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Photochemical Cross-Linking of Yeast tRNA^{Phe} Containing 8-Azidoadenosine at Positions 73 and 76 to the *Escherichia coli* Ribosome†

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ABSTRACT: The 3′-terminal -A-C-C-A sequence of yeast tRNA^{Phe} has been modified by replacing either adenosine-73 or adenosine-76 with the photoreactive analogue 8-azidoadenosine ($8N_3A$). The incorporation of $8N_3A$ into tRNA^{Phe} was accomplished by ligation of 8-azidoadenosine 3′,5′-bisphosphate to the 3′ end of tRNA molecules which were shortened by either one or four nucleotides. Replacement of the 3′-terminal A76 with $8N_3A$ completely blocked aminoacylation of the tRNA. In contrast, the replacement of A73 with $8N_3A$ has virtually no effect on the aminoacylation of tRNA^{Phe}. Neither substitution hindered binding of the modified tRNAs to *Escherichia coli* ribosomes in the presence of poly(U). Photoreactive tRNA derivatives bound noncovalently to the ribosomal P site were cross-linked to the 50S subunit upon irradiation at 300 nm. Nonaminoacylated tRNA^{Phe} containing $8N_3A$ at either position 73 or position 76 cross-linked exclusively to protein L27. When *N*-acetylphenylalanyl-tRNA^{Phe} containing $8N_3A$ at position 73 was bound to the P site and irradiated, 23S rRNA was the main ribosomal component labeled, while smaller amounts of the tRNA were cross-linked to proteins L27 and L2. Differences in the labeling pattern of nonaminoacylated and aminoacylated tRNA^{Phe} containing $8N_3A$ in position 73 suggest that the aminoacyl moiety may play an important role in the proper positioning of the 3′ end of tRNA in the ribosomal P site. More generally, the results demonstrate the utility of $8N_3A$-substituted tRNA probes for the specific labeling of ribosomal components at the peptidyltransferase center.

Photoaffinity labeling has proven to be a useful method for identifying components of the *Escherichia coli* ribosome that are located in or near the two main tRNA binding sites (Ofengand, 1980; Ofengand et al., 1986). In most cases, such studies have been performed with aminoacyl-tRNA molecules containing photolabile substituents either on the aminoacyl moiety or on one of the several naturally occurring bases containing reactive thiol or amino groups. As the reagents used for tRNA modification are typically 10–20 Å in length—and often quite bulky—there is always a possibility that the substituents may perturb the structure of the tRNA molecule, interfere with proper tRNA–ribosome binding, or cross-link to ribosomal components at some distance from the actual site of tRNA interaction (Cooperman, 1980). Although photochemical reaction of a specific tRNA base with a unique ribosomal site to form a "zero-length" cross-link would be highly desirable in these investigations, such cross-links have been completely characterized only for the attachment of the 5′-anticodon base of tRNA$_1^{Val}$ to the cytosine residue at position 1400 of *E. coli* 16S rRNA (Prince et al., 1982) and to the equivalent base in yeast 18S rRNA (Ehresmann et al., 1984). The generality of the above approach is limited by the absence of suitable photoreactive moieties in the vast majority of tRNA molecules. In an effort to overcome this difficulty, while preserving the advantages of zero-length cross-linking, we have investigated the utility of replacing normal nucleosides with photolabile nucleoside analogues to produce tRNA derivatives which can be cross-linked to ribosomes under conditions that are unlikely to adversely affect tRNA structure, ribosome integrity, or tRNA–ribosome association.

The 8-azidopurine analogues developed by Czarnecki et al. (1979) are probably the most common photolabile nucleosides in use today. While they have been applied to the exploration of nucleotide binding sites in a wide variety of proteins (Haley, 1983), they have rarely been employed in the study of nucleic acids. We believe that the 8-azidopurines have great potential for elucidating tRNA–ribosome contacts in cases where they can be specifically incorporated into the RNA chain. In this report, we described the introduction of 8-azidoadenosine ($8N_3A$)[1] into positions 73 and 76 at the 3′ end of yeast tRNA^{Phe}. When bound to the ribosomal P site in the presence of poly(U), the modified tRNA^{Phe} molecules form essentially zero-length cross-links to the ribosome upon irradiation of the complexes with light of 300 nm. The particular components

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[1] Abbreviations: $8N_3A$, 8-azidoadenosine; pNp, nucleoside 3′,5′-bisphosphate; [$8N_3A73$]tRNA^{Phe} and [$8N_3A76$]tRNA^{Phe}, tRNA^{Phe} derivatives containing $8N_3A$ at positions 73 and 76, respectively; tRNA^{Phe}(–A), tRNA^{Phe}(–CA), tRNA^{Phe}(–CCA), and tRNA^{Phe}(–ACCA), tRNA^{Phe} from which one, two, three, or four 3′-terminal nucleotides have been removed, respectively; [^{32}P]tRNA, tRNA containing ^{32}P adjacent to the $8N_3A$ residue; AcPhe, *N*-acetylphenylalanyl; PEI, poly(ethylenimine); EDTA, ethylenediaminetetraacetic acid; TCA, trichloroacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid.

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labeled depend both on the position of the $8N_3A$ residue and on the presence or absence of the aminoacyl group, in the tRNA.[2]

Experimental Procedures

Materials. Yeast tRNA^{Phe}, calf intestine alkaline phosphatase, and poly(U) were purchased from Boehringer Mannheim. [^{3}H]Phenylalanine (48.3 Ci/mmol) and [5′-^{32}P]pCp (3000 Ci/mmol) were products of New England Nuclear. [α-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham. Crude [γ-^{32}P]ATP was from ICN. Puromycin, adenosine 3′-monophosphate, and 8-azidoadenosine 3′,5′-cyclic monophosphate were from Sigma. T4 polynucleotide kinase and T4 RNA ligase were obtained from Pharmacia. RNase T_1 was purchased from Calbiochem-Behring. Snake venom phosphodiesterase was from Worthington. PEI–cellulose plates were purchased from Macherey & Nagel. [5′-^{32}P]p$8N_3$Ap and [5′-^{32}P]pAp were synthesized as described by Wower et al. (1988). Partially purified yeast phenylalanyl-tRNA synthetase was prepared by the method of Schmidt et al. (1971). Yeast tRNA nucleotidyltransferase (ATP[CTP]:tRNA nucleotidyltransferase) was prepared according to Sternbach et al. (1971). 70S tight-couple ribosomes were isolated from *E. coli* MRE600 as described by Rheinberger and Nierhaus (1980).

Preparation of tRNA^{Phe} Derivatives. tRNA^{Phe} truncated by either one or four nucleotides at the 3′ terminus was prepared by stepwise Whitfield degradation according to Paulsen and Wintermeyer (1984a). Addition of ^{32}P-labeled nucleoside bisphosphates to the 3′ terminus of the truncated tRNA molecules was performed in reaction mixtures containing 50 mM HEPES, pH 7.5, 15 mM $MgCl_2$, 10 μM ATP, 0.01 mg/mL serum albumin, 0.5–1.0 μM truncated tRNA as the acceptor, 1 μM [5′-^{32}P]p$8N_3$Ap, [5′-^{32}P]pAp, or [5′-^{32}P]pCp as the donor, and 200 units/mL RNA ligase. The mixtures were incubated for 1 h at 37 °C or for 15 h at 4 °C in the dark. In order to remove the 3′-terminal phosphate from the [^{32}P]tRNAs, alkaline phosphatase was added to a final concentration of 20 units/mL, and incubation was continued for 30 min at 37 °C. The labeled tRNA^{Phe} species were purified by electrophoresis through 10% polyacrylamide gels [acrylamide–*N*,*N*′-methylenebis(acrylamide), 19:1 by weight] in 100 mM Tris–100 mM H_3BO_4, pH 8.3, containing 2.5 mM EDTA and 8 M urea. The 3′-terminal -C-C-A sequence of [$8N_3$A73]tRNA^{Phe}(–CCA) was regenerated by incubating the tRNA derivative at a concentration of 10 A_{260} units/mL with 0.2 mM ATP (or [α-^{32}P]ATP), 0.4 mM CTP, and an appropriate amount of yeast nucleotidyltransferase in 20 mM glycine, pH 9.5, 17 mM KCl, and 7 mM $MgCl_2$ for 30 min at 37 °C. After incubation, the tRNA was precipitated with ethanol and isolated by denaturing polyacrylamide gel electrophoresis as above.

Aminoacylation of tRNA^{Phe} and Its Derivatives. Up to 0.1 A_{260} unit of unmodified or modified tRNA^{Phe} was aminoacylated in a 0.1-mL reaction mixture containing 100 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 15 mM ATP, 10 μM [^{3}H]phenylalanine, and an appropriate amount of yeast aminoacyl-tRNA synthetase. The reaction was carried out for 1 h at 37 °C. The extent of aminoacylation was determined by the method of Hirst Bruns and Philipps (1970) after repair of the -C-C-A terminus using [α-^{32}P]ATP. In the case of unlabeled tRNA, aminoacylation was assessed by measurement of TCA-precipitable ^{3}H radioactivity. Phe-[$8N_3$A73]-tRNA^{Phe} was acetylated according to Haenni and Chapeville (1966).

Formation and Analysis of tRNA–Ribosome Complexes. Noncovalent binding of photoreactive derivatives of tRNA^{Phe} to 70S ribosomes was performed by incubating 150 pmol/mL either [^{32}P]tRNA^{Phe}, Ac[^{3}H]Phe-tRNA^{Phe}, or Ac[^{3}H]Phe-[^{32}P]tRNA^{Phe} with 100 μg/mL poly(U) and 620 pmol/mL 70S tight couples in 50 mM Tris-HCl, pH 7.4, 100 mM NH_4Cl, and 20 mM $MgCl_2$ for 10–15 min at 37 °C. Mixtures were then chilled to 0 °C for subsequent treatment. The amount of tRNA^{Phe} bound noncovalently to the ribosomes was judged from the retention of ^{32}P or ^{3}H on membrane filters in the presence of 20 mM $MgCl_2$ (Nirenberg & Leder, 1964). Association of aminoacylated tRNA with the ribosomal P site was verified by the reduction in ^{3}H bound to the filter after the ribosomal complexes were incubated with 1 mM puromycin for an additional 15 min at 25 °C.

Cross-linking of nonaminoacylated tRNA^{Phe} or AcPhe-tRNA^{Phe} containing $8N_3A$ to the ribosomes was accomplished by irradiating noncovalent 70S complexes for 10 min at 4 °C in a Rayonet photochemical reactor (Model RPR-100) equipped with four RPR-3000-Å lamps. The percentage of cross-linking was calculated as the ratio of radioactivity bound to the filter in 0.1 mM $MgCl_2$ to that retained at 20 mM $MgCl_2$ (Ofengand et al., 1979). The irradiated complexes were treated with 10 mM DTT, precipitated with ethanol, resuspended in 10 mM Tris-HCl, pH 7.6, 50 mM KCl, 0.25 mM $MgCl_2$, and 0.05% (v/v) 2-mercaptoethanol, and centrifuged through a 27-mL 10–30% (w/v) sucrose gradient in the same buffer for 100 min at 40 000 rpm in a Beckman VTi50 rotor. The gradient effluent was monitored for absorbance at 260 nm and ^{32}P radioactivity (Čerenkov). The subunits were pooled, precipitated with ethanol, resuspended in 10 mM sodium acetate, pH 5.0, 400 mM LiCl, 2.5 mM EDTA, and 0.5% (w/v) SDS, and centrifuged through a 15-mL 5–20% (w/v) sucrose gradient in the same buffer for 145 min at 40 000 rpm in a Beckman VTi50 rotor to separate tRNA–protein and tRNA–rRNA complexes. The absorbance was monitored, and fractions were analyzed for radioactivity. Fractions containing ^{32}P radioactivity were pooled and precipitated with ethanol.

Identification of Ribosomal Proteins Cross-Linked to tRNA^{Phe} Species Labeled with [5′-^{32}P]p$8N_3$Ap. [^{32}P]tRNA–protein complexes (2000–5000 cpm) were resuspended in 10–50 μL of 10 mM Tris-HCl, pH 7.8, 0.5 mM EDTA, and 0.1% (w/v) SDS and digested with 25 units of RNase T_1. The mixtures were first incubated for 1 h at 37 °C followed by an additional 10 min at 60 °C. After incubation, the complexes were precipitated with ethanol, and the pellets were dissolved in a buffer appropriate for either electrophoresis or immunological tests.

Preliminary identification of protein(s) cross-linked to the tRNA derivatives was performed by one-dimensional polyacrylamide gel electrophoresis according to Laemmli and Favre (1973). Further analysis of the cross-linked proteins was carried out by two-dimensional gel electrophoresis. The first dimension was as described by Mets and Bogorad (1974), and the second dimension was according to the method of Kaltschmidt and Wittmann (1970). Final identification of the cross-linked protein(s) was achieved by the immunological spot test described by Gulle et al. (1987).

Results

Preparation of tRNA^{Phe} Containing 8-Azidoadenosine. A scheme for the preparation of yeast tRNA^{Phe} containing $8N_3A$

[2] A preliminary report of this work was presented in a poster at the 76th Annual Meeting of the American Society of Biological Chemists, Washington, DC, June 8–12, 1986 (Wower et al., 1986).

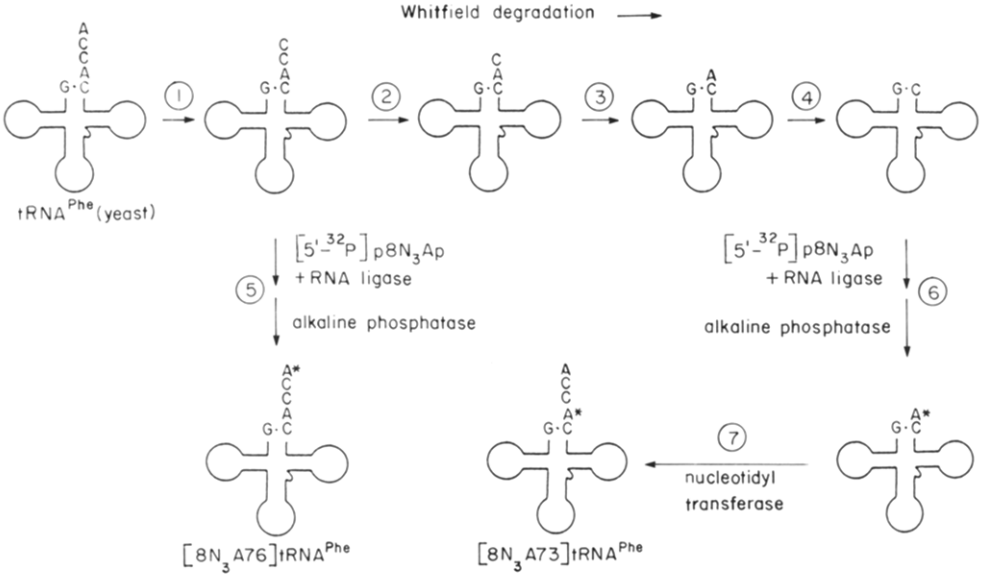

FIGURE 1: Preparation of yeast $tRNA^{Phe}$ containing $8N_3A$ at positions 73 and 76. Samples of $tRNA^{Phe}$ were shortened by one or four nucleotides using the Whitfield degradation procedure (steps 1–4). The truncated molecules were then ligated with $p8N_3Ap$, and the 3′-terminal phosphate was removed with alkaline phosphatase (steps 5 and 6). Finally, the -C-C-A end of $[8N_3A73]tRNA^{Phe}$(–CCA) was repaired with nucleotidyltransferase (step 7). A* denotes 8-azidoadenosine.

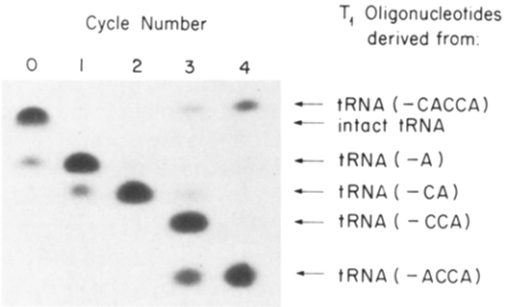

FIGURE 2: Analysis of oligonucleotides from the 3′ end of $tRNA^{Phe}$ after each of four cycles of the Whitfield degradation. Intact and truncated $tRNA^{Phe}$s, prepared as described under Experimental Procedures, were labeled at the 3′ end with [5′-^{32}P]pCp using RNA ligase, digested to completion with RNase T_1, and subjected to electrophoresis through a 20% polyacrylamide gel in 100 mM Tris–100 mM H_3BO_4, pH 8.3, containing 2.5 mM EDTA and 8 M urea. The tRNA variants from which the oligonucleotides were derived are indicated at the right.

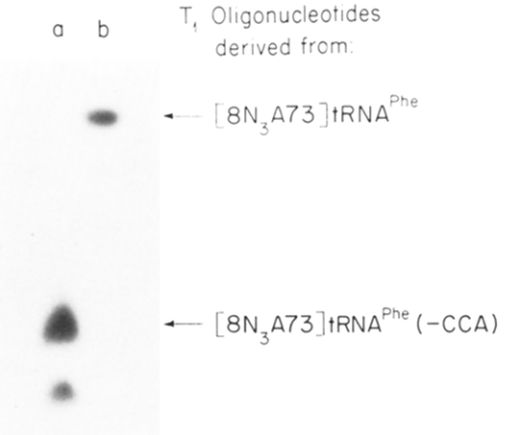

FIGURE 3: Electrophoretic analysis of 3′-terminal oligonucleotides from $[8N_3A73]tRNA^{Phe}$. (a) $[^{32}P][8N_3A73]tRNA^{Phe}$(–CCA) and (b) $[^{32}P][8N_3A73]tRNA^{Phe}$ after repair of the 3′ end with nucleotidyltransferase were digested with RNase T_1 and subjected to gel electrophoresis as described in the legend to Figure 2. tRNA variants from which the oligonucleotides were derived are indicated at right.

at positions 73 and 76 is shown in Figure 1. $tRNA^{Phe}$(–A) and $tRNA^{Phe}$(–ACCA) were prepared by subjecting yeast $tRNA^{Phe}$ to one or four cycles of the Whitfield degradation which consists of periodate oxidation of the 3′-terminal ribose, cleavage of the oxidized nucleoside with aniline, and removal of the 3′-terminal phosphate with RNase-free alkaline phosphatase. RNase T_1 digests of the products of each cycle were examined by gel electrophoresis as illustrated in Figure 2. As some of the tRNA in the original preparation lacked the 3′-terminal adenosine, the desired products, $tRNA^{Phe}$(–A) and $tRNA^{Phe}$(–ACCA), contained a small amount of tRNA molecules shortened by an extra nucleotide.

The $tRNA^{Phe}$(–A) and $tRNA^{Phe}$(–ACCA) preparations were extended at the 3′ end with [5′-^{32}P]$p8N_3Ap$ in the presence of T4 RNA ligase. The reaction was monitored by measuring the radioactivity incorporated into tRNA after the tRNA had been separated from unreacted [5′-^{32}P]$p8N_3Ap$ by gel electrophoresis. As a control, the incorporation of [5′-^{32}P]pAp into the truncated tRNAs was also followed. The yield of the reaction was estimated to be 85–95% in all cases. As observed by Paulsen and Wintermeyer (1984a), the incorporation of nucleoside bisphosphates into $tRNA^{Phe}$(–ACCA) proceeded faster and more efficiently at 37 °C than at lower temperatures. We did not, however, note any differences between pAp or $p8N_3Ap$ with respect to the extent of ligation obtained with any of the truncated tRNA substrates.

The -C-C-A end of $tRNA^{Phe}$ containing $8N_3A$ at position 73 was restored by the action of yeast nucleotidyltransferase, and the resulting $[8N_3A73]tRNA^{Phe}$ was separated from contaminating $[8N_3A72]tRNA^{Phe}$(–ACCA), which is not a substrate for nucleotidyltransferase, by electrophoresis through denaturing polyacrylamide gels. The homogeneity of the $[8N_3A73]tRNA^{Phe}$ and $[8N_3A76]tRNA^{Phe}$ preparations was determined by analysis of their RNase T_1 digestion products. The digest of $[8N_3A73]tRNA^{Phe}$ contained ^{32}P only in the pentanucleotide C-$8N_3$A-C-C-A while that of unrepaired tRNA yielded the labeled dinucleotide C-$8N_3$A (Figure 3). Hydrolysis of $[8N_3A76]tRNA^{Phe}$ with RNase T_1 demonstrated that the ^{32}P-labeled pentanucleotide C-A-C-C-$8N_3$A was contaminated with less than 5% of the ^{32}P-labeled tetranucleotide C-A-C-$8N_3$A (data not shown). The presence of the

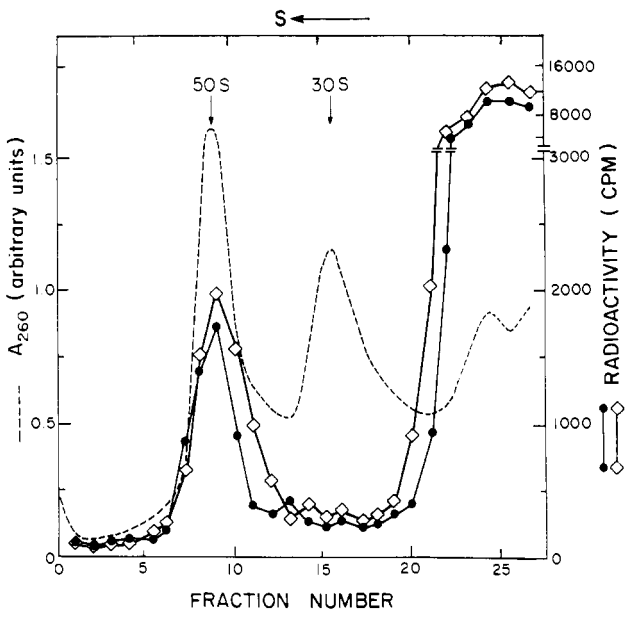

FIGURE 4: Cross-linking of AcPhe-[8N_3A73]tRNA^{Phe} and [8N_3A73]tRNA^{Phe} to the 50S subunit. Noncovalent complexes of AcPhe-[^{32}P][8N_3A73]tRNA^{Phe} and [^{32}P][8N_3A73]tRNA^{Phe} with poly(U) and 70S ribosomes were formed as described under Experimental Procedures, irradiated at 300 nm, and centrifuged through 10–30% sucrose gradients in 10 mM Tris-HCl, pH 7.6, 50 mM KCl, 0.25 mM $MgCl_2$, and 0.05% 2-mercaptoethanol at 40000 rpm for 100 min at 4 °C in a Beckman VTi50 rotor. (●) AcPhe-[8N_3A73]tRNA^{Phe}; (◇) nonaminoacylated [8N_3A73]tRNA^{Phe}.

latter product in the digest reflected the fact that a small fraction of the initial tRNA preparation lacked the 3′-terminal adenosine.

Both [8N_3A73]tRNA^{Phe} and [8N_3A76]tRNA^{Phe} were tested for their amino acid acceptor activity. In the case of [8N_3A76]tRNA^{Phe}, no aminoacylation was detected even when a number of experimental conditions such as incubation time, pH, buffers, ionic strength, and the presence of dimethyl sulfoxide were varied. Others have also noted that certain modifications of the 3′-terminal A residue impair or abolish tRNA acceptor activity (Sprinzl & Cramer, 1979; Paulsen & Wintermeyer, 1984a). By contrast, the specific acceptance of [8N_3A73]tRNA^{Phe}, which ranged from 1075 to 1180 pmol/A_{260} unit, was only 8% less than that of unmodified tRNA^{Phe} prepared in the same way except that pAp was used instead of p8N_3Ap in the ligation reaction. Phe-[8N_3A73]-tRNA^{Phe} was acetylated when required by the method of Haenni and Chapeville (1966).

Cross-Linking of Modified tRNA^{Phe} to E. coli Ribosomes. Either nonaminoacylated [8N_3A73]tRNA^{Phe} or AcPhe-[8N_3A73]tRNA^{Phe} was bound nonenzymatically to the P site of 70S tight-couple ribosomes in the presence of poly(U). All experiments were conducted at a 1:4 molar input ratio of tRNA to ribosomes. The extent of poly(U)-dependent binding in the case of the modified tRNAs was essentially quantitative and was comparable to that of unmodified AcPhe-tRNA^{Phe} (data not shown). The noncovalent complexes were irradiated with 300-nm lamps for different lengths of time. Following irradiation, the complexes were filtered through nitrocellulose membranes under conditions in which noncovalently bound tRNAs were dissociated from the ribosome. The covalent attachment of nonaminoacylated [8N_3A73]tRNA^{Phe} and AcPhe-[8N_3A73]tRNA^{Phe} was then estimated from the ^{32}P radioactivity that remained associated with the filters. A standard irradiation time of 10 min was adopted as cross-linking reached its maximum of 12–13% within that period.

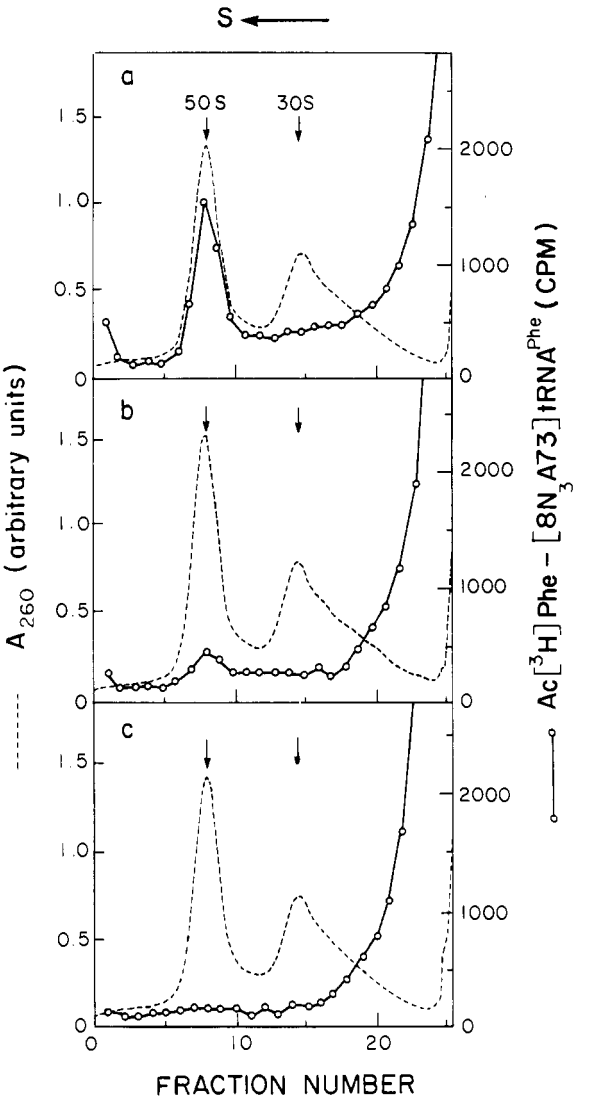

FIGURE 5: Effects of puromycin on complexes containing Ac[^{3}H]-Phe-[8N_3A73]tRNA^{Phe}. Noncovalent Ac[^{3}H]Phe-[8N_3A73]-tRNA^{Phe}-poly(U)·70S ribosome complexes were irradiated at 300 nm and separated into subunits by sucrose gradient centrifugation as described in the legend to Figure 4. (a) Complete incubation mixture; (b) complete incubation mixture treated with puromycin after irradiation; (c) complete incubation mixture treated with puromycin before irradiation.

Control experiments showed that a 10-min irradiation of ribosomes alone with 300-nm light does not affect their activity in poly(U)-directed poly(Phe) synthesis. The extent of cross-linking was essentially the same for both nonaminoacylated [8N_3A73]tRNA^{Phe} and AcPhe-[8N_3A73]tRNA^{Phe}.

The irradiated ribosomal complexes were separated into subunits by sucrose gradient centrifugation in a buffer containing 0.25 mM Mg^{2+}. Figure 4 shows that nonaminoacylated tRNA^{Phe} and AcPhe-tRNA were both cross-linked exclusively to 50S ribosomal particles. In contrast, no covalent attachment occurred when poly(U) or irradiation was omitted (see Figure 8). As demonstrated in Figure 5, P-site binding of Ac[^{3}H]Phe-[8N_3A73]tRNA^{Phe} was confirmed by the release of over 95% of the bound radioactivity with puromycin either before or after cross-linking (see also Figure 8). The ribosomal binding site occupied by nonaminoacylated tRNA^{Phe} cannot be directly assessed using the puromycin reaction. However, since it is known that nonaminoacylated tRNA has a strong preference for P-site binding (de Groot et al., 1971; Wurmbach & Nierhaus, 1979; Lill et al., 1986), we assume

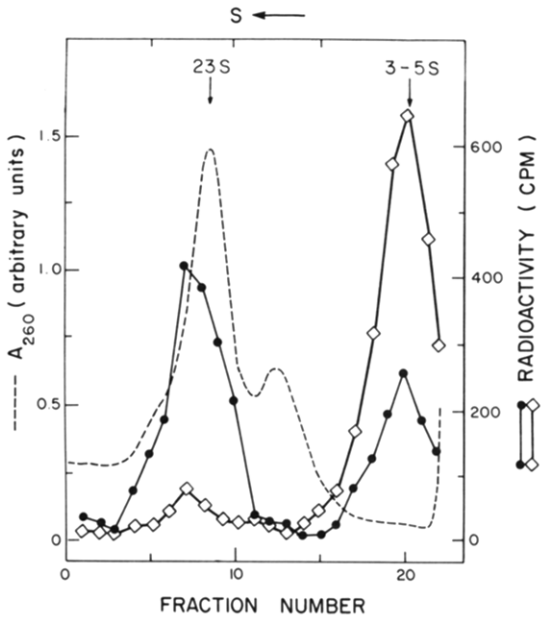

FIGURE 6: Distribution of cross-linked [$8N_3$A73]tRNA^{Phe} between 23S rRNA and 50S ribosomal subunit proteins. AcPhe-[^{32}P]-[$8N_3$A73]tRNA^{Phe}– and [^{32}P][$8N_3$A73]tRNA^{Phe}–50S ribosomal subunit complexes were isolated as in Figure 4, treated with 10 mM sodium acetate, pH 5.0, 100 mM LiCl, 0.25 mM EDTA, and 0.5% (w/v) SDS, and centrifuged through a 5–20% sucrose gradient in the same buffer at 40000 rpm for 145 min at 10 °C in a Beckman VTi50 rotor. (●) AcPhe-[$8N_3$A73]tRNA^{Phe}; (◇) nonaminoacylated [$8N_3$A73]tRNA^{Phe}.

that [$8N_3$A73]tRNA^{Phe} was also bound and cross-linked to the ribosomal P site.

Because yeast tRNA^{Phe} containing $8N_3$A at its 3′ terminus could not be aminoacylated, this derivative was used only in its nonaminoacylated form. All cross-linking experiments were performed under the conditions described for [$8N_3$A73]-tRNA^{Phe}. Although the extent of poly(U)-dependent binding of [$8N_3$A76]tRNA^{Phe} was comparable to that of unmodified tRNA^{Phe}, only 4.3% of the modified tRNA became cross-linked to ribosomes. As in the case of [$8N_3$A73]tRNA^{Phe}, covalent attachment was dependent on the presence of poly(U) and occurred only to 50S subunits (data not shown).

Identification of the Cross-Linking Site on the 50S Subunits. 50S subunits containing cross-linked, nonaminoacylated [$8N_3$A73]tRNA^{Phe} or AcPhe-[$8N_3$A73]tRNA^{Phe} were treated with LiCl–SDS, and the 23S rRNA was separated from proteins by centrifugation through SDS-containing sucrose gradients. In the case of AcPhe-[$8N_3$A73]tRNA^{Phe}, the ^{32}P radioactivity was distributed between RNA and proteins in a ratio of about 3:2 (Figure 6). By contrast, nonaminoacylated [$8N_3$A73]tRNA^{Phe} was cross-linked exclusively to ribosomal proteins. Polyacrylamide gel electrophoresis of RNase T_1 digests of AcPhe-[$8N_3$A73]tRNA^{Phe}–protein complexes revealed two radioactive bands, labeled I and II in Figure 7, lane a. Only band II was noted when complexes containing nonaminoacylated [$8N_3$A73]tRNA^{Phe} (Figure 7, lane b) or [$8N_3$A76]tRNA^{Phe} (data not shown) were examined by the same method. Since the AcPhe-[$8N_3$A73]tRNA^{Phe} preparations contained some deacylated [$8N_3$A73]tRNA^{Phe}, it was necessary to determine whether band II in digests of the AcPhe-[$8N_3$A73]tRNA^{Phe}–protein complexes represented, at least in part, cross-linking from the deacylated material. Accordingly, AcPhe-[$8N_3$A73]tRNA^{Phe} was bound to ribosomes and irradiated before and after reaction with puromycin. Analysis of the tRNA–ribosome complexes that resulted when irradiation was performed after puromycin treatment revealed

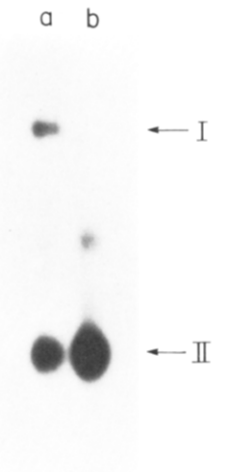

FIGURE 7: Electrophoresis of proteins cross-linked to [$8N_3$A73]-tRNA^{Phe} on a one-dimensional polyacrylamide gel. Protein–tRNA complexes from fractions 17–22 of the sucrose gradient illustrated in Figure 6 were digested with RNase T_1 as described under Experimental Procedures and subjected to polyacrylamide gel electrophoresis according to Laemmli and Favre (1973). The autoradiogram depicts ribosomal proteins cross-linked to (a) AcPhe-[^{32}P]-[$8N_2$A73]tRNA^{Phe} and (b) nonaminoacylated [^{32}P][$8N_3$A73]-tRNA^{Phe}. The results for nonaminoacylated [^{32}P][$8N_3$A76]tRNA^{Phe} were the same as in (b).

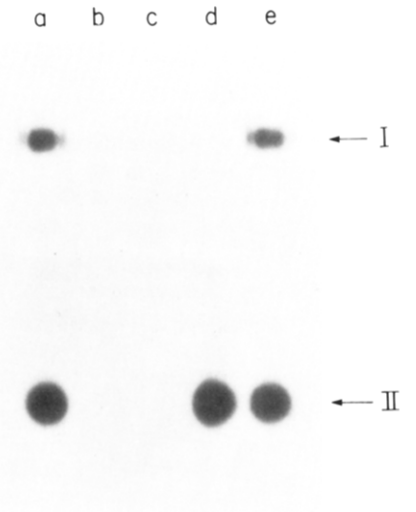

FIGURE 8: Effects of irradiation, template, and puromycin on the pattern of protein–AcPhe-[^{32}P][$8N_3$A73]tRNA^{Phe} cross-linking. Samples of protein–tRNA complexes prepared as described in Figure 6 were analyzed by the gel system of Laemmli and Favre (1973). (a) Complete incubation mixture; (b) poly(U) omitted; (c) irradiation omitted; (d) complete incubation mixture treated with puromycin before irradiation; (e) complete incubation mixture treated with puromycin after irradiation.

that only band II was present (Figure 8). Furthermore, no cross-linking to 23S RNA was observed in this case. The results suggest that some if not all of the label incorporated into band II in experiments with AcPhe-[$8N_3$A73]tRNA^{Phe} did indeed stem from the presence of deacylated tRNA. Moreover, the fact that both nonaminoacylated tRNA and tRNA deacylated after binding to the P site labeled the same ribosomal component supports the assumption that nonaminoacylated tRNA^{Phe} containing $8N_3$A was bound and cross-linked to the P site.

Identification of the Cross-Linked Proteins. RNase T_1 digests of tRNA–protein complexes isolated as in Figure 6 were mixed with unlabeled ribosomal proteins and subjected to two-dimensional polyacrylamide gel electrophoresis. The

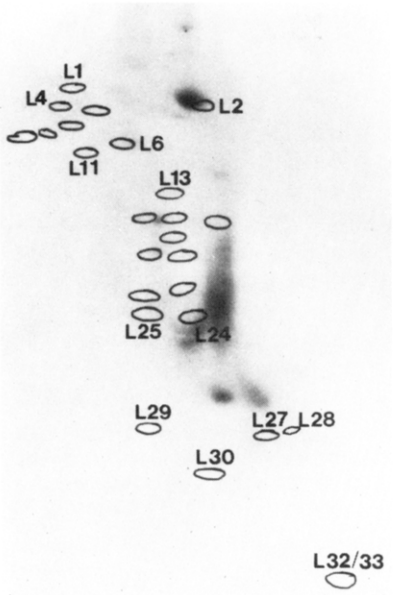

FIGURE 9: Two-dimensional polyacrylamide gel analysis of protein–oligonucleotide complexes. Protein–AcPhe-[^{32}P][8N_3A73]tRNA^{Phe} complexes prepared as described in Figure 6 were digested with RNase T_1 and analyzed by two-dimensional polyacrylamide gel electrophoresis as described under Experimental Procedures. The gels were then stained with Coomassie brilliant blue and subjected to autoradiography. The positions of the stained protein spots are encircled on the autoradiogram. The direction of electrophoresis is from left to right (first dimension) and from top to bottom (second dimension). Several 50S subunit proteins are identified by number.

^{32}P-labeled complexes were located by autoradiography of the gel after it had been stained with Coomassie brilliant blue. This method was sufficient for an unequivocal identification of band I as protein L2 in the covalent complex formed by AcPhe-[8N_3A73]tRNA^{Phe} (Figure 9). Due to the substantial influence of cross-linked oligonucleotides on the electrophoretic mobility of the smaller ribosomal proteins, it was only possible to identify a group of candidates for the protein component of the ^{32}P-labeled complexes designated as band II in Figures 7 and 8 and located in the vicinity of ribosomal protein L24 on the two-dimensional gel. Taking into account the "northwest" shift in protein mobility that should be caused by cross-linked oligonucleotides, the most probable candidates were L27 and L28.

Final identification of the protein present in band II was made by the immunological spot test, carried out with RNase T_1 digested protein–tRNA complexes and antibodies to individual 50S subunit proteins. Figure 10 shows an autoradiograph of an immunological test plate which demonstrates that protein L27 was the sole ribosomal protein attached to nonaminoacylated [8N_3A73]tRNA^{Phe}.

The protein cross-linked to nonaminoacylated [8N_3A76]-tRNA^{Phe} was analyzed only by two-dimensional gel electrophoresis. Because the radioactively labeled material in RNase T_1 digests of protein–[8N_3A76]tRNA^{Phe} complexes migrated on the gel with the same mobility as that from the L27–[8N_3A73]tRNA^{Phe} complexes, the cross-linked protein was assumed to be L27 in this case as well.

Discussion

In our search for a mild, general procedure for establishing "zero-length" cross-links between tRNA and its ribosomal binding sites, we have developed a new approach based on the incorporation of the photolabile nucleoside 8N_3A at specific positions within the tRNA chain. As 8N_3A can be photolyzed efficiently at wavelengths of 300 nm and above (Wower et al., 1988), the harmful effects of 254-nm irradiation, which has been employed previously to form zero-length tRNA–ribosome bonds (Abdurashidova et al., 1979, 1981), can be avoided. Moreover, as the replacement of adenosine with its 8-azido analogue should cause little if any change in tRNA structure, and because the latter, when photoactivated, has the potential to react with a wide variety of chemical groups, the use of 8N_3A should permit the identification of ribosomal protein and RNA components immediately adjacent to the tRNA when it is bound at its functional sites on the ribosome.

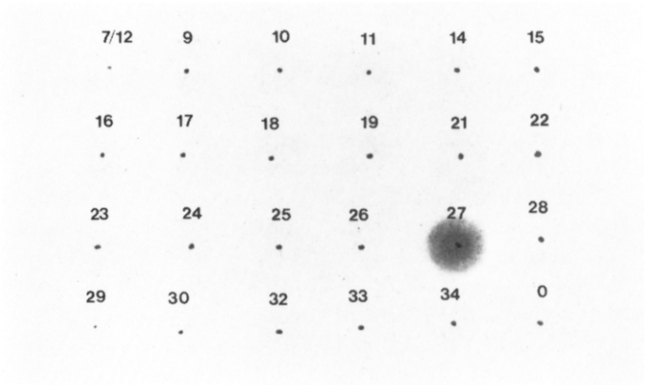

FIGURE 10: Identification of the ribosomal protein in the cross-linked complexes with [8N_3A73]tRNA^{Phe} by the immunological spot test. Antibodies against the individual 50S ribosomal proteins used in the test are indicated on the autoradiogram. "0" denotes a control minus antiserum. The antiserum against protein L27 gives a positive reaction that is seen as a radioactive spot on the film.

Since attempts to incorporate 8N_3A into the 3′-terminal position of tRNA^{Phe} with the aid of nucleotidyltransferase have been unsuccessful (Sprinzl et al., 1977), we investigated the utility of T4 RNA ligase for accomplishing this goal. RNA ligase has already been used to attach various modified nucleotides and other compounds to the 3′-hydroxyl of RNA molecules (Gumport & Uhlenbeck, 1981; Uhlenbeck & Gumport, 1982; Richardson & Gumport, 1983; Paulsen & Wintermeyer, 1984a). The donor substrate, p8N_3Ap, was prepared in radioactive form both to monitor the incorporation of 8N_3A into tRNA and to simplify the analysis of the resulting tRNA–ribosome complexes (Wower et al., 1988). In this work, [5′-^{32}P]p8N_3Ap was incorporated into yeast tRNA^{Phe} at position 73 or 76.

The photoreactive tRNA^{Phe} species were noncovalently bound to the P site of 70S ribosomes programmed with poly(U) and then irradiated with 300-nm light to produce covalent cross-links. The nonaminoacylated, 8N_3A-labeled tRNA^{Phe} derivatives cross-linked exclusively to 50S subunit protein L27. When AcPhe-[8N_3A73]tRNA^{Phe} was located in the P site, most of the covalently bound tRNA was attached to 23S rRNA, while a smaller amount was cross-linked to proteins L2 and L27. The latter protein was most likely labeled by deacylated tRNA present in the AcPhe-[8N_3A73]tRNA^{Phe} preparation. The availability of the 8N_3A-labeled tRNA^{Phe} derivatives described here has thus allowed us to study for the first time the contacts between ribosomes and the 3′ end of tRNA molecules in both their aminoacylated and their nonaminoacylated forms. The differences observed in the cross-linking patterns of AcPhe-[8N_3A73]tRNA^{Phe} and [8N_3A73]tRNA^{Phe} suggest that the 3′ terminus of P-site-bound tRNA may undergo a structural change at or near nucleotide 73 that is related to the presence or absence of the aminoacyl moiety. Although it is possible that nonaminoacylated tRNA^{Phe} could cross-link to the ribosomal E site (Rheinberger & Nierhaus, 1983), we consider

this to be unlikely in the present case. Because nonaminoacylated $tRNA^{Phe}$ has a 10–30-fold higher affinity for the P site than the E site (Lill et al., 1986), the latter would not be occupied at the low molar tRNA:ribosome ratios used.

In light of previous information on the accessibility of the 3′-terminal -A-C-C-A sequence of P-site-bound $tRNA^{Phe}$, it is interesting to note that $tRNA^{Phe}$ containing $8N_3A$ in position 73 cross-links to the ribosome in relatively high yield. Tritium exchange measurements (Farber & Cantor, 1980), chemical modification studies (Peattie & Herr, 1981), and experiments with fluorescent tRNA derivatives (Paulsen & Wintermeyer, 1984b) all indicated that the 3′-terminal -C-C-A sequence is in contact with the ribosome at the P site but that A73 is not. The substantial yield of cross-linking from $8N_3A73$, however, implies that the base is in close proximity to protein L27 or to 23S rRNA.

Previous labeling experiments with tRNA containing chemically or photochemically reactive groups attached to the aminoacyl moiety have suggested that at least 10 proteins, including L2, L11, L14, L15, L16, L18, L23, and L27 in the 50S subunit and S14 and S18 in the 30S subunit, are located in the vicinity of the 3′ end of tRNA and, consequently, that they are constituents of the peptidyltransferase center [for reviews, see Ofengand (1980) and Ofengand et al. (1986)]. It is apparent from an inspection of current ribosome models derived by immune electron microscopy (Stöffler & Stöffler-Meilicke, 1986), however, that the labeled proteins encompass a much larger portion of the ribosome surface than would be expected for the peptidyltransferase center. The observed heterogeneity of labeling may be caused in large measure by differences in the size, reactivity, and flexibility of the substituents attached to the aminoacyl moiety of the modified aminoacyl-tRNAs. In particular, most of the substituents used were quite long, ranging from 6 to 20 Å, and could therefore access ribosomal components at considerable distance from the aminoacyl group. This may also explain why many of the derivatized aminoacyl-tRNAs cross-linked to more than two—and in some cases up to six—ribosomal proteins which, in several instances, were situated at some distance from one another on the ribosome surface (Ofengand, 1980). In addition, it has been noted that the erroneous identification of cross-linked proteins may have contributed to the reported heterogeneity of cross-linking in studies where gel electrophoresis alone was used for this purpose (Ofengand, 1980). By contrast, we have shown that tRNAs containing $8N_3A$ are highly specific labeling agents, cross-linking to only one or two components which are likely to be immediately adjacent to their site of interaction with the ribosome.

Exclusive cross-linking of L27 by $8N_3A$ at positions 73 and 76 of $tRNA^{Phe}$, as well as its frequent labeling by various affinity probes attached to the aminoacyl moiety of aminoacyl-tRNA, indicates that this protein is one of the main constituents of the peptidyltransferase center. Since the yield of the covalent L27–[$8N_3A73$]$tRNA^{Phe}$ complex is relatively high, it should be possible to identify the cross-linked amino acid in the protein. The labeling of L2 by $8N_3A$ at position 73 of $tRNA^{Phe}$ suggests that this protein is also located at or near the peptidyltransferase center. This inference is supported by other affinity labeling studies in which reactive groups were attached to the aminoacyl moiety (Kuechler & Ofengand, 1979; Ofengand, 1980; Abdurashidova et al., 1984). The fact that L2 represents only a minor site of cross-linking in our experiments may indicate that it is relatively inaccessible to $8N_3A$ at position 73. This could be due to the short range of the $8N_3A$ cross-link in that L2 might be shielded by the primary cross-linking target in 23S rRNA.

Cross-linking of chemically reactive or photoreactive derivatives of aminoacyl-tRNA to 23S rRNA has been demonstrated in several cases [for a review, see Ofengand (1980)]. The site of attachment to the 23S rRNA has been defined in only one of these experiments in which 3-(4-benzoylphenyl)-propionyl-Phe-$tRNA^{Phe}$ was located in the ribosomal P site (Barta et al., 1984). We are presently determining the segment to which AcPhe-[$8N_3A73$]$tRNA^{Phe}$ is cross-linked in 23S rRNA and anticipate that our results will further elucidate the role that 23S rRNA plays in the binding of tRNA to the ribosome and in the formation of the peptidyl bond during protein biosynthesis.

The present experiments are the first in which azidopurines have been used to probe the topography of tRNA binding sites on the ribosome. These compounds offer a number of advantages in the study of tRNA–ribosome interaction. Owing to recent advances in the biochemistry of nucleic acids (Cedergren & Grosjean, 1987), azidopurines can be incorporated at virtually any position within the tRNA molecule. Because they contain no bulky substituents and are similar in size and structure to the residues they replace, they should lead to little or no distortion of the secondary and tertiary structures of the resulting tRNA derivatives, which can therefore be expected to retain normal or nearly normal biological activity. Moreover, photolabile azide groups can be placed at various positions of the heterocyclic nucleotide bases. These considerations, together with the fact that the protein–tRNA or tRNA–rRNA cross-links produced are no more than a few angstroms in length, suggest that tRNAs substituted with azidopurines will prove very useful in acquiring detailed information about the contacts that each portion of the tRNA molecule makes with the ribosome during the various stages of protein synthesis as well as with tRNA synthetases and transport factors such as elongation factor Tu and initiation factor 2.

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