SELECTIVE LABELLING OF THE ß-SUBUNIT OF L-PHENYLALANYL-tRNA SYNTHETASE FROM E.COLI WITH N-BROMOACETYL-L-PHENYLALANYL-$tRNA^{Phe}$

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SUMMARY: L-Phenylalanyl-tRNA synthetase has been reacted with N-bromoacetyl-[^{14}C] Phe-$tRNA^{Phe}$ to yield covalently linked enzyme-N-acetyl-[^{14}C] Phe-$tRNA^{Phe}$. The labelled enzyme was dissociated in the presence of 4M guanidinium chloride and the subunits subsequently separated by gel chromatography. The elution pattern is indicative of covalent binding of the tRNA to the ß-subunit of the enzyme.

A recent reinvestigation of the structure of L-phenylalanyl-tRNA synthetase from E.coli provided evidence for an $\alpha_2\beta_2$ subunit composition (1,2). Despite the symmetry of the subunit structure only a single active site has been reported for the enzyme (3,4). The present paper reports a first step in identifying the location of the various substrate binding sites with respect to the different subunits.

MATERIALS AND METHODS:

The N-hydroxysuccinimide ester of bromoacetic acid was prepared according to de Groot et al.(5). The product had mp. 114°C, gave the theoretically expected combustion analysis and was further identified by its NMR spectrum. Purified $tRNA^{Phe}$ having an acceptance of 1090 pmol L-phenylalanine per A_{260}unit was obtained from Boehringer (Mannheim). [^{14}C] Phe-$tRNA^{Phe}$ was prepared as described by Bartmann et al.(4). N-Bromoacetyl-[^{14}C] Phe-$tRNA^{Phe}$ was obtained

by reacting 1 nmol [^{14}C]Phe-tRNA^{Phe} in 500 µl 0.1M triethanolamine pH 7.8 with 10 µmol of the ester dissolved in 150 µl dioxane for 20 minutes at room temperature (6). The yield of N-acylation was determined by the method developed by Schofield & Zamecnik (7) and was found to be 90-95%. L-Phenylalanyl-tRNA synthetase (sp.act. 53600 nmol·mg^{-1}·h^{-1}) was prepared as described previously (1).

RESULTS AND DISCUSSION:

In order to demonstrate that the substrate properties of the tRNA are not affected by the modification procedure, the following experiments were performed: Non-acylated tRNA^{Phe} was treated with the reagent under the same conditions as described for [^{14}C]Phe-tRNA^{Phe}. The charging capacity and the kinetics of the aminoacylation reaction of the tRNA remained unchanged after the treatment, thus indicating that the modification procedure does not result in important changes within the tRNA. This was confirmed by the result that N-bromoacetyl-Phe-tRNA^{Phe} was a competitive inhibitor of tRNA^{Phe} with an inhibition constant similar to the K_m for tRNA^{Phe}.

The reaction of N-bromoacetyl-[^{14}C]Phe-tRNA^{Phe} with L-phenylalanyl-tRNA synthetase was performed as described in the legend of Figure 1. The appearance of a peak containing radioactivity at the elution position of the enzyme indicates that the label had reacted with the enzyme. When the elution was repeated after incubation with a 20-fold excess of unlabelled tRNA^{Phe} the same amount of radioactivity was found, indicating that the radioactive label was not reversibly bound. In contrast, when the unlabelled tRNA^{Phe} was present in the reaction mixture during the incubation time, only a small fraction of radioactivity was incorporated into the enzyme (Figure 1).

The amount of radioactivity in the enzyme fraction usually accounted

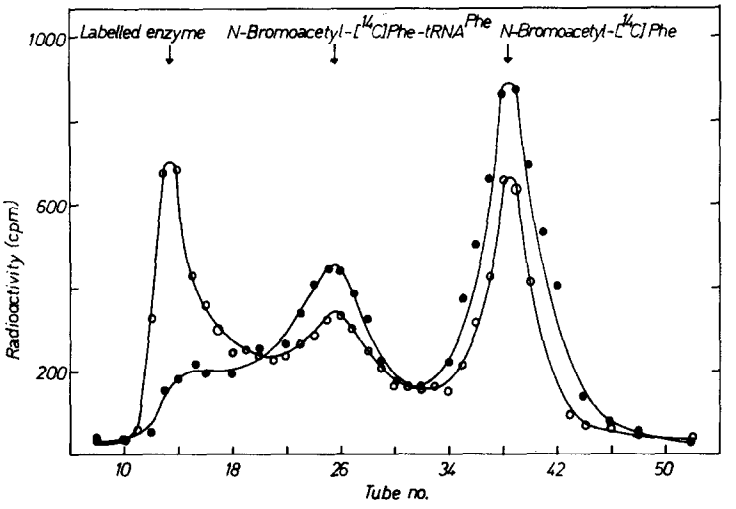

Figure 1: Reaction of L-phenylalanyl-tRNA synthetase with N-bromoacetyl-[^{14}C]Phe-tRNA^{Phe}. 300 pmol N-bromoacetyl-[^{14}C]Phe-tRNA^{Phe} and 400 pmol of L-phenylalanyl-tRNA synthetase were added to a solution containing 10mM potassium phosphate pH 7.5 and 5mM $MgCl_2$. The final volume was 750 µl. The solutions were incubated for 24 hours at 25°C. Separation of the reactants was performed on a Sephadex G 100 column (60 x 0.8cm) run with a buffer containing 20mM Tris-HCl, 0.2mM EDTA, 5mM $MgCl_2$, 0.2mM dithioerythritol, and 0.4M KCl. Fractions of 15 drops were collected at a rate of 8ml/h. (●—●) 6nmol of tRNA^{Phe} added to the mixture before the incubation. (○—○) 6nmol of tRNA^{Phe} added to the solution prior to gel filtration.

for less than 20% of the original amount of enzyme used. This was confirmed by the observation of a 10% to 20% inhibition of the enzyme activity in the standard aminoacylation assay (2).

It was not possible to follow saturation kinetics of the inactivation for two reasons: First the low level of affinity labelling and second the complication of the reaction by the spontaneous deacylation of N-bromoacetyl-[^{14}C]Phe-tRNA^{Phe} during the long incubation time (30% loss of label after 24 hours under reaction conditions).

The labelled enzyme was dissociated into subunits by incubation in 4M guanidinium chloride (2). Separation of the subunits was per-

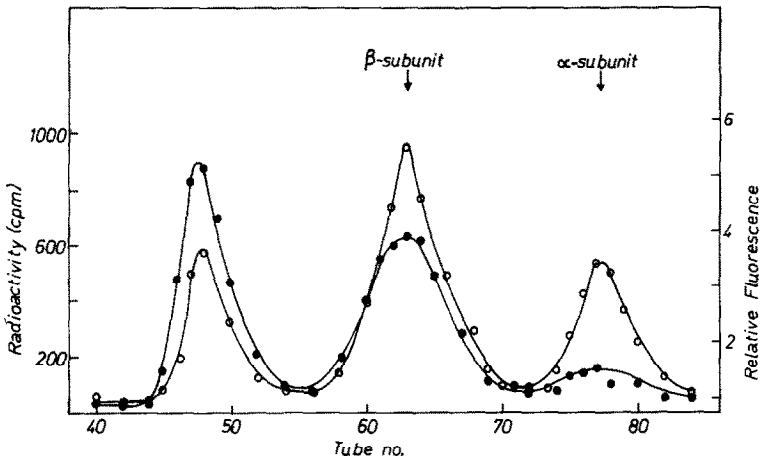

Figure 2: Analysis of the reaction products of N-bromoacetyl-[^{14}C]Phe-tRNA^{Phe} with L-phenylalanyl-tRNA synthetase. 550 pmol L-phenylalanyl-tRNA synthetase and 400 pmol N-bromoacetyl-[^{14}C]Phe-tRNA^{Phe} were added to a solution containing 10mM potassium phosphate pH 7.5 and 5mM $MgCl_2$ to give a final volume of 1100 µl. The mixture was incubated for 24 hours at 25° C. Then guanidinium chloride was added to give a final concentration of 4M. After an incubation of 15 min at room temperature, the solution was applied to a Biogel A5m column (80 x 1.7cm) equilibrated with a buffer containing 4M guanidinium chloride, 0.2M KCl, 20mM Tris-HCl, 0.2mM EDTA, 0.2mM dithioerythritol, and 10% glycerin. Elution at a rate of 3ml/h was performed with the same buffer and fractions of 15 drops were collected. Protein concentration (o—o) was determined by measuring the tryptophan fluorescence of the eluate (2). Radioactivity (●—●) was counted using dioxane containing 0.5% PPO as scintillation liquid. The peak which is first eluted corresponds to a ß-subunit which is covalently attached to tRNA.

formed on a Biogel A5m column as described. The elution profile (Figure 2) indicates three protein peaks which contained variable amounts of radioactivity. The two slower fractions emerged at positions corresponding to the α- and ß-subunit as determined from the dissociation pattern of unlabelled enzyme. The first peak is at a position reconcilable with the molecular weight of a tRNA-ß-subunit conjugate. Accordingly, most radioactivity (relative to protein) is found in the fast moving fraction. The radioactivity found at the position of the ß-subunit is almost certainly due to bound label which has been hydrolysed from the tRNA after reaction with the

protein. The radioactivity migrating in the position of the α-subunit comprises only a minute fraction of total radioactivity and may reflect nonspecific labelling.

The results are consistent with a specific binding of N-bromoacetyl-Phe-$tRNA^{Phe}$ to the ß-subunit of the enzyme followed by covalent binding of the N-bromoacetyl-L-phenylalanine moiety to the protein. Whether both subunits can be labelled simultaneously remains to be determined.

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