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Affinity Labeling of *Escherichia coli* Ribosomal Proteins with an Analog of the Natural Initiator tRNA†

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ABSTRACT: Bromoacetylmethionyl-tRNA^{fMet} is an analog of the peptide chain initiator formylmethionyl-tRNA^{fMet}. Its binding to unwashed 70S *Escherichia coli* ribosomes is enhanced 18-fold in the presence of bacteriophage f2 RNA. f2 RNA does not promote the binding of the analog to salt-washed ribosomes. This presumably indicates that the process requires initiation factors. Addition of alanyl-tRNA, in the form of an alanyl-tRNA · EF-Tu · GTP complex, to the reaction mixture containing the analog bound to ribosomes in the presence of f2 RNA results in the attachment of about 0.6 mol of alanyl residues/mol of analog attached. This is taken as evidence for the formation of a substituted methionylalanyl dipeptide and is the expected result since alanine is the second amino acid in the coat protein translated from f2 RNA *in vitro*. About 5% of the labeled analog and of the labeled alanyl residues were found to be covalently linked to 50S proteins. One protein, L2, contained four times as much covalently attached label as any other 50S protein. In another experiment in which only the labeled analog was bound to ribosomes L2 again was the most heavily labeled. A smaller amount of label was attached to L27. In the absence of f2 RNA or 30S subunits only 5% as much analog became linked to 50S proteins as in a complete system. These results are in line with earlier conclusions from experiments with poly(uridylic acid) and a phenylalanyl-tRNA analog. They both indicate that L2 and perhaps L27 may be part of the peptidyl-tRNA binding site (P site) of the 50S subunit or are at least close to this site.

The determination of the location within the ribosome and function in protein synthesis of particular ribosomal proteins is under way. One of the approaches to this problem is based on affinity labeling. This involves the use of certain analogs of substrates or inhibitors of reactions occurring on the ribosome. Such a substrate analog carries a chemically reactive group. Upon binding of the analog to the ribosome the reactive group is potentially able to form a covalent linkage between the analog and particular ribosomal components. Any ribosomal component which reacts with the analog can then be identified. If other explanations can be ruled out, it is assumed that such a ribosomal component is part of the binding site or is located close to the binding site of the substrate. The validity of this assumption is greatly supported if it can be shown that the substrate analog functions correctly after it has been linked covalently to the ribosome.

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One of the compounds which has been used for affinity labeling of ribosomal components is BrAcPhe-tRNA, an analog of peptidyl-tRNA (Pellegrini *et al.*, 1972). This compound has been found to become covalently linked mainly to the L2 and L27 proteins of the large ribosomal subunit in the presence of poly(uridylic acid) (Oen *et al.*, 1973). BrAcPhe-tRNA was found to participate in peptide linkage formation with Phe-tRNA and with the antibiotic puromycin. The products of these reactions have not been identified. These results were taken to indicate that BrAcPhe-tRNA can function similarly to normal peptidyl-tRNA and that the proteins L2 and L27 are part of (or are at least located close to) the peptidyl-tRNA binding site of the large ribosomal subunit.

Many of the affinity labeling experiments in the past were performed with Phe-tRNA analogs and the synthetic homopolymer poly(uridylic acid) (Oen *et al.*, 1973; Czernilofsky *et al.*, 1974; Bispink and Matthaei, 1973; Bochkareva *et al.*, 1971). This is an artificial system in which translation occurs without proper peptide chain initiation. In this communication we report experiments with affinity labeling in which a natural mRNA (f2 bacteriophage RNA) and an analog of the natural chain initiator tRNA (BrAcMet-tRNA^{fMet}) were used.

Experimental Section

Published procedures were used for preparing elongation factor Tu (EF-Tu, previously designated as S_3, apparently a homogeneous protein), elongation factor Ts (EF-Ts, previously designated as S_1, "purified peak S_1") from *Bacillus stearothermophilus* (Beaud and Lengyel, 1971), f2 bacteriophage RNA (Eoyang and August, 1968; Gupta *et al.*, 1970), [^{3}H]Ala-tRNA, ribosomes (Gupta *et al.*, 1971), and salt-washed ribosomes (*i.e.*, ribosomes washed in a solution of high ionic strength to remove initiation and elongation factors (Nishizuka and Lipmann, 1966). [^{35}S]Met-tRNA^{fMet} and [^{3}H]Met-tRNA^{fMet} were prepared by charging purified tRNA^{fMet} on *Escherichia coli* (a gift of A. D. Kelmers, Oak Ridge National Laboratory) using an aminoacyl-tRNA synthetase preparation purified by fractionation on DEAE-cellulose (Muench and Berg, 1966).

Preparation of Derivatives of Met-tRNA^{fMet}. [^{35}S]Met-tRNA^{fMet} and [^{3}H]Met-tRNA^{fMet} were prepared by charging purified tRNA^{fMet} from *E. coli* (a gift of A. D. Kelmers, Oak Ridge National Laboratory) using an aminoacyl-tRNA synthetase preparation purified by fractionation on DEAE-cellulose (Muench and Berg, 1966). The tRNA was 85–100% aminoacylated. N-Acetylation and N-bromoacetylation of Met-tRNA^{fMet} to give AcMet-tRNA^{fMet} and BrAcMet-tRNA^{fMet} were performed as described for Phe-tRNA using the *N*-hydroxysuccinimide ester of acetic or bromoacetic acid (Pellegrini *et al.*, 1972). Met-tRNA^{fMet} was 70% acetylated by this method. The amount of N-acetylation was determined as previously described (Pellegrini *et al.*, 1972). The reaction of the succinimide esters at positions on the tRNA other than the α-amino group of methionine was not excluded. However, covalent products of the resulting affinity label are detected by the incorporation of radioactivity into ribosomal proteins. Only the methionine is radioactive. Also, we have observed that the rest of the tRNA molecule is dissociated from the methionine residue at the time of the final analysis of the ribosomal proteins (Pellegrini, 1973). The only way radioactivity could be incorporated into ribosomal components is through covalent reaction of the α-bromocarbanyl moiety attached directly to the methionine residue.

Initiation Complex Preparation. The reaction mixture (1 ml) contained the following components: 50 mM Tris-Cl (pH 7.4), 50 mM NH_4Cl, 5 mM magnesium acetate, 0.4 mM GTP, 0.56 μM BrAcMet-tRNA^{fMet} (unlabeled or labeled with [^{35}S]methionine (sp act. 20 Ci/mmol) or [^{3}H]-methionine (sp act. 2.6 Ci/mmol), 30 A_{260} units of ribosomes (or salt-washed ribosomes if so indicated), and in some cases 20 A_{260} units of f2 RNA. After incubation at 37° for 40 min the reaction mixture was cooled to 0° and made 6 mM in 2-mercaptoethanol.

Assay of the Reactivity of the Initiation Complex Preparations with Puromycin. Initiation complex preparations (0.075 ml) prepared with BrAcMet-tRNA^{fMet} were made 2 mM in puromycin, incubated at 0° for 10 min, and filtered through Millipore filters. The filters were washed with an ice cold solution containing 50 mM Tris-Cl (pH 7.4), 50 mM NH_4Cl, 7.5 mM magnesium acetate, and 6 mM 2-mercaptoethanol, dried, and counted in a toluene based scintillator.

EF-Tu · GTP · Ala-tRNA Complex Preparation. The reaction mixture (1 ml) contained the following components: 50 mM Tris-Cl (pH 7.4), 50 mM NH_4Cl, 10 mM magnesium acetate, 6 mM 2-mercaptoethanol, 0.05 mM GTP, 8.5 A_{260} units of Ala-tRNA (sp act. 30.3 Ci/mmol; carrying 400 pmol of [^{3}H]Ala residues), 40 μg of EF-Tu, and 40 μg of EF-Ts (needed to regenerate EF-Tu). The reaction mixture was incubated at 37° for 5 min.

Pretranslocation Complex Preparation. Three volumes of initiation complex preparation was mixed with 2 vol of EF-Tu · GTP · Ala-tRNA complex preparation and incubated at 0° for 40 min.

Dissociation of the Pretranslocation Complex Preparation into Subunits and Separation of the Subunits by Sedimentation through a Sucrose Gradient. The pretranslocation complex preparation (1.25 ml) was dissociated into subunits by dialyzing it against a solution (2 l.) containing 20 mM Tris-Cl (pH 7.4), 10 mM NH_4Cl, 0.5 mM magnesium acetate, and 1mM dithiothreitol at 0° for 12 hr. During this time the dialyzing buffer was changed four times. The dissociated pretranslocation complex preparation (0.5 ml) was applied to the top of an isokinetic sucrose gradient (5–20% w/v, total volume 12 ml) (Noll, 1969), prepared in 20 mM Tris-Cl (pH 7.4), 100 mM NH_4Cl, 0.5 mM magnesium acetate, and 1 mM dithiothreitol. The gradient was centrifuged at 40,000 rpm (180,000g) in the SB 283 rotor of the IEC B60 ultracentrifuge at 2° for 4.5 hr. After centrifugation the gradient was collected in 0.5-ml fractions. Aliquots (0.2 ml) of each were counted in Bray's scintillation fluid. The fractions in the 50S region and in the 30S region were pooled for further analysis.

Processing of Pooled 50S Fractions for Two-Dimensional Gel Electrophoresis. To the pooled fractions from the 50S region of the sucrose gradient, 10 mg of unlabeled carrier 50S subunits (Pellegrini *et al.*, 1972) was added. The subunits were sedimented by centrifugation at 190,000g for 5 hr and the pellet obtained was resuspended in 0.25 ml of 10 mM Tris-Cl (pH 7.4), 50 mM NH_4Cl, 0.25 mM magnesium acetate, and 6 mM 2-mercaptoethanol. To this was added 0.25 ml of an 8 M urea–4 M LiCl solution. The mixture was allowed to stand at 4° for 72 hr. The precipitated RNA was sedimented by centrifugation at 3000g for 18 min and the supernatant fraction containing ribosomal proteins was dialyzed against 4 M urea at 4° for 4 hr. The solution (0.5 ml) was supplemented with 100 μg of pancreatic

ribonuclease and incubated at room temperature for 1 hr. Thereafter the ribosomal proteins were fractionated by two-dimensional gel electrophoresis according to the procedure of Kaltschmidt and Wittmann (1970). The gels were stained in a solution of 0.01% w/v Coomassie Blue in 10% trichloroacetic acid (Chrambach *et al.*, 1967) for 2 hr. Each protein spot was cut out, and trichloroacetic acid was removed by extracting the gel pieces with ether. The pieces were dried in a vacuum oven at 80° overnight. For the double label experiment each piece of gel was supplemented with 0.75 ml of a 30% hydrogen peroxide solution and incubated at 60° for 2 hr. The dissolved gels were counted in Bray's scintillation fluid. When labeled proteins contained only [^{3}H]methionine the corresponding gel pieces were oxidized in a Packard sample oxidizer and counted in Permafluor II (Packard). One-dimensional gel electrophoresis was performed as previously described (Pellegrini *et al.*, 1972; Fogel and Sypherd, 1968). Identical protein samples were electrophoresed on duplicate gels. One gel was stained in 10% trichloroacetic acid containing 0.01% Coomassie Blue; the other was immediately sliced into 2-mm slices and each of these was oxidized in a Packard sample oxidizer. It should be noted that proteins L26 and L27 coelectrophorese when analyzed by this one-dimensional gel technique.

Results

BrAcMet-tRNA^{fMet} is a structural analog of the natural peptide chain initiator fMet-tRNA^{fMet}. The functional analogy between the two compounds is revealed by the following series of observations.

BrAcMet-tRNA^{fMet} Can Substitute for fMet-tRNA^{fMet} in Initiation Complex Formation. f2 RNA promotes the binding of BrAcMet-tRNA^{fMet} to ribosomes carrying bound initiation factors but not to ribosomes from which the initiation factors had been removed by washing with salt solutions. The behavior of BrAcMet-tRNA^{fMet} and AcMet-tRNA^{fMet} is quite similar (Table I). This indicates that the reactive bromo group is not causing any drastic interference with tRNA recognition or placement on the ribosome. The binding characteristics are similar to those established earlier for the binding of fMet-tRNA^{fMet} to ribosomes (Iwasaki *et al.*, 1968; Anderson *et al.*, 1967). A direct comparison is not possible because 2-mercaptoethanol had to be absent in our incubation mixtures while it was present in reported studies with fMet-tRNA^{fMet}.

Eighty per cent of the fMet residues in an initiation complex with f2 RNA can be released from the ribosomes upon treatment with puromycin (Gupta *et al.*, 1971). Under the same conditions 50% of the BrAcMet residues are released (unpublished data). These results indicated that the bulk of the BrAcMet-tRNA^{fMet} residues are apparently correctly situated at the peptidyl (P) site.

The Initiation Complex Containing BrAcMet-tRNA^{fMet} Can Be Converted into a Pretranslocation Complex. Under the proper conditions only one initiation complex is formed on the f2 RNA molecule. This is formed at the site specifying the initiation of translation of f2 coat protein (Gupta *et al.*, 1971). The second amino acid in the coat protein is alanine. When Ala-tRNA (in the form of the Ala-tRNA · GTP · EF-Tu complex) is added to the initiation complex it is bound to the ribosome. Subsequently, peptide bond formation takes place and a fMet-Ala-dipeptide is formed which is not reactive with puromycin, *i.e.*, is located at the aminoacyl (A) site. The resulting ribosomal complex is designated as the pretranslocation complex (Gupta *et al.*, 1971).

TABLE I: Binding of Analogs of fMet-tRNA^{fMet} to Ribosomes and Salt-Washed Ribosomes in the Presence and Absence of f2 RNA.[a]

	pmol of [^{3}H]Met Residue for	
	BrAc[^{3}H]Met-tRNA Retained on Filter	Ac[^{3}H]Met-tRNA Retained on Filter
Initiation complex	5.5	9.0
Initiation complex without f2	0.30	0.09
Initiation complex (except with salt-washed ribosomes)	0.36	0.12

[a] The initiation complex was prepared according to the procedure in the Experimental Section. f2 RNA was omitted where indicated and either ribosomes or salt-washed ribosomes were used as indicated; 0.075-ml aliquots of the initiation complex were applied to Millipore filters. The filters were washed with an ice cold solution containing 50 mM Tris-Cl (pH 7.4), 50 mM $(NH_4)Cl$, 7.5 mM magnesium acetate, and 6 mM 2-mercaptoethanol, dried, and counted in a toluene based scintillator.

We examined whether an initiation complex formed with BrAcMet-tRNA^{fMet} would also direct Ala-tRNA binding. In the course of these experiments we attempted to maximize the possibility of forming a covalent linkage of the affinity label to the ribosomes prior to the addition of Ala-tRNA and minimize the possibility of the formation of this linkage after adding Ala-tRNA. Since the analog reacts with SH groups (free BrAcMet-tRNA^{fMet} reacts to completion at 37° within 40 min with 13 μM cysteine), the initiation complex was formed in a reaction mixture with no added SH compound. The incubation was performed at 37° for 40 min. We hoped that these conditions would allow the affinity analog to form a covalent linkage to properly situated reactive groups on the ribosome. To diminish the probability of covalent linkage formation between the affinity analog and ribosomes after the addition of Ala-tRNA the following procedure was followed. The reaction mixture containing the initiation complex was cooled to 0° and supplemented with 6 mM mercaptoethanol. Thereafter, the Ala-tRNA · GTP · EF-Tu complex was added to the reaction mixture and incubated with it at 0° for 40 min. At these low temperatures the reaction of free BrAcMet-tRNA^{fMet} with cysteine is 20 times slower than at 37° (Pellegrini, 1973). Moreover, since the concentration of 2-mercaptoethanol in the reaction mixture was several thousand-fold higher than that of the ribsomes any free BrAcMet-tRNA^{fMet} could have reacted with 2-mercaptoethanol. This experimental procedure offers a reasonable chance that any Met residues from BrAcMet-tRNA^{fMet} which are found covalently attached to ribosomes became covalently linked to them prior to the binding of Ala-tRNA.

The results in Table II reveal that (1) Ala residues (from an Ala-tRNA · GTP · EF-Tu complex) became attached to an initiation complex in which BrAcMet-tRNA^{fMet} was substituted for fMet-tRNA^{fMet} and (2) the amount of Ala residues bound was diminished by over 80% when the "initi-

TABLE II: Dependence on BrAcMet-tRNA^{fMet} of the Binding of Ala-tRNA to Ribosomes in the Presence of f2 RNA.[a]

	Ala-tRNA Retained on Filter (pmol of [^{3}H]Ala Residue)
Pretranslocation complex	5.1
"Pretranslocation complex" (except without BrAcMet-tRNA^{fMet}	0.92

[a] The initiation complex was prepared according to the procedure in the Experimental Section (except that BrAcMet-tRNA^{fMet} was omitted if so indicated). Both complexes were converted into pretranslocation complexes according to the procedure in the Experimental Section. Aliquots (0.125 ml) of the complexes were applied to Millipore filters. These were prepared for counting as indicated in Table I.

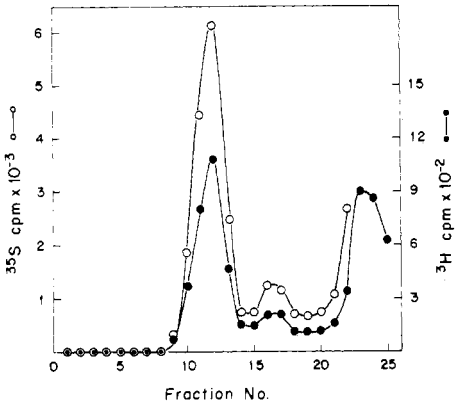

FIGURE 1: Amount of Met and Ala residues bound to separated 50S and 30S subunits of a pretranslocation complex preparation. The complex was prepared with BrAc[^{35}S]Met-tRNA^{fMet} and [^{3}H]Ala-tRNA and was dissociated, and its subunits were separated from each other according to the procedures described in the Experimental Section.

TABLE III: Amount of Met and Ala Residues in a Pretranslocation Complex, Bound to the Separated 50S Subunits (Obtained by Centrifugation of the Dissociated Pretranslocation Complex through a Sucrose Gradient) and Linked Covalently to 50S Proteins.[a]

	Pretranslocation Complex		50S Subunit		30S Subunit	
	Met	Ala	Met	Ala	Met	Ala
(1) pmol of aminoacyl residues retained on filters	66.3	45.7	3.3	2.0	0.10	0.08
(2) Ala/Met	0.69		0.61		0.80	
			50S Subunit Proteins			
			Met	Ala		
(3) pmol of aminoacyl residues linked covalently to			3.1	1.9		

[a] The pretranslocation complex was prepared with BrAc-[^{35}S]Met-tRNA^{fMet} and [^{3}H]Ala-tRNA, dissociated and fractionated into 50S and 30S subunit-containing regions by centrifugation through a sucrose gradient according to procedures in the Experimental Section. The pooled fractions in the 50S and 30S regions of the gradient were filtered through Millipore filters. The filters were prepared for counting as indicated in the legend to Table I. Proteins of the 50S subunit were processed as described for two-dimensional gel electrophoresis through the treatment with pancreatic ribonuclease and counted. The picomoles shown are total amounts calculated to be present in a pretranslocation complex preparation, or in the subunits obtained by dissociating it or in the 50S subunit proteins obtained from the total 50S subunits. The latter values were corrected for the loss of protein during the separation of the proteins from the ribosomal RNA. Total protein was determined by the procedure of Lowry *et al.* (1951).

ation complex" was prepared without BrAcMet-tRNA^{fMet}

The ratio of Ala to Met residues in the resulting pretranslocation complex was 0.69 (Table III), *i.e.*, similar to that in a pretranslocation complex containing fMet-tRNA^{fMet} in which it was 0.5 (Gupta *et al.*, 1971). By analogy we assume then that in the pretranslocation complex prepared with BrAcMet-tRNA^{fMet} the Ala residues were also in the form of BrAcMet-Ala-tRNA^{Ala}.

Covalent Reaction of BrAcMet-tRNA^{fMet} Appears to Occur Mainly with the 50S Subunit. The pretranslocation complex was dissociated into ribosomal subunits and these were separated from each other by centrifugation through a sucrose gradient (Figure 1). Approximately 5% of the net residues bound in the pretranslocation complex were retained on the 50S subunit and 0.1% on the 30S subunit (Table III). These are first indications that a covalent reaction might have occurred between the BrAcMet-tRNA^{fMet} and the ribosomes. Moreover, in this case over 30 times more covalent reaction occurred with components of the 50S subunit than with those of the 30S subunit. It should be noted that in the previous studies in which BrAcPhe-tRNA was used the same ratio was as low as 4 (Eilat *et al.*, 1974).

The data in Table III reveal that 60% as many Ala residues were bound to the 50S subunit as Met residues. This makes it likely that the majority of the covalently linked product is a dipeptide. Since the amount of labeled Met and Ala residues bound to the 30S subunits was small, these were not characterized further.

Analysis of BrAcMet-tRNA^{fMet} Reacted 50S Proteins. 50S particles were isolated from BrAcMet-tRNA^{fMet} treated 70S ribosomes. Ribosomal proteins and rRNA were separated by treatment with LiCl–urea as described in the Experimental Section. Ribosomal RNA contained less than 5% of the total [^{3}H]- or [^{35}S]Met covalently incorporated into the ribosome. This was not further characterized. Ribosomal proteins contained the remainder of the covalently incorporated amino acids as shown in Table III. The pattern of incorporation into individual 50S ribosomal proteins was analyzed by two-dimensional gel electrophoresis. Results from ribosomes incubated with BrAc[^{3}H]Met-tRNA^{fMet} alone (Figure 2C) and BrAc[^{35}S]Met-tRNA^{fMet} (Figures 2A,B) followed by enzymatic addition of [^{3}H]Ala-tRNA are given in Figure 2. The major reaction product in both cases is clearly protein L2. The relative amount of labeling of L2 varies with respect to L26–L27 as do the amounts of labeling of other proteins. A similar variation has been found for labeling with BrAcPhe-tRNA and it ap-

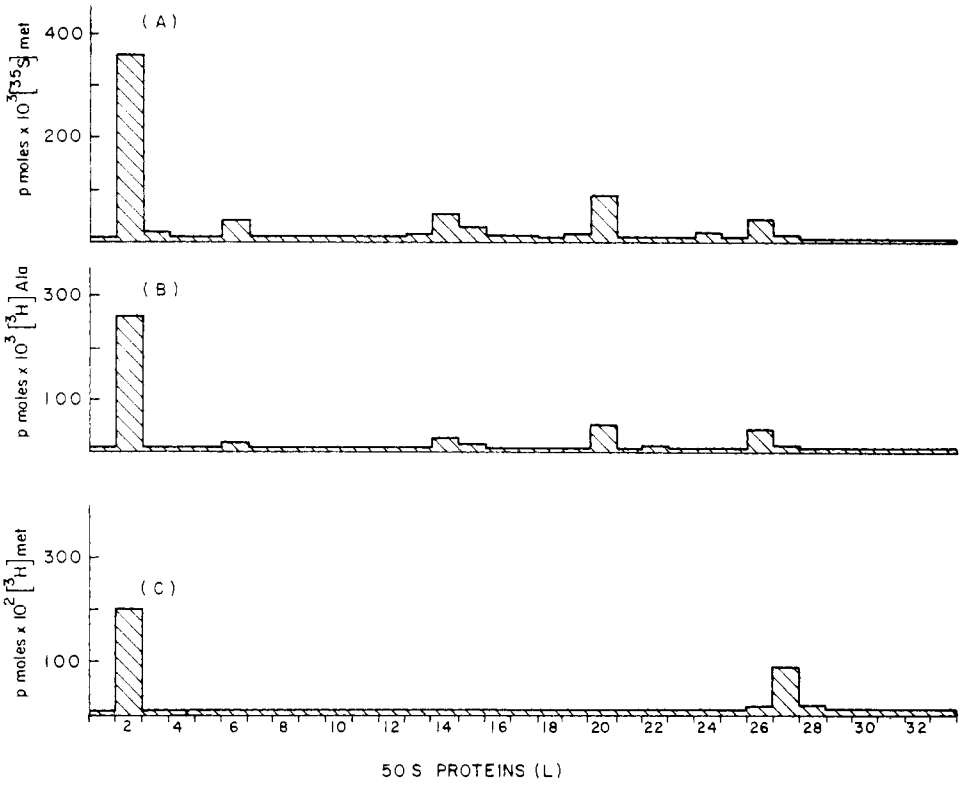

FIGURE 2: Amount of Met and Ala residues linked covalently to 50S proteins in initiation complex and pretranslocation complex preparations as analyzed by polyacrylamide gel electrophoresis in two dimensions: (A) and (B) the pretranslocation complex was prepared according to the procedure in the Experimental Section; the protein sample applied to the gel contained 31,400 cpm of [^{35}S]Met residues and 11,250 cpm of [^{3}H]Ala residues; (C) the initiation complex was prepared according to the procedure in the Experimental Section. The protein sample applied to the gel contained 33,000 cpm of [^{3}H]Met residues. For further details see the Experimental Section. For the experiments shown in A and B only a small region of the two-dimensional gel around L27 was cut and counted. Subsequently to these experiments we learned that the alkylated L27 protein tends to spread into neighboring regions in the gels. Thus, it is conceivable that in this experiment we may have detected only part (we estimate from other experiments about one-third) of the total label present in L27. However, it is clear from the results that this would not alter any of the conclusions drawn from the experiment. For the experiment in C, L27 was analyzed in a larger region of the gel. This may account, at least in part, for finding a larger percentage of the total labeling in L27.

pears to be a function of the ribosome preparation used (Pellegrini *et al.*, 1974). However, the majority of the reaction takes place with L2.

In the standard BrAc[^{3}H]Met-tRNA^{fMet} labeling reaction the only significantly reactive ribosomal component besides protein L2 is protein L26–L27. Resolution of radioactive products in the L26–L27 region of the gel leaves much to be desired (Czernilofsky *et al.*, 1974; Pellegrini *et al.*, 1974). Hence, from gel analysis alone, one cannot unequivocally identify whether the radioactive component is L26, L27, or both. By analogy to previous work where specific antisera were used (Czernilofsky *et al.*, 1974) it is likely that the actual product seen in this region is alkylated L27. Overall, the labeling pattern seen with BrAc[^{3}H]Met-tRNA^{fMet} is very similar to labeling obtained with BrAcPhe-tRNA (Oen *et al.*, 1973).

For clearer identification of modified proteins, both one- and two-dimensional gel analyses were performed on the same sample of 50S proteins labeled with BrAc[^{3}H]Met-tRNA^{fMet}. The one-dimensional gel lacks the resolving power of two-dimensional separating. However, the entire gel can be conveniently sliced and counted. Therefore, all radioactive products are sure to be found.

A set of one-dimensional results is shown in Figure 3. The ratio of [^{3}H]Met incorporated into proteins L2 and L26–L27 is identical with that found by two-dimensional analysis. Therefore, these products represent the only major sites of radioactive incorporation.

As seen in Figure 2, the pattern of attachment of [^{3}H]Ala residues to 50S proteins is similar to that for [^{35}S]Met. L2 is again the main reaction product. The yield of dipeptide bound to L2 is 75%, that is, 75% of the Met residues found covalently attached to this protein were able to participate in peptide bond transfer to an aminoacyl-tRNA in the A site.

Proteins labeled to a lesser extent in this dipeptide experiment are L20, L26–L27, L6, and L14. Of these proteins L26–L27 contains the highest ratio of dipeptide, 75%. This is comparable to that found in L2. L14 and L20 contain approximately 50% dipeptide, while L6 has less than 40%. These results suggest that BrAcMet-tRNA^{fMet} that has reacted with proteins L2 and L26–L27 is in the best position to participate in peptide bond formation and by this criterion these sites are the most likely to be functionally significant. The major difference between these results and those obtained from dipeptide experiments with BrAc[^{1}H]Phe-tRNA and [^{3}H]Phe-tRNA (Oen *et al.*, 1973; Eilat *et al.*, 1974) is the extent of labeling of proteins L15–L16. Consistently 25% of the total [^{3}H]Phe incorporated into ribosomal protein in these experiments is found with L15–L16. No labeling of L15–L16 is found in the BrAcMet-tRNA^{fMet} dipeptide experiment. This may be due to a different binding mode of BrAcPhe-tRNA. It has been suggested that the 3′-(Ac-AA-CCA) end of bound peptidyl-tRNA is flexible (Pestka, 1969; Celma *et al.*, 1971). The Phe residue is very hydrophobic and may well

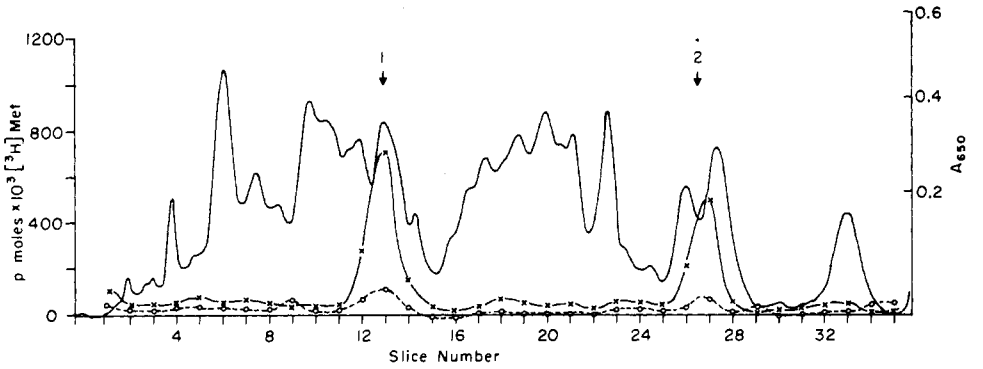

FIGURE 3: Amount of Met residues linked covalently to 50S proteins in an initiation complex preparation and in a preparation obtained under conditions of the formation of the initiation complex but with no f2 RNA added. Analysis by polyacrylamide gel electrophoresis in one dimension. The initiation complex was prepared with BrAc[^{3}H]Met-tRNA^{fMet} according to the procedures described in the Experimental Section except that f2 RNA was omitted from the preparation if so indicated. The protein sample from the initiation complex applied to the gel contained 10,200 cpm of [^{3}H]Met residues; that from the complex prepared without f2 RNA contained 1,680 cpm. Gels were stained with Coomassie Brilliant Blue (Chrambach *et al.*, 1967) and scanned at 650 nm in a Gilford spectrophotometer with a linear transport attachment. Gels to be analyzed for radioactivity were not stained but were cut into 2 mm thick slices which were oxidized in a Packard sample oxidizer and counted in Permafluor II (Packard): (1 and 2) the areas of the one-dimensional gel in which proteins L2 and L26–L27 migrate, respectively; (—) A_{650}; (x–x) picomoles of ^{3}H-labeled residues (from BrAcMet-tRNA^{fMet}) attached to 50S proteins from the initiation complex;)O--O([^{3}H]Met residues (from BrAcMet-tRNA^{fMet}) attached to 50S proteins from the complex formed in the absence of f2 RNA.

TABLE IV: Amounts of Met Residues from BrAc[^{3}H]Met-tRNA^{fMet} Linked Covalently to 50S Proteins in an Initiation Complex, in a Complex Prepared in the Same Way as the Initiation Complex but without f2 RNA, and in Salt-Washed 50S Subunits Incubated with BrAc[^{3}H]Met-tRNA^{fMet}.[a]

	pmol of [^{3}H]Met residues/mg of protein retained on filters
Initiation complex	1506
"Initiation complex" (without f2 RNA)	218
Salt-washed 50S subunits incubated with BrAc[^{3}H]Met-tRNA^{fMet}	82

[a] The initiation complex was prepared with BrAc[^{3}H]Met-tRNA^{fMet} according to the procedure in the Experimental Section except that f2 RNA was omitted if so indicated. Salt-washed 50S subunits were prepared according to the procedure of Pellegrini *et al.* (1972). The reaction mixture (0.5 ml) contained 12.5 mg of salt-washed 50S subunits which were treated with 15 nmol of BrAc[^{3}H]Met-tRNA^{fMet} (an amount equivalent to that found bound to whole 50S particles isolated from the "initiation complex" reaction). The mixture included 50 μM Tris-HCl (pH 7.4), 50 μM NH_4Cl, 0.3 mM magnesium acetate, and 6 mM 2-mercaptoethanol. The components of the reaction mixture were mixed at 4°. Immediately after adding BrAcMet-tRNA^{fMet}, 0.5 ml of a solution containing 4 M LiCl and 8 M urea was added. The solution was incubated at 4° for 72 hr. The amount of BrAcMet-tRNA^{fMet} added to the reaction mixture, 17 nmol, corresponded to that usually found in the standard reaction mixture at the time of its treatment with the LiCl–urea containing solution. 50S proteins were prepared from the initiation complex and the "initiation complex" (without f2 RNA) according to the procedure in the Experimental Section.

bind in a somewhat different way to ribosomal proteins (Harris *et al.*, 1971; Nathans and Neidle, 1963; Rychlik *et al.*, 1970).

Since L15–L16 is highly reactive toward A site bound BrAcPhe-tRNA, it may be that the different binding made of BrAcPhe-tRNA in the P site brings it closer to the A site than BrAcMet-tRNA^{fMet} (Eilat *et al.*, 1974). Although the binding of BrAcPhe-tRNA does not exactly parallel that of BrAcMet-tRNA^{fMet} in this case, the fact that BrAcPhe-tRNA reacted with L15–L16 can still participate in peptide formation argues that this binding is functionally significant for that system.

Reaction of BrAcMet-tRNA^{fMet} Requires Ribosome Binding and Does Not Take Place with Isolated Ribosomal Proteins. The dipeptide experiment mentioned above is the strongest evidence we have that BrAcMet-tRNA reacts with ribosomes while in a functionally significant site.

A supporting piece of evidence for affinity labeling is the demonstration that the extent of covalent reaction correlates with the extent of binding. This is usually done by competition with natural substrate. However, for ribosome studies, our previous work with BrAcPhe-tRNA^{Phe} indicated that mRNA dependence of covalent reaction is a more reliable test than tRNA competition (Pellegrini *et al.*, 1974). Therefore, the effect of f2 RNA on the covalent incorporation of [^{3}H]Met was examined. In the absence of f2 RNA, binding of BrAcMet-tRNA^{Met} is greatly reduced (see Table I) as is covalent reaction (see Table IV). A comparison of the one-dimensional gel labeling patterns of BrAcMet-tRNA^{fMet} in the presence and absence of f_2 RNA is given in Figure 3. f2 RNA stimulates the covalent reaction of BrAcMet-tRNA^{fMet} into particular proteins. Reaction with L2 is stimulated tenfold. This is comparable to the stimulation of binding of BrAcMet-tRNA^{fMet} in the initiation complex (see Table I). As found with BrAcPhe-tRNA (Pellegrini *et al.*, 1974), covalent reaction of the affinity analog directly parallels binding.

Reaction of BrAcMet-tRNA^{fMet} with isolated 50S subunits at the time of protein stripping is negligible (see Table IV). This strongly suggests that the reaction of BrAcMet-tRNA^{fMet} during isolation of 50S proteins is most unlikely. It is certainly not sufficient to account for the results presented here in Figures 2 and 3.

Discussion

The yield of the covalent reaction of BrAcMet-tRNA^{fMet} with ribosomal proteins in the initiation complex is only 5%

(Table III). This is comparable to the yield observed previously with BrAcPhe-tRNA in the presence of poly(uridylic acid) (Pellegrini *et al.*, 1974). Such low yields raise the suspicion that covalent reaction could have occurred either through a nonspecific reaction or from tRNAs which were not bound correctly to ribosomes or were bound to nonfunctional ribosomes. The following considerations can be marshalled to dispel this suspicion.

The best evidence for the occurrence of the covalent reaction with BrAcMet-tRNA^{fMet} correctly bound at the peptidyl site is the following result. Seventy-five per cent of the BrAcMet-tRNA^{fMet} which was found covalently attached to L2 reacted with [^{3}H]Ala-tRNA (Figure 2). This means that the very same tRNA molecules which can covalently react with ribosomal proteins can also bind correctly at the peptidyl site. We cannot prove, however, whether covalent attachment to the ribosome occurred before or after peptide bond formation. It should be noted, though, that our experimental protocol was designed to maximize the chances of the covalent reaction prior to peptide bond formation. Thus, a 40-min incubation of the BrAcMet-tRNA^{fMet} ribosome complex at 37° preceded a 40-min incubation with Ala-tRNA at 0°. This procedure makes it probable, though not certain, that the bulk of the peptide bond formation may have occurred with moieties of BrAcMet-tRNA^{fMet} which were already covalently attached to protein.

The origin of the low yield of the covalent reaction is still not clear. Possibilities include some destruction of the bromoacetyl moiety by reaction with any traces of 2-mercaptoethanol which may have survived our extensive dialysis procedures or by Tris buffer or even water. Bruton (1969) has suggested that α-haloacetylmethionyl-tRNA can react intramolecularly to yield a cyclic sulfinium compound which might then be incapable of functioning as an affinity label. Another possibility is a heterogeneity of ribosome binding modes so that only some fraction of affinity label is situated within reach of the reactive nucleophiles of ribosomal proteins. The two 50S proteins we label (*i.e.*, L2 and L27) are known to have very reactive sulfhydryls (Moore, 1971; R. R. Crichton, personal communication). However, S18 and L17 also have very reactive sulfhydryls and these are not labeled to any significant extent. In addition, the covalent reaction we observe is strongly dependent on the presence not only of the reagent, BrAcMet-tRNA^{fMet}, but also on that of the correct messenger RNA and, apparently, of the initiation factors. These facts make a nonspecific reaction seem unlikely.

The most reasonable conclusion from these results is that L2, and probably also L27, lie in the peptidyl site adjacent to the 3′ end of the initiator peptidyl-tRNA. Whether one or both of these proteins actually form part of the peptidyl transferase cannot be stated, but their spatial location in the ribosome makes this conceivable. The BrAc moiety does not appear to interfere substantially with the correct placement of the initiator tRNA analog in the P site of the ribosome. Neither does it appear to affect the subsequent Ef-Tu and GTP dependent binding of Ala-tRNA.

After this work was concluded, Hauptmann *et al.* (1974) reported similar studies with a different initiator tRNA analog, *p*-nitrophenylcarbamyl-Met-tRNA^{Met}. They find initiation factor dependent covalent reaction, although the ability of the bound product to participate in peptide transfer was not investigated. The main covalent product is protein L27, which was unequivocally identified by the use of specific antisera. The difference between our results and those of Hauptmann *et al.* (1974) can probably be explained most easily by the different chemical specificity of the two reagents used. BrAc will favor sulfhydryl reaction while *p*-nitrocarbamyl will favor amines.

These results add BrAcMet-tRNA^{fMet} to the list of affinity analogs which have been used successfully to identify ribosomal proteins at or near particular functional sites on the *E. coli* ribosome (Czernilofsky *et al.*, 1974; Pongs *et al.*, 1972, 1973; Sonenberg *et al.*, 1973; Oen *et al.*, 1973). The functioning of BrAcMet-tRNA^{fMet} in a natural messenger-directed system and the good agreement between these affinity labeling results and those with BrAcPhe-tRNA support the idea that AcPhe-tRNA (at least at its 3′ end) can bind to 70S ribosomes in a similar, if not identical, manner to fMet-tRNA^{fMet}. These results should encourage the use of BrAcPhe-tRNA, BrAcMet-tRNA^{fMet}, and similar analogs in studies of antibiotic action. Those antibiotics which are believed to distort the P site or alter peptidyl-tRNA binding are good candidates for further study.

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Isolation and Properties of Human Plasma α-1-Proteinase Inhibitor†

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ABSTRACT: Human α-1-proteinase inhibitor has been purified to homogeneity in excellent yield (60%) by a simple four-step procedure. The protein has a molecular weight of 53,000 by sedimentation equilibrium centrifugation and contains four half-cystine residues. The N-terminal has been identified as either glutamic acid or glutamine. In contrast to the results of others, the molecule has only two sialic acid residues, indicating that differences in charge between various phenotypes of α-1-proteinase inhibitor cannot be due solely to differences in sialic acid content. Isoelectric focussing indicates that the protein is elicitied mainly as two major isoinhibitors. The protein inhibits 2 mol of trypsin or 2 mol of chymotrypsin, suggesting that it acts as a "multiheaded inhibitor" with overlapping inhibitory sites.

Human α-1-proteinase inhibitor (α-1-PI),[1] more commonly called α-1-antitrypsin, is a glycoprotein which has been shown to be responsible for approximately 90% of the trypsin inhibitory capacity of plasma (Heimburger *et al.,* 1971). The broad specificity and high concentration of this protein in plasma and tissue fluids reflect its important role in preventing tissue proteolysis. The high incidence of phagocyte mediated proteolysis of lung tissue observed in some α-1-PI deficient individuals correlates with this function (Laurell and Eriksson, 1963; Fagerhol, 1972). This has resulted in an increased interest in both the properties and function of this protein.

During the past several years many laboratories have published methods for the isolation of human α-1-PI in various states of purification (Moll *et al.,* 1958; Bundy and Mehl, 1959; Schultze *et al.,* 1962; Shamash and Rimon, 1966; Liener *et al.,* 1973; Murthy and Hercz, 1973; Crawford, 1973). Unfortunately, none of the procedures used are desirable because the isolated inhibitor is obtained either in low yield or in a partially inactive form.

The isolation of α-1-PI is complicated primarily by two other plasma proteins, albumin and orosomucoid (α-1-acid glycoprotein). Albumin has the same molecular weight behavior as α-1-PI in gel exclusion chromatography; its ionic properties, too, mimic those of α-1-PI so closely as to render them unresolvable by ion exchange chromatography, particularly with the high proportion of albumin present in plasma. Orosomucoid is poorly resolved from α-1-PI by disc electrophoresis (Ornstein, 1964); detection of this impurity is further obscured since it stains very weakly with conventional protein stains. Contamination with orosomucoid is also particularly inconvenient for sugar analysis since it contains some 40% polysaccharide.

Recently, we described a technique for the preparation of albumin-depleted plasma (Travis and Pannell, 1973). By using this procedure, together with other conventional steps, we have succeeded in purifying α-1-PI to homogeneity with good recovery of inhibitory activity. This report describes the techniques used for the isolation of the inhibitor as well as some of the properties of the purified protein.

Experimental Section

Materials

Human plasma was obtained from St. Mary's Hospital, Athens, Ga. Sepharose-Blue Dextran was synthesized by the method of Travis and Pannell (1973). The final product had a capacity to bind 8 mg of albumin/ml of packed derivative. Porcine and human trypsins were prepared as previously described (Travis and Liener, 1965; Travis and Roberts, 1969). Both trypsin preparations were titrated with *p*-nitrophenyl guanidinobenzoate (Chase and Shaw, 1967) to determine the number of active sites. The porcine and human enzymes were found to have 82.5 and 86.5% active sites, respectively. Bovine trypsin, bovine α-chymotryp-

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[1] Abbreviations used are: α-1-PI, α-1-proteinase inhibitor; NP, normal plasma; SDS, sodium dodecyl sulfate.