ENZYMATIC HYDROLYSIS OF N-SUBSTITUTED AMINOACYL-tRNA

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The mechanism of the release of polypeptide chains from the ribosome-messenger RNA complex is not fully understood. It has been reported that free polypeptide chains are formed in cell-free protein-synthesizing systems, directed by polyribonucleotides, only if these polynucleotides contain statistically high frequencies of UAA codons.1-5 However, we do not know how the chain is released from the tRNA ribosome-mRNA complex after interruption of the translation by the UAA triplet. This release implies a hydrolysis of the ester bond between polypeptide and tRNA which could be catalyzed by a specific enzyme.

The search for such an enzymatic activity necessitates the use of the relatively unstable polypeptidyld-tRNA’s. It is difficult and laborious to prepare them in measurable quantities. In contrast, the chemically N-substituted aminoacyl-tRNA’s, although having similar characteristics in other respects, are stable and readily synthesized.6,7 An enzyme capable of hydrolyzing this ester linkage between N-acetylamino-acids and tRNA’s has now been found in extracts of Escherichia coli. This enzyme was partially purified and several of its characteristics were studied. The enzyme also catalyzes the hydrolysis of di-phenylalanyl-tRNA and N-substituted oligopeptidyld-tRNA’s.

Material.—C14-amino acids were obtained from the Commissariat à l’Energie Atomique (France); E. coli B tRNA, from General Biochemicals; crystalline pancreatic DNase and RNase, from Mann Research Laboratories; snake venom phosphodiesterase, from British Drug Houses Ltd.; T1 RNase, from Sigma Corp.

E. coli leucine-specific tRNA of about 50% purity was a gift from Dr. M. Yaniv; and a sample of H2-diphenylalanyl tRNA, from Dr. C. Ganoza.

The tRNA was charged with different C14-amino acids in the presence of an E. coli 105,000 × g supernatant. The C14-aminoacyl-tRNA was acetylated with acetic anhydride, as described by Haenni and Chapeville.7 In all cases it was shown that after acetylation all amino groups of the tRNA-bound amino acids were substituted. When serine and threonine are used it is possible that the OH groups also react with acetic anhydride, forming the corresponding esters.

C14-diphenylalanyl-tRNA was prepared according to Nakamoto and Kolakofsky4 by incubating C14-phenylalanyl-tRNA in the presence of ribosomes and 105,000 × g supernatant without addition of GTP. C14-polylysyl-tRNA was prepared from an incubation mixture of E. coli ribosomes with C14-lysyl-tRNA, poly A, GTP, and E. coli supernatant.3

Methods.—Analysis of the degradation products of N-acetylaminoacyl-tRNA: For most of the N-acetylaminoacyl-tRNA’s, the method described below for N-acetylleucyl-tRNA was used.

N-acetylleucine, leucine, N-acetylleucyladenosine (obtained after digestion of N-acetylleucyl-tRNA with pancreatic ribonuclease), and N-acetylleucyl-tRNA were separated by paper electrophoresis (Fig. 1). Under the same conditions, after treatment with RNase T1, two N-acetylleucyloligonucleotides were separated, one of which migrates with N-acetylleucine (Fig. 6). If a similar mixture had to be analyzed, both N-acetylleucyloligonucleotides would be converted to N-acetylleucyladenosine by treatment with pancreatic RNase before electrophoresis.

N-acetylleucyl-tRNA, N-acetylleucyladenylate (N-acetylleucyl AMP, obtained after digestion with purified venom phosphodiesterase of N-acetylleucyl-tRNA), N-acetylleucyladenosine, and
N-acetylleucine were also separated by paper chromatography (n-butanol, acetic acid, water; 78:5:17). The Rf's were, respectively, 0.0, 0.17, 0.70, and 0.92.

Preparation of E. coli extracts: The RNase I-deficient mutant MRE 600 of E. coli, obtained through the kindness of Dr. R. Traut, was grown in complete medium and harvested during the logarithmic phase. Cells were suspended in equal volume of Tris-HCl 0.01 M buffer, pH 7.4, and disrupted using a French press. Cell debris was removed by centrifugation at 30,000 × g for 30 min and the DNA hydrolyzed by the addition of 2 μg/ml of DNase.

Purification of the hydrolyase from E. coli: To 77 ml of 30,000 × g supernatant prepared from 60 gm of cells, were added: 25 ml of 30% solution of polyethylene glycol (PEG 6000, purchased from Union Carbide Inc.), 8.5 ml of a 20% solution of Dextrane 500 (Pharmacia), and 0.14 ml of 4 M NaCl. The mixture was shaken for 1 hr and centrifuged at 15,000 × g, for 10 min. The upper phase was discarded, and 40 ml of Tris-HCl, 0.01 M, pH 7.4, were added to the lower phase. After mixing, 16 ml of the PEG 6000 solution and 23.5 gm of NaCl were added. After 1 hr of shaking, the mixture was centrifuged at 15,000 × g, for 10 min. The upper phase, containing the enzymatic activity, was dialyzed against three changes of 1 liter of 0.01 M Tris-HCl, pH 7.4.

The total volume (105 ml) of the dialyzed solution (fraction I) was passed through a DEAE-cellulose column (8 × 3 cm) previously equilibrated with a solution of Tris-HCl, 0.01 M, pH 7.4. The filtrate and 50 ml of washing Tris-HCl buffer were combined and concentrated to 23 ml (fraction II); then 4.1 gm of ammonium sulfate were added. After centrifugation, the upper phase containing most of the PEG was discarded. The lower phase, containing the enzyme activity was dialyzed, and could be kept at −20°C for long periods, or at 0°C for several days.

All operations were accomplished at 0–4°C. Table 1 shows the values for protein content and enzyme activity of the different fractions.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Enzymatic Activity during Purification</th>
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<tbody>
<tr>
<td>Total activity (U)</td>
<td>Total protein (mg)</td>
</tr>
<tr>
<td>30,000 × g supernatant</td>
<td>1.7 × 10⁶</td>
</tr>
<tr>
<td>Fraction I</td>
<td>2.3 × 10⁴</td>
</tr>
<tr>
<td>Fraction II</td>
<td>8.5 × 10³</td>
</tr>
</tbody>
</table>

The concentration of protein was determined with the method of Lowry et al. with bovine serum albumin as a standard. Enzyme activity was determined using paper electrophoresis as described under Methods. A unit (U) is defined as 1 μM of N-acetylleucine from N-acetylleucyl-tRNA in 1 min at 37°C.

Assay for enzymatic activity: The volume of the assay mixture was 0.05 ml and contained: enzyme; 1.7 μmoles barbital-acetate buffer at pH 8.0; E. coli tRNA containing 75 μmoles of N-acetylleucyl-tRNA. After 5 min of incubation at 37°C, the results of the reaction were determined by paper electrophoresis. The radioactive spots were located either by autoradiography or by strip-counting, and then were counted in a Tri-Carb liquid scintillation spectrometer.

When the nuclease activity of the enzyme preparation was negligible, the following method was used: after incubation of the reaction mixture, 5 μl of a 20% solution of potassium acetate at pH 5 and 0.5 ml of cold ethanol were added. After centrifugation, the radioactivity of an aliquot of the supernatant (containing the free N-acetylleucine) was determined at infinite thinness.

One unit of activity is defined as the amount of enzyme necessary for the formation of 1 μmole of N-acetylleucine from N-acetylleucyl-tRNA in 1 min at 37°C.

Results.—Enzymatic hydrolysis of the N-acetylleucyl-tRNA: After incubation of N-acetylleucyl-tRNA, either with the 105,000 × g supernatant of E. coli MRE 600 or with the purified fractions, the analysis of the mixture showed free N-acetylleucine (Fig. 1). In the experiment depicted in Figure 2, about 70 out of 90 μmoles of N-acetylleucyl-tRNA were hydrolyzed in 30 minutes. When the enzyme preparations were heated to 95°C for ten minutes, prior to the incubation, all activity was destroyed.

The activity of the enzyme was not changed by addition of Mg++ ions to a con-
The measurement of enzymatic activity is possible up to pH 8.5 but at higher pH's, nonenzymatic hydrolysis becomes important, making the determination of activity difficult. It was observed that, with several N-acetylaminoacyl-tRNA's,
the rates of enzymatic hydrolysis increased with pH, the greatest rates being observed at pH 8.5.

Approximation of $K_m$ value: A Michaelis constant was determined, using about 50 per cent pure N-acetylleucyl-tRNA (Fig. 4) and the stated conditions of assay. The $K_m$ value is approximately $5 \times 10^{-10} M$.

Recharge of tRNA after enzymatic deacylation: If the enzymatic deacylation of the tRNA results solely from the hydrolytic cleavage of the ester bond between N-acetylamino-acid and the 3'-hydroxyl group of the terminal nucleotide in the tRNA molecule, then the products of the reaction would only be N-acetylleucine and an intact tRNA molecule. We have already shown that N-acetylaminoacid is produced. Following the enzymatic reaction, production of a tRNA molecule (with an intact...pCpCpA terminal sequence) could be demonstrated by the ability to recharge the enzymatically stripped tRNA in the presence of amino acid, ATP, and aminoacyl-tRNA-synthetase.

Two identical preparations of N-acetylleucyl-tRNA were deacylated, respectively, by alkaline hydrolysis at pH 10 and enzymatically in the manner described previously. From these two preparations, tRNA was recovered by phenol treatment and ethanol precipitation. In each case, the leucine acceptor activity of 70 $\mu$g of tRNA was determined in the presence of purified leucyl-tRNA-synthetase. Eighty $\mu$moles of leucine were esterified with tRNA stripped at pH 10, and 43 $\mu$moles, with tRNA prepared by enzymatic hydrolysis. This shows that about one half of the accepting capacity of the enzyme-treated tRNA has been lost, as compared with the nonenzymatically stripped tRNA. The contamination of the hydrolyase preparation with a nuclease could explain such a difference. Nevertheless it may be concluded that the reaction catalyzed by the enzyme produces a

![Table 2](image)

**TABLE 2**

<table>
<thead>
<tr>
<th>Incubation of Fraction II with N-Acetylleucyladenosine</th>
<th>N-acetyl-C14-leucyl-tRNA</th>
<th>N-acetyl-C14-leucine</th>
<th>N-acetyl-C14-leucyladenosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation of N-acetyl-C14-leucyl-tRNA in the presence of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNase alone 40 min</td>
<td>0</td>
<td>740</td>
<td>4260</td>
</tr>
<tr>
<td>RNase alone 10 min then fraction</td>
<td>0</td>
<td>715</td>
<td>4285</td>
</tr>
<tr>
<td>II 30 min</td>
<td>4585</td>
<td>415</td>
<td>0</td>
</tr>
<tr>
<td>Buffer 40 min</td>
<td>870</td>
<td>3650</td>
<td>480</td>
</tr>
</tbody>
</table>

20 $\mu$moles (about 10,000 cpm) of N-acetyl-C14-leucyl-tRNA were incubated at pH 8 for 10 min in the presence of pancreatic RNase (20 $\mu$g/ml). The incubation mixture was then divided into two parts, one of which was supplemented with enzymatic fraction II (50 $\mu$g of protein), and both were maintained at 37°C for an additional 30 min. As a control, 10 $\mu$moles of N-acetyl-C14-leucyl-tRNA were incubated in buffer for 40 min and for another control, 10 $\mu$moles N-acetyl-C14-leucyl-tRNA were incubated with enzymatic fraction II alone for 30 min. The products of the reactions were, in each case, separated by paper electrophoresis and counted. All values were normalized to 5000 total cpm per incubation mixture.

The slight difference observed in the amounts of N-acetylleucine formed in the presence or absence of RNase is significant. It is likely to be due to a contamination of RNase by polypeptidyl-tRNA-hydrolase.
tRNA molecule that is intact at least in those of its structural elements required for recognition by aminoacyl-tRNA-synthetase.

**Substrate specificity:** (I) **Polynucleotide part:** The activity of the purified fraction II has been tested using different substrates obtained from N-acetylleucyl-tRNA by different enzymatic degradations of the tRNA moiety. These substrates are: (1) N-acetylleucyl-adenosine, produced by treatment of N-acetylleucyl-tRNA with pancreatic RNase;¹² (2) N-acetylleucyl-adenosine-5'-P (N-acetylleucyl-AMP) produced by venom phosphodiesterase;¹² (3) N-acetylleucyloligonucleotides: as previously shown by Berg, Lagerkvist, and Dieckmann with leucyl-tRNA,¹³ enzymatic cleavage of N-acetylleucyl-tRNA by T₁ RNase gave two N-acetylleucyloligonucleotides; the sequences of these two products, which correspond to two different tRNAₐₜₐ's, are, respectively, CpApCpCpA- and UpApCpCpA-N-acetylleucine. These can be separated by paper electrophoresis under the conditions described in Figure 6.

![Image of Figure 5](image_url)

**Fig. 5.—Incubation of fraction II with N-acetylleucyl-AMP.** The incubation mixture contained, in 80 μl: venom phosphodiesterase, 15 μg; MgCl₂, 0.4 μmole; barbital-acetic acid pH 8, 4 μmoles; N-acetyl-C⁴-leucyl-tRNA, 100 μmoles. After 10 min incubation at 37°C, it was divided into two parts: to one of them 40 μg of proteins of fraction II were added and both were incubated for additional 20 min. The mixtures were then analyzed by paper chromatography as described in Methods. The figure shows the corresponding autoradiogram. (I) As a control, incubation in the presence of fraction II alone for 30 min; (II) phosphodiesterase alone; (III) phosphodiesterase and fraction II.

![Image of Figure 6](image_url)

**Fig. 6.—Incubation of enzymatic fraction II with T₁ RNase digest of N-acetyl-C⁴-leucyl-tRNA.** Incubation mixture (total vol 100 μl): barbital-acetic acid pH 8, 4 μmoles; N-acetyl-C⁴-leucyl-tRNA, 250 μmoles; T₁ RNase, 100 units. After 15 min incubation at 37°C, an aliquot was analyzed by paper electrophoresis (A); the autoradiogram shows three spots: from left to right, two major spots containing respectively 35 and 63%, and a minor spot containing 9% of the total radioactivity. The first two are N-acetyl-C⁴-leucyloligonucleotides, and the third, N-acetyl-C⁴-leucyladenosine. The remaining mixture was divided into two parts (B and C), one of which (C) was supplemented with enzymatic fraction II (30 μg of proteins). After an additional 30 min incubation, 2 μg of pancreatic RNase were added to both mixtures. They were again incubated for 5 min and subjected to electrophoresis. The autoradiogram shows a minor spot of N-acetyl-C⁴-leucine (II), and a major spot of N-acetyl-C⁴-leucyladenosine (III). The proportions of N-acetylleucine were, in (B), 9%, and in (C), 24% of the total. (D) was the same control as (C) in Fig. 1.
The experiments reported in Table 2 and in Figure 5 show that free N-acetylleucine was not formed, either from N-acetyl-leucyladenosine, or from N-acetyl-leucyl-AMP.

In contrast, in the presence of enzymatic fraction II, the N-acetyl-leucylpentanucleotides produced after T1 RNase treatment were hydrolyzed with the formation of N-acetylleucine (Fig. 6). However, the extent of the hydrolytic reaction was less with these as substrates than with undegraded N-acetyl-leucyl-tRNA as substrate; five times more N-acetylleucine was produced under the same conditions from N-acetyl-leucyl-tRNA.

Thus, the enzyme acts most efficiently on the relatively long polynucleotide chain of tRNA.

(II) Aminoacyl residue: All the experiments described above have been performed using either N-acetyl-leucyl-tRNA, or a derivative of this compound as a substrate. Using the same procedure, it has also been possible to show an extensive enzymatic cleavage of different N-acetyl-aminoacyl-tRNA's. Figure 7 shows the kinetics of the reaction catalyzed by the purified fraction II for N-acetylseryl-, N-acetylvalyl-, N-acetyltreonyl-, and N-acetyl-leucyl-tRNA.

It is not possible to conclude from these results whether fraction II contains a unique enzyme with low specificity for the aminoacyl residue, or several enzymes, each specific for a given amino acid. However, using the crude extract or fraction II, we observed that the ratios of activities toward N-acetylvalyl-, N-acetyl-leucyl-, and N-acetylphenylalanyl-tRNA's were identical, suggesting that a single enzyme was acting on these different substrates.

(III) Unacylated aminoacyl-tRNA's: Spontaneous hydrolysis of the ester bond occurs very rapidly at pH 8 for most of the aminoacyl-tRNA's; only isoleucyl-, valyl-, and threonyl-tRNA are stable enough so that a test for enzymatic cleavage at that pH is possible.

As shown in Figure 8, in five minutes, the hydrolysis of N-acetyltreonine-tRNA was at least 100 times greater in the presence of the enzyme than in its absence. With threonyl-tRNA the enzymatic hydrolysis was only double and this difference may be due to the action of the nuclease which, we know, is present as a contaminant of fraction II. Similar results were obtained with valyl-tRNA and isoleucyl-tRNA. It appears that the enzyme does not catalyze the hydrolysis of the ester bond in the presence of free -NH2 group of tRNA-esterified amino acid.
(IV) *Polypeptidyl-tRNA's*: N-acetyl-C\(^{14}\)-diphenylalanyl-tRNA and N-acetyl-leucyl-glycyl-C\(^{14}\)-phenylalanyl-tRNA, both prepared according to Lapidot *et al.*\(^{14}\) and C\(^{14}\)-diphenylalanyl-tRNA and C\(^{14}\)-polylysyl-tRNA were incubated with fraction II for 30 minutes. After incubation, free di- or polypeptides were separated from tRNA-bound material by paper electrophoresis or by chromatography. In all cases, except for polylysyl-tRNA, the enzyme catalyzed the hydrolysis of polypeptidyl-tRNA's. The details of these experiments will be reported later.\(^{15}\)

![Graph showing enzymatic hydrolysis of acylated and unacylated C\(^{14}\)-threonyl-tRNA](image)

**Discussion.**—The enzyme described in this report is a specific hydrolase, which cleaves the ester linkage between tRNA and N-substituted aminoacyl residues (either N-acetyl- or polypeptidyl-tRNA). The enzyme requires, in the substrate, the presence of a relatively long polynucleotide chain, providing evidence that it is not a nonspecific hydrolase. It shows a low level of specificity with respect to particular amino acids, except that it does not cleave polylysyl-tRNA (perhaps because of the presence of free e-amino groups). However, unsubstituted aminoacyl-tRNA's are not acted on to a significant extent.

All these properties are in good agreement with those expected for an enzyme involved in the mechanism of chain termination during protein biosynthesis. Whether enzymatic cleavage of the polypeptidyl-tRNA occurs on the ribosome or after its release in the soluble fraction has not yet been firmly established. However, Ganosa\(^{4}\) reported that the release of the polypeptide chain was lost during purification and presented some arguments for cleavage while the polypeptidyl-tRNA is situated on the ribosome. If the hydrolase acts at that level, an efficient control mechanism must operate in order to avoid any interruption of chains during the growth. The UAA triplet would not only stop the translation but in some unknown way would trigger the cleavage by the hydrolase of the ester linkage between the polypeptide and tRNA.

Another possible role for the hydrolase would be to regenerate the tRNA's from unfinished polypeptides which could have "accidentally" escaped from the ribosomal complex.

In the second model, it has to be assumed that the chain associated with the ribosome-messenger complex is protected against enzymatic hydrolysis. One may note that nascent polypeptide chains are protected against proteolytic activity.\(^{16}\)
The properties of the enzyme described in this paper are very similar to those of the D-tyrosyl-tRNA-deacetylase recently described by Calendar and Berg. Since these authors did not check possible hydrolytic activity against N-acetyl-L-tyrosyl-tRNA, a direct comparison of the two enzymes cannot be made. It would not be too surprising if both enzymes would prove to be analogous or even identical: the D-tyrosyl-tRNA-deacylase cleaves the ester bond between D-amino acids (either tyrosine or phenylalanine) and tRNA's. The structure of the D-amino acid, with its NH₂-group in opposite configuration, as compared with the L form, could be operationally equivalent to a substitution of the amino group for the recognition of the substrate by the enzyme molecule.

Capecchi has recently isolated a protein factor, the charge properties of which are opposed to those of the hydrodase described here and which is required for the chain determination triggered by the UAG triplet. It remains to be shown whether there is any relation between these two enzymes in polypeptide chain termination.

Abbreviations used: poly A, polyadenylic acid; poly U, polyuridylic acid; ATP, adenosine 5'-triphosphate; GTP, guanosine 5'-triphosphate; AMP, adenosine 5'-phosphate; DEAE-cellulose, O-(diethylaminoethyl) cellulose.

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