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Peptidyl Transfer Ribonucleic Acid Hydrolase Activity of Proteinase *k*†

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ABSTRACT: Proteinase *k*, a seryl-protease obtained from *Tritirachium album*, is able to specifically hydrolyze N-blocked aminoacyl transfer ribonucleic acids (tRNAs). The blocked amino acid is released, and the tRNA molecule remains able to be recharged by its cognate amino acid. Aminoacyl-tRNAs are highly resistant to hydrolysis by the protease. This activity is not due to contamination of the protease preparation. A commercial protease from *Streptomyces griseus* displayed a similar activity, while trypsin, chymotrypsin, and papain unspecifically hydrolyzed all charged tRNAs tested. The characteristics of the hydrolysis performed by proteinase *k* closely resemble the peptidyl-tRNA hydrolase activity described in different cells as a scavenger for the peptidyl-tRNA that eventually falls from the polysomes. Our results warn about a hasty identification of any N-blocked aminoacyl-tRNA hydrolase activity in the cytoplasm as an independent peptidyl-tRNA hydrolase.

In previous experiments studying the sensitivity of ribosomal functions to proteases (Bernabeu et al., 1979), it was found that proteinase *k*, a protease obtained from *Tritirachium album* (Ebeling et al., 1974), was able to release ethyl acetate soluble radioactivity from the 3′-terminal fragment *N*-Ac-[^{3}H]Leu-ACCAC(U),[1] mimicking the "fragment reaction" catalyzed by ribosomal peptidyl transferase (Monro, 1971).

Although most proteases display a certain level of esterolytic activity, to our knowledge the sensitivity of the ester bond in the aminoacyl transfer ribonucleic acids (tRNAs) to the catalytic action of these enzymes has not been studied extensively before. Given the esterolytic activity of proteinase *k*, we considered it of interest to study in detail the characteristics of the reaction that takes place in the catalytic hydrolysis of aminoacyl-tRNA by proteases. The resulting data are reported here and show a surprising similarity between the specificity of substrate displayed by proteinase *k* and that of some peptidyl-tRNA hydrolases reported in the literature (Cuzin et al., 1967; Vogel et al., 1968; De Groot et al., 1969).

Materials and Methods

Labeled aminoacyl-tRNAs were obtained by charging commercial tRNA from *Escherichia coli* and yeast with the appropriate radioactive amino acids, using the homologous supernatant fraction (S-100) as a source of aminoacyl-tRNA synthetases. Separation of the [^{35}S]Met-tRNA$_f$ and [^{35}S]-Met-tRNA$_m$ was achieved on a column of BD-cellulose as described elsewhere (Carrasco et al., 1976). The 3′-terminal fragments were prepared from the charged aminoacyl-tRNA by treatment with RNase T_1 as described by Monro (1971). Proteinase *k* was obtained from Merck. All the other proteases were purchased from Sigma Chemical Co.

Activity Tests. Protease activity was tested on radioactive ribosomal proteins by measuring the loss of radioactivity precipitable by 5% trichloroacetic acid (Cl_3AcOH). The reaction was carried out in 50 μL of 30 mM PO_4^{3-}, pH 7.4,

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[1] Abbreviations used: *N*-Ac-Leu-A, *N*-acetylleucyladenosine; PMFS, phenylmethanesulfonyl fluoride.

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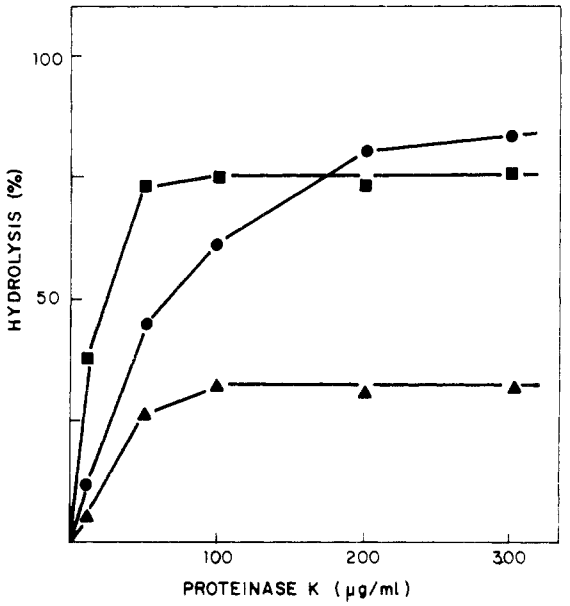

FIGURE 1: Hydrolysis of *N*-Ac-Leu-tRNA (●) and *N*-Ac-Leu-ACCAC(U) (▲, ■) by proteinase *k*. The experiments were carried out as indicated under Materials and Methods at 0 °C in the presence of 1–2 nM substrate. 33% methanol (■) stimulates the hydrolysis of the fragment.

containing 200 μg/mL protease with ~2500 cpm of precipitable proteins. It was incubated for 20 min at 25 °C.

Unless otherwise stated, aminoacyl-tRNA hydrolase tests were carried out under conditions similar to those of the protease activity tests. The extent of hydrolysis was determined in two ways, either by extracting the mixture, adding 150 μL of 0.3 M sodium acetate, and shaking it in a vortex for 1 min with 1 mL of ethyl acetate or by precipitating the sample with 1.5 mL of 5% Cl_3AcOH at 0 °C after supplementing it with 2 μL of carrier tRNA (50 mg/mL). In the first case the release of extractable radioactivity was measured, and in the second case the loss of precipitable aminoacyl-tRNA was followed.

Chromatography and Electrophoresis Techniques. The products of hydrolysis were analyzed by ascending chromatography on Whatman No. 1 paper by using a mixture of butanol–acetic acid–water (4:1:1 v/v). Electrophoresis was carried out following the technique described by Leboy et al. (1964). Electrofocusing of protease samples was done following the method of Catsimpoolas (1968) using LKB ampholine, pH 3.5–10.0.

Results

Hydrolysis of N-Ac-[^{3}H]Leu-ACCAC(U) and N-Ac-[^{3}H]Leu-tRNA by Proteinase k. Incubation of either *N*-Ac-[^{3}H]Leu-tRNA or its RNase T_1 product *N*-Ac-[^{3}H]-Leu-ACCAC(U) in the presence of proteinase *k* results in the release of radioactivity. These results were tested by trichloroacetic acid (Cl_3AcOH) precipitation and ethyl acetate extraction, respectively. The extension of the hydrolysis is proportional to the concentration of the protease, as shown in Figure 1, and can reach up to 80% of the *N*-Ac-Leu-tRNA present. The 3′-terminal fragment is apparently more resistant (Figure 1), but its hydrolysis can be strongly stimulated by 30% methanol.

Characterization of the Hydrolysis Products. Analysis of the material released from *N*-Ac-Leu-ACCAC(U) by chromatography showed the presence of only one compound that coincides with *N*-Ac-Leu and is clearly distinguishable, under the conditions used, from leucine and *N*-Ac-Leu-Ap, two of the other possible hydrolysis products (Figure 2).

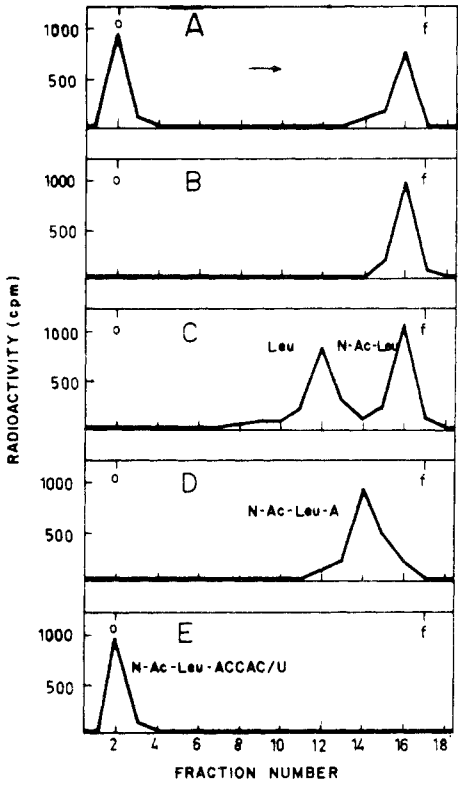

FIGURE 2: Chromatography of the hydrolysis products of *N*-Ac-Leu-ACCAC(U) by proteinase *k* [(0) origin, (f) front]. (A) Total reaction mixture; (B) ethyl acetate phase; (C) markers of Leu and *N*-Ac-Leu; (D) *N*-Ac-Leu-A; (E) *N*-Ac-Leu-ACCAC(U).

The identification of *N*-Ac-Leu as a product of the hydrolysis seems to indicate that the release of radioactivity is the consequence of splitting the ester bond between the amino acid and the 3′-terminal adenine of the tRNA molecule. If this is so, the resulting tRNA molecule has to remain intact and therefore be susceptible to recharging with the cognate amino acid. This was probed by supplementing a sample of *N*-Ac-Leu-tRNA, previously treated with proteinase *k*, with an excess of [^{3}H]leucine and the S-100 supernatant fraction as well as with the other components required for tRNA charging. After 10 min of incubation a level of Cl_3AcOH-precipitable radioactivity similar to that attained previously with proteinase *k* treatment was obtained (Figure 3).

Substrate Specificity of the Hydrolysis. With respect to the hydrolysis of aminoacyl-tRNAs, proteinase *k* displays a strict specificity for the NH_2-blocked molecules. This specificity is also maintained when the 3′-terminal fragments are used as substrates. We have tested different amino acids under two different conditions, and the results are summarized in Table I. Except for Arg-tRNA, which is resistant to the hydrolysis in both of its forms, only the N-blocked derivatives, including Pro-tRNA, are sensitive to proteinase *k*.

Purity of the Proteinase k Preparation. The unexpected characteristics of aminoacyl-tRNA hydrolase activity associated with proteinase *k* forced us to check the purity of our enzyme preparation in order to eliminate the possibility of contamination. Only one protein band could be detected in the different protease preparations used through polyacrylamide gel electrophoresis, using either electrofocusing or the system of Leboy et al. (1964) (Figure 4).

Inactivation of Protease and Hydrolase Activities. As a possible means of differentiating between the two activities associated with our preparation, we followed their inactivation with either phenylmethanesulfonyl fluoride (PMFS) or thermal

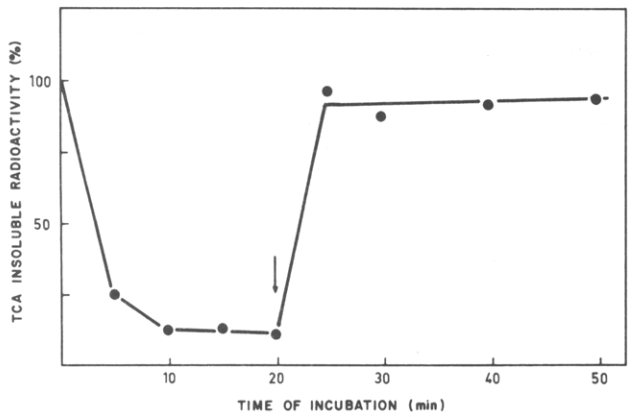

FIGURE 3: Recharging of proteinase *k* treated *N*-Ac-Leu-tRNA. A mixture (0.5 mL) containing 67 mM Tris-HCl, pH 7.8, 200 μg/mL proteinase *k*, and 3.25 nM *N*-Al-Leu-tRNA (60 Ci/mmol) was incubated at 0 °C. The hydrolysis was followed by Cl_3AcOH precipitation of 25-μL aliquots. After 20 min the sample was supplemented with 125 μL of a mixture of the composition required to give 20 mM Mg_2Cl, 40 mM NH_4Cl, 50 mM Tris-HCl, pH 7.8, 2 mM dithiothreitol (DTT), 5 mM ATP, and 0.66 μM [^{3}H]leucine (60 Ci/mmol). 50 μL of a tRNA-free supernatant fraction (S-100) was also added, and the sample was incubated at 37 °C.

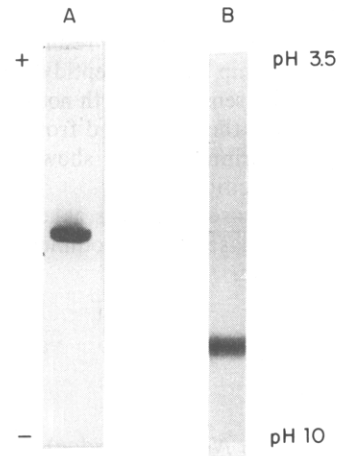

FIGURE 4: 20 μg of proteinase *k* was analyzed by electrophoresis (A) and by electrofocusing (B) between pH 3.5 and 10.

denaturation. In both cases (parts A and B of Figure 5) the two activities are lost in parallel. The high resistance to heat denaturation displayed by both activities is worth noting.

Table I: Hydrolysis of Different Aminoacyl-tRNAs by Proteinase *k*[a]

substrate	hydrolysis at 0 °C: substrate concn (μM)	hydrolysis at 0 °C: % hydrolysis[b]	hydrolysis at 37 °C: substrate concn (μM)	hydrolysis at 37 °C: % hydrolysis[b]
N-Ac-[^{14}C]Ala-tRNA	0.053	79	0.533	57
[^{14}C]Ala-tRNA	0.044	6	0.675	5
N-Ac-[^{14}C]Arg-tRNA	0.062	7	0.465	2
[^{14}C]Arg-tRNA	0.037	0	0.275	0
N-Ac-[^{3}H]Leu-tRNA	0.061	87	0.169	86
[^{3}H]Leu-tRNA	0.042	2	0.024	7
N-Ac-[^{35}S]Met-tRNA$_f$	0.010	68	0.185	55
[^{35}S]Met-tRNA$_f$	0.009	7	0.094	2
N-Ac-[^{35}S]Met-tRNA$_m$			0.049	44
[^{35}S]Met-tRNA$_m$			0.031	3
N-Ac-[^{3}H]Phe-tRNA	0.044	40	0.208	43
[^{3}H]Phe-tRNA	0.049	0	0.274	4
[^{3}H]Pro-tRNA	0.041	1	0.219	0
N-Ac-[^{3}H]Tyr-tRNA	0.063	57	0.211	55
[^{3}H]Tyr-tRNA	0.039	2	0.341	1

[a] The tests were carried out as described under Materials and Methods by using 200 and 8 μg/mL proteinase *k* in the experiments at 0 and 37 °C, respectively. The extent of hydrolysis was followed by Cl_3AcOH precipitation. [b] The 100% values correspond to Cl_3AcOH-precipitable radioactivity in the controls incubated in the absence of protease.

Aminoacyl-tRNA Hydrolase Activity of Several Proteases. We have extended our studies to other proteases to see if they show characteristics similar to those of proteinase *k* as far as aminoacyl-tRNA hydrolysis is concerned. As summarized in Table II, protease from *Streptomyces griseus* shows specificity similar to that of proteinase *k*. On the contrary, other commercially available proteases are able to hydrolyze aminoacyl-tRNA unspecifically. Elastase did not show hydrolase activity under the conditions tested.

Discussion

The results presented in this report indicate that proteinase *k*, a seryl-protease obtained from *T. album* (Ebeling et al., 1974), is able to specifically hydrolyze N-blocked aminoacyl-tRNAs. The catalytic reaction splits the bond between the aminoacyl residue and the 3′-terminal adenosine in the

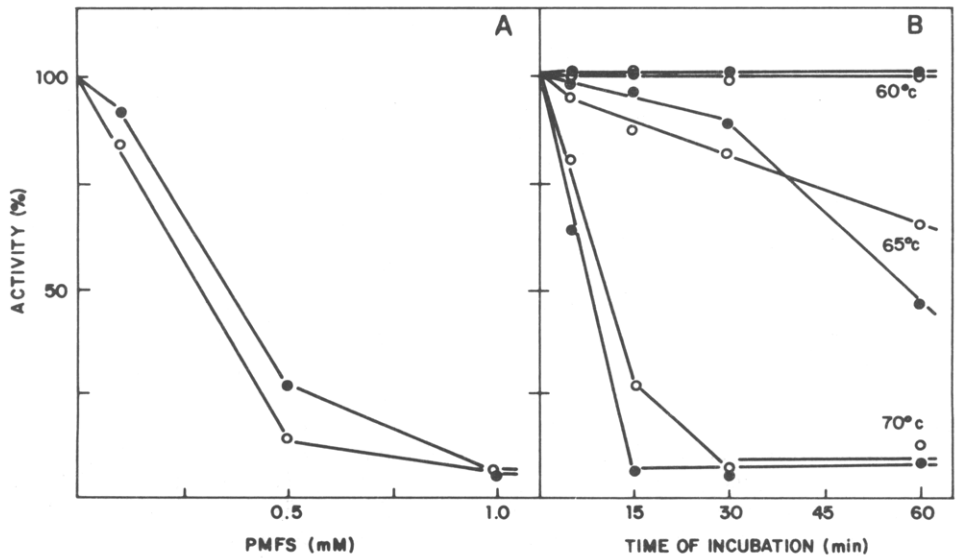

FIGURE 5: Inactivation of proteinase *k*. A sample of proteinase *k* (200 μg/mL) in 30 mM PO_4^{3-}, pH 7.4, was either treated with phenylmethanesulfonyl fluoride (PMFS) at 25 °C for 5 min (A) or incubated at high temperature (B). The protease (●) and aminoacyl-tRNA hydrolase (○) activities of the samples were tested.

Table II: Aminoacyl-tRNA Hydrolase Activity of Several Proteases[a]

protease	cleavage of substrates (%)					
	N-Ac-Phe-tRNA	Phe-tRNA	*N*-Ac-Leu-tRNA	Leu-tRNA	*N*-Ac-Leu-ACCAC(U)	Leu-ACCAC(U)
proteinase *k*	45	3	90	9	71	10
protease *S. griseus*	19	6	45	6	27	4
papain	9	11	32	46	32	11
elastase	0	1	3	2	13	7
trypsin	91	94	79	76	82	69
chymotrypsin A	91	93	80	90	78	39
control	5	7	0	7	0	2

[a] The tests were carried out under the same conditions given in Table I for the experiments performed at 0 °C. Data are corrected as in Table I.

tRNA, and it does not seem to be due to contamination by other proteins.

Although esterolytic activity is normal in proteases, proteinase *k* and probably the protease from *S. griseus* (though in this case contamination cannot be excluded) are unique among the enzymes tested (Table II) with respect to their specificity toward the aminoacyl-tRNAs. Only N-blocked molecules are sensitive to hydrolysis by these two proteases. The resistance of the Arg-tRNA to hydrolysis by proteinase *k* is in agreement with the specificity displayed by the enzyme in the hydrolysis of peptide bonds (Ebeling et al., 1974).

It is interesting to note the similarities between the results obtained with proteinase *k* and those reported for some peptidyl-tRNA hydrolases (De Groot et al., 1969; Chapeville et al., 1969). These enzymes are supposed to hydrolyze the peptidyl-tRNA molecules that eventually detach from the polysomes during protein synthesis (Menninger, 1976; Atherly, 1978). Thus, they are only active on N-blocked aminoacyl-tRNAs with the *N*-(formylmethionyl)-tRNA being relatively more resistant to hydrolysis, although some of the peptidyl-tRNA hydrolases reported can also under some conditions hydrolyze this initiator tRNA (Kossel, 1970; Menninger et al., 1974).

A comparison of the specific activities of peptidyl-tRNA hydrolases and that of proteinase *k* is limited by the few studies reported using pure hydrolases. It seems clear, however, that under comparable conditions the extent of hydrolysis achieved by the two enzymes is similar. In fact, data from Kossel (1970) allow us to calculate an approximate specific activity of 0.67 nmol/(min mg) for a highly purified peptidyl-tRNA hydrolase using *N*-Ac-Leu-tRNA as substrate, and our results (Table I) give a specific activity of 0.60 nmol/(min mg) for proteinase *k* acting on the same substrate.

The different susceptibilities of *N*-Ac-Leu-tRNA and *N*-Ac-Leu-ACCAC(U) toward hydrolysis by proteinase *k* (Figure 1) have similarly been reported in the case of peptidyl-tRNA hydrolases (Cuzin et al., 1967). The reason for this difference is not clear. A change in the optimal ionic conditions for the catalysis due to the polycationic character of the tRNA molecule that could affect the electrostatic potentials near the catalytic center, as has been reported for other enzymes (Douzou & Maurel, 1977), is a possibility that is going to be explored. The stimulation of the 3′-terminal fragment hydrolysis by methanol, perhaps through changes in the dielectric constant of the medium, is compatible with this explanation, although a direct effect on the substrate and/or the enzyme conformation cannot be excluded.

These similarities question the identity of at least some of the reported hydrolases since to our knowledge none of the reports on peptidyl-tRNA hydrolases tested for the protease activity of the preparations. At most, deductions made on the lack of peptidase activity of the preparation were based on the integrity of the peptide released from the peptidyl-tRNA used as substrate for the hydrolases (Menninger et al., 1970).

We do not question the existence of real peptidyl-tRNA hydrolases. In fact, there are clear differences between some characteristics of these enzymes and those found for proteinase *k*, such as the sensitivity of hydrolases (Vogel et al., 1968) and the resistance of proteases to heat inactivation. However, our results strongly recommend a check of the protease activity of purified peptidyl-tRNA hydrolases before characterizing them as independent activities.

From a different point of view, our results suggest a possible evolutionary relationship between peptidyl-tRNA hydrolases and proteases. In this sense it is worth noting that among the proteases tested, only those obtained from microorganisms, therefore the most primitive ones, show a peptidyl-tRNA hydrolase type of specificity.

In addition, proteinase *k* appears as a suitable source of peptidyl-tRNA hydrolase activity commercially available in a highly purified form.

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