PHOTOAFFINITY LABELING OF RAT LIVER RIBOSOMES BY PHENYLALANINE-tRNA N-ACYLATED BY 2-NITRO-4-AZIDOBENZOIC ACID

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1. Introduction

Aminoacyl-tRNA molecules NH_2-substituted by different reagents able to attach to ribosomal proteins and RNA have proved suitable tools for elucidation of the components involved in organization of the peptidyltransferase (PTase) region of bacterial ribosomes (reviewed in [1]).

On eukaryote ribosomes, however, there are still very few comparable studies. *p*-Nitrophenoxycarbonyl-(PNPC)-Phe-tRNA after poly(U)-dependent attachment to rat liver ribosomes attacked a number of proteins located after two-dimensional electrophoresis mainly in three areas of the gel slab. Some of these proteins are most likely involved in binding of the aminoacyl-terminus of the tRNA molecule to the ribosome [2].

When applying PNPC-Phe-tRNA to yeast ribosomes [3] two proteins were preferentially labeled whereas *N*-iodoacetyl-Phe-tRNA reacted with a higher number of proteins of the large ribosomal subunit. After binding of *N*-bromoacetyl-puromycin to rat liver ribosomes [4], proteins L28 and L29, according to the new proposed international nomenclature [5], that will be used throughout here (corresponding to L27 and L29 of our former nomenclature [6]), were found labeled indicating their localization near or within the peptidyltransferase centre.

In this paper Phe-tRNA N-terminally substituted with the photoreagent 2-nitro-4-azidobenzoic acid (NAB) was used as an affinity label to detect ribosomal protein(s) near or within the PTase centre of rat liver ribosomes. In dependence on poly(U) mainly protein L10 and, to a minor extent, proteins L17 and L23/23a were labeled.

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2. Materials and methods

Ribosomes were prepared from a postmitochondrial supernatant of rat liver homogenate by addition of Triton X-100 and sodium deoxycholate to 2% and 1.3% final conc., respectively, and centrifugation for 90 min at 105 000 × *g*. The ribosomes were resuspended in a medium of 10 mM triethanolamine (TEA), 500 mM KCl, 1 mM $MgCl_2$, 10 mM β-mercaptoethanol (pH 7.5) to 10 mg/ml and puromycin was added to 0.5 mM. After incubation for 20 min at 0°C and 10 min at 37°C 25 ml of the mixture was layered over a cushion of 25 ml 20% sucrose in buffer A (10 mM TEA, 500 mM KCl, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, pH 7.5). Derived subunits were pelleted for 20 h at 25 000 rev./min in rotor SW 25.2 at 20°C. The pellets containing 60 S and 40 S particles were resuspended to 400–500 A_{260} units/ml in 10 mM TEA, 50 mM KCl 5 mM $MgCl_2$ (pH 7.5) and either used directly or stored with 6% sucrose and 10 mM β-mercaptoethanol in liquid nitrogen until use. Ribosomes were passed through a Sephadex G-25 column equilibrated with buffer B (10 mM TEA, 100 mM KCl and 18 mM $MgCl_2$, pH 7.5) to remove β-mercaptoethanol.

E. coli tRNA (Boehringer) was charged with [^{3}H]Phe (spec. act. 15–50 Ci/mmol, Fa. Amersham) by synthetases present in a Sephadex G-25 passed postribosomal supernatant of *E. coli* K12 in the

presence of 25 mM Tris, 10 mM $MgCl_2$, 0.5 mM EDTA, 5 mM ATP (pH 7.1) yielding 2–15% charged Phe-tRNA.

N-Acylation of Phe-tRNA by NAB-*N*-hydroxy-succinimide ester prepared from 2-nitro-4-amino-benzoic acid according to [7] followed the method in [8]. The extent of *N*-acylation was determined by thin-layer chromatography on Silufol (Kavalier, Czechoslovakia) in butanol–H_2O–acetic acid (90:25:10) after 135 min development at room temperature. Radioactivity was scanned with a 'Dünnschicht'-Scanner, Bertold, Wildbad (fig.1). The R_F value of Phe was 0.30, that of NAB-Phe 0.80.

Elongation factors EF-1 and EF-2 were prepared from rat liver basically following the method in [9] by stepwise elution from calcium phosphate gel followed by DE-52 cellulose chromatography or Sephadex G-200 filtration.

Puromycin reactivity of NAB-[^{3}H]Phe-tRNA in ribosome complexes was tested according to [3] in buffer B for 30 min at 37°C.

For detection of ribosomal proteins being labeled by NAB-[^{3}H]Phe-tRNA the following procedures were applied:

(1) Incubation in the dark of 500–800 pmol NAB-[^{3}H]Phe-tRNA with 4–5 nmol ribosomes in buffer B with or without 2 mg poly(U) for 20 min at 37°C (fig.2). To remove unbound Phe-tRNA and possible traces of β-mercaptoethanol in some cases poly(U) containing samples were filtered through a Sephadex G-100 column but omission of this step led to identical results.

(2) Irradiation for 20 min at 4°C with an HQV 125 ultraviolet lamp, Narva, Berlin, emitting light from 305–420 nm.

(3) Adjustment of β-mercaptoethanol to 10 mM, KCl to 500 mM, $MgCl_2$ to 10 mM, puromycin to 0.5 mM and incubation for 10 min at 37°C.

(4) Centrifugation through gradients of 10–30% sucrose in buffer A in an SW 25.2 rotor at 20°C according to [10] with subsequent fractionation via a flow-through cell of a Gilford-2400 spectrophotometer.

(5) Pooling of 60 S and 40 S particles.

(6) Precipitation of ribosomal subunits by addition of 0.8 vol. ethanol containing 50 mM $MgCl_2$ for 30 min at –20°C and centrifugation for 20 min at 15 000 × *g*.

(7) Resuspension of the pellets in water to 5–10 mg ribosomal particles/ml.

(8) Hydrolysis of the ester-bond between the NAB-Phe and the tRNA moiety by adjusting the solution to pH 10–11 by addition of NaOH, triethylamine or Tris. This step was omitted in the last two preparations, because application of 67% acetic acid (step 9) causes cleavage of this bond.

(9) Extraction of ribosomal proteins, and one- and two-dimensional PAA gel electrophoresis were performed according to [6,11]. Protein samples were treated with iodoacetamide [11]. After electrophoresis gel pieces were digested by hyamine treatment, toluene-based scintillation fluid was added and radioactivity measured as in [4].

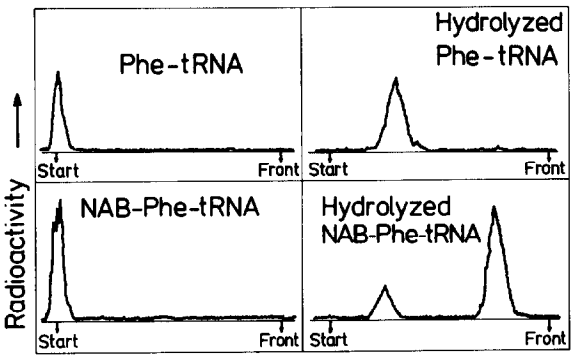

Fig.1. Analysis of *N*-acylation of [^{3}H]Phe-tRNA by *N*-hydroxy-succinimide ester of NAB by thin-layer chromatography (see section 2).

3. Results and discussion

Ribosomes prepared by puromycin treatment as in section 2 are expected to have free A- and P-sites and thus a high capacity for poly(U)-dependent binding of Phe-tRNA or NAB-Phe-tRNA.

Binding of Phe-tRNA to poly(U) coded ribosomes at 6 mM $MgCl_2$ requires the presence of elongating factor EF-1 and GTP for the formation of stable complexes that can be isolated by sucrose gradient centrifugation or Sephadex gel filtration. However, binding of NAB-Phe-tRNA to ribosomes is not catalyzed by EF-1 (table 1). Therefore, conditions for non-enzymatic

Table 1
Influence of elongation factor EF-1 on binding of [^{3}H]Phe-tRNA and NAB-[^{3}H]Phe-tRNA to ribosomes

	pmol [^{3}H]Phe bound	
	– EF 1	+ EF 1
[^{3}H]Phe-tRNA	0.5	3.0
NAB-[^{3}H]Phe-tRNA	0	0.2

40 pmol ribosomes were incubated with 10 pmol [^{3}H]Phe-tRNA or NAB-[^{3}H]Phe-tRNA with 20 μg poly(U), 20 mM TEA, 60 mM KCl and 6 mM $MgCl_2$ (pH 7.5) for 20 min 37°C. After centrifugation through 10–30% sucrose gradients in the same buffer for 50 min at 50 000 rev./min in Spinco SW56 rotor the ribosomes were collected from the 80 S region. The values in the table refer to the amount of [^{3}H]Phe bound by 16 pmol 80 S

binding of NAB-Phe-tRNA to ribosomes were studied. From fig.2 it is obvious that poly(U)-dependent binding of NAB-Phe-tRNA to ribosomes increases with increasing concentrations of Mg^{2+} reaching maximal values > 15 mM $MgCl_2$ in the reaction mixture. When omitting poly(U) unspecific binding remains sufficiently low (~15%). Under these optimized conditions ~80% of the radioactivity is bound to the ribosomes which roughly matches the extent of NAB-substitution of the α-amino group of the Phe-tRNA (see fig.1). 65% of NAB-Phe-tRNA bound to ribosomes was found sensitive towards puromycin which points to a preferred P-site location. Addition of EF-2 and GTP increased puromycin reactivity only slightly, up to 75%.

For detection of ribosomal protein(s) attacked by NAB-[^{3}H]Phe-tRNA experiments were performed according to the scheme given in section 2 which all led to very similar results. Ribosomes were applied in ~10-fold excess over NAB-[^{3}H]Phe-tRNA in order to bind all the affinity label. By irradiation between 0.2 and 0.8% of the ribosome-bound radioactivity was covalently linked to ribosomal proteins.

Experiments with poly(U) yielded ribosomal proteins of the large subunit that, when analyzed by one-dimensional electrophoresis in 5% PAA gels at pH 8.3, contained the bulk of radioactivity in a relatively narrow zone having migrated ~6–7 cm away from the spacer gel. Without poly(U), however, the corresponding area contained only very little radioactivity.

Analysis of the amido black-stained spots of large

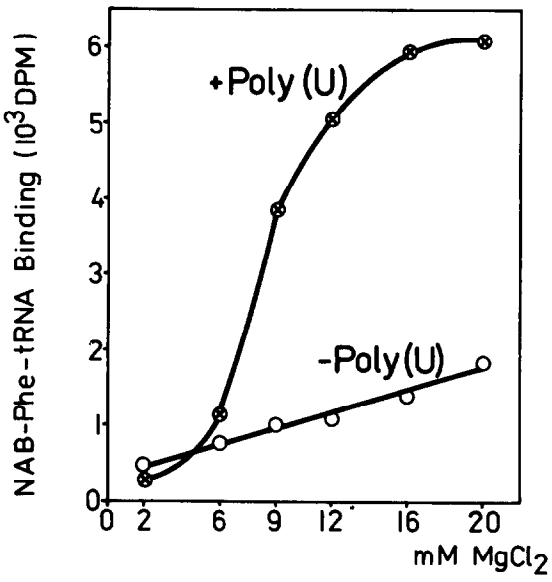

Fig.2. NAB-[^{3}H]Phe-tRNA binding to ribosomes in buffer B at different $MgCl_2$ concentrations. Samples containing 40 pmol ribosomes and 1 pmol NAB-[^{3}H]Phe-tRNA in 100 μl were incubated at 37°C for 15 min, cooled, after dilution with 2 ml corresponding buffer passed through membrane filters (0.45 μm, Sartorius) and washed with 15 ml of the same buffers. Filters were dried and counted in toluene-based scintillation fluid. 10 000 dpm correspond to 1 pmol NAB-[^{3}H]Phe-tRNA.

subunit proteins after two-dimensional separation on PAA gels (fig.3) led to the result that in the presence of poly(U) protein L10 is strongly labeled. Part of the radioactivity is shifted slightly anionically in comparison to the stained spots of unlabeled L10. Besides, proteins L17 and L23/23a contained always some radioactivity which, however, was < 20% of L10 labeling. Additional radioactivity in other areas of the two-dimensional gel was not found in agreement with the analyses of the one-dimensional gels where labeling was restricted to one zone only, in which proteins L10, L17 and L23/23a are located.

Proteins prepared from control samples without poly(U) revealed only a small ^{3}H-labeling of protein L10.

In proteins from 40 S ribosomal subunits neither with nor without poly(U) was any radioactivity found.

The results obtained with NAB-Phe-tRNA have proved the advantage of NAB as a reactive group for

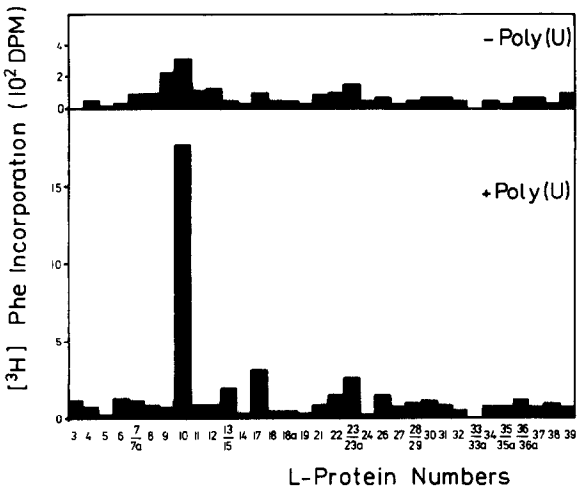

Fig.3. Two-dimensional PAA gel electrophoresis of large subunit proteins after incubation of ribosomes with NAB-[^{3}H]Phe-tRNA according to the scheme given in section 2. Ribosomal proteins (2–3 mg) were subjected to two-dimensional electrophoresis. Amido black-stained zones representing the ribosomal proteins were cut out of the two-dimensional gel slabs and the radioactivity was determined. dpm values are means of 4 independent experiments.

specific labeling of ribosomal components. The label allows:

(1) Proper binding of the Phe-tRNa analogue to the ribosome;
(2) Separation of not specifically bound material;
(3) Specific crosslinking to the surrounding by irradiation.

Under the conditions applied, radioactive Phe is covalently bound mainly to protein L10 and, to a much lower degree, to L17 and L23/23a. The reaction is strictly dependent on the presence of poly(U) that is required to place the NAB-Phe-tRNA into the right position at the ribosome. From experiments analyzing the puromycin reactivity of the NAB-Phe-tRNA-molecule at the ribosome it is reasonable to assume that the NAB-Phe residues of the affinity label are located at the ribosomal P-site. Therefore, ribosomal protein L10 and possibly L17 and L23/23a seem to be positioned very near or within the ribosomal P-site.

Comparing our results with the findings in [2] obtained by affinity labeling of rat liver ribosomes with PNPC-Phe-tRNA it is obvious that L10 and L23/23a are also among the proteins found labeled by these authors. Labeling of additional proteins may be explained by the properties of the reactive group used for substitution of Phe-tRNA.

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