THE SITE OF REACTION ON RIBOSOMAL PROTEIN L27 WITH AN AFFINITY LABEL DERIVATIVE OF $tRNA_f^{met}$

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1. Introduction

PNPC-Met-$tRNA_f^{met}$** was recently used for affinity labelling of ribosomal proteins from the P-site in *E. coli* and the predominantly labelled proteins were L27 and L15 [1,2]. The specificity of the labelling reaction was proved by its dependence on natural mRNA [1] and initiation factors [2] and by the inhibition with puromycin [3].

To further investigate the site of the reaction, analyses of the tryptic peptides from labelled protein L27 were performed by a fingerprinting technique. Radioactivity was detected in only two spots. Complete enzymatic hydrolysis was obtained by digesting the labelled protein sequentially with either trypsin–thermolysine–pronase or trypsin–chymotrypsin–pronase yielding a unique endproduct which, by its relative Rf-values, compares to a synthesized Lys-NH-CO-NH-Met compound.

It was concluded that the affinity labelling reaction occurs at one or two distinct sites of protein L27 with the ε-amino groups of lysine.

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**Abbreviations: PNPC-Met-$tRNA_f^{met}$, *p*-Nitrophenyl-carbamyl-methionyl-transfer ribonucleic acid (formylatable, methionine specific); Lys-NH-CO-NH-Met, *N*-(5-Amino-5-carboxypentyl)-*N'*-(1-carboxy-3-methylmercaptopropyl)urea; α-BOC-Lys, α-*t*-Butyloxycarboryl lysine; HEPES, *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

2. Materials and methods

'Preincubated' ribosomes were isolated [4,5]. PNPC-Met-$tRNA_f^{met}$ was synthesized, the affinity labelling was carried out and the 50S subunits and proteins were prepared as described [1].

Large subunit proteins (1 mg) from affinity labelled ribosomes were dissolved in 0.5 ml of the following buffer: 6 M urea, 20 mM H_3PO_4 and 6 mM mercaptoethanol adjusted to pH 6.5 with methylamine. Unlabelled 50S proteins (20 mg) were added as carrier. This solution was applied to a Sephadex G100 column (1 × 150 cm) equilibrated with the same buffer. The fractions were collected and the radioactivity was determined in aliquots. Fractions containing labelled protein L27 were pooled, dialyzed against 2% acetic acid and lyophilized.

This protein preparation was subjected to trypsin digestion in water, adjusted to pH 8 with NH_3, at 37°C for two hours at a protein-to-enzyme weight ratio of 35 to 1. The digest was either analyzed by a fingerprinting technique on thin-layer plates (see below) and subsequent autoradiography or, for further purification of the radioactive material, it was applied to a paper strip (Whatman 3 MM) and high voltage electrophoresis was performed at pH 1.9 in 2% formic acid and 8% acetic acid in water. The radioactive peptides were localized, eluted and further digested either with chymotrypsin under similar conditions as described for trypsin or with thermolysine at pH 8.5 in a solution of 20 mM ammonium acetate and 0.5 mM $CaCl_2$ for 2 h at 60°C with a substrate-to-enzyme weight ratio of 20 to 1. In either case further digestion was done with pronase

at pH 8 to 8.5 at 37°C for 12 h. At three-hour intervals equal amounts of pronase were added. The final substrate-to-enzyme weight ratio was 5 to 1.

The digest was analyzed by a fingerprinting technique on thin layer plates (20 × 20 cm, Polygramfolie, Macherey-Nagel and Co, MN, Cel. 400). The electrophoresis in the first dimension was in pyridine:acetic acid:acetone:water = 5:10:37:198, pH 4.4, at 400 V for 2 h. Ascending chromatography was in acetic acid:pyridine:*n*-butanol:water = 1.5:5:7.5:6. Autoradiography of the dried plates was performed with a Kodak RP/R54 Royal X-O mat X-ray film.

The synthesis of Lys-NH-CO-NH-[^{35}S]Met was achieved by dissolving 10 mg of α-BOC-Lys (Bachem, Switzerland) in 0.5 ml of 0.6 M HEPES buffer, pH 7.2. PNPC-[^{35}S]Met-tRNA (10 μg) and carrier tRNA (100 μg) were added and this mixture incubated for 12 h at 0°C. Then the solution was adjusted to pH 5 with acetic acid and the α-BOC-Lys-NH-CO-NH-[^{35}S]Met-tRNA and the carrier tRNA were precipitated in and washed with ethanol. The precipitate was dried in vacuo. The tRNA was cleaved off the α-BOC-Lys-NH-CO-NH-[^{35}S]Met in 0.5 M triethylamine at 37°C for 15 min and the triethylamine was evaporated in vacuo. The protective group ('BOC') was removed by treatment with trifluoracetic acid at 0°C for 1 h and the products were dried again.

3. Results and discussion

Under the conditions used, the PNPC-[^{35}S]Met-tRNA$^{met}_{f}$ reacts only with proteins L15 and L27 [1]. Because of the difference in their mol. wts, 17 500 and 12 700 respectively [6], these two proteins are readily separated by gel filtration on Sephadex G100. After elution of some radioactive material in the void volume, due to protein aggregation, two peaks of radioactivity labelled proteins were eluted. The larger protein, L15, was contained in the first peak and the smaller protein, L27, was contained in the second one (fig.1). For the following experiments, the fractions containing L27 were used. Other proteins present in these fractions can be ignored because they are not affinity labelled and hence not radioactive. Additional evidence for the complete separation of L15 and L27 was obtained from Ouchterlony double diffusion tests (data not shown).

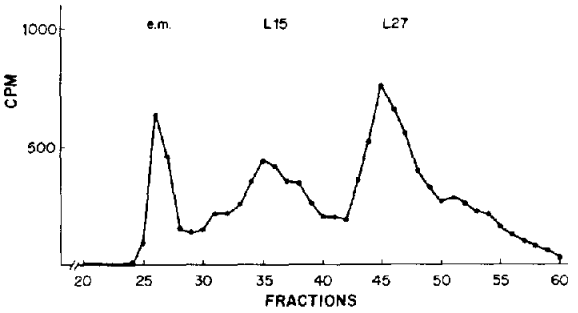

Fig.1. Gel filtration of affinity labeled protein of 50S subunit on Sephadex G100. For details see Materials and methods. Fractions 1 to 26 correspond to the void volume. Fractions 43 to 49 were used for further analysis. e.m.: excluded material.

The fractions containing affinity labelled protein L27 were subjected to digestion with trypsin. This was done in order to determine whether the label is randomly distributed over the whole protein, in which case radioactivity would be expected in a large number of peptides, or if it is associated with only one or few peptides, indicating a more specific reaction with the protein. After separation of the protein digest by a fingerprinting technique on thin layer plates, autoradiography revealed two predominant spots (fig.2). It should be mentioned that under the

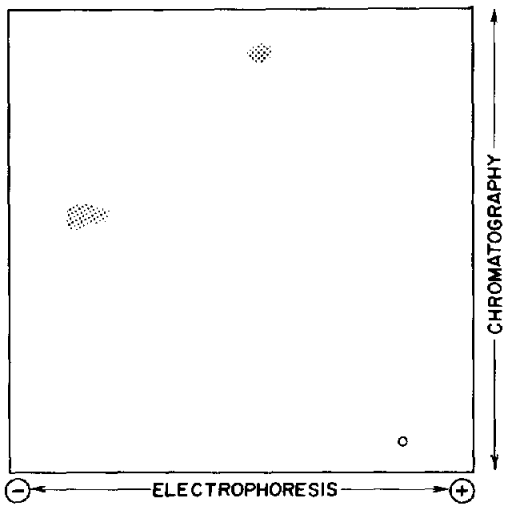

Fig.2. Fingerprint analysis of affinity labelled protein L27. Schematic drawing from an autoradiograph. For details see Materials and methods. (o) Origin.

same conditions the single protein L27, after tryptic digestion, yields about twenty ninhydrine reactive spots (our unpublished results) which is in agreement with recent results of Chen et al. [7].

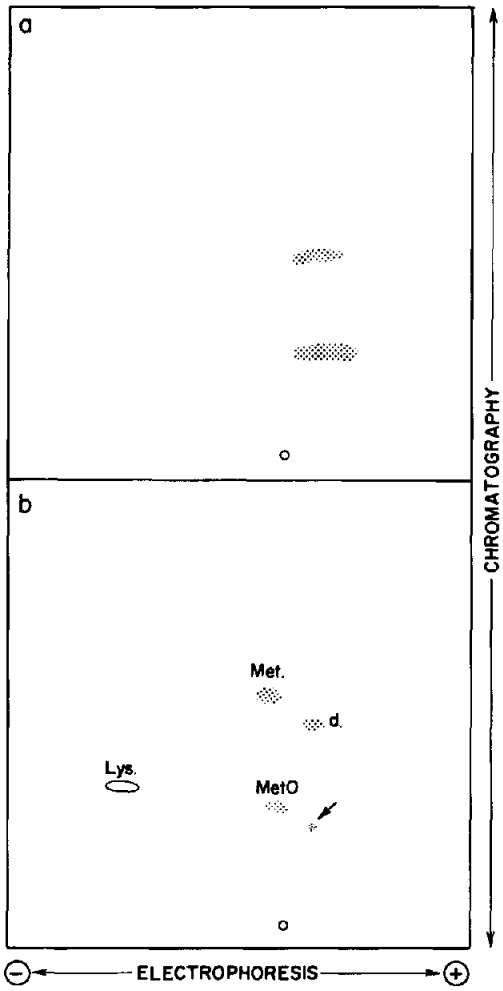

Fig.3. Two-dimensional analysis by fingerprinting technique, as described in Materials and methods. Schematic drawings from autoradiographs. (a), Two-dimensional separation of the product of complete enzymatic hydrolysis of affinity labelled protein L27 with trypsin–thermolysine–pronase. A similar result was obtained with trypsin–chymotrypsin–pronase. For details see Materials and methods. (b), Two-dimensional separation of synthesized Lys-NH-CO-NH-[^{35}S] Met and of lysine (Lys), methionine (Met) and methionine sulfoxide (MetO). The shaded circles represent spots seen on the autoradiograph. Methionine, methionine sulfoxide and lysine were identified by the ninhydrine reaction (see Results and discussion).

Next an attempt was made to determine the amino acid in L27 involved in the labelling reaction. This was done by complete enzymatic hydrolysis of the labelled protein. Two combinations of four different enzymes were used. Under either condition, i.e. sequential treatment with trypsin–chymotrypsin–pronase or trypsin–thermolysine–pronase, radioactivity was found in two distinct spots (fig.3a). The radioactively labelled tryptic peptides were separated from trypsin by high-voltage electrophoresis before further digestion with either thermolysine or chymotrypsin. Then excess amounts of pronase were added without removing the enzymes. Preliminary experiments [8] had suggested the formation of amide rather than ester bonds between the label and the protein. Because of the resistance of the bond to hydroxylamine treatment at pH 10 it was assumed that the reaction took place with ε-amino groups of lysines [8]. Therefore, it appeared reasonable to synthesize Lys-NH-CO-NH-[^{35}S]Met and to compare this compound electrophoretically and chromatographically with the end product of the complete hydrolysis described above. PNPC-[^{35}S]Met-tRNA$^{met}_{f}$ was reacted with α-BOC lysine under mild conditions. Carrier tRNA was added, the reaction stopped by adjusting the pH to 5 and the derivative coprecipitated in ethanol. The ester bond between tRNA and methionine was cleaved by triethylamine treatment and the protecting group of the α-amino function was removed with concentrated trifluoracetic acid. The compound was then analyzed by the fingerprinting technique. Three dominant spots can be seen after autoradiography (fig.3b, shaded spots). Two of these are methionine and methionine sulfoxide which were identified by the ninhydrine reaction of the two amino acids, added before electrophoresis (non-radioactive lysine was also added for reference; fig.3b). Therefore, the third spot derives from Lys-NH-CO-NH-[^{35}S]Met. The position after electrophoresis of the synthesized derivative is in accordance with that expected from its theoretical pK value. A very faint spot indicated by the arrow (fig.3b) most probably derives from the compound containing sulfoxide which is accidentally created by oxidation in the same way as methionine sulfoxide. From the similarity in the migration behavior of the two spots in fig.3a and of the derivative and its oxidation product shown in fig.3b, it was concluded that the

products of hydrolysis and of synthesis are identical. This would indicate that the affinity label indeed reacts with lysine.

Only two out of twenty possible peptides are labelled radioactively. Whether these two peptides are related is presently unknown. It follows, therefore, that the aminoacyl end of PNPC-[^{35}S]Met-tRNA$^{met}_{f}$ bound to the P-site interacts with one or very few confined regions of protein L27; that is, the labelling reaction does not take place at random, which provides additional evidence of the specificity of this particular affinity labelling procedure.

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