Vol. 109, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1982 Pages 341-347

AMINOPEPTIDASE Co, A NEW YEAST PEPTIDASE

Tilman Achstetter, Claudia Ehmann and Dieter H. Wolf

Biochemisches Institut der Universität Freiburg, Hermann-Herder-Straße 7, D-7800 Freiburg im Breisgau, West Germany

Received October 5, 1982

Summary: A new aminopeptidase - aminopeptidase Co - has been detected in the yeast Saccharomyces cerevisiae. The enzyme is only active in the presence of Co^{2+}ions. Zn^{2+}- and Mn^{2+}ions are inhibitory. The enzyme activity is also inhibited by chelating agents. Of the p-nitroanilide derivatives tested only those containing basic amino acids are cleaved.

Introduction: It is gradually becoming clear that peptide bond hydrolysis represents an essential mechanism in the regulation of cell metabolism at the posttranslational level. Complete understanding of this regulatory mechanism depends on a knowledge of the intracellular peptidases and their characteristics, and of the cellular processes which they catalyze. Eight peptidases - two endoproteinases (1-3), two carboxypeptidases (1,4,5), one dipeptidase (6) and three aminopeptidases (6,7) have so far been characterized in the yeast Saccharomyces cerevisiae (for review see (8)). In addition mutants lacking the two endoproteinase activities and the two carboxypeptidase activities led to the detection of a variety of new endoproteinases and carboxypeptidases (9,10, for review see 11) which are under current investigation (12). A X-prolyl-dipeptidyl aminopeptidase was also recently reported (13). Here we describe the discovery of a new aminopeptidase, the activity of which is strictly dependent on Co^{2+}ions.

0006-291X/82/220341-07$01.00/0

This property clearly differentiates the new aminopeptidase from all similar activities so far known in yeast.

Materials and Methods: Yeast growth media were from Difco (Roth, Karlsruhe, F.R.G.). Aminopeptidase substrates (L-aminoacyl-p-nitroanilides) were obtained from Bachem (Bubendorf, Switzerland). DEAE Sepharose CL-6B, Sephadex G-150, catalase, bovine serum albumin and hen egg ovalbumin were from Pharmacia (Freiburg, F.R.G.). Proteinase inhibitors were purchased from the Peptide Institute (Osaka, Japan). Rabbit muscle aldolase was from Boehringer (Mannheim, F.R.G.). All other chemicals of highest purity available were from Roth (Karlsruhe, F.R.G.). Saccharomyces cerevisiae strain ABYS 1 (pra1 prb1 prc1 cps1) (B.Mechler and D.H.Wolf, unpublished) was grown on complete medium (1% yeast extract, 2% peptone, 2% glucose supplemented with 50 mg per l of adenine and uracil) for 36 h at 30°C into stationary phase. Extracts were prepared by passing cells suspended in 0.1 M Tris/HCl buffer pH 7.2 (cell mass:buffer volume, 1:1) twice through a French pressure cell at 10,000 psi. Subsequent centrifugation at 15,000 rpm for 15 min (Sorvall centrifuge, SS 34 rotor) and at 45,000 rpm for 30 min (Kontron centrifuge, 50 Ti rotor) yielded a clear cell free extract. Before separating the enzyme activities by ion exchange chromatography, proteins were fractionated by ammonium sulfate precipitation. Proteins precipitating in the concentration range of 0 to 40% ammonium sulfate were discarded. The remaining supernate was saturated up to 66% ammonium sulfate and the precipitate formed was dissolved in 0.02 M Tris/HCl buffer pH 7.2 and dialyzed against the same buffer for 15 h. The dialyzed protein solution (150 mg of protein) was applied to a DEAE-Sepharose CL-6B column (10 ml of gel) and the proteins were eluted using a gradient of 0 to 0.3 M sodium chloride in 0.02 M Tris/HCl buffer pH 7.2. Fractions of 0.8 ml were collected. Molecular weight of aminopeptidase Co was determined in cell free extracts in the absence of externally added Co^{2+}ions using Sephadex G-150 gel permeation chromatography. Column size was 1.0 x 100 cm. Molecular weight markers used were ovalbumin (M_r = 43,000), bovine serum albumin (M_r = 67,000), aldolase (M_r = 158,000) and catalase (M_r = 232,000). In addition aminopeptidase II (M_r = 85,000 (6)) was used as internal molecular weight marker of the cell extract. Aminopeptidase activities were tested using L-aminoacyl-p-nitroanilides as substrates. Enzyme-catalyzed hydrolysis was measured spectrophotometrically at 405 nm and 30°C as p-nitroaniline appearance. Test was done in 1 ml of 0.1 M Tris/HCl pH 8.5. Substrate concentration was 1 mM. Units are expressed as µmoles p-nitroaniline (ε_{405nm} = 9,500 M^{-1} x cm^{-1} (14)) per min and ml of enzyme. Protein was determined after the method of Lowry et al.(15) using bovine serum albumin as standard.

Results and Discussion: Three aminopeptidases have unambiguously been shown to exist in Saccharomyces cerevisiae and have been characterized to different extents. 1) A vacuolar aminopeptidase (7) of M_r = 640,000, later named aminopeptidase I (6) has the following characteristics: the enzyme is highly active against leucine-p-nitroanilide but shows very little activity against lysine-p-nitroanilide (6). Its activity is dependent on Zn^{2+} -

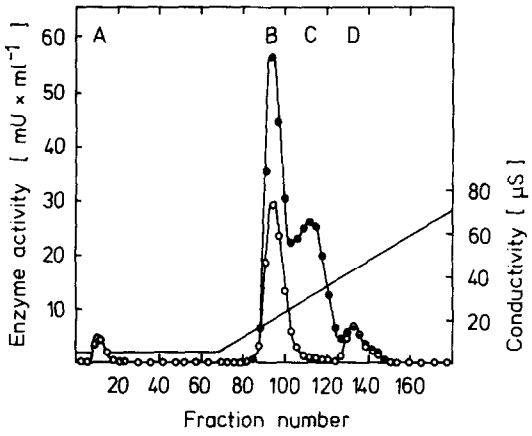

Figure 1 Aminopeptidase activity pattern determined with lysine-p-nitroanilide of an ammonium sulfate fractionated (40-66%) yeast extract following ion exchange chromatography on DEAE Sepharose CL-6B. For test, 0.01 ml of the eluted fractions were incubated. Test pH was 7.5. o——o, activity without addition; ●—●, activity with addition of 2 x 10^{-4}M $CoCl_2$; ——, conductivity (1:500).

but not Co^{2+}ions (6,7). 2) Aminopeptidase II (M_r = 85,000) cleaves leucine-p-nitroanilide and lysine-p-nitroanilide at nearly the same rate (6). Even though the enzyme activity may be dependent on Zn^{2+}ions, it is not inactivated by 1.6 mM nitrilotriacetic acid (16). 3) Aminopeptidase III is considered to be an enzyme of considerable lower molecular weight than aminopeptidase II and the enzyme is active against leucine-p-nitroanilide but not against lysine-p-nitroanilide (6).

We separated the aminopeptidase activities of an ammonium sulfate fractionated cell extract on DEAE Sepharose and visualized the eluted activities by their action on lysine-p-nitroanilide (Fig. 1). When tested without added Co^{2+}ions an activity profile consisting of two minor (A and D) and one major (B) lysine-p-nitroanilide cleaving activity was found. Activity under peak A very likely corresponds to aminopeptidase I since activity found against leucine-p-nitroanilide is ten-fold higher (not shown), a characteristic typical of aminopeptidase I. Activity of peak B most likely corresponds to aminopeptidase II since leucine-p-nitroanilide as substrate resulted in an activity profile of iden-

Table 1 Divalent metal ion dependency of aminopeptidase Co activity. The metal salts used were chlorides. The enzyme was preincubated in the presence of the respective metal ion(s) for 5 min at room temperature. Each metal ion concentration was 1 x 10^{-4} in the test. Enzyme activity (control) was 11.9 mU per mg.

Ion added	% activity	Ion added	% activity
Co^{2+} (control)	100		
None	9		
Zn^{2+}	7	Co^{2+}/Zn^{2+}	9
Mn^{2+}	5	Co^{2+}/Mn^{2+}	24
Cu^{2+}	10	Co^{2+}/Cu^{2+}	103
Ni^{2+}	15	Co^{2+}/Ni^{2+}	63
Hg^{2+}	5	Co^{2+}/Hg^{2+}	62
Ca^{2+}	10		
Mg^{2+}	9		

tical position and similar height as measured for lysine-p-nitroanilide (not shown), characteristics published for aminopeptidase II (6). Activity D is different from aminopeptidase III since no activity corresponding to aminopeptidase III should be detectable when tested against lysine-p-nitroanilide (6). This activity remains to be characterized in future studies.

When Co^{2+}ions were included in the test using lysine-p-nitroanilide as substrate a high activity peak occured at a position where no activity could be visualized without addition (Fig. 1, peak C). Also the activity of peak B increased, which may be due to an as yet unreported stimulation of aminopeptidase II by Co^{2+}ions. We concentrated our studies on the newly appearing, Co^{2+} stimulated activity of peak C. Table 1 shows that the enzyme activity is dependent on Co^{2+}ions. No other divalent ion tested can substitute for Co^{2+}. Interestingly, Zn^{2+}ions, necessary for catalytic function of aminopeptidase I and possibly aminopeptidase II (6,7) are highly inhibitory to the new enzyme. These characteristics, strong Co^{2+}-dependence and Zn^{2+}-inhibition, may be the reasons why the enzyme has remained undetected until now. Because of its unique metal ion dependence we will call the new enzyme aminopeptidase Co. Optimum Co^{2+}ion concentration for activity was determined to

Table 2 Substrate specificity of aminopeptidase Co

Substrate	Specific activity [mU x mg^{-1}]	
	without addition	1 x 10^{-4}M Co^{2+} added
L-Lysine-p-nitroanilide	4.8	43.3
L-Arginine-p-nitroanilide	1.8	14.5
L-Leucine-p-nitroanilide	5.8	6.4
L-Phenylalanine-p-nitroanilide	2.0	1.1
L-Alanine-p-nitroanilide	1.9	2.6
L-Glutamic acid-p-nitroanilide	0.4	0.6
L-Proline-p-nitroanilide	< 0.2	< 0.2
N-α-Benzoyl-D,L-lysine-p-nitroanilide	1.4	1.5

be around 5 x 10^{-5} M. Preincubation of the enzyme with the metal ions is necessary for maximum activity. Recently a carboxypeptidase involved in enkephalin processing responding similarly to Co^{2+} - and Zn^{2+} ions has been described (17). Cobalt stimulated cleavage was only detected with p-nitroanilides containing aminoterminal unsubstituted basic amino acids like lysine and arginine (Table 2). We believe that the background (non-cobalt-stimulated) cleavage of p-nitroanilide derivatives of the amino acids tested is due to some contamination with other peptidases, which are not completely separated by the chromatography step. Optimum pH for cleavage of lysine-p-nitroanilide by aminopeptidase Co was found to be around 8.5. The catalytic activity of aminopeptidase Co is highly sensitive to action of the metal ion chelating agent EDTA. o-Phenanthroline and nitrilotriacetic acid, two other metal ion chelators, are weekly or not effective when tested at a concentration of 10^{-4} M (Table 3). Only a great excess (10^{-3} M) of these two inhibitors gave a complete loss of aminopeptidase Co activity (not shown). Other proteinase inhibitors proved ineffective. Gel filtration indicated the molecular weight of the enzyme to be about 100,000 (data not shown). Separation of a cell-free extract into the membrane fraction and the soluble

Table 3 Action of proteinase inhibitors on aminopeptidase Co activity. Enzyme activity was determined in the presence of 1 x 10^{-4}M Co^{2+}. 11.9 mU per mg of enzyme were included in the test.

Inhibitor added	concentration	% activity remaining
None		100
EDTA	10^{-4}M	1
o-Phenanthroline	10^{-4}M	78
Nitrilotriacetic acid	10^{-4}M	100
p-Hydroxymercuribenzoate	10^{-4}M	76
Phenylmethylsulfonyl fluoride	10^{-3}M	93
Bestatin	50 µg/ml	82
Antipain	50 µg/ml	81
Leupeptin	50 µg/ml	96

content showed the aminopeptidase Co activity to be in the soluble fraction (12).

The main characteristics of aminopeptidase Co, namely strong cobalt dependence, which is not replaced by any other divalent cation tested, inhibition by Zn^{2+} ions and activity against lysine-p-nitroanilide but not leucine-p-nitroanilide, clearly distinguish the enzyme from all three previously known yeast aminopeptidases. The function of the enzyme has to be elucidated in further studies.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft, Bonn, and the Fonds der Chemischen Industrie, Frankfurt, F.R.G. The expert help of Mrs. R.Ch. Wilke and Mrs. H. Gottschalk during the preparation of this manuscript is gratefully acknowledged.

References:

1. Hata, T., Hayashi, R., and Doi, E. (1967) Agr. Biol. Chem. 31, 150-159.
2. Meussdoerffer, F., Tortora, P., and Holzer, H. (1980) J. Biol. Chem. 255, 12087-12093.
3. Kominami, E., Hoffschulte, H., and Holzer, H. (1981) Biochim. Biophys. Acta 661, 124-135.
4. Kuhn, R.W., Walsh, A.K., and Neurath, H. (1974) Biochemistry 13, 3871-3877.
5. Wolf, D.H. and Weiser, U. (1977) Eur. J. Biochem. 73, 553-556.
6. Frey, J. and Röhm, K.H. (1978) Biochim. Biophys. Acta 527, 31-41.
7. Matile, P., Wiemken, A., and Guyer, W. (1971) Planta (Berl.) 96, 43-53.

8. Wolf, D.H. (1980) Advances in Microbial Physiology (A.H. Rose and J. Gareth Morris, eds.) Vol.21, pp.267-338, Academic Press, London-New York.
9. Achstetter, T., Ehmann, C., and Wolf, D.H. (1981) Arch. Biochem. Biophys. 207, 445-454.
10. Wolf, D.H. and Ehmann, C. (1981) J. Bacteriol. 147, 418-426.
11. Wolf, D.H. (1982) Trends Biochem. Sci. 7, 35-37.
12. Achstetter, T., Emter, O., Ehmann, C., and Wolf, D.H. (in preparation).
13. Suarez Rendueles, M.P., Schwencke, J., Garcia Alvarez, N., and Gascon, S. (1981) FEBS-Lett. 131, 296-300.
14. Erlanger, B.F., Kokowsky, N., and Cohen, W. (1961) Arch. Biochem. Biophys. 95, 271-278.
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
16. Knüver, J. (1979) Diplomarbeit, Fachbereich Chemie, Universität Marburg, F.R.G..
17. Fricker, L.D. and Snyder, S.H. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 3886-3890.