Suc-Ala-Ala-Pro-Phe-NA was noted in cells growing on mineral medium containing leucine.

Activities Measurable in the Presence of Aminopeptidase M in Extracts of Sporulating Cells—When activities in the presence of externally added aminopeptidase M were measured in sporulating diploid cells, values higher than for any of the vegetative growth conditions tested were found for Ac-Ala-Ala-Pro-Ala-NA, Ac-Ala-Ala-Ala-NA, MeO-Suc-Ala-Ala-Pro-Met-NA, Suc-Ala-Ala-Pro-Phe-NA, Bz-Pro-Phe-Arg-NA, H-D-Pro-Phe-Arg-NA, Tos-Gly-Pro-Arg-NA, H-D-Val-Leu-Lys-NA, and Tos-Gly-Pro-Lys-NA. Activities against Suc-Ala-Ala-Val-NA and Suc-Phe-Pro-Phe-NA specifically disappear in sporulating cells. No activity was detected against the chymotrypsin substrate Suc-Phe-NA in these mutant cells (Table II) under any conditions.

Activities Detectable in the Membrane Fraction—Proteolytic activity in highly purified membranes was also measured. The membrane fractions used had been thoroughly washed at high salt concentration. This washing procedure reduced the contamination of the membrane fraction with the cytoplasmic enzyme glucose-6-phosphate dehydrogenase to below 0.5% of its activity as compared to the soluble cell extract. Activity which considerably exceeds the limitations of the test was found only against Cbz-Ala-Ala-Leu-NA and Ac-Ala-Ala-Pro-Ala-NA in the presence of aminopeptidase M. Values of 0.21 and 0.12 milliunits/mg, respectively, amounting to 50 and 1.5% of the activity present in cell extracts, were found in stationary phase cells grown on glucose. Cleavage of the two chromogenic substrates by membrane-associated proteolytic...
activity occurred, at least in part, at some site other than the 4-nitroanilide bond (not shown). Whether some cleavage occurs also at the 4-nitroanilide bond of the peptides is unclear, as aminopeptidase activity cleaving aminocarbonyl-4-nitroanilides can be found in purified membranes (Table III) (27). No membrane-bound aminopeptidase activity in yeast has been described until recently. Whether Leu-NA and Phe-NA activity is due to a single enzyme has to be elucidated. Both activities are inhibited by nitritotriacetic acid (2 mM, not shown). When the salt wash is omitted more abundant and considerably higher proteolytic activities against chromeogenic substrates are found in the membrane fraction (25) which must be due to additional proteinase(s) bound loosely to the membranes investigated.

From the above experiments the following general conclusions can be drawn. 1) Yeast cells lacking endoproteinas A and B as well as carboxypeptidases Y and S contain proteolytic activities against all substrates tested except Suc-Ala-Ala-NA and Suc-Phe-NA. 2) Peptides with similar sequence but different amino-terminal blocking groups are attacked proteolytically to very different extents (compare activities against Bz-Pro-Phe-Arg-NA and H-D-Pro-Phe-Arg-NA as well as Suc-Ala-Ala-NA and Ac-Ala-Ala-NA). 3) In addition to cleavage at the 4-nitroanilide bond, most substrates were cleaved at an amino acid residue different from AA. Activities at such sites seem to be more abundant and in some cases are very high relative to those cleaving at the 4-nitroanilide bond of the peptides. 4) Many of the activities varied considerably with the growth conditions of the cells. 5) The variations measured with and without aminopeptidase M may point to different enzyme activities. The variations in activity with different substrates under different growth conditions strongly indicate the presence of multiple proteolytic activities. The idea of multiple proteolytic activities in yeast was previously suggested after testing cell extracts separated by gel filtration against four different substrates without addition of aminopeptidase M (25).

**Separation of Proteinases by Ion-exchange Chromatography**—To gain more information about the proteinases, we separated the extracts on DEAE-Seepharose and measured activities against nine selected substrates (Figs. 2–4, Miniprint Supplement). Both variations of the test (Fig. 1) were used when necessary. A 40 to 66% ammonium sulfate fraction of the cell extract in which most of the proteolytic activity was retained preceded the ion-exchange chromatography step. Extracts of cells grown to logarithmic and stationary phase on glucose complete medium, two growth conditions in which considerable differences in proteolytic activity can be noted (Table II) were used. Substrates were chosen that showed considerable sensitivity in cells in either one or both growth phases and that can be grouped into the following classes. 1) Substrates with different sequences except for AA; Bz-Ile-Glu-Gly-Arg-NA, Bz-Pro-Phe-Arg-NA, and Bz-Phe-Val-Arg-NA. 2) Substrates with the same sequence except for AA. These include: Ac-Ala-Ala-Pro-ALA, Suc-Ala-Ala-Pro-Leu-NA, Suc-Ala-Ala-Pro-Phe-NA, and Suc-Ala-Ala-Pro-Met-NA. 3) The peptide Cbz-Gly-Gly-Leu-NA, which had revealed a unique activity on gel filtration of a cell extract (25). 4) The oxytocinase substrate BzL-Cys-NA. Tests against all substrates except Cbz-Gly-Gly-Leu-NA and BzL-Cys-NA were done with and without addition of aminopeptidase M.

Ion-exchange chromatography (Figs. 2 to 4, Miniprint Supplement) in each case separated several activity peaks. In no case was the profile identical for the different substrates. Furthermore, considerable alterations in the profile were visible when activities against a single substrate were measured in extracts of logarithmic and stationary phase cells. In addition, big differences in the activity pattern were noted when cleavage of a substrate was followed with and without aminopeptidase M (Figs. 2 to 4, Miniprint Supplement). Clearly, multiple proteolytic activities can be seen.

These studies give an impression of the possible number of neutral proteinase activities in the yeast cell but not the exact number of enzymes. Activities eluting at the same NaCl concentration and detectable in the same variant of the assay with different substrates of similar sequence may be due to the same enzyme. On the other hand, we may assume that activity peaks measured with a particular substrate are due either to different enzymes or to different forms or fragments of the same enzyme, while activities that cleave substrates of different sequence may partly be due to different enzymes.

As in crude extracts (Table II) dramatic differences can be found in the activity patterns after chromatography when activity is measured with and without aminopeptidase M. Presuming that no intrinsic yeast aminopeptidase activity is present in the respective fractions, such differences must be due to at least two different cleavage steps and are likely due to different proteolytic enzymes. Enzymes cleaving at sites other than the 4-nitroanilide bond represent the highest activities. At present, we cannot unequivocally attribute 4-nitroanilide bond hydrolysis to any of the proteolytic activities separated by ion-exchange chromatography. Intrinsic aminopeptidase activities are eluted from DEAE-Seepharose over the whole range of NaCl concentrations needed to elute the proteolytic activities (27). Based on the high stimulatory power of aminopeptidase M, such intrinsic aminopeptidase activity must, however, be rather small. It may nevertheless lead to some 4-nitroaniline release from aminocarbonyl-4-nitroanilides generated by endoproteolytic cleavage at a site other than the 4-nitroanilide bond.

As studied in previous gel filtration experiments (25), identification of a single enzyme in such complex mixtures by using proteinase inhibitors of different specificities is impossible. Therefore, the actual number of proteinases must await careful dissection by purification and characterization. First attempts in this direction have led to the purification and characterization of proteinase yscD (29, 29) and to a partial characterization of proteinase yscE (29) for nomenclature see Ref. 28.

In a previous study we reported a considerable increase in some proteolytic activity against Bz-Pro-Phe-Arg-NA and Bz-Ile-Glu-Gly-Arg-NA when Co²⁺ ions were added to extracts of stationary phase cells separated by gel filtration (25). Recently, we detected a new aminopeptidase activity which is strongly Co²⁺ ion-dependent and which cleaves lysine-4-nitroanilide and arginine-4-nitroanilide with high efficiency.
Table IV

| Addition               | Specific activity (cpm/min x mg protein) |
|-----------------------|-----------------------------------------|
| None                  | 100                                     |
| ATP                   | 150                                     |
| ATP (γ-S)             | 174                                     |
| GTP                   | 142                                     |
| UTP                   | 109                                     |
| CTP                   | 101                                     |
| ATP + phosphoenolpyruvate | 207                                |
| + pyruvate kinase     |                                         |
| Phosphoenolpyruvate   | 203                                     |
| ADP                   | 153                                     |
| AMP                   | 156                                     |
| cAMP                  | 82                                      |
| K phosphate           | 160                                     |
| Na pyrophosphate      | 180                                     |
| Na2SO₄                | 221                                     |

(15). This aminopeptidase activity, which we named aminopeptidase Co, is high in stationary phase cells, whereas very little of it can be found in logarithmically growing cells (not shown). As activity against Bz-Pro-Phe-Arg-NA and Bz-Ile-Glu-Gly-Arg-NA can be considerably enhanced by addition of aminopeptidase M (Table II and Fig. 2, 1B, 2B, Miniprint Supplement), we consider Co⁺² activation with both peptides at least in part due to the activation of the Co⁺²-dependent aminopeptidase which subsequently cleaves arginine-4-nitroanilide generated by previous endoproteolytic cleavage.

Activity Measurable Against [³H]Methylcasein—In mutant strains lacking both endoproteinas A and B, the ability to cleave nonspecific substrates such as the collagen derivatives Hide Powder Azure or Azocoll and acid-denatured hemoglobin is almost completely lost (20, 24). The question arose whether some activity still exists in the mutant lacking endoproteinas A and B as well as the two carboxypeptidase Y and S that is able to cleave a nonspecific substrate. In order to have high sensitivity we chose [³H]-radiolabeled methylcasein as substrate. Whereas extracts of stationary phase wild-type cells grown on glucose complete medium led to a degradation rate of 15,200 cpm/min x mg (S.D. ± 2%; mean of two determinations) extracts of the quadruple mutant resulted in a degradation rate of 45 cpm/min x mg (S.D. ± 15%; mean of three determinations) under the same conditions. Thus, the mutant has lost more than 99.7% of the casein degrading activity due to the absence of these enzymes. Casein degrading specificity had been reported for the two endoproteinas A and B and for carboxypeptidase Y (6).

Is the [³H]Methylcasein Splitting Activity ATP-dependent?—The detection of ATP-dependent proteolytic activity has been described in other organisms (30-32) using [³H]methylcasein as substrate. However, differences were observed in other organisms (30-32) using [³H]methylcasein as substrate. Whereas extracts of stationary phase wild-type cells grown on glucose complete medium led to a degradation rate of 15,200 cpm/min x mg (S.D. ± 2%; mean of two determinations) extracts of the quadruple mutant resulted in a degradation rate of 45 cpm/min x mg (S.D. ± 15%; mean of three determinations) under the same conditions. Thus, the mutant has lost more than 99.7% of the casein degrading activity due to the absence of these enzymes. Casein degrading specificity had been reported for the two endoproteinas A and B and for carboxypeptidase Y (6).

Separation of [³H]Methylcasein Splitting Activities—Ion-exchange chromatography of crude cell extracts of logarithmically growing or stationary phase cells on DEAE-Sepharose, the method employed for the separation of activities against chromogenic substrates, led to the detection of a major activity splitting [³H]methylcasein that is not adsorbed by the ion-exchange column (not shown). However, this activity did not exhibit the activation characteristics observed in crude extracts nor could any activity affected by ATP be found in the salt gradient used for ion-exchange chromatography. In contrast, gel filtration on Sepharose CL-6B uncovered a proteolytic activity (Fig. 5, A and B, peak II, Miniprint Supplement) which shows the activation characteristics found in crude extracts (Table II). The activation of this proteolytic activity by ATP and GTP as well as pyrophosphate (Miniprint Supplement) and the rather high molecular weight of the ATP-stimulated activity (greater than that of thyroglobulin, M₉ = 668,000) might imply a similarity of this enzyme with an ATP-stimulated casein cleaving proteinase found by De Martino and Goldberg (31) in rat liver cells. Differences between the enzymes of yeast and rat liver cells are found in their responses to effectors. In contrast to the rat liver enzyme, the activity of the yeast enzyme is increased by phosphatase (Miniprint Supplement). Measurements of the effect of sulfate have not been reported for the rat liver enzyme. The activation of the caseinolytic activity in yeast by such unrelated compounds as ATP and sodium sulfate leaves us with the question whether the enzyme might be activated in vitro by ATP or some other ion or whether the activation observed is simply due to an in vitro salt effect.

At least two other caseinolytic activities (Fig. 5, A and B, peaks I and III, Miniprint Supplement) could be identified by gel chromatography. These two activities show no activation by ATP (Fig. 5, A and B, Miniprint Supplement) but by sulfate (not shown). The activity shoulders of the major peaks I, II, and III of the gel chromatography (Fig. 5, Miniprint Supplement) imply that there might be more than three activities against [³H]methylcasein in mutant extracts.

The inability to detect any caseinolytic activity subject to activation by ATP after ion-exchange chromatography of crude cell extracts may point to the fact that none of the

In addition to breakdown products of [³H]methylcasein which were trichloroacetic acid-soluble we also detected trichloroacetic acid-insoluble fragments which had molecular weights in the range of 19,000 to 20,500 and 19,500 as measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (not shown). In contrast to proteinases A and B, the residual activity in crude extracts releasing trichloroacetic acid-soluble material from [³H]methylcasein is partly dependent on divalent cations, Ca⁺², Mg⁺², and Co⁺² being the most effective (not shown). After desalting the crude extracts by dialysis or by passage through a Sephadex G-25 column, activity can be increased by about 1.5-fold by addition of ATP when Mg⁺² ions are present (Table IV). Optimum Mg⁺² concentration for activation was measured to be between 10 and 20 mM (not shown). ATP resembles ATP in its activating capacity. UTP and CTP are not effective (Table IV). An ATP-regenerating system using phosphoenolpyruvate and pyruvate kinase increased the activity in cell-free extracts above the value found for ATP alone. However, phosphoenolpyruvate itself showed a similar activation effect (Table IV). Activation of [³H]methylcasein splitting activity could also be brought about by ADP and AMP, whereas cAMP was not effective. In addition, potassium phosphate, sodium pyrophosphate, and sodium sulfate led to a considerable increase of [³H]methylcasein cleaving activity.

Separation of [³H]Methylcasein Splitting Activities—Ion-exchange chromatography of crude cell extracts of logarithmically growing or stationary phase cells on DEAE-Sepharose, the method employed for the separation of activities against chromogenic substrates, led to the detection of a major activity splitting [³H]methylcasein that is not adsorbed by the ion-exchange column (not shown). However, this activity did not exhibit the activation characteristics observed in crude extracts nor could any activity affected by ATP be found in the salt gradient used for ion-exchange chromatography. In contrast, gel filtration on Sepharose CL-6B uncovered a proteolytic activity (Fig. 5, A and B, peak II, Miniprint Supplement) which shows the activation characteristics found in crude extracts (Table II). The activation of this proteolytic activity by ATP and GTP as well as pyrophosphate (Miniprint Supplement) and the rather high molecular weight of the ATP-stimulated activity (greater than that of thyroglobulin, M₉ = 668,000) might imply a similarity of this enzyme with an ATP-stimulated casein cleaving proteinase found by De Martino and Goldberg (31) in rat liver cells. Differences between the enzymes of yeast and rat liver cells are found in their responses to effectors. In contrast to the rat liver enzyme, the activity of the yeast enzyme is increased by phosphatase (Miniprint Supplement). Measurements of the effect of sulfate have not been reported for the rat liver enzyme. The activation of the caseinolytic activity in yeast by such unrelated compounds as ATP and sodium sulfate leaves us with the question whether the enzyme might be activated in vitro by ATP or some other ion or whether the activation observed is simply due to an in vitro salt effect.

At least two other caseinolytic activities (Fig. 5, A and B, peaks I and III, Miniprint Supplement) could be identified by gel chromatography. These two activities show no activation by ATP (Fig. 5, A and B, Miniprint Supplement) but by sulfate (not shown). The activity shoulders of the major peaks I, II, and III of the gel chromatography (Fig. 5, Miniprint Supplement) imply that there might be more than three activities against [³H]methylcasein in mutant extracts.

The inability to detect any caseinolytic activity subject to activation by ATP after ion-exchange chromatography of crude cell extracts may point to the fact that none of the
proteolytic activities against chromogenic substrates and separated by this chromatographic step is due to the ATP-dependent caseinolytic proteinase. No decision can be made as yet as to whether or not any identity exists between activities against chromogenic peptide substrates and the activities against \([^{3}H]methylcasein\). Because of the multitude of proteolytic activities which can be separated in mutant extracts, an unequivocal assignment of substrate specificities will only be possible after purification of the respective enzymes.

\[^{3}H\]Methylcasein Cleavage Activity Detectable in the Membrane Fraction—Activity hydrolyzing \([^{3}H]methylcasein\) was also detected in the purified membrane fraction. A value of 15 cpm/min x mg (S.D. ± 20%; mean of two determinations) was found in stationary phase cells grown on glucose. The caseinolytic activity present in the membrane fraction amounts to about 9% of the total. Whether this activity is identical with the caseinolytic activity of high molecular weight (Fig. 5B, peak I, Miniprint Supplement) separated by gel filtration has to be elucidated in future studies.

Specificity and Function of the Newly Detected Proteinases.

A Speculative View—What can we speculate about the specificity of the new proteinases? The loss of more than 98.7% of the \([^{3}H]methylcasein\) splitting activity in mutants lacking the two endoproteinases A and B and the two carboxypeptidases Y and S points to the fact that these enzymes are the major unspecific proteolytic activities in the yeast cell. As expected from their broad specificity in vitro, the physiological consequence of proteinase A and proteinase B absence in mutants is reduced intracellular protein degradation (20, 23, 24) and a disturbed sporulation process (20-24) that upon imposition of the additional deficiency in carboxypeptidase Y and carboxypeptidase S is nearly completely abolished (26; for review, see Ref. 5). In accordance with their in vitro specificity and their in vivo function all four enzymes have been found to be localized in the vacuole (33, 34), the lysosome-like digestive organelle of the yeast cell. One might argue that the enzyme activities representing the residual less than 0.3% of the \([^{3}H]methylcasein\) splitting activity found in mutant cells are also of unspecific nature. However, this is not necessarily so. Splitting of \([^{3}H]methylcasein\) at the low rate found might be an in vitro artifact unrepresentative of the actual in vivo function of the respective enzymes. This view might be supported by the fact that in contrast to the two endoproteinases A and B and the two carboxypeptidases Y and S the \([^{3}H]methylcasein\) hydrolyzing activity in the mutant is not enriched in the vacuole (34).

We may probably expect proteinases of rather narrow specificity among the enzymes active against a particular chromogenic substrate. The observation that no significant activity can be found in the mutant strain against Suc-Phe-NA, a standard substrate for chymotrypsin, one of the pancreatic proteinases of broad specificity, might support this view. Obviously, many of the proteinases require more than one amino acid to recognize a synthetic peptide as substrate.

The majority of the proteolytic activity described here cannot be found in vacuoles (34). It is clear that the amount of activity of an enzyme found with a certain chromogenic substrate may not at all be representative of its in vivo activity. For instance, low activity in cell extracts against a substrate might be due to a low intracellular concentrations of the enzyme, which may, however, be biologically highly active because of substrate channeling. On the other hand, synthetic peptide substrates may simply be very inefficient substrates for an enzyme as compared to the in vivo substrates.

We do not know anything about the in vivo function of the enzymes. Apart from understanding of general protein degradation to provide amino acids for protein synthesis, knowledge about other processes in yeast which require more specific proteinases has recently been accumulating. These processes include, for instance, the presumptive signal peptide cleavage of secreted proteins (35), pro-enzyme cleavage of vacuolar proteinases (19, 36, 37), pre-protein cleavage of nuclear-coded mitochondrial proteinases (38), processing of the polyprotein precursor of the mating pheromone \(a\)-factor (39, 40), inactivation of \(a\)-factor (41), and maturation of the yeast killer toxin (42). Since the endoproteinases A and B can be excluded from being vital catalysts in the activation process of chitin synthetase and the catabolite inactivation of gluconeogenic enzymes (for review, see Ref. 5) involvement of these proteinases in these processes has to be considered. A proteinase located in the mitochondrial matrix involved in cleavage of mitochondrial precursor polypeptides has been partly purified recently (43, 44). Whether this enzyme is present among those enzyme identified here remains to be established.

We may assume that the proteolytic processes found in yeast until now represent only the tip of the iceberg. The multiple proteinases found in yeast make this organism especially suited to investigate the role of this class of enzymes in the regulation of cell metabolism at the post-translational level. Purification and characterization of the enzymes will facilitate the future approach of isolating mutants devoid of the respective enzymes in an attempt to answer the question of their intracellular function.

Acknowledgments—We thank Drs. P. Ries and L. Svendsen, Pentapharm AG, Basel, Switzerland, for their generous gift of the chromogenic substrates Bz-Pro-Phe-Arg-NA, Chz-Val-Gly-Arg-NA, Tos-Gly-Pro-Arg-NA, and Tos-Gly-Pro-Lys-NA used in our experiments. We thank Dr. J. Phillips for critical reading of the manuscript. The expert help of H. Gottschalk and W. Fritz during the preparation of this manuscript is gratefully acknowledged.

REFERENCES

1. Reich, E., Rifkin, D. B., and Shaw, E. (eds) (1975) *Proteases and Biological Control*, pp. 1-397, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
2. Steiner, D. F., Quinn, P. S., Chan, S. J., Marsh, J., and Tager, H. S. (1980). *Ann. N. Y. Acad. Sci.* 343, 1-16
3. Wolf, D. H. (1986) *Adv. Microb. Physiol.* 21, 267-338
4. North, M. J. (1982) *Biological Control*, pp. 1-987, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
5. Kuhn, R. W., Walsh, K. A., and Neurath, H. (1974) *Biochemistry* 13, 3871-3877
6. Wolf, D. H., and Weiser, U. (1977) *Eur. J. Biochem.* 73, 553-556
7. Wolf, D. H., and Ehmann, C. (1978) *FEBS Lett.* 91, 59-62
8. Mattile, P., Wiemken, A., and Gutter, W. (1971) *Planta (Berl)* 96, 45-53
9. Frey, J., and Röhm, K. H. (1978) *Bioclim. Biophys. Acta* 527, 261-261
10. Achstetter, T., Ehmann, C., and Wolf, D. H. (1982) *Bioclim. Biophys. Res. Commun.* 109, 341-347
11. Holzer, H., and Seheki, T. (1976) *Tokai J. Exp. Clin. Med.* 1, 115-125
12. Molano, J., and Gancedo, C. (1974) *Eur. J. Biochem.* 44, 213-211
13. Ulane, R. E., and Cabib, E. (1976) *J. Biol. Chem.* 251, 3367-3374
Yeast Proteinases

Yeast Storage and Growth Conditions - The yeast Saccharomyces cerevisiae strain ATCC 10135 (3 g dry weight/m 3) was cultured in a submerged culture in a 5-litre fermenter. The fermentation medium was a glucose-limited chemostat system consisting of 8% glucose as the carbon source, 1% yeast extract, and a minimal nutrient solution. The fermentation was conducted at 30°C with a H2SO4 concentration of 0.2 M. The cultivation time was 24 h, and the oxygen concentration was maintained at 20%.

Preparation of Protein Extracts - For the yeast proteinase extraction, the yeast cells were harvested by centrifugation at 10,000 x g for 10 min at 4°C and resuspended in 0.1 M Tris-HCl buffer (pH 7.5). The cell suspension was then homogenized with a teflon pestle and homogenate was centrifuged at 15,000 x g for 30 min at 4°C. The supernatant was used as a crude proteinase extract.

Protein Determination - Protein concentration was determined by the method of Lowry et al. (11). The protein was dissolved in 0.1 M sodium hydroxide and measured at 280 nm. The protein concentration was calculated from a standard curve of bovine serum albumin (BSA).

Chromatography - For separation of proteinase activities by ion exchange chromatography, the dialyzed yeast proteinase extract (150 mg of protein) was applied onto a column (1.6 x 40 cm) of DEAE-Sepharose FF in 0.1 M Tris-HCl buffer, pH 7.5. The column was eluted with a linear gradient of 0 to 0.5 M NaCl in 0.1 M Tris-HCl buffer, pH 7.5. The fractions were collected and the enzyme activity was determined.

Results - The yeast proteinase extract contained three proteinase activities, designated A, B, and C. Activity A eluted from the DEAE-Sepharose column as a single peak at 0.25 M NaCl, whereas activities B and C showed two distinct peaks at 0.35 M and 0.45 M NaCl, respectively. The specific activities of the proteinases were 1.8 U/mg for A, 0.6 U/mg for B, and 0.4 U/mg for C.

Discussion - The yeast proteinase extract contained three distinct proteinase activities. Activity A was characterized as an endopeptidase, while activities B and C were exopeptidases. Further studies are needed to elucidate the molecular properties and biological functions of these proteinases.
New Yeast Proteinases

Separation of New Proteinases Produced by Chromogenic Substrates by Ion Exchange Chromatography

Ion Exchange Chromatography (IC) was performed using the DEAE Sephadex A-50 column. The activities of proteinases in the yeast extract were separated into two peaks. One peak eluted at 115 mM NaCl concentration, while the other peak eluted at 175 mM NaCl concentration.

The activities in the yeast extract were separated into two peaks by IC using a column of NAP-5 or NAP-10. The activities were eluted at 100 and 125 mM NaCl concentrations.

When the yeast extract was applied to a column of NAP-5, two peaks were observed in the NaCl gradient elution. The activity of the first peak was eluted at 110 mM NaCl, while the activity of the second peak was eluted at 115 mM NaCl. The activities in the yeast extract were separated into three peaks by IC using a column of NAP-5 or NAP-10. The activities were eluted at 100, 125, and 150 mM NaCl concentrations.

When the yeast extract was applied to a column of NAP-5, two peaks were observed in the NaCl gradient elution. The activity of the first peak was eluted at 110 mM NaCl, while the activity of the second peak was eluted at 115 mM NaCl. The activities in the yeast extract were separated into three peaks by IC using a column of NAP-5 or NAP-10. The activities were eluted at 100, 125, and 150 mM NaCl concentrations.

When the yeast extract was applied to a column of NAP-5, two peaks were observed in the NaCl gradient elution. The activity of the first peak was eluted at 110 mM NaCl, while the activity of the second peak was eluted at 115 mM NaCl. The activities in the yeast extract were separated into three peaks by IC using a column of NAP-5 or NAP-10. The activities were eluted at 100, 125, and 150 mM NaCl concentrations.

When the yeast extract was applied to a column of NAP-5, two peaks were observed in the NaCl gradient elution. The activity of the first peak was eluted at 110 mM NaCl, while the activity of the second peak was eluted at 115 mM NaCl. The activities in the yeast extract were separated into three peaks by IC using a column of NAP-5 or NAP-10. The activities were eluted at 100, 125, and 150 mM NaCl concentrations.

When the yeast extract was applied to a column of NAP-5, two peaks were observed in the NaCl gradient elution. The activity of the first peak was eluted at 110 mM NaCl, while the activity of the second peak was eluted at 115 mM NaCl. The activities in the yeast extract were separated into three peaks by IC using a column of NAP-5 or NAP-10. The activities were eluted at 100, 125, and 150 mM NaCl concentrations.

When the yeast extract was applied to a column of NAP-5, two peaks were observed in the NaCl gradient elution. The activity of the first peak was eluted at 110 mM NaCl, while the activity of the second peak was eluted at 115 mM NaCl. The activities in the yeast extract were separated into three peaks by IC using a column of NAP-5 or NAP-10. The activities were eluted at 100, 125, and 150 mM NaCl concentrations.

When the yeast extract was applied to a column of NAP-5, two peaks were observed in the NaCl gradient elution. The activity of the first peak was eluted at 110 mM NaCl, while the activity of the second peak was eluted at 115 mM NaCl. The activities in the yeast extract were separated into three peaks by IC using a column of NAP-5 or NAP-10. The activities were eluted at 100, 125, and 150 mM NaCl concentrations.

When the yeast extract was applied to a column of NAP-5, two peaks were observed in the NaCl gradient elution. The activity of the first peak was eluted at 110 mM NaCl, while the activity of the second peak was eluted at 115 mM NaCl. The activities in the yeast extract were separated into three peaks by IC using a column of NAP-5 or NAP-10. The activities were eluted at 100, 125, and 150 mM NaCl concentrations.

When the yeast extract was applied to a column of NAP-5, two peaks were observed in the NaCl gradient elution. The activity of the first peak was eluted at 110 mM NaCl, while the activity of the second peak was eluted at 115 mM NaCl. The activities in the yeast extract were separated into three peaks by IC using a column of NAP-5 or NAP-10. The activities were eluted at 100, 125, and 150 mM NaCl concentrations.

When the yeast extract was applied to a column of NAP-5, two peaks were observed in the NaCl gradient elution. The activity of the first peak was eluted at 110 mM NaCl, while the activity of the second peak was eluted at 115 mM NaCl. The activities in the yeast extract were separated into three peaks by IC using a column of NAP-5 or NAP-10. The activities were eluted at 100, 125, and 150 mM NaCl concentrations.

When the yeast extract was applied to a column of NAP-5, two peaks were observed in the NaCl gradient elution. The activity of the first peak was eluted at 110 mM NaCl, while the activity of the second peak was eluted at 115 mM NaCl. The activities in the yeast extract were separated into three peaks by IC using a column of NAP-5 or NAP-10. The activities were eluted at 100, 125, and 150 mM NaCl concentrations.
Fig. 3 Separation of proteinase activities by ion exchange chromatography on DEAE Sepharose CL-68. Strain ABY81 was grown on YPD medium. Extracts were prepared, ammonium sulfate fractionation, chromatography of extracts and tests were done as outlined under Materials and Methods. 1A, Suc-Ala-Ala-Pro-Phe-NA; 2A, Nα-Ala-Ala-Pro-Val-NA tested with addition of aminopeptidase N; 2B, Nα-Ala-Ala-Pro-Leu-NA tested without addition of aminopeptidase N. 3A, Suc-Ala-Ala-Pro-Phe-NA; 3B, Nα-Ala-Ala-Pro-Leu-NA tested with addition of aminopeptidase M.

Fig. 4 Separation of proteolase activities by ion exchange chromatography on DEAE Sepharose CL-68. Strain ABY81 was grown on YPD medium. Extracts were prepared, ammonium sulfate fractionation, chromatography of extracts and tests were done as outlined under Materials and Methods. 1A, Nα-Ala-Ala-Pro-Leu-NA tested without addition of aminopeptidase N; 2A, Suc-Ala-Ala-Pro-Leu-NA tested without addition of aminopeptidase N; 2B, Suc-Ala-Ala-Pro-Leu-NA tested with addition of aminopeptidase N. 3A, Nα-Ala-Ala-Pro-Leu-NA tested with addition of aminopeptidase M. M, R, N and NaCl concentration. The arrow marks the start of the salt gradient.

Fig. 5 Separation of 1Hmethyl-casein splitting activity by gel filtration on Sepharose CL-68. Strain ABY81 was grown on YPD medium into logarithmic (I) and stationary (II) phase. Extracts were prepared in 50 mM Tris buffer, pH 7.4, in the presence of 10 mM MgCl2 and 200 mM NaCl. 300 μg of protein was applied on the column. Chromatography was done as outlined under Materials and Methods. 10 ml of each fraction were tested. Elution time was 500 ml. Peak II was collected and used for both parameters. Fraction number and proteinase activity are shown. 1Hmethyl-casein splitting activity of peak III in logarithmic and stationary growth phase (Fig. 5A,B), indicates that they represent at least two different enzymes. Activity should be stable between peaks I and III as well as between peaks II and III (Fig. 5A,B) indicates the presence of additional casein splitting proteases.

1) Synthesized for our purpose.
2) Genetic markers are: gph - protease A deficiency; gph2 - protease B deficiency; gph1 - carboxypeptidase Y deficiency; grg2 - carboxypeptidase S deficiency; ger - adenyl deaminase; cpe - carboxypeptidase S3; histidine auxotrophy; gph - well mating type. W. J. B. Whelan, C. M. and Wolf, D. H. unpublished.