Photo-Affinity Labeling of tRNA Binding Sites in Macromolecules. I. Linking of the Phenacyl-p-azide of 4-Thiouridine in (Escherichia coli)

Valyl-tRNA to 16S RNA at the Ribosomal P Site

(p-azidophenacyl bromide/tRNA function/nitrenes)

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ABSTRACT. The phenacyl-p-azide of 4-thiouridine in (E. coli) tRNA was prepared for use as a photo-affinity probe of tRNA binding sites on ribosomes. The derivatized tRNA was 90–100% as active as control tRNA for aminoacylation, nonenzymatic binding to the ribosomal P site, elongation factor Tu(EFTu)-dependent binding to the A site, EFTu-GTP-aa-tRNA ternary complex formation, and transfer of valine into polypeptide. Irradiation of p-azidophenacyl-[3H]valyl-tRNA bound noncovalently to the ribosomal P site resulted in covalent attachment of 15–20% of the noncovalently bound tRNA to the ribosomes. The binding occurred exclusively to the 16S RNA of the 30S ribosomal subunit, thus suggesting that the region of the ribosome within 9 Å of the 4-thiouridine of tRNA, when it is bound in the P site, is solely 16S RNA.

Chemical (1–3) and photochemical (4) affinity-labeled tRNA has been used by several groups to identify the ribosomal components which constitute tRNA binding sites on the ribosome. So far, this approach has been confined to the study of the peptidyl transferase center of the ribosome by the use of aa-tRNA suitably derivatized at the amino group of the aminocoyl moiety. The conflicting results obtained have been due at least in part to the use of affinity labels of different chemical specificity. By the use of chemical affinity labeling probes, Oen et al. (1) have identified the 30S proteins, L2 and L27, as being at or near the peptidyl-transferase center, and Kuechler’s laboratory (2, 3) have found L27, L15, and L16 to be near this site. On the other hand, Dospink and Matthei (4), using a nonspecific carbene photoaffinity label at the same place on the tRNA, found that reaction occurred only with 23S tRNA.

For our experiments we sought a reagent which, on the one hand, would show a general reactivity for a wide variety of functional groups in both RNA and protein but which, on the other hand, could be placed at different but defined sites in tRNA. The latter requirement suggested derivatization of minor nucleotides in tRNA since they occur at known loci, and in appropriate cases, only once in a given tRNA, while the former requirement dictated the use of photoaffinity labeling derivatives.

In this paper, we report the derivatization of the 4-thiouridine (Srd) residue in tRNA with p-azidophenacyl bromide, the formation of a covalent link with the ribosome, and the demonstration that 16S RNA is the only ribosomal component to react.

MATERIALS AND METHODS

Chemicals. (E. coli) tRNA was obtained from Boehringer-Mannheim or was prepared as previously described (5). The specific activity varied between 1200–1600 pmol/A260 unit. The tRNA was 92–95% free of the 4-thiouridine (position 8)-cytidine (position 13) light-induced crosslink, which was detected as previously described (5, 6). tRNA and unfractionated tRNA were prepared as described (6). Unfractionated E. coli aminoacyl-tRNA synthetase was prepared as described by Muench and Berg (7). Elongation factors EFTu and EFTs from E. coli (8) were kindly donated by Dr. D. Miller. Ribosomes from E. coli B were prepared according to Brot et al. (9) except that 1.0 M NH4Cl was used for the final wash. By analytical ultracentrifugation in 10 mM Mg++, they contained <2% free 30S particles, but a substantial amount, 35–40%, of free 50S particles.

[pH]Valine (30.6 Ci/mmole) was purchased from Amersham/Searle, and phenacyl bromide was obtained from Aldrich.

p-Azidophenacyl bromide (Fig. 1) was synthesized by the Chemical Research Division of Hoffmann-La Roche according to an unpublished procedure of Dr. S. Hixon, University of Massachusetts. We are extremely grateful to Dr. Hixon for a description of this procedure prior to publication. 4-Thiouridine disulfide was obtained from Cyclo Chemical Corp. and converted to 4-thiouridine as previously described (10).

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** Scheme for the reaction of p-azido phenacyl bromide with 4-thiouridine. In the maximally extended form the distance from the sulfur atom to the azido group is 9 Å and in the direction indicated.
Phenacyl (or p-Azidophenacyl) tRNA\textsuperscript{Val}. The procedure was that of Sestric \textit{et al.} (11) as modified by Yang and Soll (12). Twenty \(A_{490}\) units of purified tRNA\textsuperscript{Val}, in 1.0 ml of 0.1 M phosphate buffer at pH 7.4 was added to 9.5 ml of 4.9 mM (2000 X molar excess) phenacyl bromide or p-azidophenacyl bromide in 100% dimethylsulfoxide (Me\textsubscript{2}SO). The resultant reaction mixture (90% in Me\textsubscript{2}SO) was homogeneous. The reaction was terminated by addition of a 25-fold molar excess of mercaptoethanol over phenacyl bromide. After dialysis, derivatized tRNA was isolated by ethanol precipitation (2.5 volumes) from 0.2 M KOAc, pH 5.

Valyl-tRNA. Control and derivatized valyl-tRNA were prepared by scaling-up the standard charging assay (see below) except that [\textsuperscript{3}H]valine (30.6 Ci/mmol) was used.

\textbf{Photochemical Cross-Linking of \textasciitilde{S}rd (Position 8) and Cytidine (Position 13).} Irradiation, subsequent reduction, and assay of the fluorescent binucleotide produced was performed (6) in a final volume of 1.5 ml.

Assays for aminoclylation, valyl-tRNA-EFTu-GTP ternary complex formation, and EFTu-dependent binding to ribosomes were performed as described previously (13, 14). Synthesis of a (Val,Phe) copolypeptide was also assayed as described (14) except that reaction was for 10 min. Poly(U\textsubscript{5},G) was used in all assays in place of the poly(G,U,A) or poly(U\textsubscript{5},G) used previously. Nonenzymatic binding of the P site was assayed as described (14) except that tetracycline was omitted and 20 mM Mg(OAc)\textsubscript{2}, 8 \(\mu\text{g}\) of poly(U\textsubscript{5},G) and 10 \(A_{490}\) units of ribosomes were used. The extent of reaction was measured in all of the above assays, and in every case, it was proportional to the amount of tRNA or valyl-tRNA added to the mixture.

\textbf{Irradiation of [\textsuperscript{3}H]Val-p-azidophenacyl-tRNA–ribosome Complexes.} Ribosomal P site binding mixtures, described above, containing 20-30 pmol of [\textsuperscript{3}H]Val-p-azidophenacyl-tRNA in 0.5-1.0 ml total volume were incubated for 15 min at 30\(^\circ\). Ribosomes were in a 5- to 10-fold functional excess over tRNA as determined by preliminary titration. The Val-tRNA (70-75\%) was complexed under these conditions. The extent of tRNA binding to ribosomes was the same in the presence or absence of \(4 \times 10^{-4}\) M tetracycline, thus indicating that virtually all of the tRNA was bound at the ribosomal P site. The mixtures, in Pyrex vessels, were irradiated at 0\(^\circ\) for the times indicated, by means of a Rayonet RPR-100 photochemical reactor equipped with 350-nm lamps. Under these conditions, less than 0.05% of the light energy is transmitted below 310 nm.

\textbf{RESULTS}

\textbf{Reaction of tRNA\textsuperscript{Val} with phenacyl or p-azidophenacyl bromide}.

Reaction of \textasciitilde{S}rd with primary halides adjacent to a carbonyl or vinyl group results in the type of addition product illustrated in Fig. 1 (11, 15–17). When \textasciitilde{S}rd was mixed with a 2-fold excess of p-azidophenacyl bromide at pH 7.4 in 60% methanol, a rapid reaction occurred and produced a new compound with \(R_F\) (0.45) in CHCl\textsubscript{3}:MeOH (9:1) on silica gel thin-layer chromatography, intermediate between \textasciitilde{S}rd (0.20) and the bromide (0.75). The structure of the product was characterized by its absorption spectrum, which did not vary between pH 2 and 10, and showed no evidence of the 330-nm band characteristic of N\textsubscript{6}-substituted 4-thiouridine (17). Azide (2120 cm\textsuperscript{–1}), acetophenone ketone (1690 cm\textsuperscript{–1}), and ribose hydroxyl (33–3400 cm\textsuperscript{–1}) infrared absorption bands were also present. A detailed characterization of this structure will be presented elsewhere.

Reaction of \textasciitilde{S}rd at position 8 in \textit{E. coli} valine tRNA with the p-azidophenacyl group could not be monitored spectrally due to interference from the tRNA, and the probe was not available in radioactive form. Consequently, an indirect method was used to measure the extent of reaction with the probe. Photochemically-induced crosslinking of \textasciitilde{S}rd (position 8) with cytidine (position 13) in tRNA yields a binucleotide which, after chemical reduction, is highly fluorescent (6). If, however, the sulfur atom of the \textasciitilde{S}rd is blocked due to reaction with phenacyl bromide, crosslink formation cannot occur (18) and there will be no fluorescent product. A decrease in the specific fluorescence of tRNA after irradiation is, therefore, a measure of the loss of \textasciitilde{S}rd.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Reaction mixture} & \textbf{Reaction time*} (min) & \textbf{Irradiation} & \textbf{tRNA\textsuperscript{Val}} & \textbf{tRNA\textsuperscript{Met}} & \textbf{tRNA\textsuperscript{Mixed}} \\
\hline
Complete & 0 & + & 15.1 & 11.4 & 10.5 & 11.6\textsuperscript{†} \\
Complete & 5 & + & 3.9 & 3.3 & 2.1 & 2.3\textsuperscript{†} \\
Complete & 5 & - & 1.5 & 1.6 & 0.6 & 0.6\textsuperscript{†} \\
Minus phenacyl Br & 0 & + & 18.2 & 13.2 & 13.3 & 13.3 \\
Minus phenacyl Br & 5 & + & 16.1 & 13.3 & 13.3 & 13.3 \\
Minus phenacyl Br & 5 & - & 1.6 & 1.6 & 1.6 & 1.6 \\
\% Reaction & 83 & 83 & 84 & 84 \\
\hline
\end{tabular}
\caption{Reaction of phenacyl bromide with 4-thiouridine in tRNA}
\end{table}

\* For 0 time, tRNA was added after a 2-min reaction with mercaptoethanol (25 X molar excess).

\textsuperscript{†} Mixture preincubated 5 min before adding tRNA.

\textsuperscript{†} We thank Mr. S. Traiman of the Physical Chemistry Department, Chemical Research Division, Hoffmann-La Roche, for the IR analysis.
The reaction with Srd in tRNA was very rapid, unlike the one described by Yang and Soll (12), being complete in less than 5 min at room temperature (Table 1). The table also shows that there was no loss of Srd if phenacyl bromide was added, and that trnaVal, tRNA\(^{Met}\), and unfractionated tRNA, all behaved similarly. On lines 3 and 6 are indicated values for unirradiated samples which are measurements of the amount of binucleotide contaminating the tRNA samples, and can be considered blank values. The extent of reaction was 83–84% by this assay in all three cases, and could not be further increased by increasing the reaction time, reagent concentration, or by denaturation of the tRNA at 70° for 4 min in 1 mM EDTA. The last column of the table shows that the phenacyl bromide was not unstable since 5-min preincubation in the 90% Me2SO solution before the addition of a small volume of concentrated tRNA did not affect the extent of reaction. At the present time, we do not have an explanation for the apparent lack of reactivity of 16% of the tRNA.

The above results showed that Srd in tRNA could react with p-azidophenacyl bromide. Model studies with similar compounds (11, 12, 15, 16) have shown that the major nucleotides do not react, and pseudouridine is also unreactive under these conditions (unpublished experiments). Nevertheless, a direct test of the specificity of the reaction with nucleotides in tRNA using radioactive p-azidophenacyl bromide would be desirable.

**Functional activity of derivatized tRNAs**

Derivatization of Srd in tRNA with various chemical agents does not affect amino-acid acceptor activity (literature cited in ref. 17). In order to be sure that reaction of Srd with this probe did not alter the function of the tRNA molecule, the activity assays listed in Table 2 were examined. The values for amino acid acceptor activity, binding to the ribosomal P site, EFTu-GTP ternary complex formation, and the ability to donate aminoacyl-tRNA to ribosomes were all 90–100% of untreated tRNA control values. It is interesting to note that the presence of the photolabile azido group on the phenacyl moiety restored fully the ability of the derivatized tRNA to accept amino acid.

**Covalent link formation between [3H]Val-p-azidophenacyl-tRNA and ribosomes**

The kinetics of covalent crosslink formation between p-azidophenacyl-tRNA and ribosomes is presented in Fig. 2. Covalent link formation was dependent on irradiation as well as the presence of both polynucleotide and ribosomes. These controls show that the tRNA was not activated while free in solution, with subsequent reaction occurring randomly with the ribosome, polynucleotide, or itself, to give a filter-adsorbable product. Phenacyl-tRNA, which lacks the photolabile azido group, did not show any crosslink formation to ribosomes on irradiation of a complete reaction mixture (data not shown). Crosslinking was essentially complete at 4 hr, with an efficiency of covalent bond formation of 15–20% of the noncovalently bound tRNA in 20 mM Mg\(^{++}\) or 10–15% of the total input tRNA. The reaction is probably so slow because of inefficient absorption of energy; the absorption maximum for the 4-thiouridine derivative is at 305 nm, whereas the lamp emission maximum is at 350 nm and Pyrex glass was used to filter out light below 305 nm so that radiation damage to the ribosomes or tRNA would be avoided. The relatively low (20%) yield probably is due to competition between buffer molecules and ribosomal components for the reactive nitrene. A similar slow rate of covalent linking and a lower efficiency (3%) was observed by Bispink and Matthei (4).
FIG. 3. Sucrose density gradient separation of 30S and 50S ribosomal subunits following covalent attachment of [3H]valyl-phenacyl tRNA. Incubation mixtures of tRNA and ribosomes (1.0 ml) were irradiated for 4 hr at 0° as in Fig. 2, dialyzed overnight against 2 liters of 10 mM Tris-HCl at pH 7.0, 50 mM KCl, 6 mM mercaptoethanol, and 0.3 mM MgCl₂, and applied to 36 ml of a 10%-30% sucrose gradient in the same buffer and centrifuged for 16 hr at 24,000 rpm in an SW 27 rotor. 1.0 ml fractions were collected, 0.1 ml aliquots were diluted 1:10, and the A₂₆₀ and radioactivity of each fraction were determined. C, A₀₋₀₀; ⋄, △ (different scale), complete incubation mixture; Δ, incubation mixture minus poly(U₉₋₀); ■, complete incubation mixture but unirradiated.

Separation of 30S and 50S subunits containing attached [PH]Val-tRNA

The [3H]Val-phenacyl-tRNA-70S ribosome covalent complex was dissociated into 30S and 50S subunits and separated on a sucrose gradient as shown in Fig. 3. When polynucleotide was present in the original reaction mixture, all of the ribosome-associated radioactivity migrated with the 30S peak. There was no detectable radioactivity associated with the 50S subunit. If polynucleotide was omitted from the reaction, or the complete incubation mixture was not irradiated, no radioactivity was associated with either subunit. The radioactivity near the top of the gradient corresponds to unreacted tRNA which was present in the reaction mixture.

Separation of the 30S subunit-[PH]Val-tRNA complex into 30S proteins and 16S RNA

The 30S subunit-[3H]Val-phenacyl-tRNA covalent complex was isolated and applied to a sucrose gradient which was 0.5% in sodium dodecylsulfate, in order to separate the 30S ribosomal proteins from the 16S RNA. Under these conditions, the proteins complex with the sodium dodecylsulfate and do not migrate into the gradient. The results are shown in Fig. 4. The A₀₋₀₀ peak corresponds to 16S RNA and all the radioactivity sediments with this peak. From these results, we conclude that only 16S RNA is within 9 Å of the sulfur atom of the thioridine residue when tRNA is bound at the ribosomal P site.

DISCUSSION

Photo-affinity labeling has a number of advantages over chemical affinity labeling. Since the photo-affinity probe is not reactive until irradiated, a tRNA, derivatized with such a label, can first be bound to the ribosome under the appropriate conditions with subsequent activation of the probe by irradiation. This allows control of the point in time at which covalent link formation occurs. The carbene or nitrene formed upon irradiation is very reactive as well as nonspecific (19), and is, therefore, capable of reaction with almost any component of either the ribosomal RNA or protein which might be within range of the probe. Aryl azides are preferred to alkyl azides or diazo compounds because of the longer lifetime of arylnitrenes in aqueous solution (19) and because the red-shifted absorption spectrum of the parent aryl azide means that photo-activation can be accomplished at wavelengths that are not deleterious to either the ribosome or tRNA.

For these initial studies, we chose the minimal length probe that would incorporate the needed bifunctional groups in order to detect only that part of the binding site closest to the tRNA. The compound we settled on, p-azidophenacyl bromide, was independently chosen by Hixson and coworkers for different affinity labeling studies (20).

As shown in Fig. 1, the active nitrene atom extends 9 Å from the sulfur atom of the thioridine and in a line angled approximately 120° from the C₅-S₅ vector. In the 3-dimensional model of tRNA described by Kim et al. (21), rotation about this vector would be restricted when the p-azidophenacyl group is attached, so that the active nitrene is probably constrained to a region below the aminoacyl acceptor arm and to the rear of the plane of the structure as given in the 3-dimensional representation. (Fig. 4 of ref. 21.) (We thank S. Kim for helpful discussion and analysis of the probable position of this probe in the 3-dimensional structure of tRNA.) A more detailed description of the position of the active nitrene with respect to the rest of the tRNA molecule must await further analysis of the position of the 4-thioridine base in the crystal structure and evidence that this structure is maintained when tRNA is bound to the ribosome. Irrespective of the actual structure on the ribosome, exclusive (>99%) labeling of the 16S tRNA implies that no component of the 50S subunit nor the 30S proteins is within range of the probe. If they had been, at least some crosslinking should have been found in view of the high reactivity and lack of chemical specificity of nitrene reactions (19).
The only other nonselective affinity probe used up to now was attached to tRNA at the aminoacyl end and resulted in exclusive labeling of the 23S rRNA of the 50S subunit (4). Although there is too much flexibility inherent in the combined C-C-A unpaired terminal nucleotides plus the derivatized aminoacyl group, to allow any strong conclusions to be drawn about tRNA orientation from a comparison of our results with these findings, it does seem clear that more of the tRNA structure must be in contact with the 30S subunit than has been supposed up to now.

Numerous other studies have implicated 30S components in tRNA binding sites (22–27). However, all suffer from two disadvantages. First, it is not possible to distinguish between a direct effect on the tRNA binding site and an allosteric effect which indirectly alters the binding site, and second, none of the previous approaches give any information about what region of the tRNA is involved. We believe the approach described in this paper is direct, and within the normal geometric constraints, unambiguous, and will prove to be of considerable utility.

We chose tRNAVal for study of the P binding site in order to be able to compare P and A site binding with the same tRNA. Preliminary results at the A site already show differences in the attachment pattern. Both 50S and 30S subunits were about equally labeled, and the attached tRNA was partitioned differently between the rRNA and the protein. The contrast between this result and that reported above reinforces our conclusion that the binding studied in the body of this paper is predominantly P site binding. In addition, preliminary studies with N-acetyl[3H]Val-phenacyl tRNA gave results identical to those presented here, further indicating that the binding occurred to the P site.

The amount of irradiation needed for activation does not appear to be deleterious since ribosomes isolated after such treatment were as active as controls for binding Val-tRNA.

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