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Hormone processing and membrane-bound proteinases in yeast

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A search for maturating peptidases of the precursor protein of the mating hormone (pheromone) α-factor of *Saccharomyces cerevisiae* was performed using short model peptides representing those sequences of the precursor protein, where cleavage is thought to occur *in vivo*. This search was done in a mutant lacking several of the unspecific vacuolar peptidases. The chromogenic peptide Cbz-Tyr-Lys-Arg-4-nitroanilide led to the detection of a membrane-bound enzyme called proteinase yscF. Cleavage of the synthetic peptide derivative occurs after the basic amino acid pair, a proposed signal for hormone processing. Optimum pH for the reaction is 7.2. The enzyme does not cleave after single basic amino acid residues indicating that it is distinct from trypsin-like proteinases. Proteolytic activity is enhanced by Triton X-100. The enzyme is strongly inhibited by EGTA, EDTA and mercurials but insensitive to phenylmethylsulfonyl fluoride. The enzyme activity is strongly dependent on Ca^{2+} ions. In a mutant (*kex2*), which accumulates an over-glycosylated α-factor precursor, no proteinase yscF activity can be found. Membrane-bound peptidase activity possibly involved in removal of the arginyl and lysyl residues remaining at the carboxy terminus of the α-factor pheromone peptide after the initial cut of the precursor molecule could be identified by using the model peptides Cbz-Tyr-Lys-Arg and Cbz-Tyr-Lys.

Key words: α-factor/maturation/pheromone/proteinases/yeast

Introduction

In the yeast *Saccharomyces cerevisiae* α-factor, one of the two oligopeptide hormones (pheromones) triggering sexual conjugation of the haploid cell types *a* and α (Betz *et al.*, 1981) is synthesized as a high mol. wt. precursor protein which must undergo proteolytic maturation to yield the active pheromone (Emter *et al.*, 1983; Julius *et al.*, 1984a). The cloned α-factor precursor gene (MFα1) revealed that α-factor is present in four repeats in the precursor molecule (Kurjan and Herskowitz, 1982). The repeats are flanked by peptide spacers each starting with the amino acid sequence Lys-Arg (Kurjan and Herskowitz, 1982). Sequences of two basic amino acids have been found in many mammalian hormone precursor molecules and are thought to represent the initial processing sites (Steiner *et al.*, 1980; Docherty and Steiner, 1982; Mains *et al.*, 1983). To date no proteolytic enzyme could be unequivocally assigned to the catalysis of the initial processing event (Docherty and Steiner, 1982). Based on our finding that chromogenic peptide substrates represent an extremely powerful tool to uncover proteinases in yeast (Achstetter *et al.*, 1981, 1984a) we introduced the chromogenic peptide substrate Cbz-Tyr-Lys-Arg-NA (Cbz-, benzyloxycarbonyl; -NA, -4-nitroanilide), which harbours the carboxy-terminal tyrosine residue of the α-factor molecule and the consecutive Lys-Arg sequence of the spacer peptide, to search for the processing endoproteinase in yeast (Figure 1). Carboxypeptidase activity possibly responsible for the removal of the basic amino acid(s) remaining at the carboxy terminus of each α-factor molecule after the initial endoproteolytic cut of the precursor protein was sought with the aid of model peptides also containing the carboxy-terminal tyrosine residue of the pheromone and, in addition, either both or only one of the consecutive basic amino acids (Figure 1).

Results

When searching for processing enzymes of hormone (pheromone) precursor proteins, it is desirable to work on cells devoid of unspecific peptidases, which may interfere with the processing peptidases *in vitro* (Docherty and Steiner, 1982). We have isolated mutants of the yeast *S. cerevisiae* lacking the activities of the major unspecific proteinases (Wolf, 1982) and used them for this search. A report, which described that the dipeptidyl aminopeptidase responsible for the removal of part of the spacer peptide generated after initial cleavage of the α-factor precursor was localized in the membrane fraction (Suárez Rendueles *et al.*, 1981; Julius *et al.*, 1983a), focused our attention on membrane-bound enzymes possibly involved in the initial cleavage of the pheromone precursor. Proteolytic activity specifically recognizing the basic amino acid pair Lys-Arg was monitored using the chromogenic peptide derivative Cbz-Tyr-Lys-Arg-NA reflecting an amino acid sequence at which the initial cleavage of the precursor molecule is thought to occur. The test allows detection of proteolytic activity cleaving at the 4-nitroanilide bond by direct liberation of 4-nitroaniline (A in Tables I and III) or at some site other than the 4-nitroanilide bond (B in Tables I and III). Such an

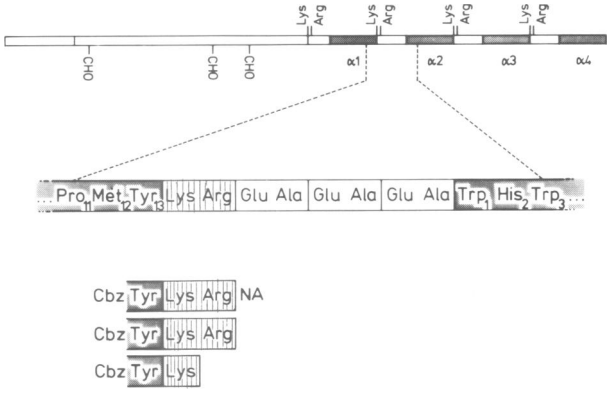

Fig. 1. Structure of α-factor pheromone precursor from *S. cerevisiae* (according to Kurjan and Herskowitz, 1982) and model peptides used in this study for detection of putative maturases. The four α-factor sequences α1 to α4 are represented by dotted bars, the pre- and pro-sequences as well as the spacer sequences (each starting with a Lys-Arg pair) are white. CHO, carbohydrate.

Table I. Membrane-bound proteolytic activity and cleavage of substrates containing basic amino acid residues

Peptide substrate	Specific activity mU/mg	
	A	B
Cbz-Tyr-Lys-Arg-NA	0.51	0.55
Cbz-Lys-Arg-NA	<0.05	<0.05
Cbz-Arg-Arg-NA	<0.05	<0.05
Bz-Pro-Phe-Arg-NA	<0.05	<0.05
Bz-Ile-Glu-Gly-Arg-NA	<0.05	<0.05
Tos-Gly-Pro-Arg-NA	<0.05	<0.05
Bz-Val-Gly-Arg-NA	<0.05	<0.05
D-Val-Leu-Arg-NA	<0.05	<0.05
Bz-Asp-Trp-Arg-NA	<0.05	<0.05
Lys-NA	<0.05	–
Arg-NA	>0.05	–

Proteolytic activity was measured as outlined in Materials and methods. 50–100 μg of protein were included in the test. A, test without aminopeptidase M; B, test with aminopeptidase M.

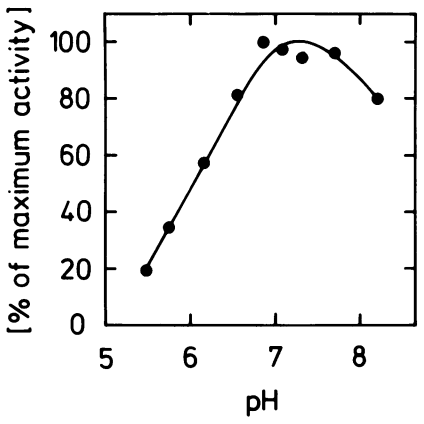

Fig. 2. Dependence of proteinase yscF activity on pH. 17.5 mU of proteinase yscF were included in the test. Test was done as outlined in Materials and methods. Buffer was 0.2 M Tris/maleate/NaOH.

internal cut can be visualized by addition of aminopeptidase M to the test, which liberates 4-nitroaniline from the amino-terminally unblocked peptidyl-4-nitroanilide generated (Achstetter *et al.*, 1984a).

Table I shows proteolytic activity in the purified membrane fraction of a strain lacking the unspecific vacuolar peptidases proteinase yscB, carboxypeptidase yscY and carboxypeptidase yscS. Of the model peptides measured, only Cbz-Tyr-Lys-Arg-NA is cleaved to a considerable extent. Cbz-Lys-Arg-NA and Cbz-Arg-Arg-NA containing a basic amino acid pair but lacking the tyrosine residue, a variety of trypsin substrates containing only one basic amino acid (arginine), and the aminopeptidase substrates Lys-NA and Arg-NA, are not hydrolyzed. These results indicate the presence of an enzyme specific for the sequence Tyr-Lys-Arg. Cleavage of Cbz-Tyr-Lys-Arg-NA occurs preferentially after the basic amino acid pair at the 4-nitroanilide bond as no significant increase in enzyme activity is visible upon addition of aminopeptidase M to the test (Table I, lane B). The intrinsic membrane-bound aminopeptidase yscP cannot be responsible for further cleavage of a peptidyl- or aminoacyl-4-nitroanilide possibly generated by internal peptide bond cleavage of the model substrate since the activity cannot be blocked by bestatin, a potent inhibitor of the aminopeptidase yscP (Achstetter *et al.*, 1983). According to the nomenclature for yeast proteinases (Achstetter *et al.*, 1984b) we call the new enzyme proteinase yscF.

Table II. Effect of inhibitors on proteinase yscF activity

Inhibitor	Final concentration	Activity (% of control)
None	–	100
Phenylmethylsulfonyl fluoride	1 mM	100
Bestatin	50 μg/ml	123
Pepstatin	50 μg/ml	100
Chymostatin	50 μg/ml	100
E64a	50 μg/ml	133
Soy bean trypsin inhibitor	50 μg/ml	102
EGTA	0.5 mM	5
EDTA	0.5 mM	23
o-Phenanthroline	1 mM	91
Dithiothreitol	1 mM	8
Tosyl-L-lysine chloromethyl ketone	2 mM	75
$HgCl_2$	0.1 mM	8
$ZnCl_2$	1 mM	1
$CoCl_2$	1 mM	84
$CaCl_2$	1 mM	113
$MgCl_2$	1 mM	87
EGTA	0.5 mM	
+ $CaCl_2$	1.0 mM	80
EGTA	0.5 mM	
+ $MgCl_2$	1.0 mM	2

Proteolytic activity was measured as outlined in Materials and methods. 50–100 μg of protein were included in the test. Pre-incubation with the single compounds was done for 60 min at 30°C. Reactivation experiments (30 min at 30°C) with Ca^{2+}- and Mg^{2+}-ions were performed after a 60 min inactivation step brought about by EGTA. Appropriate controls were run in all cases.

Proteinase yscF is activated >2-fold by treatment of the membrane fraction with 0.1% Triton X-100 (not shown). The enzyme is present in logarithmically growing and stationary phase cells (not shown). It is also present in cells of α- and *a* mating type and in diploid cells indicating that it is not regulated by the mating type (not shown). pH optimum of the enzyme for cleavage of Cbz-Tyr-Lys-Arg-NA is in the range of 7.2 (Figure 2).

The response of proteinase yscF activity to a variety of agents known to influence proteolytic activity is shown in Table II. Proteinase yscF activity is strongly inhibited by the metal chelating agents EGTA and EDTA and the thiol proteinase inhibitor $HgCl_2$. Ca^{2+} ions lead to a strong reactivation of enzyme activity after EGTA treatment. Dithiothreitol reduces proteinase yscF activity considerably. Phenylmethylsulfonyl fluoride (PMSF), an inhihitor of serine proteinases and the trypsin inhibitor tosyl-L-lysine chloromethyl ketone had no significant effect on proteinase yscF activity. Thus, proteinase yscF can be classified as a Ca^{2+}-dependent metallo-thiol-proteinase.

Strong evidence that proteinase yscF might be the maturase of α-factor pheromone precursor responsible for the initial cut of the precursor molecule comes from a mutation (*kex2*) (Leibowitz and Wickner, 1976) which causes a defect in killer toxin secretion and is thought to lead to loss of a specific processing proteinase (Bussey, 1981). Interestingly, the mutation has been shown also to block secretion of mature α-factor

Table III. Membrane-bound proteinase activities in *kex2* mutant strains and a strain wild-type for *KEX2* functions

Strain	Specific activity mU/mg				
	Proteinase yscF Cbz-Tyr-Lys-Arg-NA	Proteinase yscG Cbz-Ala-Ala-Leu-NA	Proteinase yscH Ac-Ala-Ala-Pro-Ala-NA	Dipeptidyl aminopeptidase yscIV Ala-Pro-NA	Aminopeptidase yscP Leu-NA
	A	B	B		
BYS 232-31-42 (α *prb1-1 prc1-1 cps1-3 his7 lys2 leu2*)	0.51	0.21	0.17	1.73	0.36
78 (α *kex2-1 his7*)	0.11	n.d.	n.d.	n.d.	n.d.
TA1 (α *kex2-1 prb1-1 prc1-1 cps1-3 ura1*)	<0.01	0.24	0.26	1.86	0.38

Proteolytic activity was measured as outlined in Materials and methods. 50–100 μg of protein were included in the test. A, test without aminopeptidase M; B, test with aminopeptidase M. Proteinase yscG, proteinase yscH and aminopeptidase P were measured in the absence of 0.1% Triton X-100.

(Bussey, 1981) and to result in the accumulation of an over-glycosylated α-factor precursor (Julius *et al.*, 1983b). When testing the membrane fraction of the *kex2* mutant strain 78, activity against the model substrate Cbz-Tyr-Lys-Arg-NA was reduced ~80% as compared with the strain wild-type for *KEX2* but was deficient in the three vacuolar peptidases proteinase yscB, carboxypeptidase yscY and carboxypeptidase yscS (strain BYS 232-31-42) (Table III). When the *prb1, prc1* and *cps1* mutations leading to the deficiency in these three peptidases were recombined with the *kex2* mutation, activity in the recombinant strain TA1 against Cbz-Tyr-Lys-Arg-NA was almost completely lost (Table III). This indicates that some contaminating vacuolar enzyme, and not proteinase yscF, is responsible for the residual 20% of the Cbz-Tyr-Lys-Arg-NA splitting activity in the membrane fraction of *kex2* mutant strain 78. Thus, the *kex2* mutation leads to proteinase yscF deficiency (Table III). The following membrane-associated proteinases were found to be unaffected by the *kex2* mutation (Table III): dipeptidyl aminopeptidase yscIV [also called X-prolyl dipeptidyl aminopeptidase (Suárez Rendueles *et al.*, 1981)], an enzyme activity which is thought to be involved in removal of the amino-terminally located part of the spacer peptide of the α-factor sequence generated after the initial cut of the pheromone precursor molecule (Julius *et al.*, 1983a); aminopeptidase yscP (Achstetter *et al.*, 1983); two activities, which in contrast to proteinase yscF are not inhibited by mercurials (not shown), one splitting Cbz-Ala-Ala-Leu-NA (Achstetter *et al.*, 1984a) and tentatively called proteinase yscG, and the other splitting Ac-Ala-Ala-Pro-Ala-NA (Achstetter *et al.*, 1984a), tentatively called proteinase yscH.

The specific lack of proteinase yscF activity caused by the *kex2* mutation, as well as the block to secretion of mature α-factor with the accumulation of an over-glycosylated α-factor pheromone precursor, might imply that proteinase yscF is the maturating enzyme responsible for initial splitting of the pheromone precursor. Until we know, however, whether the *KEX2* gene represents the structural gene for proteinase yscF, this conclusion remains tentative. Loss of proteinase yscF activity and concomitant accumulation of over-glycosylated α-factor precursor, the phenotypic events observed, need not necessarily be linked to each other genetically.

Splitting of the α-factor precursor molecule after the Lys-Arg amino acid pair leaves this amino acid sequence carboxy-terminally connected to the α-factor molecule (Figure 1). We therefore searched for peptidase activity in the membrane fraction able to remove the two amino acids lysine and arginine carboxy-terminally, using the two model substrates Cbz-Tyr-Lys-Arg and Cbz-Tyr-Lys (Figure 1). As Table IV shows, considerable activity cleaving these substrates can be found. At least in the case of Cbz-Tyr-Lys, cleavage must be due to carboxypeptidase activity, which we call carboxypeptidase yscα. We believe that hydrolysis of Cbz-Tyr-Lys-Arg is brought about by the same enzyme; however, cleavage of this substrate by a dipeptidyl carboxypeptidase cannot be excluded. Cleavage of these model peptides cannot be due to the action of already known carboxypeptidases, as the strain used is devoid of the two vacuolar carboxypeptidases yscY and yscS and as substrates of the newly detected carboxypeptidases yscγ (Cbz-Ser-Phe), carboxypeptidase yscδ (Cbz-Ala-Phe) and carboxypeptidase yscε (Cbz-Ala-Phe) (Wolf and Ehmann, 1981), were not hydrolyzed by the membrane fraction (Table IV).

Table IV. Carboxypeptidase activity in the membrane fraction of *S. cerevisiae* strain BYS 232-31-42 (α *prb1-1 prc1-1 cps1-3 his7 lys2 leu2*)

Peptide tested	Specific activity mU/mg
Cbz-Tyr-Lys	0.41
Cbz-Tyr-Lys-Arg	0.96
Cbz-Ser-Phe	<0.1
Cbz-Ala-Phe	<0.1

Carboxypeptidase activity was measured as outlined in Materials and methods.

Discussion

The enzymes described here, proteinase yscF and carboxypeptidase yscα, are novel enzymes thought to be involved in propheromone processing. Strong evidence for this physiological function is available for proteinase yscF. The enzyme is strictly specific towards the basic peptidase pair Lys-Arg and its activity is absent in a strain carrying the *kex2* mutation, which leads to accumulation of an over-glycosylated α-factor precursor. After our work had been completed, a report appeared describing the identification of a membrane-bound proteinase using Boc-Gln-Arg-Arg-MCA (Boc-, butoxycarbonyl; MCA, 7-amino-4-methyl-coumarin) as a substrate (Julius *et al.*, 1984b). It appears from the characteristics described for this enzyme by Julius *et al.* (1984b) (substrate cleavage after the basic amino acid pair, pH optimum, membranous location, activation by Triton X-100 and Ca^{2+} ions, inhibition by Zn^{2+} and other heavy metal ions, resist-

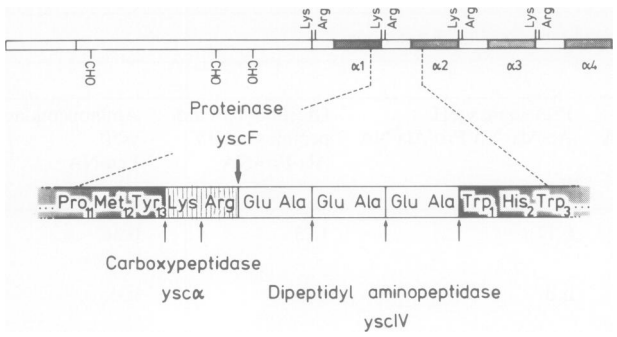

Fig. 3. Processing and processing enzymes of the α-factor precursor. The sequence of one of four otherwise very similar spacer peptides (Kurjan and Herskowtiz, 1982) (white bars) with its flanking α-factor sequences (dotted bars) is taken as an example.

ance to the action of PMSF and absence in *kex2* mutant cells) that it is identical with proteinase yscF. On the basis of gene dosage experiments using the cloned *KEX2* gene, these authors provide evidence that *KEX2* might be the structural gene for the enzyme (Julius *et al.*, 1984b). Our studies extend these findings in the following way.

(i) Problems with the identification of the α-factor precursor processing proteinase due to contaminating proteinases [which account for up to 40% of the total activity as shown by Julius *et al.* (1984b)] can be avoided by using a mutant deficient in several of the vacuolar peptidases (Table III).

(ii) Proteinase yscF actually cleaves a peptide which harbors a part of the α-factor precursor sequence that is thought to be cleaved *in vivo*.

(iii) Proteinase yscF is highly specific for cleavage after the pair of basic amino acids. The enzyme does not cleave any substrate tested that contains only one basic amino acid (Arg) (Table I).

(iv) The dibasic amino acid pair Lys-Arg or Arg-Arg is not sufficient for cleavage to occur. Proteinase yscF requires at least one additional amino acid at the amino-terminal side of the Lys-Arg pair for hydrolysis (Table I).

(v) In contrast to the three spacer peptides of the α-factor precursor located between the four α-factor sequence repeats (Figure 1), where the amino acid preceding the Lys-Arg pair of each spacer peptide is the carboxy-terminal tyrosine of α-factor, the Lys-Arg pair of the first spacer peptide is preceded by aspartic acid (Kurjan and Herskowitz, 1982). Comparison of the peptide derivative used by us (Cbz-Tyr-Lys-Arg-NA) and by Julius *et al.* (1984b) (Boc-Gln-Arg-Arg-MCA) to test the maturase activity, shows that enzymatic cleavage is not strictly dependent on the nature of the amino acid at the amino-terminal side of the basic amino pair. This indicates that the enzyme should be able to recognize all four Lys-Arg sequences of the α-factor precursor molecule as signals for processing.

(vi) Yeast cells contain membrane-associated carboxypeptidase activity able to remove the basic amino acids from the model peptides used. This activity is thus a strong candidate for further processing at the carboxy terminus of each α-factor molecule after the initial endoproteolytic cut of the precursor protein.

The finding of a membraneous location of proteinase yscF and carboxypeptidase yscα fits quite well all other data presently available on the maturation of the α-factor pheromone precursor. Based on data of Julius *et al.* (1984a) the precursor is thought to remain membrane attached to dictate its correct proteolytic maturation. Dipeptidyl aminopeptidase yscIV (X-prolyl dipeptidyl-aminopeptidase), an enzyme, which removes the Glu-Ala and Asp-Ala residues remaining at the amino-terminal of the α-factor sequences after the initial cut of the precursor at the Lys-Arg pairs, is membrane bound (Julius *et al.*, 1983a). The proposed maturation steps of the α-factor precursor protein and their catalysts are summarized in Figure 3.

Recently, a serine proteinase located in the soluble fraction of yeast lysates was identified on the basis of its ability to cleave the [Leu]-enkephalin derivative Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Phe-Ala-NH_2 between the dibasic Arg-Arg sequence (Mizuno and Matsuo, 1984). Proteinase yscF is completely different from this enzyme. Whether the enzyme described by Mizuno and Matsuo (1984) is involved in prohormone processing remains to be seen.

Materials and methods

Yeast strains

Construction of proteinase triple mutant strains as is BYS 232-31-42 (α *prb1-1 prc1-1 cps1-3 his7 lys2 leu2*) lacking the vacuolar peptidases proteinase yscB, carboxypeptidase yscY and carboxypeptidase yscS, has been described (Wolf and Ehmann, 1981). Strain 78 (α *kex2-1 his7*) (Leibowitz and Wickner, 1976) was obtained from the Yeast Genetics Stock Center, Berkeley. Strains RC629 (*a sst1-2 ade2-1 ura1 his6 met1 can1 cyh1 rme*) and RC757 (α *sst2-1 his6 met1 can1 cyh2 rme*) (Chan and Otte, 1982) were a generous gift of Dr R.K.Chan. Strain TA1 (α *kex2-1 prb1-1 prc1-1 cps1-3 ura1*) defective in *KEX2* gene product and lacking three vacuolar peptidases was obtained by crossing strain 78 with strain BYS 232-31-4 (*a prb1-1 prc1-1 cps1-3 ade ura1 leu2*) on Petri plates containing 1% yeast extract, 2% peptone, 3% glycerol, 50 μg/ml of adenine and uracil and 2% agar buffered with citrate phosphate to pH 4.7. Mating was induced by addition of 4 μg/ml synthetic α-factor. Diploids were obtained by complementation and sporulated. Tetrads were isolated, spores were dissected and tested for α-factor secretion on a lawn of strain RC629 and for *a*-factor secretion on a lawn of strain RC757 according to Manney *et al.* (1983). α-Cells defective in α-factor secretion were picked.

Growth of cells and membrane preparation

Strain BYS 232-31-42 (α *prb1-1 prc1-1 cps1-3 his7 lys2 leu2*) was grown into late stationary phase at 30°C in a medium containing 1% yeast extract, 2% peptone, 2% glucose and 50 μg/ml of adenine and uracil. Membranes were prepared in 20 mM Tris-HCl buffer, pH 7.2, as described (Achstetter *et al.*, 1984a) with the following modifications. Freshly prepared cell extracts were centrifuged for 10 min at 2000 *g*. The resulting supernatant was centrifuged for 30 min at 150 000 *g*. The pellet was resuspended in twice the starting volume of 20 mM Tris-HCl buffer, pH 7.2, containing 200 mM KCl. After removal of loosely bound protein by homogenization, the membranes were sedimented by 150 000 *g* for 40 min. This procedure was repeated using six times the starting volume of 20 mM Tris-HCl buffer, pH 7.2, containing 200 mM KCl and sedimentation of membranes was carried out at 260 000 *g* for 50 min. Membranes were suspended in 20 mM Tris-HCl buffer, pH 7.2, and stored in liquid nitrogen. The membranes were essentially free of glucose-6-phosphate dehydrogenase, a marker enzyme for the cytoplasmic fraction (Achstetter *et al.*, 1984a).

Enzyme assays

Endoproteolytic activity using the chromogenic substrates indicated was assayed in 100 mM Tris-HCl buffer, pH 7.5, 0.1% Triton X-100 in a total volume of 0.2 ml. Substrate concentrations were 0.5 mM. Test modification A: without aminopeptidase M; test modification B: 1 μg (6 mU) of aminopeptidase M was included. Liberation of 4-nitroaniline was measured at 405 nm in an Eppendorf spectrophotometer. For details of the assay and definition of units see Achstetter *et al.* (1984a). Aminopeptidase and dipeptidylaminopeptidase activity was measured using the 4-nitroanilide derivatives indicated. For details of the test see Achstetter *et al.* (1983). Carboxypeptidase activity was determined by measuring the increase of fluorescence brought about by carboxy-terminal liberation of amino acids from peptides and subsequent reaction with *o*-phthaldialdehyde (Benson and Hare, 1975). Enzymatic hydrolysis was carried out at 30°C in a total volume of 0.2 ml containing 50 mM potassium phosphate buffer, pH 7.5 and 2 mM of substrate. Peptide stock solutions were 40 mM in dimethylsulfoxide. 20–100 μg of protein were included in the test. Aliquots of 10 μl were removed after different test times and diluted into 0.2 ml of 10 mM potassium phosphate buffer, pH 7.3. 100 μl of a solution containing 0.05% *o*-phthaldialdehyde, 1% ethanol, 0.1% Brij-

35, 0.2% 2-mercaptoethanol and 10 mM potassium phosphate buffer, pH 7.3, were added. Fluorescence was measured after 25 min at 25°C (334 nm/400–3000 nm). Controls without substrate were run in parallel. Calibration of the increase in fluorescence was done for each amino acid. Glucose-6-phosphate dehydrogenase was measured according to Kato *et al.* (1979).

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