Biochimica et Biophysica Acta, 370 (1974) 530–540

BBA 67358

PARTIAL PURIFICATION AND CHARACTERIZATION OF TWO PEPTIDASES FROM *NEUROSPORA CRASSA*

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(Received July 9th, 1974)

SUMMARY

Two peptidases from *Neurospora crassa* have been partially purified and characterized with respect to substrate specificity, requirements for activity, and molecular size Both enzymes appear to be dipeptidases (EC 3 4 13) One enzyme catalyzes the preferential hydrolysis of certain methionyl dipeptides The second enzyme hydrolyses only dipeptides containing proline carboxy-terminal residues In this paper these enzymes are referred to as methionyl dipeptidase and imidodipeptidase, respectively Of the substrates tested, methionylalanine and methionylserine are most rapidly hydrolyzed by methionyl dipeptidase, and methionylprolinesi the preferred substrate for imidodipeptidase The possibility that these enzymes may function in the removal of HN_2-terminal methionine from newly initiated *N crassa* proteins is discussed

INTRODUCTION

The synthesis of cytoplasmic proteins of *Neurospora crassa* and other eukaryotic cells is initiated by a specific methionyl tRNA [1, 2] Methionine, however, is subsequently removed from the NH_2-terminal of a large percentage of newly initiated eukaryotic proteins For example, only 5% of the soluble proteins from *N crassa* retain methionine as their NH_2-terminal residue [3] The hydrolysis of methionine from nascent proteins after they have reached a specific length has been reported in several different systems including rabbit reticulocytes, rat liver, ascites cells, and HeLa cells [4–7] NH_2-terminal modification of another class of proteins appears to occur by a different mechanism in which removal of NH_2-terminal methionine occurs only after the protein is completed and released from the ribosome Protamines are representative of the latter class of proteins, i e , protamine from trout testes is initiated by methionine and the methionine removed only after the protein has been released from the ribosome [8]

This study began in an attempt to identify peptidases which may function in removal of NH_2-terminal methionine from newly initiated proteins in *N crassa* A number of investigators have reported enzymes which may function in the removal of NH_2-terminal methionine from newly initiated proteins in eukaryotes [9, 10],

however, the physiological roles of these enzymes remains unknown It is probable that there are two peptidases that are involved in the modification of newly synthesized proteins One peptidase is involved in the modification of the NH_2-terminal region of proteins that are still nascent A second enzyme may be involved in the NH_2-terminal modification of proteins which are completed and released from the ribosome before modification occurs In this communication we report the partial purification and characterization of two *N crassa* peptidases which preferentially hydrolyse certain methionyl dipeptides

METHODS

Materials

The sources of peptide substrates and other chemicals were the same as described previously [11] Hypatite C (hydroxyapatite) was a product of Clarkson Chemical Co Ampholine carrier ampholytes for electrofocusing were purchased from LKB

Growth of cells

N crassa IA was a gift from Dr Gene A Scarborough, Department of Biochemistry, University of Colorado 16 L of Fries media containing sucrose was inoculated with conidia [12] and maintained for 3 days at 37 °C with continuous aeration The average yield was 300–400 g washed, pressed dry *N crassa*

Enzyme assays

Hydrolysis of the peptide substrates was measured by the fluorescence assay of Roth [13] using the conditions described previously [11] Briefly, 0 1-ml aliquots of the reaction mixture were withdrawn at appropriate time intervals and added to 0 1 ml of ice-cold absolute ethanol to stop the reaction 3 ml of the reagent (0 05 M sodium borate buffer (pH 9 5), 0 125 mM *o*-phthalaldehyde and 0 125 mM 2-mercaptoethanol) were added to each aliquot and the mixture was incubated for 5 min at room temperature Fluorescence was measured after excitation at 310 nm using a Beckman ratio fluorometer In addition, hydrolysis of tripeptides was monitored by comparison of the ninhydrin-positive material in the reaction mixture with appropriate standard amino acids and dipeptides after electrophoresis (pH 1 9, 3000 V, 30 min) and staining The amount of protein in the final reaction mixture varied from 150 μg to 0 2 μg depending on the stage of purification of each enzyme

After each purification step the fraction(s) containing peptidase activity was separated by electrophoresis in polyacrylamide gels [14] and the peptidase activity measured directly in the gels by the L-amino acid oxidase reaction coupled with the peroxidase-catalysed oxidation of *o*-dianisidine [11, 15, 16]

Protein was measured by the method of Lowry et al [17] using bovine serum albumin as a standard

Preparative polyacrylamide gel electrophoresis

Preparative polyacrylamide-gel electrophoresis was conducted as described earlier [11]

Electrofocusing

The column used for electrofocusing was an WKB8100-1 ampholine column, 110 ml Electrofocusing was conducted using ampholytes with a pH range of pH 3–10 or pH 3–6 using the procedure described by Haglund [17]

RESULTS

Partial purification of methionyl dipeptidase and imidodipeptidase

For the purification of methionyl dipeptidase and imidodipeptidase frozen *N crassa* were allowed to partially thaw at room temperature, and then homogenized in 2 5 vol of 10 mM Tris–HCl buffer (pH 7 4) containing 1 mM 2-mercaptoethanol, and 2 5 mM $MnCl_2$ (Buffer A) in a Waring Blender using glass beads [19] Nucleic acids were precipitated by addition of 1 M $MnCl_2$ to a final concentration of 50 mM [20] The precipitate was removed by centrifugation at 10 000 × *g* for 20 min The supernatant fractions, referred to as the crude extracts, contained the peptidase activity The specific activities of the methionyl dipeptidase and imidodipeptidase in the crude extracts were 2 7 and 2 3 units/mg protein, respectively

$(NH_4)_2SO_4$ fractionation

This step and all subsequent steps were carried out at 0–4 °C The crude extract was brought to 50% satn by addition of solid $(NH_4)_2SO_4$ and centrifuged at 10 000 × *g* for 20 min The supernatant fraction (pH 6 1) contained the major peptidase activities The supernatant fraction was then brought to 75% $(NH_4)_2SO_4$ saturation by further addition of solid $(NH_4)_2SO_4$ The peptidase activity was localized in the pellet obtained by centrifugation as described above The specific activities of the peptidases precipitating between 50 and 75% $(NH_4)_2SO_4$ satn were 2 9 and 2 5 units/mg protein for the methionyl dipeptidase and imidodipeptidase, respectively

DEAE-cellulose chromatography

For purification of methionyl dipeptidase the material from the preceding step was thoroughly dialyzed against Buffer A and applied to a DEAE-cellulose column (5 cm × 45 cm) equilibrated with Buffer A containing 50 mM KCl The adsorbed protein was eluted with a 4-l linear gradient of 50–200 mM KCl in Buffer A The fractions having the highest enzyme activity were combined The specific activity of methionyl dipeptidase in the combined fraction was 10 units/mg protein

The separation of methionyl dipeptidase from imidodipeptidase was first observed after this procedure Methionyl dipeptidase eluted between 150 and 170 mM KCl and imidodipeptidase eluted between 90 and 120 mM KCl During subsequent purifications of imidodipeptidase, material from the 50–75% $(NH_4)_2SO_4$ fraction was chromatographed by a batch procedure using DEAE-cellulose equilibrated with 100 mM KCl in Buffer A The column was then washed with the same buffer Under these conditions imidodipeptidase passed directly through the column 100-ml fractions were collected and the first 600 ml were combined The specific activity of imidodipeptidase in the combined fraction was 11 units/mg protein

Hydroxyapatite chromatography

Material from the preceding DEAE-cellulose chromatography procedures was

dialyzed against 10 mM Tris–HCl buffer–1 mM 2-mercaptoethanol (pH 7 4) to remove the Mn^{2+}, and then against 20 mM potassium phosphate buffer (pH 7 4) Methionyl dipeptidase was then applied to a hydroxyapatite column (2 5 cm × 40 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7 4) The protein was eluted with a 2 l linear gradient of 20–60 mM potassium phosphate The major methionyl dipeptidase activity was eluted between 40 and 50 mM potassium phosphate Fractions having the highest specific activity were combined The specific activity of methionyl dipeptidase in the combined fraction was 50 units/mg protein

Imidodipeptidase preparations were applied to a hydroxyapatite column (2 5 cm × 40 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7 4) and the protein eluted with a 2-l linear gradient from 20–150 mM potassium phosphate buffer (pH 7 4) The major imidodipeptidase activity was eluted between 90 and 130 mM potassium phosphate buffer Fractions having the highest specific activity were combined The specific activity of imidodipeptidase in the combined fraction was 21 6 units/mg protein

A large amount of protein and enzyme units were lost at this stage in the purification of both methionyl dipeptidase and imidodipeptidase Changes in pH, and ionic strength did not improve the recovery of either enzyme, nor did use of other adsorbants such as CM-cellulose result in additional purification of either enzyme

Preparative polyacrylamide-gel electrophoresis of methionyl dipeptidase

After chromatography on hydroxyapatite aliquots of the enzyme preparation containing 5 mg of protein were applied to the preparative electrophoresis unit as described previously [11] Approx 8 h were required for elution of the peptidase The specific activities of each fraction were determined and those fractions with similar specific activities were combined Analytical gel electrophoresis showed the presence of 4 bands when stained with Coomassie brilliant blue and destained as described by Swank and Munkres [21] Hydrolysis of methionylalanine was observed

TABLE I

PARTIAL PURIFICATION OF METHIONYL DIPEPTIDASE

1 5 kg of *N crassa* were used for the extraction of methionyl dipeptidase One unit of enzyme activity equals the hydrolysis of 1 μmole of methionylalanine per min under standard assay conditions described under Methods

Procedure	Protein		Methionyl dipeptidase		
	(mg/ml)	Total mg	Peptidase activity (total units)	Spec act (units/mg)	Yield (% recovery)
(1) Crude extract	1	5500	15 000	2 7	—
(2) 50–75% $(NH_4)_2SO_4$ precipitate	2	2700	8 000	2 9	53
(3) DEAE-cellulose chromatography	2 5	500	5 000	10	33
(4) Hydroxyapatite chromatography	1	20	1 000	50	6 7
(5) Preparative gel electrophoresis	0 4	0 3	60	200	0 4

in only one of the 4 bands The specific activity of methionyl dipeptidase in the combined fraction was 200 units/mg protein with a final yield of 0 4% The data for the partial purification of methionyl dipeptidase are summarized in Table I

Electrofocusing of imidodipeptidase

The isoelectric point of imidodipeptidase was determined to be at pH 5 1 using samples from the fraction which precipitated between 50 and 75% $(NH_4)_2SO_4$ saturation Material from the hydroxyapatite chromatography procedure was electrofocused for 48 h in pH gradient of pH 3–6 at 1000 V Aliquots of 0 5 ml were collected and assayed for enzyme activity Fractions with similar specific activities were combined The specific activity of imidodipeptidase in the combined fraction was 152 units/mg protein with a final yield of 0 5% This procedure provided a 10-fold purification of imidodipeptidase and was easily reproducible Further purification of imidodipeptidase was not attempted The data for the partial purification of imidodipeptidase are summarized in Table II

TABLE II

PARTIAL PURIFICATION OF IMIDODIPEPTIDASE

15 kg of *N crassa* were used for the extraction of imidodipeptidase One unit equals the hydrolysis of 1 μmole of methionylproline per min using the assay conditions described under Methods

Procedure	Protein		Imidodipeptidase		
	mg/ml	Total mg	Peptidase activity (total units)	Spec act (units/mg)	Yield (% recovery)
(1) Crude extract	0 2	12 800	29 400	2 3	—
(2) 50–75% $(NH_4)_2SO_4$ precipitate	3 6	5 800	14 500	2 5	49
(3) DEAE-cellulose chromatography	21	1 090	12 000	11	41
(4) Hydroxyapatite chromatography	12 3	42	910	21 6	3
(5) Electrofocusing	0 18	0 7	152	217	0 5

Enzyme properties

Divalent metal ion requirements To examine the possible divalent cation requirement of methionyl dipeptidase and imidodipeptidase, aliquots from the 50–75% fractions or side fractions from the hydroxyapatite chromatography were dialyzed against 1 mM EDTA in 10 mM Tris buffer–1 mM 2-mercaptoethanol (pH 7 4) The effect of various divalent cations on the activity of both enzymes is shown in Table II Mn^{2+} of all divalent cations tested restored activity of both enzymes to the highest level Complete loss of both enzyme activities was obtained by dialysis against EDTA Addition of 1 mM Mn^{2+} required approx 1 h to restore the activity of both methionyl dipeptidase and imidodipeptidase Heat denaturation experiments using both enzymes after treatment with EDTA showed that Mn^{2+} did not act to stabilize either enzyme during incubation at 50 °C for time periods up to 1 h No change in substrate specificity could be shown with either enzyme when substrates

TABLE III

DIVALENT CATION REQUIREMENT

Enzyme fractions were dialyzed against 1 mM EDTA in 10 mM Tris buffer–1 mM 2-mercaptoethanol (pH 7 4) for 24 h and then dialyzed against 10 mM Tris buffer–1 mM 2-mercaptoethanol (pH 7 4) for an additional 8 h Divalent cations were added to enzyme aliquots to give a final concentration of 1 mM The enzyme solution was then allowed to incubate for at least 1 h before assaying for enzyme activity using either methionylalanine or methionylproline as substrate The rates of hydrolysis are relative to the initial rates of hydrolysis of the appropriate substrates obtained in presence of the native enzymes

Methionyl dipeptidase		Imidodipeptidase	
Additions	(Dialysis) Relative rates of hydrolysis	Additions	(Dialysis) Relative rates of hydrolysis
None	4	none	9
Mn^{2+}	75	Mn^{2+}	69
Mg^{2+}	9	Mg^{2+}	15
Ca^{2+}	8	Ca^{2+}	12
Zn^{2+}	15	Zn^{2+}	48
Co^{2+}	39	Co^{2+}	17
Fe^{2+}	16	Fe^{2+}	13

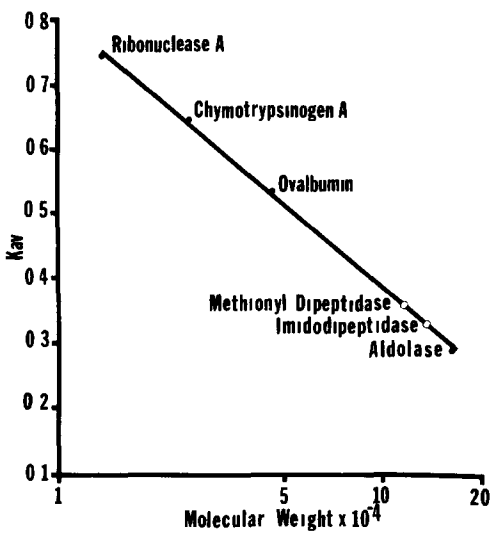

Fig 1 Estimation of the molecular weight of methionyl dipeptidase and imidodipeptidase Aliquots from the 75% $(NH_4)_2SO_4$ fractions, DEAE-cellulose chromatography, or hydroxyapatite chromatography were dialysed against the standard Tris buffer containing 0 1 M KCl A Sephadex G-150 column (2 cm × 35 cm) equilibrated with Buffer A containing 0 1 M KCl was used 1 ml of the sample was applied and a flow rate of 12 5 ml/h was maintained using the upward flow technique The column was calibrated with aldolase (M_r 158 000), ovalbumin (M_r 45 000), chymotrypsinogen (M_r 25 000), and ribonuclease (M_r 13 700) Molecular weight determinations for methionyl dipeptidase were 110 000 and 114 000 Molecular weight determinations for imidodipeptidase were 128 000 and 133 000

that were either cleaved slowly or not at all under standard assay conditions were tested with six divalent cations listed in Table III

Sulfhydryl requirements

Methionyl dipeptidase was completely inactivated by dialysis against 1 mM *p*-chloromercuribenzoate In contrast, imidodipeptidase activity was not altered by 1 mM *p*-chloromercuribenzoate, suggesting that methionyl dipeptidase but not imidodipeptidase has a requirement for a free sulfhydryl group for enzyme activity

pH requirements Methionyl dipeptidase was active between pH 5 5 and 9 5 with a pH optimum of pH 8 5 The enzyme is inactive at pH extremes of 4 5 and 10 Enzyme activity could not be restored after exposure to pH 4 5 Reactivation after

TABLE IV

SUBSTRATE SPECIFICITY OF METHIONYL DIPEPTIDASE AND IMIDODIPEPTIDASE

Assay conditions were those described under Methods using 1 mM final concentration of each substrate The initial rates of hydrolysis relative to either methionylalanine or methionylproline were measured

Methionyl dipeptidase		Imidodipeptidase	
Peptide	Relative rates of hydrolysis	Peptide	Relative rates of hydrolysis
Met–Ala	100	Met–Pro	100
Met–Ser	75	Ala–Pro	60
Met–Ile	60	Val–Pro	40
Met–Thr	50	Gly–Pro	8
Met–Met	50	Pro–Met	0
Met–Leu	30	Pro–Ala	0
Met–Val	0	Pro–Val	0
Met–Pro	0	Pro–Leu	0
Met–Glu	5	Pro–Ile	0
Met–His	5	Met–Ala	0
Ala–Ala	38	Ala–Met	0
Ala-Met	25	Met–Met	0
Nle–Ala*	40	Met–Val	0
Eth-Ala**	36	Thr–Val	0
Leu–Ala	32	Leu–Ala	0
Thr–Ala	26	Gly–Gly	0
Ser–Ala	20	Met–Gly–Met–Met	0
Gly–Gly	20	Gly–Pro–Ala	0
Gly–Ala	0	Ala–Pro–Gly	0
Ala–Gly	0		
Ala–Leu	0		
Gly–Ser	0		
Gly–D-Ser	0		
fMet–Ala***	0		
Met–Ala–Ser	0		
Met–Leu–Gly	0		
Met–Gly–Met-Met	0		

* Norleucylalanine
** Ethionylalanine
*** Formylmethionylalanine

exposure to upper pH extremes was not attempted Imidodipeptidase was active between pH 4 5 and 9 5 with a pH optimum of pH 7 5 The effect on enzyme activity of changes in pH beyond the range indicated was not investigated

Enzyme size The molecular weight of methionyl dipeptidase and imidodipeptidase was estimated by molecular seive chromatography on a Sephadex G-150 column as shown in Fig 1 [22] The molecular weight of methionyl dipeptidase was approx 110 000–114 000, and for imidodipeptidase 128 000–133 000

Substrate specificity The relative rates of hydrolysis of 27 peptides in the presence of methionyl dipeptidase are shown in Table IV The relative rates of hydrolysis of all substrates except methionylproline were similar in the different stages of purification The K_m values for 3 methionine containing dipeptides is shown in Table V The K_m values indicate the different substrates tested may have different

TABLE V

KINETIC CONSTANTS FOR METHIONYL DIPEPTIDASE AND IMIDODIPEPTIDASE

The initial rates of hydrolysis of the substrates were measured under standard assay conditions described in Methods Fractions were used which showed activity in only one region of a polyacrylamide gel The data was analyzed statistically using a computer provided by Dr F W Briese

Methionyl dipeptidase		Imidodipeptidase	
Substrate	K_m (mM)	Substrate	K_m (mM)
Met–Ala	0 25 ± 0 02	Met–Pro	0 81 ± 0 14
Met–Ser	0 57 ± 0 18	Val–Pro	0 69 ± 0 24
Met–Ile	1 02 ± 0 21	Gly–Pro	1 3 ± 0 24

abilities to bind to the enzyme Differences in substrate affinity for methionyl dipeptidase partially accounts for the differences in the relative rates of hydrolysis indicated in Table IV In addition, there is a difference in V of the substrates tested For example, at a concentration of 2 mM the initial rate of hydrolysis of methionylisoleucine was only 65% that of methionylalanine Hydrolysis of methionylvaline could not be detected Initial rates of hydrolysis of methionylserine were not altered by the presence of methionylvaline or formylmethionylalanine These results suggest that methionyl dipeptidase does not bind either of the latter two substrates

The relative rates of hydrolysis of 19 peptides by imidodipeptidase is shown in Table IV Of the substrates tested only dipeptides containing carboxyterminal proline were hydrolyzed by imidodipeptidase The K_m values for 3 proline containing dipeptides is shown in Table V The K_m values are similar indicating the differences in relative rates of hydrolysis shown in Table IV are due to differences in the efficiency of hydrolysis of the substrates tested

DISCUSSION

The partial purification and some of the properties of two peptidases from *N crassa* are described The yields are low, and both enzymes appear to be unstable under a variety of routine isolation procedures Due to the instability of the enzymes, the estimates of specific activities and yields are probably minimal

Both peptidases have a requirement for Mn^{2+}, however, further studies are required to establish that they are both metalloenzymes Methionyl dipeptidase but not imidodipeptidase appears to have a free sulfhydryl group requirement for enzyme activity

Of the peptides tested as substrates for methionyl dipeptidase, methionylalanine and methionylserine were the most rapidly hydrolyzed Hydrolysis of substrates larger than dipeptides could not be demonstrated

Interest in characterization of methionyl dipeptidase resulted from previous studies on *Escherichia coli* peptidases which may be involved in removal of NH_2-terminal methionine from newly initated proteins [3, 11, 23] Methionyl dipeptidase is similar to the *E coli* enzyme recently purified in this laboratory [11] Both enzymes require Mn^{2+}, both have a free sulfhydryl group requirement for enzyme activity, the pH requirements are similar, and they both have a similar molecular size The substrate specificity of methionyl dipeptidase from *N crassa* is also very similar to that of the purified *E coli* peptidase Both enzymes have the highest activity toward methionylalanine and methionylserine and also have similar K_m values for both substrates The substrate specificity of methionyl dipeptidase differs from the *E coli* peptidase in respect to (1) methionyl dipeptidase is unable to cleave methionylvaline, whereas the *E coli* peptidase cleaves methionylvaline at a slow but measurable rate (2) methionyl dipeptidase cleaves methionylisoleucine at a rate 60% that of methionylalanine, but with the *E coli* peptidase, methionylisoleucine is cleaved slowly and acts as a noncompetitive inhibitor of the hydrolysis of methionylalanine, (3) the relative rate of hydrolysis of other dipeptides (Met–Leu, Gly–Ala, Gly–Gly) differ significantly

The rationale for using simple peptide substrates to examine enzymes which may be involved in NH_2-terminal modification of newly initated proteins was based on the possible substrate specificity of such enzymes determined by the studies of NH_2-terminal residues of *N crassa* proteins synthesized in vivo [3] For a discussion of the possible models for NH_2-terminal modification of newly initiated proteins see Vogt [24] The NH_2-terminal amino acids of native *N crassa* proteins indicate that the removal of the initiating methionine is approx 95% complete Alanine is the NH_2-terminal amino acid of about 1/3 of the proteins extracted from *N crassa* Abnormally large amounts of other NH_2-terminal amino acids are not observed [3] These results suggest that the peptidase catalyzing the removal of NH_2-terminal methionine from newly initiated *N crassa* proteins has the ability to cleave a variety of methionyl peptide bonds, but may be especially efficient in hydrolyzing methionine from nascent chains beginning with methionylalanine The methionyl dipeptidase examined in the present studies has only some of these properties For instance the initial rate of hydrolysis of methionylalanine was greater than that of any other substrate tested Also, methionylalanine was cleaved 4 times faster than alanylmethionine indicating preferential hydrolysis of peptides beginning with methionine However, only five of the ten methionyl dipeptides (Met–Ala, Met–Ser, Met–Ile, Met–Thr, and Met–Met) used as substrates were hydrolyzed at significantly greater rates than non-methionyl containing dipeptides In addition, methionylvaline was not hydrolyzed at a measurable rate by this enzyme but valine is the NH_2-terminal residue of 10% of the soluble *N crassa* protein [3] The substrate specificity of methionyl dipeptidase determined by using small peptide substrates, therefore

appears to be inconsistent with it being involved in NH_2-terminal modification

Interest in characterization of imidodipeptidase resulted from its ability to hydrolyze methionylproline more rapidly than any of the other proline containing peptides tested This observation suggested that imidodipeptidase may function to remove methionine from the NH_2-terminal of a class of proteins that are modified after their release from the ribosome Protamine is an example of this class of proteins When first released from the ribosome, protamine begins with the sequence methionylproline The methionine is subsequently hydrolyzed to produce the proline NH_2-terminal residue observed in the mature protein [8] For this reason, it was believed that methionylproline may serve as a substrate for the enzyme involved in modification of the class of proteins characterized by protamine However, imidodipeptidase from *N crassa* appears to be similar to the prolidase isolated from swine kidney which is specific for dipeptides containing proline as the carboxy-terminal residue [24]

Both methionyl dipeptidase and imidodipeptidase have some of the properties required for the enzymes involved in the removal of NH_2-terminal methionine from newly initiated and newly completed *N crassa* proteins In both cases, however, there are obvious discrepancies in the properties of the enzymes described in this communication and the properties required of the enzymes which catalyze the hydrolysis of NH_2-terminal methionine It appears both enzymes are dipeptidases and lack the substrate specificity apparently required of the enzymes involved in removal of NH_2-terminal methione from newly initiated proteins The physiological roles of dipeptidases are not understood and even though many dipeptidases have been reported they remain poorly characterized It appears that simple substrates such as those used in this study are not adequate to study the enzymes involved in NH_2-terminal modification It is possible that nascent polypeptides 30–50 amino acid residues long which appear to be the substrate for NH_2-terminal modification [25–27] will have to be used, further complicating the search for the methionine cleavage enzyme

ACKNOWLEDGMENTS

We wish to express our gratitude to Miss Lynn Deisher for her excellent technical assistance

Supported by Public Health Service Training Grant No GM01983-05S1, Department of Health, Education, and Welfare, and by Grant No 5 R01 CA12574-03 from the National Institutes of Health

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