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Evidence for a ribosome-associated thiol protease cleaving wheat germ methionyl-tRNA synthetase

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A wheat germ protease is responsible for M_r 105000 methionyl-tRNA synthetase hydrolysis, generating two fragments of M_r 82000 (harbouring the catalytic domain) and 20000, respectively. Specificity of the protease was sought for using different kinds of protein substrates. It turned out that charged peptides were preferentially cleaved and that no proteolysis occurred when proteins were replaced by small synthetic substrates, harbouring target sites similar to those cleaved in proteins. The protease could be a ribosomal protein, since it remained associated to ribosomal structure, even after treatment by deoxycholate, Triton X-100, 800 mM KCl and puromycin. Nevertheless, it was still active after ribonuclease treatment of the ribosomes. An identical protease activity was found in rat liver, but not in *E. coli*.

Methionyl-tRNA synthetase; Proteolytic cleavage; Ribosome-associated thiol protease; Wheat germ

1. INTRODUCTION

In mammalian cells, several aminoacyl-tRNA synthetases were found in high molecular weight complexes [1–4], where the methionyl-tRNA synthetase component was identified as a M_r 105000 monomer. In prokaryotes, methionyl-tRNA synthetase appears as a free dimer constituted of two identical subunits of M_r 85000 [5,6]. In wheat germ, a methionyl-tRNA synthetase of M_r 105000 has been purified as 'free' enzyme [7,8], but was also found included in a complex [8,9]. Nevertheless, the 'free' enzyme was always the most abundant form, certainly because of the weakness of bonds in the complex, the latter being probably broken up during enzyme purification. The wheat germ MetRS was also obtained as a dimeric enzyme of M_r 2 × 82000, similar to the *E. coli* one, when extraction was performed without thiol protease inhibitor [8,10]. We have shown that a protease, inhibited by ClHgBzOH, was responsible for the conversion of M_r 105000 methionyl-tRNA synthetase to a M_r 82000 form, by releasing a M_r 20000 polypeptide, constituted of numerous charged amino acids [8]. Two inactive M_r 82000 proteolysed forms bound together, giving an active dimer submitted to an association–dissociation equilibrium between inactive monomers and functional dimers, as already described for such enzymes [10,11].

Abbreviations: ClHgBzOH, *p*-hydroxymercuribenzoate; MetRS, methionyl-tRNA synthetase (EC 6.1.1.10)

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The aim of the present report is to study the location, the distribution and the activity of the protease which exhibits such a high specificity towards methionyl-tRNA synthetase.

2. MATERIALS AND METHODS

2.1. *Chemicals*

ClHgBzOH, *Nα-p*-tosyl-L-lysine chloromethylketone (TLCK), leupeptin, pepstatin, bestatin, soybean trypsin inhibitor, proteins and synthetic substrates were purchased from Sigma. *o*-Phenanthroline, EDTA, diisopropylfluorophosphate (iPr_2P-F), and 2-mercaptoethanol were from Merck. Sepharose CL-4B and polyacrylamide gels were from Pharmacia and Biogel A-5m was from Bio-Rad.

2.2. *Native methionyl-tRNA synthetase*

The M_r 105000 methionyl-tRNA synthetase was purified as described in [8].

2.3. *Preparation of ribosomes*

Wheat germ cells were disrupted at 4°C using a mortar. 600 mM mannitol was added to extraction buffer to avoid mitochondria disruption. The 40000 × *g* supernatant was supplemented with 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100 and applied to a Sepharose CL-4B column (2.4 × 100 cm), equilibrated with 25 mM potassium phosphate buffer (pH 7.2), containing 14 mM 2-mercaptoethanol and 5 mM $MgCl_2$ (buffer A). 2 ml fractions were collected and those corresponding to the ribosomes were centrifuged at 100000 × *g* for 150 min. Ribosomes were resuspended in buffer B corresponding to buffer A supplemented with 800 mM KCl and 1 mM puromycin, gently stirred for 15 min and applied to a Sepharose CL-4B column (1.9 × 140 cm) equilibrated at 20°C with the same buffer B (except puromycin). 1 ml fractions were collected. Ribosomal subunits were eluted as two peaks, centrifuged separately at 100000 × *g* for 3 h at 4°C and resuspended in buffer A. Determination of protease activity was carried out with 80 S ribosomes, suspended in buffer A (0.2 $A_{260}/\mu l$).

2.4. *Enzyme assays*

MetRS proteolysis. It was performed as indicated in [8].

Casein proteolysis. Protease activity was assayed in a 200 μl reaction mixture containing 10 mM potassium phosphate buffer, pH 6.8, 5 mM 2-mercaptoethanol, 2 mM $MgCl_2$ and 260 mM (5 mg/ml) $\varkappa$-casein. After equilibration at 35°C, the reaction was initiated by the addition of 10 μl ribosome suspension (see above). Assays were performed during 15–30 min. To the cleaved off peptide of $\varkappa$-casein is attached a short carbohydrate unit, terminated by *N*-acetylneuraminidate, that can be quantitated by measuring sialic acid in the supernatant at 550 nm [13].

With β-casein exhibiting Tyr residues on cleaved-off soluble acid peptide, enzyme activity was determined by measuring absorbance of the supernatant at 280 nm, according to [14].

2.5. *N-terminal sequence determination*

N-terminal amino acids were determined by the microsequencing method of Chang et al. [15]. Identification of DABTH amino acids was obtained by TLC on micropolyamide sheets (Schleicher and Schüll, Dassel, Germany).

2.6. *Preparation of* E. coli *and rat liver ribosomes*

E. coli P2200 was obtained from the French Institut National de la Recherche Agronomique. The bacterial membranes were disrupted by treatment with 0.1% (w/v) lysozyme and then 20% sucrose. Rat liver was suspended in 100 mM Tris-HCl buffer, pH 8.0, containing 25 mM KCl, 25 mM $MgCl_2$, 14 mM 2-mercaptoethanol and 600 mM mannitol. Cells were disrupted using a Potter homogeniser and the ribosome suspensions were recovered by centrifugation (40000 × *g* for 30 min). *E. coli* and rat liver ribosome preparations were added with sodium deoxycholate and Triton X-100, and purified by chromatography on Sepharose CL-4B, according to the method used for wheat germ ribosomes.

2.7. *Ribonuclease treatment*

The reaction mixture contained, in a total volume of 0.1 ml: 20 mM potassium phosphate buffer, pH 7.2, 2 mM $MgCl_2$, 25 mM KCl, 5 mM 2-mercaptoethanol and 20 μl of ribosome suspension in buffer A (1 A_{260}/μl). The mixture was incubated at 35°C. The reaction was started by addition of pancreatic ribonuclease (75 μg/ml) and performed for 30 min.

2.8. *Proteolysis of peptide substrates*

Proteolysis of the hexapeptide was performed at 35°C in 100 mM potassium phosphate buffer, pH 6.8, and the absorbance change was monitored at 310 nm as indicated in [16].

Proteolysis of other peptide substrates was performed at 35°C in 100 mM Tris-HCl buffer, pH 7.6, containing 5 mM 2-mercaptoethanol and 1 mM substrate, in a final volume of 2 ml. The reaction was initiated by the addition of 5 μl of ribosome suspension (see section 2.3) and enzyme activity was determined by measuring absorbance at 405 nm for substrate including *p*-nitroanilide and at 256 nm for the others.

3. RESULTS

3.1. *Identification of an endoprotease hydrolysing native methionyl-tRNA synthetase*

Several proteolytic activities present in a 40000 × *g* supernatant, prepared from a crude extract, were analysed. The sample was applied to a column of Biogel A-5m and protease activities eluted from the column were monitored, using specific substrates (Fig. 1). On the one hand, numerous protease activities (such as endoprotease, aminopeptidase, trypsin-like activities) were eluted, in the range of M_r 25000 to 100000, but none of them was active on MetRS. On the other hand, another endoprotease activity was found in a high molecular weight form. The latter was able to cleave only one peptide bond in MetRS of M_r 105000 and in β- and $\varkappa$-caseins (Fig. 2).

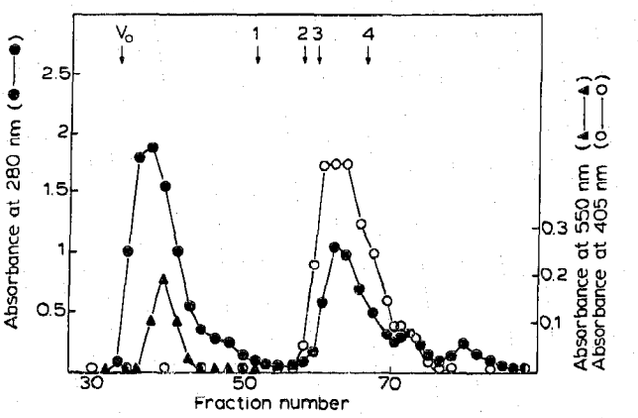

Fig. 1. Chromatography on Biogel A-5m. Gel filtrations were performed on a 2.4 × 100 cm column which was calibrated using (1) thyroglobulin (M_r 670000), (2) catalase (232000), (3) aldolase (160000) and bovine serum albumin (67000). V_0 indicates location of the void volume (M_r 5 × 10^6). 4.5 ml fractions were collected. $\varkappa$-Casein was used to monitor the high specific protease activity followed by measuring absorbance at 550 nm (▲). Among other protease activities, only trypsin-like activity is displayed by the absorbance at 405 nm (○).

3.2. *Characterization and localisation of the protease*

The MetRS specific protease was purified by chromatography on Sepharose CL-4B. Fig. 3 shows the elution profiles. The protease activity always appeared associated to the smaller ribosome subunit.

The protease activity remains unaffected by ribonuclease treatment of the ribosomal fraction. As indicated in Table I, the ribosomal protease, inhibited only by ClHgBzOH and leupeptin, behaves as a thiol protease.

3.3. *Specificity of ribosomal protease*

Methionyl-tRNA synthetase, β- and $\varkappa$-caseins were specifically cleaved by the ribosome-associated protease (Fig. 2), whereas other proteins like α-casein, haemoglobin, catalase, urease and ovalbumin, were not proteolysed (data not shown). Proteolysis was also performed on $\varkappa$-casein denatured by heating (100°C for 30 min), freezing and thawing, prior to its addition to the assay mixture. Denaturation was detected spectrophotometrically at 280 nm. Under these experimental conditions, denatured $\varkappa$-casein exhibited an identical hydrolysis pattern. However, protease activity on that substrate was reduced by half, comparatively to untreated $\varkappa$-casein. We also noticed that a modified casein-like azocasein was not hydrolysed either.

After proteolysis, peptides were separated by HPLC gel filtration on a TSK G2000 SW column (60 × 0.75 cm), equilibrated with a P_i/NaCl buffer. Suc-

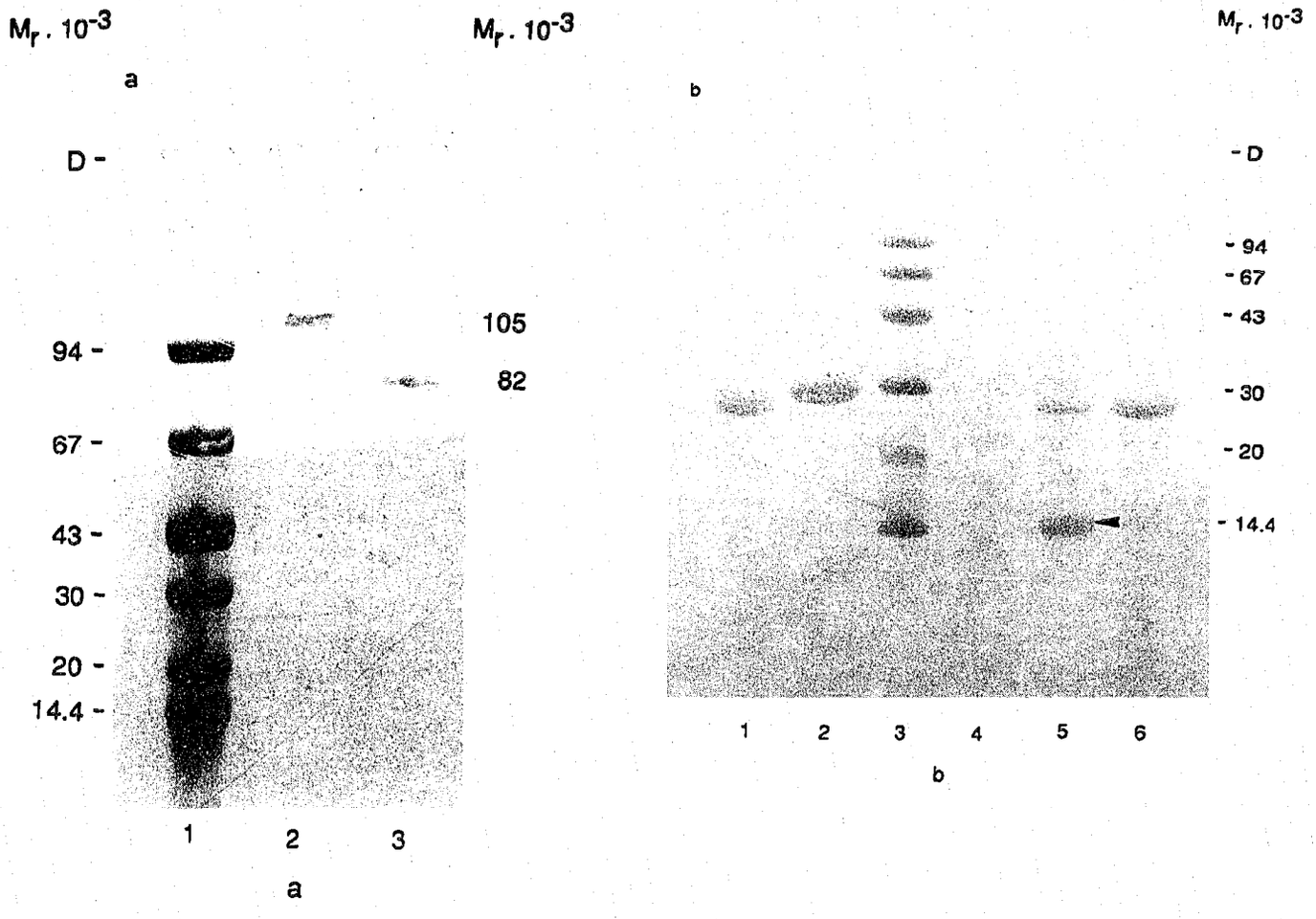

Fig. 2. Gel electrophoresis patterns of MetRS, β- and $\varkappa$-caseins submitted to ribosomal thiol protease hydrolysis. (a) Electrophoresis of markers (lane 1), purified native (lane 2) or proteolysed (lane 3) MetRS was performed on polyacrylamide gels (7.5% T, 2.6% C, 1% SDS) prepared according to Fairbanks et al. [17]. (b) Native and protease-treated β- and $\varkappa$-caseins were subjected to automatic SDS-PAGE Phast System Pharmacia in homogeneous gels (20% T, 2% C, 2.5% SDS). Proteolysed (lane 1) and native (lane 2) β-casein, markers (lane 3), ribosomal protease alone, staying located at the origin (lane 4), proteolysed (lane 5) and native (lane 6) $\varkappa$-casein (the arrow indicates the truncated M_r 14400 peptide).

cessively, 50 μl (500 μg) of proteolysed caseins (or native proteins, as control of M_r and purity) were injected. Then, three Edman cycles were carried out and the DABTH-amino acids identification showed the sequences Phe-Leu-Leu for β-casein and Ala-Ser-Gly for $\varkappa$-casein. This indicated that cleavages occurred between Ala-189 and Phe-190 for β-casein, and between Ile-125 and Ala-126 for $\varkappa$-casein. The M_r of the resulting cleaved peptides were respectively 2340 and 21642 for β-casein and 4568 and 14437 for $\varkappa$-casein. In both cases, these results are in agreement with gel electrophoresis patterns (Fig. 2).

Small synthetic substrates, suitable for the determination of hydrolysed peptide bonds involving the carboxyl group of Ala and Ile, such as *N*α-benzoyl-L-alanyl-*p*-nitroanilide, Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-methyl ester and *N*-benzoyl-L-alanyl-methyl ester, Ala-Ala-*p*-nitroanilide and *N*-benzoyl-L-isoleucyl-methyl ester, were subjected to ribosomal protease hydrolysis. None of these potential substrates was hydrolysed.

3.4. *Distribution of ribosomal protease in eukaryotic and prokaryotic cells*

Various assays were carried out to identify any protease activity linked to respectively *E. coli*, rat liver and 15-day-old wheat seedling ribosomes, in comparison to that displayed by quiescent wheat germ ones. No protease activity specific for β- and $\varkappa$-caseins was detected associated to *E. coli* ribosomes under pH conditions from 6.0 to 8.5. However, we noticed a similar activity in rat liver, as well as in wheat seedling ribosomes (Table II).

4. DISCUSSION

When β and $\varkappa$ milk caseins were subjected to proteolysis by ribosomal protease, a behaviour similar to

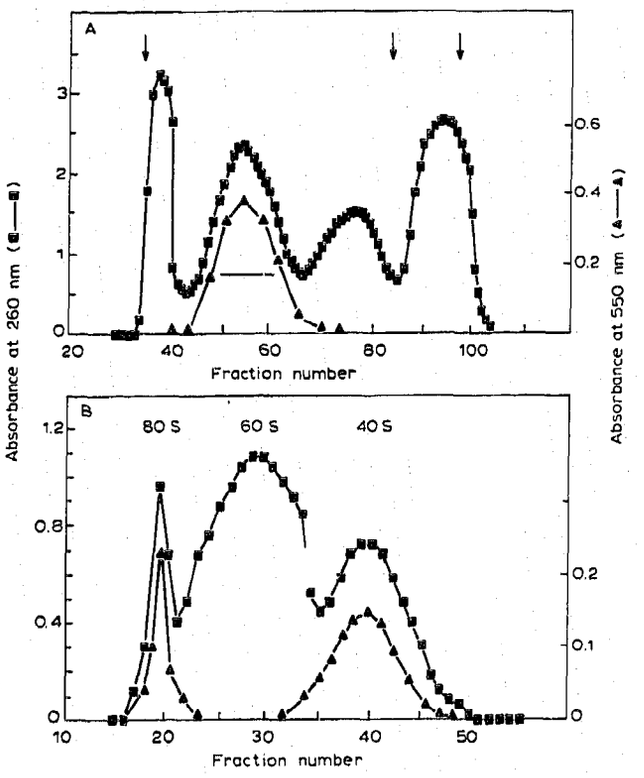

Fig. 3. Sepharose CL-4B chromatography. Gel filtration was performed as indicated in section 2. Absorbance was monitored at 260 nm (■) and 2 ml fractions were collected. (A) The horizontal bar indicates the fractions pooled, corresponding to ribosomes, with an absorbance ratio A_{260}/A_{280} of about 1.8–2 and exhibiting protease activity (▲). Vertical arrows indicate, from left to right, respectively, the location of the void volume, of thyroglobulin (M_r 670000) and of bovine serum albumin (M_r 67000). (B) Corresponds to the isolation of ribosomal subunits.

Table I

Inhibition of protease activity[a] by various protease inhibitors

Inhibitors	Conc. (mM)	% inhibition[b]	% activation[b]
iPr_2P-F	2	10 ± 8	–
Soybean trypsin inhibitor	1.4×10^{-3}	0 ± 5	–
TLCK	1	0 ± 10	–
o-Phenanthroline	10	0 ± 5	–
EDTA	10	0 ± 5	–
ClHgBzOH	2	100 ± 5	–
Leupeptin	4×10^{-2}	50 ± 10	–
Pepstatin	3×10^{-2}	0 ± 5	–
Bestatin	0.1	0 ± 5	–
Bovine α_2-macroglobulin	6×10^{-4}	0 ± 5	–
2-Mercaptoethanol	10	–	250 ± 20
Na^+, K^+, Mg^+	100	0 ± 5	0 ± 5

[a] Protease activity was determined by measuring spectrophotometrically the κ-casein hydrolysis in the presence of the 80 S ribosomal fraction, as indicated in section 2

[b] 0% inhibition and 100% activation corresponded to the value of the initial rate of the proteolysis reaction, in the absence of reducing agent and protease inhibitor; whereas 100% inhibition was defined when no absorbance at 550 nm was detected

the MetRS one was observed. We noticed that the ribosomal protease cleaved one peptide bond, on β- and κ-caseins, which exhibit an amphipolar character, giving, on the one hand, charged peptides, and on the other hand, hydrophobic ones (the latter ones bound together). In contrast, α-casein was not proteolysed. Interestingly, this protein which does not exhibit an amphipolar character is very sensitive to proteolysis processes, because of its relaxed secondary structure.

Moreover, small synthetic substrates exhibiting peptide bonds similar to those cleaved in β- and κ-caseins, but unable to mimic secondary structure of such proteins, were not proteolysed. Lastly, we also noticed that the hydrolysed peptide bonds on β- and on κ-casein, were not identical.

These results indicate that the specificity of such protease would be rather based on the interactions between the enzyme and a well-structured part of the protein-substrate. Consequently, the nature of the amino acids located near the hydrolysed bond could play a very minor role. In other words, these results imply that the proteolytic specificity is not limited to the primary structure, but includes spatial conformation of the substrate.

Two types of results concerning the location and the distribution of the protease appeared important for an eventual role of the protease in vivo.

First, the detection of the protease activity was coincident with the presence of the MetRS of M_r 105000. In higher eukaryotic cells (such as hepatocytes), where MetRS is present as a monomeric form of M_r 105000, included in a high molecular weight complex, ribosomes exhibit the protease activity, whereas in *E. coli* (where MetRS is only present as a M_r 2×85000 structure) no ribosomal protease activity was ever detected.

Second, the protease activity was located on ribosomes and the use of deoxycholate, Triton X-100, KCl and puromycin did not remove it. This indicates that the protease activity is tightly associated to the 40 S ribosomal subunit. MetRS and ribosomes are two components of the protein synthesis machinery. They may be close to each other in vivo and consequently they could react together in the cytosol.

Proteolysis of aminoacyl-tRNA synthetases, including MetRS, has already been described in the literature [18,19]. However, we are reporting here an action due to a protease tightly bound to ribosomes, that generates a 'prokaryotic-like' MetRS (M_r 2×82000), i.e. a free enzyme (we previously noticed that a multisynthetasic complex exists in wheat germ [8,9]), from the M_r 105000 form. Therefore, we envisage that such a proteolysis leads to the removal of a polypeptide possibly involved in the association of the eukaryotic MetRS to a multisynthetasic complex. Upon removal of this segment, the proteolysed MetRS could be removed from the complex and as a consequence would be

Table II

Ribosomal protease activity[a] in eukaryotic and prokaryotic cells

	E. coli	Rat liver	Quiescent wheat germ	15-day-old wheat seedlings
Protease activity: ΔA_{550} ($min^{-1} \cdot unit^{-1}$)[b]	0	0.015 ± 0.001	0.025 ± 0.001	0.020 ± 0.001

[a] Enzyme activity was determined using κ-casein as substrate. Conditions for purification and activity measurements are described in section 2

[b] One unit of ribosome corresponds to the value $A_{260} = 1$

unable to participate in protein synthesis, since channeling seems necessary to such a metabolic pathway [20]. In that case, the consequences of such a proteolysis could be very important on account of the initiator role played by the methionine in the protein synthesis.

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