IDENTIFICATION OF A PROTEIN AT THE RIBOSOMAL DONOR-SITE BY AFFINITY LABELING

R. Hauptmann, A. P. Czernilofsky, H. O. Voorma*, G. Stöffler**, and E. Kuechler

Institut für Biochemie, Universität Wien, Austria; *Biochemisch Laboratorium, Rijksuniversiteit Leiden, Holland; and **Max-Planck-Institut für Molekulare Genetik, Berlin-Dahlem, GFR.

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SUMMARY: p-nitrophenylcarbamyl-methionyl-$tRNA_f^{Met}$ is shown to act as an analogue of fMet-$tRNA_f^{Met}$ in initiation complex formation. It binds to E. coli ribosomes in the presence of initiation factors and R 17-RNA as messenger. Covalent bond formation occurs in the complex between the Met-$tRNA_f^{Met}$ derivative and protein of the 50 S ribosomal subunit. The protein labeled predominantly in the reaction has been identified as L 27 indicating that this protein is located at the donor-site of the ribosome.

Recently affinity labeling has been employed by several groups for the study of ribosomal topology. Derivatives of phenylalanyl-tRNA have been synthesized (1 - 5) which bind to Escherichia coli ribosomes at high Mg^{2+} concentration in the presence of polyuridylic acid and react with ribosomal proteins or RNA under covalent bond formation. Analogues of antibiotics have also been used as affinity labels (6 - 8).

In order to reduce the possibility of artifacts due to labeling under unnatural binding conditions we have now carried out the affinity labeling in a natural protein synthesizing system. Radioactively labeled PNPC-Met-$tRNA_f^{Met}$ [1]) was bound to ribosomes at 5 mM Mg^{2+} concentration in the presence of phage R 17-RNA as messenger and purified initiation factors. Under these conditions binding should occur exclusively at the donor-site of the ribosome. The affinity label was found to attach primarily to protein L 27 indicating that this protein is located at the donor-site. A small amount of label was also found in protein L 15.

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1) PNPC-Met-$tRNA_f^{Met}$, p-nitrophenylcarbamyl-methionyl-transfer ribonucleic acid (formylatable, methionine specific).

MATERIALS AND METHODS: [^{35}S]methionine (Amersham, England) had a specific activity of 300 Ci/mmole; $tRNA_f^{Met}$ (94 - 98 % formylatable) was obtained from Boehringer Mannheim, Germany; purified initiation factors IF-1, IF-2, IF-3 were isolated according to Benne et al. (9); non-formylated [^{35}S]Met-$tRNA_f^{Met}$ was prepared according to Bretscher (10) at a specific activity of 10^{10} cpm /mg of $tRNA_f^{Met}$; synthesis of PNPC-[^{35}S]Met-$tRNA_f^{Met}$ was carried out as described previously for PNPC-Phe-tRNA[2] (3); ribosomes were washed twice with high NH_4Cl buffers (3); antisera were prepared and characterized as described by Stöffler and Wittmann (11).

The affinity labeling reaction was carried out in 60 mM NH_4Cl, 20 mM HEPES (pH 7.2), 5 mM magnesiumacetate, 0.2 mM mercaptoethanol, 0.2 mM GTP containing per ml: 4 mg ribosomes, 0.5 mg R 17-RNA, 2 µg IF-1, 12 µg IF-2, 6 µg IF-3 and 50 µg PNPC-[^{35}S]Met-$tRNA_f^{Met}$. Samples were kept at 25° C for 10 min before being incubated at 37° C for 80 min. 50 S ribosomal proteins were prepared as described previously (3); the specific radioactivity was 1.8×10^5 cpm/mg of protein.

Two-dimensional polyacrylamide gel electrophoresis was perfomed according to Kaltschmidt and Wittmann (12). For immunological identification 40 µg of labeled 50 S ribosomal protein was incubated with 0.25 ml of antiserum in a total volume of 0.5 ml containing 0.5 M LiCl, 50 mM Tris-HCl pH 8.2, 1 mM dithiothreitol for 30 min at 0° C. The mixture was then layered on to 5 - 40 % (w/w) sucrose gradient in 0.5 M LiCl, 0.5 M urea, 50 mM Tris-HCl pH 8.2, 10 mM mercaptoethanol containing 0.01 % bovine serum albumin. Samples were centrifuged for 36 h at 27,000 rpm in a SW 27 rotor and the radioactivity of the fractions determined (5).

RESULTS: PNPC-aminoacyl-tRNA reacts with aminogroups of proteins under formation of amide bonds with elimination of p-nitrophenol (13). In the resulting linkage the aminoacyl-tRNA is attached to the protein only via

[2] PNPC-Phe-tRNA, p-nitrophenylcarbamyl-phenylalanyl-transfer ribonucleic acid.

one carbonylgroup. The affinity labeling reaction can therefore occur solely on a ribosomal protein which is in close proximity to the aminoacyl-moiety of the tRNA.

The initiation complex was formed with PNPC-[^{35}S]Met-tRNA$_f^{Met}$ at 5 mM Mg^{2+} concentration on high-salt washed ribosomes in the presence of purified initiation factors IF-1, IF-2, IF-3 and R 17-RNA as messenger. Nonspecific labeling was minimized by the use of a fivefold molar excess of ribosomes over PNPC-Met-tRNA$_f^{Met}$. In order to avoid transpeptidation due to contaminating aminoacyl-tRNA, purified tRNA$_f^{Met}$ was used in the experiments. PNPC-Met-tRNA$_f^{Met}$ is bound to the ribosome in a puromycin sensitive state. Puromycin added to the incubation mixture before incubation with PNPC-Met-tRNA$_f^{Met}$ inhibits the affinity labeling by more than 80 % (data not shown); labeling is also dependent on the presence of R 17-RNA (14).

Following incubation the ribosomes were dialyzed against 0.5 mM Mg^{2+}. 50 S subunits were isolated on sucrose gradients and the RNA digested with ribonuclease which leaves the methionyl-adenosine residue covalently attached to ribosomal protein. For identification the labeled proteins were separated by two-dimensional gel electrophoresis (12). The distribution of radioactivity in 50 S ribosomal proteins is shown in Fig. 1. The label is incorporated predominantly into protein L 27. A small amount of radioactivity was recovered in two groups of proteins: L 13-L 14-L 15 and L 32-L 33. Labeling is strongly dependent on the presence of initiation factors as indicated by the control experiment in the lower panel of Fig. 1. Omission of all three initiation factors leads to a drastic reduction in incorporation.

Identification of protein L 27 as the main target of the affinity label was confirmed independently by immunochemical methods. Soluble antigen-antibody complexes were formed under conditions of antibody excess. Because of their large molecular weights these complexes can be separated on sucrose gradients from the residual ribosomal proteins. The results of an experiment employing anti-L 27 antiserum are shown in Fig. 2. A considerable amount of radioacti-

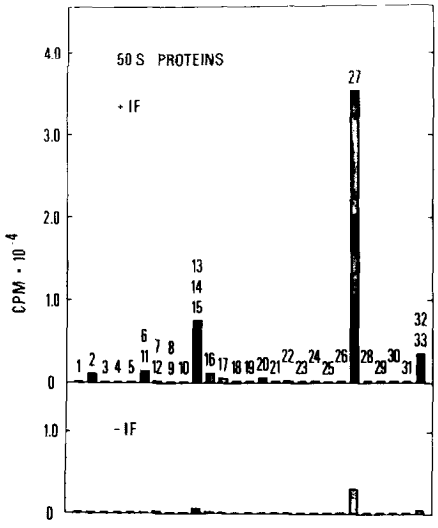

Fig. 1: Distribution of radioactivity in 50 S ribosomal proteins as determined by two-dimensional polyacrylamide-gel electrophoresis. 1 mg of carrier 50 S protein was added to 1 mg of labeled 50 S protein to facilitate identification of the stained protein spots.

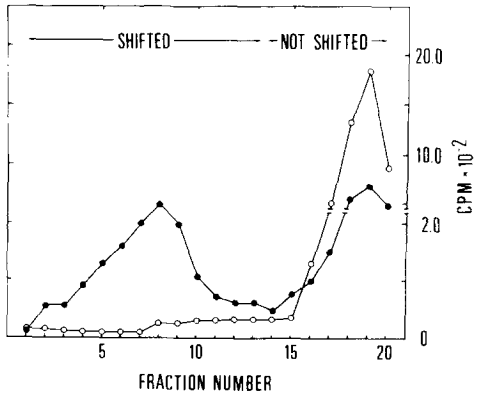

Fig. 2: Sucrose gradient centrifugation of soluble antigen-antibody complexes. The distribution of radioactivity is shown when anti-L 27 (●——●) or anti-L 21 (o——o) antiserum are used for complex formation.

vity is shifted from the top of the gradient into rapidly sedimenting material. Anti-L 21 antiserum yields no shift of labeled protein into heavy complexes (Fig. 2). The results obtained with several antisera are listed in table I.

Table I

Immunological identification of radioactively labeled proteins

specificity of antiserum preparation	radioactivity (cpm)		% shifted	% shifted corrected
	shifted	not shifted		
L 27	1436	2028	41.5	35.2
L 15	592	3905	13.2	6.9
L 16	469	4178	10.1	3.8
L 14	328	3751	8.0	1.7
L 13	328	4873	6.3	nil
L 33	278	4386	6.0	nil
L 2	319	5094	5.9	nil
L 11	283	4850	5.5	nil
L 21	249	4502	5.2	nil
L 6	238	4515	5.0	nil
nonspecific	469	7030	6.3	nil

Radioactivity in rapidly sedimenting soluble antigen-antibody complexes ("shifted") and in the slowly sedimenting fraction of the gradient ("not-shifted") was determined as indicated in Fig. 2. Nonspecific antiserum was taken from a rabbit before injecting it with a ribosomal protein. The numbers in the last column ("% shifted corrected") are obtained by subtraction of the percentage shifted with nonspecific antiserum from that shifted with the specific antiserum.

Anti-L 27 antiserum shifts 35 % of the total radioactivity into rapidly sedimenting material. Anti-L 15 antiserum shifts only 7 %. The shifts observed with anti-L 14 and anti-L 16 antisera are only marginal. No shifts of radioactivity exceeding background are obtained with antisera to the following proteins: L 2, L 6, L 11, L 13, L 21 and L 33. This indicates that the radioactivity recovered in the group of L 13-L 14-L 15 on the two-dimensional gel is mostly due to labeled L 15.

DISCUSSION: The strong dependence on initiation factors indicates that the tertiary structure of the Met-$tRNA_f^{Met}$ molecule is not significantly altered

by the substitution with the p-nitrophenylcarbamyl-group. It is likely that the enzymatic binding of PNPC-Met-$tRNA_f^{Met}$ mediated by IF-2 occurs at the donor-site as is also indicated by the strong puromycin inhibition. Nevertheless, we do not exclude the possibility of a small amount of labeling at "entry" or "pre-P" sites (15 - 18).

Previous studies on the nonenzymatic binding of PNPC-Phe-tRNA at 20 mM Mg^{2+} concentration have yielded significant amounts of label in proteins L 15, L 2 and L 16 in addition to labeling of L 27 (5). A small amount of radioactivity was also recovered in the group of proteins L 32-L 33. At high Mg^{2+} concentration PNPC-Phe-tRNA can bind to both the donor- and the acceptor-site on the ribosome. In contrast enzymatic binding of PNPC-Met-$tRNA_f^{Met}$ at 5 mM Mg^{2+} concentration leads to binding to the donor-site only. However, the experiments must not be interpreted in such a way that all proteins labeled exclusively with PNPC-Phe-tRNA at high Mg^{2+} but not with PNPC-Met-$tRNA_f^{Met}$ at low Mg^{2+} concentration are necessarily acceptor-site proteins. It is quite possible that certain species of ribosomes (19, 20) which have lost their capability to bind PNPC-Met-$tRNA_f^{Met}$ enzymatically, can still carry out nonenzymatic binding of PNPC-Phe-tRNA at high Mg^{2+} concentration. L 15 seems to be labeled in both cases but to a different extent; its position is at present unclear. However, it is plausible that the donor- and the acceptor-site may share common proteins. Recent experiments by Nierhaus and Nierhaus (21) and by Pongs et al. (8) indicate that L 16 is the chloramphenicol binding protein, which is located at the acceptor-site. This is in agreement with our results which show that significant labeling of L 16 occurs only with PNPC-Phe-tRNA at high Mg^{2+} concentration.

The present experiments are aimed at an understanding of the donor-site of E. coli ribosomes. Further exploitation of affinity labeling techniques should enable us to determine ribosomal protein neighbourhoods of the aminoacyl-moiety of tRNA at the individual steps of polypeptide synthesis.

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