N-ACETYLPHENYLALANYL-tRNA SPECIFIC HYDROLASE IN YEAST

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SUMMARY: An enzyme has been found and partially purified from yeast which hydrolyzes N-acetylphenylalanyl-tRNA and is different from the well-characterized N-substituted aminoacyl-tRNA hydrolase. The enzyme splits the ester bond between N-acetylphenylalanine and the 3' -hydroxyl group of the terminal nucleotide in the tRNA.

INTRODUCTION: Enzymes hydrolyzing both peptidyl-tRNAs and N-substituted aminoacyl-tRNAs have been found in the cytoplasm of both prokaryotic (1-4) and eukaryotic (5-7) cells. Common characteristics to these enzymes are their low levels of specificity with respect to particular amino acids (1) or peptidyl residues (8) and their inability to attack unsubstituted aminoacyl-tRNAs (1-3). The products of hydrolysis are the intact tRNA molecule and the corresponding N-substituted amino acid or peptidyl residue. An enzyme with these characteristics (N-substituted aminoacyl-tRNA hydrolase) was detected (5) and crystallized (6) from yeast extracts. We now report on the identification of a different enzymatic activity present in yeast which hydrolyzes N-acetylphenylalanyl-tRNA much more effectively than other N-acetylated aminoacyl-tRNAs. This enzyme has been partially purified and separated from the previous identified hydrolase.

MATERIALS AND METHODS: Uniformly labeled [^{14}C] L-amino acids were obtained from the Radiochemical Center (Amersham, England). Crude yeast tRNA was purchased from Boehringer. The charging of tRNA with radioactive amino acids and the isolation of the [^{14}C]aminoacyl-tRNAs was done as described (9). Acetylation of the [^{14}C]aminoacyl-tRNA was performed using acetic anhydride (10). The extent of acetylation was checked by alkali treatment of the acetylated [^{14}C] aminoacyl-tRNA followed by paper chro-

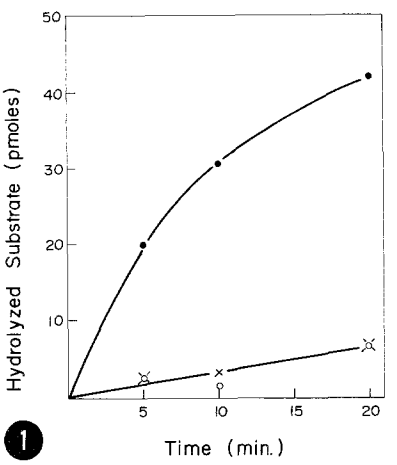
matography (9). In all cases more than 95% acetylation was achieved.

Preparation of the yeast extracts. The strain of yeast used was the hybrid Saccharomyces fragilis x Saccharomyces dobzhanskii. Cells were grown as described elsewhere (9) collected at the middle of logarithmic phase by centrifugation, washed three times with cold water and stored at -20º until use. One part of yeast was ground in a cold mortar with two parts of sand and the paste was suspended in 1-2 volumes of a cold buffer containing 50 mM Tris-HCl pH 7, 10 mM 2-mercaptoethanol, and 10 mM magnesium acetate. The homogenate was centrifuged in the cold at 10,000 xg for 10 min. and the precipitate was discarded. The supernatant was centrifuged at 105,000 xg in a Spinco ultracentrifuge at 2-5º for one and a half hour. The supernatant after this centrifugation was used as source of enzyme and is referred to as crude yeast soluble fraction. This fraction can be stored at -70º for several days without significant loss of activity.

Assay for enzymatic activity. The assay mixture (0.1 ml) contained, unless otherwise stated, the following components: 50 mM Imidazol-HCl pH 6.5, 2.5 mM magnesium acetate, 2.5 mM 2-mercaptoethanol, about 80 µg of tRNA charged with 50 pmoles of N-acetyl $[^{14}C]$ amino acids (specific activity 140 µC/µmol) and the amount of enzyme indicated in each case. The reaction mixtures were incubated at 30º for the indicated periods of time. At the end of the incubation the reaction was stopped by addition of 3 ml of 5 per cent cold trichloroacetic acid. The mixtures were kept at 0º for 10 min, and filtered through glass fiber paper (Whatman GF/C); the filters were washed three times with 3 ml each time of 5 per cent cold trichloroacetic acid and the radioactivity remaining in the filters was counted in a liquid scintillation spectrometer. Controls without added enzyme were run in parallel to estimate the spontaneous hydrolysis of the substrate.

Proteins were determined by the method of Lowry et al (11).

RESULTS AND DISCUSSION: The hydrolysis of different N-substituted aminoacyl-tRNAs by a crude yeast soluble fraction is shown in Fig 1. The rates of hydrolysis of N-acetylvalyl-tRNA and N-acetylleucyl-tRNA are similar and close to those previously reported as being due to the yeast N-substituted aminoacyl-tRNA hydrolase (6). However N-acetylphenylalanyl-tRNA is hydrolyzed at much higher rates (10 to 50 times higher, depending on the



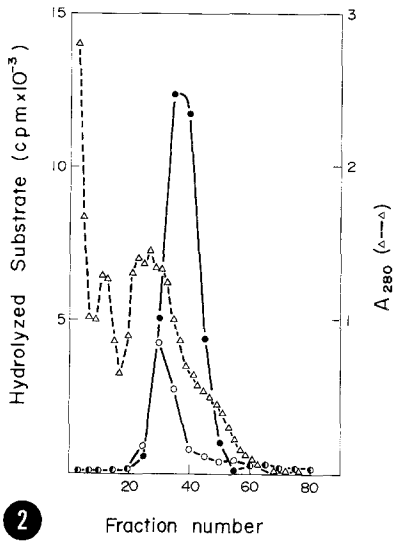


Fig 1. Hydrolysis of N-acetyl $[^{14}C]$ aminoacyl-tRNAs by the yeast soluble fraction. Reactions were carried out as described in Methods using 10 µl of yeast soluble fraction (35 mg protein/ml). N-acetyl- $[^{14}C]$ - phenylalanyl - tRNA (•—•); N-acetyl - $[^{14}C]$ valyl-tRNA (o—o); N-acetyl - $[^{14}C]$leucyl-tRNA (x—x).

Fig 2. Fractionation of the yeast hydrolases on DEAE-Sephadex A-50 column. A column of DEAE-Sephadex A-50 (2.5 x 14 cm) was equilibrated with 50 mM Tris-HCl, pH 7 containing 10 mM 2-mercaptoethanol and 10 mM magnesium acetate. The column was loaded with 15 ml of crude yeast soluble fraction (33 mg protein/ml), washed with 65 ml of the equilibration buffer, and eluted with a linear gradient of 0.1 M-0.4 M Tris-HCl pH 7 containing 10 mM 2-mercaptoethanol and 10 mM magnesium acetate (total buffer 500 ml). The flow rate was adjusted to 0.5 ml per min. and fractions of 6 ml were collected. Activity in the fractions was determined in 10 µl or 50 µl aliquots using either N-acetyl - $[^{14}C]$ phenylalanyl-tRNA (•—•) or N-acetyl $[^{14}C]$ valyl - tRNA (o—o) respectively as substrates.

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different extracts). This fact contrasts with the apparent low specificity of the yeast N-substituted aminoacyl-tRNA hydrolase towards particular amino acids residues (6) and suggested the existence in yeast of a different enzymatic activity acting preferentially on N-acetylphenylalanyl-tRNA. In a search for this activity we used the same yeast strain and followed the first steps of purification identical to those described for the crystallization of the yeast N-substituted aminoacyl-tRNA hydrolase, which basically involve

TABLE I

Separation of yeast hydrolases by fractionation with ammonim sulfate

	Volume (ml)	Proteins (mg/ml)	Activity pmoles/mg protein/10 min	
			N-acetyl-[^{14}C]Phe-tRNA	N-acetyl-[^{14}C]Val-tRNA
Crude soluble fraction	15	33	150	4.5
DEAE-Sephadex A-50, then 0-60% $(NH_4)_2 SO_4$ precipitate	5	7.7	14	34
60-80% $(NH_4)_2 SO_4$ precipitate	5	4.8	1300	0

A crude soluble fraction was chromatographed on DEAE-Sephadex A-50 as indicated in Fig 2. The pooled fractions (120 ml),containing both activities,were fractionated by adding solid ammonium sulfate to reach the percentages of saturation indicated. The precipitates were dissolved in 5 ml of a buffer containing 10 mM Tris-HCl pH 7, 10 mM potasium chloride, 10 mM 2-mercaptoethanol and 5 mM magnesium acetate. Activity was determined as specified in Methods.

chromatography on DEAE-Sephadex and fractionation with ammonium sulfate (6). The only difference was that at each step during the process of purification, the fractions were analyzed for hydrolytic activities using two substrates: N-acetyl[^{14}C] valyl-tRNA (the one routinely used in the purification of the N-substituted aminoacyl-tRNA hydrolase) and N-acetyl[^{14}C]phenylalanyl-tRNA.

Fig 2 shows the elution profile of the activities of a crude yeast preparation after chromatography on DEAE-Sephadex. In line with the results in Fig 1, the total activity on N-acetylphenylalanyl-tRNA is much greater than that on N-acetylvalyl-tRNA. Moreover, the peaks of activities on these two substrates are not coincident. Some fractions (40 to 45) which effectively hy-

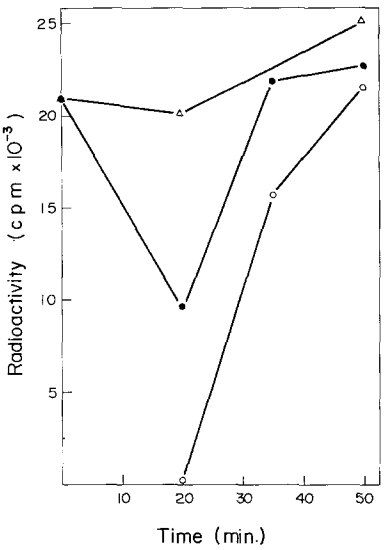

Fig 3. Hydrolysis of N-acetyl-[^{14}C]phenylalanyl-tRNA with purified hydrolase and recharging of the tRNA with [^{14}C]phenylalanine. Reaction mixtures containing: 50 mM Imidazol pH 6.5, 1 mM 2-mercaptoethanol, 1 mM magnesium acetate, 70 pmoles of N-acetyl-[^{14}C] phenylalanyl-tRNA (21,000cpm, sp. act. 140 µC/µmol) with (●—●) or without (△—△) hydrolase preparation (20 µl of the 60-80% ammonium sulfate fraction of Table I) were incubated at 30º. After 20 min incubation, 50 µl of a mixture containing 20 mM ATP, 100 mM magnesium acetate, 0.15 mM [^{14}C] phenylalanine (Sp. activity 116 µC/µmol) and 20 µl of crude yeast soluble fraction (previously passed through Sephadex G-25) as source of phenylalanyl-tRNA synthetase were added and the incubation was continued. At the time indicated acid insoluble counts were determined as indicated in Methods. Controls with on equal amount of deacylated yeast tRNA (2.6 A_{260nm} units) (o—o) instead of N-acetyl [^{14}C] phenylalanyl-tRNA were run in parallel in reactions mixtures containing the same amount of hydrolase preparation.

drolyze N-acetylphenylalanyl-tRNA have very low or no detectable activity on N-acetylvalyl-tRNA. These results contrast with the apparent equal effectivity of the yeast N-substituted aminoacyl-tRNA hydrolase to attack either of these two substrates (6).

Further separation of the enzyme acting preferentially on N-acetyl-phenylalanyl-tRNA from the hydrolase previously reported (6) can be achieved by fractionation of the pooled fractions from the DEAE-Sephadex chromatography, containing both activities, with ammonium sulfate. The results in Table I show that as reported (6) the N-substituted aminoacyl-tRNA hydro-

lase precipitates with 60% saturation of ammonium sulfate. In the 60-80% ammonium sulfate precipitate there is the hydrolytic activity very active on N-acetylphenylalanyl-tRNA but which does not hydrolyze N-acetylvalyl-tRNA to any appreciable extent. Preliminary experiments have shown that this fraction neither hydrolyzes phenylalanyl-tRNA nor the N-acetylated derivatives of leucyl- and methionyl-tRNAs. A study on the specificity of this enzyme is now in progress.

The reaction catalyzed by this enzyme involves the cleavage of the ester bond between N-acetylphenylalanine and the 3'-hydroxyl group of the 3' terminal nucleotide in the tRNA. This has been demonstrated by recharging the deacylated N-acetylphenylalanyl-tRNA, by the action of the hydrolase, with [^{14}C] phenylalanine (Fig. 3). As it is shown in the Figure the tRNA could be completely recharged. The excces of charging over the input counts is presumably due to the free tRNA specific for phenylalanine contaminating the preparations of N-acetylphenylalanyl-tRNA.

The role of this enzyme in the cell, although still unknown, might be related in someway with the initiation of protein biosynthesis. In this respect it is important to note that N-acetylphenylalanyl-tRNA acts as analogue of the N-formylmethionyl-tRNA$_F$ and is recognized by the prokaryotic initiation factor IF$_2$ or its equivalent in eukaryotic cells (12, 13).

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