AFFINITY LABELLING OF RIBOSOMAL PEPTIDYL TRANSFERASE BY A PUROMYCIN ANALOGUE

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SUMMARY: Preincubation of *Escherichia coli* ribosomes with the affinity labelling puromycin analogue, 5'-O-(N-bromoacetyl-p-aminophenylphosphoryl) 3'-N-L-phenylalanyl puromycin aminonucleoside, led to covalent attachment of the analogue to the ribosomes at the acceptor substrate site of peptidyl transferase. Bound affinity label was radioactively labelled *in situ* by the peptidyl transferase catalyzed transfer of N-acetyl-L-[^{3}H]leucine from N-acetyl-L-[^{3}H]leucyl-pentanucleotide to form a ribosome-affinity label-(acetyl-L-[^{3}H]leucine) complex. Approximately 97% of the radioactivity was covalently attached to the ribosomal RNA of the 50S subunit. This indicates that a segment of the 23S or 5S RNA forms part of, or is close to, the acceptor substrate site of peptidyl transferase.

INTRODUCTION

In a study of peptide bond formation on the ribosome, we are using the technique of affinity labelling to identify the proteins comprising peptidyl transferase and also the ribosomal components involved in the binding of antibiotics which inhibit this enzyme. In the work reported here, we show that an analogue of the acceptor substrate (A') site specific antibiotic puromycin, 5'-O-(N-bromoacetyl-p-aminophenylphosphoryl) 3'-N-L-phenylalanyl puromycin aminonucleoside (Fig. 1), can form a covalent attachment with ribosomal RNA in the A' site of peptidyl transferase on the 50S ribosomal subunit. Already several proteins related to peptidyl transferase activity have been implicated by use of affinity labelling derivatives of both aminoacyl-tRNA (1-3) and of chloramphenicol (4,5).

EXPERIMENTAL

Materials. *E. coli* tRNA and [^{3}H]leucine (specific

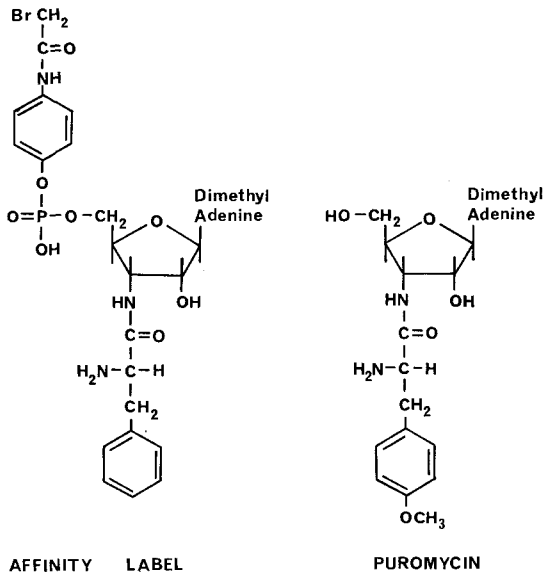

FIG. 1. Comparison of the structure of puromycin and the puromycin analogue used as an affinity label, 5'-O-(N-bromoacetyl-p-aminophenylphosphoryl) 3'-N-L-phenylalanyl puromycin aminonucleoside.

activity 54 Ci/mmol) were obtained from Schwarz-Mann and puromycin from the Nutritional Biochemical Corporation. Puromycin aminonucleoside was a generous gift of the American Cyanamid Co., Pearl River, New York. Full details of the synthesis from puromycin aminonucleoside of the affinity label, 5'-O-(N-bromoacetyl-p-aminophenylphosphoryl) 3'-N-L-phenylalanyl puromycin aminonucleoside, will be published elsewhere. The fragments, $\overset{U}{C}$pApCpCpA-(N-acetyl-L-[^{3}H]Leu), specific activity 20.6 Ci/mmol, were prepared according to Monro (6). Ribosomes from *E. coli* MRE600 were prepared essentially according to Staehelin *et al.* (7) but were washed twice with 0.5 M NH_4Cl.

Methods. All methods used are described in the legends to Figure 2 and Tables 1 and 2.

RESULTS

Inactivation of peptidyl transferase by affinity label and protection by A' site specific antibiotics

TABLE 1
ANTIBIOTIC PROTECTION OF PEPTIDYL TRANSFERASE AGAINST INACTIVATION BY AFFINITY LABEL

Additions to preincubation mixture		% Peptidyl transferase activity	% Protection by antibiotic
Antibiotic	0.2 mM Affinity label		
None	-	100	
None	+	59	
3.3 mM Chloramphenicol	+	102	100
1.0 mM Chloramphenicol	+	98	95
0.1 mM Chloramphenicol	+	85	63
4.0 mM Puromycin	+	93	83
1.0 mM Puromycin	+	82	56
4.0 mM Puromycin aminonucleoside	+	66	16
1.0 mM Puromycin aminonucleoside	+	43	0

Preincubation mixtures contained in 50 µl: 40 mM magnesium acetate, 0.4 M KCl, 40 mM sodium carbonate, pH 8.8 (21°), 75 pmol ribosomes, and affinity label, puromycin and chloramphenicol at concentrations given. After 17.5 hours at 22°, ribosomes were precipitated with 2.0 ml of 98% methanol containing 0.4 mM magnesium acetate, 4 mM KCl and 0.4 mM tris-acetate, pH 8.0 (0°). After 10 min at 0°, the ribosomes were collected by low speed centrifugation and resuspended in 40 µl of 10 mM magnesium acetate, 0.1 M KCl, 20 mM tris-acetate, pH 8.0, (0°), 0.5 mM EDTA. The precipitation was repeated three times with 75 - 100% recovery each time.

Peptidyl transferase assays contained in 50 µl: 40 mM magnesium acetate, 0.4 M KCl, 40 mM tris-acetate, pH 8.0 (0°), 30% (v/v) methanol, approx. 0.43 pmol CpApCpCpA-(acetyl-[^{3}H]Leu) (specific activity 20.6 Ci/mmol), 1 mM puromycin and 6 - 12 pmol preincubated and washed ribosomes. After incubation for 5 min at 0°, the reaction was terminated by the addition of 0.1 ml of 0.2 M sodium phosphate, pH 5.5, saturated with $MgSO_4$. The product, acetyl-[^{3}H]Leu-puromycin, was extracted and counted essentially as described by Maden and Monro (16).

Per cent peptidyl transferase activities are normalized to individual preincubated controls containing antibiotic in order to correct for incomplete removal of the antibiotic (particularly chloramphenicol) by methanol precipitation. For example, only 83 - 86% of peptidyl transferase activity was recovered from controls containing chloramphenicol.

Preincubation of ribosomes with 0.16mM affinity label for 17.5 hours followed by washing by methanol precipitation gave a 41% loss of peptidyl transferase activity as measured by the fragment reaction (Table 1). The A' site specific antibiotics, puromycin and chloramphenicol (8,9), gave substantial concentration dependent protection against inactivation by the affinity label.

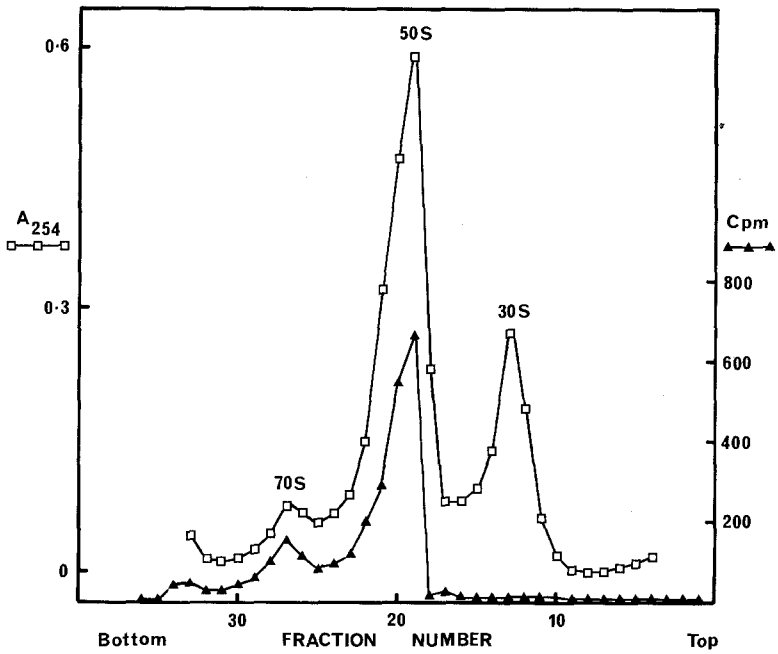

FIG. 2. Sucrose gradient analysis of dissociated ribosome-affinity label-(acetyl-[^{3}H]Leu) complex. The ribosome complex, prepared as described in Table 2, was dissociated into subunits by dialysis overnight at 4° against 20 mM tris-acetate, pH 7.4, 50 mM NH_4Cl, 0.5 mM magnesium acetate (dissociating buffer). A sample (3.6 A_{260} units, 10,200 dpm) in 0.5 ml was layered onto an 11 ml linear 10 - 30% sucrose gradient in dissociating buffer and centrifuged for 3.0 hours at 41,000 rpm and 4° in the Beckman SW41 rotor. Fractions (0.25 ml) were collected from the top of the gradient and monitored with an LKB Uvicord at 254 nm. After addition of 0.5 mg of albumin to each fraction, radioactive material was precipitated with 2.0 ml of cold 5% trichloroacetic acid and counted at 25% efficiency in 1.7 ml of toluene scintillant: NCS solubilizer: 8 M NH_4OH (88:12:1, by vol.).

The small amount of protection (15%) observed with 4mM puromycin aminonucleoside was probably due to direct reaction of the 3'-amino group of this compound with the affinity label since it is a very poor inhibitor of peptidyl transferase (10,11). This implies that the protection observed with puromycin is largely specific and not due to reaction of the affinity label with the α-amino group. Hence, these results indicate that the inactivation observed with the affinity label was due to covalent attachment of the affinity label to the A' site of peptidyl transferase.

TABLE 2
DISTRIBUTION OF RADIOACTIVITY OF THE RIBOSOME-AFFINITY LABEL-(ACETYL-[^{3}H]LEU) COMPLEX BETWEEN RNA AND PROTEIN COMPONENTS

Method of RNA-protein separation	Distribution of radioactivity			
	RNA		Protein	
	Dpm	%	Dpm	%
1. Acetic acid extraction	10,500	91.3	1,000	8.7
2. Ribonuclease digestion	24,880	97.4	670	2.6
" "	4,970	96.5	180	3.5
3. Alkaline hydrolysis	14,400	92.0	1,260	8.0

Ribosomes with bound affinity label were obtained by incubation of 500 pmol of ribosomes with 0.32 mM affinity label for 17 hours as described in Table I. The precipitated and washed ribosomes were then incubated with 19.5 pmol of UpApCpCpA-(acetyl-[^{3}H]Leu) for 3 hours at 0° under fragment reaction conditions without puromycin (Table 1). The reaction was followed by isolation and counting of the product after precipitation with cold 5% trichloroacetic acid. The ribosome-affinity label-(acetyl-[^{3}H]Leu) complex in 6 ml of 20 mM tris-acetate, pH 8.0 (0°), 10 mM magnesium acetate, 0.1 M KCl, 0.5 mM EDTA, was then isolated by centrifugation through a 3.0 ml cushion of 5% sucrose, prior to separation into RNA and protein fractions.

Acetic acid extraction for the separation of ribosomal RNA and protein was by the method of Hardy *et al.* (14). Ribonuclease digestion by the method of Czernilofsky and Kuechler (3) was followed by precipitation and washing of the protein with 5% trichoroacetic acid. Alkaline hydrolysis of RNA was carried out with 0.33 N NaOH for 1.0 hour at 37° followed by precipitation and washing of protein with 5% trichloroacetic acid. All results are corrected to dpm by use of a [^{3}H]toluene standard to allow for differences in counting efficiencies obtained when NCS and Triton-X100 solubilizers were added to the normal toluene based scintillation fluid.

Acceptor substrate activity of ribosomes with covalently attached affinity label

Ribosomes that had reacted with affinity label to form a ribosome-affinity label complex acted as acceptors of acetyl-[^{3}H]Leu from the peptidyl transferase donor substrates, ^{C}UpApCpCpA-(acetyl-L-[^{3}H]Leu). For example, ribosome-affinity

label, prepared by incubation of 9.6 pmol of ribosomes with 0.16mM affinity label for 26 hours at 26° and having 47% of peptidyl transferase activity relative to controls, was incubated with 1.9 pmol of UpApCpCpA-(acetyl-[^{3}H]Leu) for 2 hours at 0° and assayed for ribosome-affinity label-(acetyl-[^{3}H]Leu) product formation as described in Table 2. The 0.18 pmol of product (8,000 dpm) corresponded to 1.8% of the ribosome population or 3.3% of the ribosome-affinity label population. Greater incorporation could possibly have been obtained at a higher concentration of UpApCpCpA-(acetyl-[^{3}H]Leu) and longer incubation times. However, it is possible that only a small percentage of the ribosomes had peptidyl transferase activity; for example, only 11% of similarly prepared ribosomes were active in binding the A' site substrate, CpApCpCpA-L-Phe (12).

These results provide strong evidence that at least some of the affinity label is covalently bound to the A' site of peptidyl transferase. Further, the approach taken here of using non-radioactive affinity label and radioactive donor substrate means that only those molecules of covalently attached affinity label which are correctly aligned in the A' site are converted to a radioactive derivative. No estimate of the amount of non-specific attachment of affinity label can be made from the data obtained.

Sucrose gradient analysis of ribosomal subunits from the ribosome-affinity label-(acetyl-[^{3}H]Leu)complex

The sedimentation profile (Fig. 2) shows that 72% of the bound radioactivity was associated with the 50S ribosomal subunit while the remainder was associated with undissociated 70S ribosomes and unknown high molecular weight material; less than 1.5% was associated with the 30S subunit. This result provided confirmatory evidence that the radioactive affinity label derivative

was in the correct ribosomal subunit, since peptidyl transferase is known to be an integral part of the 50S subunit (13).

Distribution of radioactivity of the ribosome-affinity label-(acetyl-[^{3}H]Leu) complex between RNA and protein components

The results given in Table 2 show that 91 - 97% of the radioactivity was associated with the RNA fraction. The upper figure of 97% obtained from the ribonuclease digestion is probably the most reliable since conditions for the alkaline hydrolysis were not optimized and the acetic acid extraction procedure is known to leave some RNA (1.8%; ref. 14) associated with the protein fraction. In addition, the RNA of the ribosomes used here was partly degraded (unpublished observations) and this may prevent efficient separation. The small amount of radioactivity associated with the protein fraction has not been characterized.

DISCUSSION

The data presented clearly show that the puromycin analogue used here is an affinity label capable of binding to the acceptor substrate (A') site and inactivating peptidyl transferase on the 50S subunit of *E. coli* ribosomes. That the radioactive affinity label derivative is covalently attached to RNA indicates that part of the 5S or 23S ribosomal RNA is in, or close to, the A'; site. When the affinity label is initially bound to the A' site, the reactive alkylating group presumably must lie in roughly the same position as the 3'-penultimate cytidine residue present in all aminoacyl-tRNA's. This suggests the attractive hypothesis that the 3'-terminal -CpCpA triplet of aminoacyl-tRNA is bound to the A'; site of peptidyl transferase by conventional base pairing with a -UpGpGp- triplet of the ribosomal RNA. Such an arrangement would favour covalent reaction of the electrophilic bromoacetyl

group of the affinity label with N^7 of guanine, the most nucleophilic component of RNA (15). It is of interest that Pellegrini *et al.* (2) have labelled RNA (or a protein strongly attached to RNA) at the donor (peptidyl) site of peptidyl transferase with α-N-bromoacetyl-Phe-tRNA.

Further work is in progress to prepare radioactive affinity label in order to define more closely its reaction with *E. coli* ribosomes.

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