Photoaffinity Labeling of the Ribosomal Peptidyl Transferase Site with Synthetic Puromycin Analogues†

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ABSTRACT: A photoaffinity labeling puromycin analogue, N^{ϵ}-(2-nitro-4-azidophenyl)-L-lysinyl puromycin aminonucleoside (NAP-Lys-Pan), was synthesized and used for investigation of the peptidyl transferase center of 70S ribosomes. Visible light irradiation of NAP-Lys-Pan led to covalent linkage of the analogue with *Escherichia coli* ribosomes. In a subsequent step, poly(uridylic acid) was employed to direct Ac[^{14}C]Phe-tRNA to the P sites of the photolabeled ribosomes. Transpeptidation of Ac[^{14}C]phenylalanine to the bound NAP-Lys-Pan resulted in selective incorporation of radioactive label into the peptidyl transferase A site. Dissociation of the ribosomes into subunits, and digestion of the RNA components, indicated that the radioactive label was incorporated into a protein fraction of the 50S subunit.

Affinity labeling is a direct approach to the identification of components of biological macromolecules at specific functional sites. In its simplest form, affinity labeling requires the use of reactive analogues of natural substrates to label the substrate binding site. The specificities are dependent upon reversible complex formation resulting in a high localized concentration of reagent within the substrate binding site. Thus, the probability of covalent bond formation within this site is extensively amplified relative to a random alkylation event (Baker, 1967; Hartman, 1978). Numerous chemical affinity labels and photoaffinity labels have been developed to probe different ribosomal functional sites (for a review, see Pellegrini & Cantor, 1977). The antibiotic, puromycin, binds to the A site of ribosomal peptidyl transferase and accepts the peptide from peptidyl-tRNA. Three analogues of this antibiotic have been synthesized for the purpose of labeling the A-site proteins. The N^{α}-iodoacetyl puromycin (Pongs et al., 1973) and the *N*-(ethyl-2-diazomalonyl) puromycin (Cooperman et al., 1975) both represent peptidyl puromycin analogues and should be expected to occupy an intermediate position between what has been defined as the A and P sites (Pellegrini & Cantor, 1977). Symons and coworkers (Harris & Symons, 1973; Greenwall et al., 1974; Eckermann & Symons, 1978) have synthesized an analogue of puromycin, 5′-*O*-(*N*-bromoacetyl-*p*-aminophenylphosphoryl)-3′-*N*-L-phenylalanyl puromycin aminonucleoside (BAP-Pan-Phe). Unlike the above derivatives, BAP-Pan-Phe retains the free α-amino group of the amino acid moiety and should closely mimic the binding of the parent antibiotic. However, the reactive moiety of this analogue is at a position removed from the amino acid binding site of peptidyl transferase. BAP-Pan-Phe reacts with 23S ribosomal RNA in addition to a large amount of nonspecific binding to protein (Eckermann & Symons, 1978).

Our approach to the design of A-site labeling reagents entails the development of puromycin analogues capable of binding with A-site proteins in such a manner that the covalently bound reagent can participate in peptide transfer with Ac[^{14}C]Phe-tRNA[1] bound at the P site. A major advantage of this class of analogues involves the location of a photoreactive group in the aminoacyl moiety. This feature ensures that labeling will occur within the amino acid binding region and covalent bonding will involve the ribosomal protein(s) located at that site. In the present case, the photosensitive group, 2-nitro-4-azidophenyl, was employed because it is extremely stable in the dark and generates a highly reactive arylnitrene species upon photolysis. In addition the nitroarylazide can be photolyzed with visible radiation, eliminating photochemical damage to the ribosome which usually occurs at lower wavelengths. Thus, N^{ϵ}-(2-nitro-4-azidophenyl)-L-lysinyl puromycin aminonucleoside (NAP-Lys-Pan) was synthesized (Scheme I). This compound exhibited excellent substrate activity in the peptidyl transferase reaction and underwent photolysis upon irradiation with visible light, thus satisfying the initial criteria as a candidate photoaffinity label for the peptidyl transferase site. The present communication reports initial studies on the use of NAP-Lys-Pan for the specific incorporation of a radioactive label into the peptidyl transferase A site.

Experimental Section

Materials. Puromycin dihydrochloride was obtained from ICN Pharmaceuticals, Inc., [^{14}C]-L-phenylalanine was obtained from New England Nuclear. The polynucleotides were obtained from Miles Laboratories, and ATP, GTP, phosphoenolpyruvate, and pyruvate kinase were purchased from Sigma. Preparation of ribosomes, S-100, and factors washable from ribosomes (FWR) from *E. coli* cell paste (B, mid log), and preparation of Ac[^{14}C]-L-Phe-tRNA were as previously described (Duquette et al., 1974).

Photolysis Experiments. A standard reaction mixture consisted of 125 A_{260} units of washed 70S *E. coli* ribosomes, and NAP-Lys-Pan (1×10^{-3} M) in 1 mL of buffer containing 0.1 M Tris-Cl (pH 7.5), 0.1 M NH_4Cl (pH 7.6), 0.015 M $Mg(OAc)_2$. The reaction mixtures were photolyzed in Pyrex test tubes for 45 min at 4 °C using a General Electric DEK 500-W bulb. The tubes were placed 3 in. from the light source and were rotated 90° every 10 min. When photolysis was complete, the reaction mixtures were dialyzed against four

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[1] Abbreviations used: Ac[^{14}C]Phe-tRNA, *N*-acetyl[^{14}C]-L-phenylalanyl transfer ribonucleic acid; EDTA, ethylenediaminetetraacetic acid (tetrasodium salt); Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; NHS, *N*-hydroxysuccinimide; Pan, puromycin aminonucleoside; IR, infrared; UV, ultraviolet; ^{1}H NMR, proton magnetic resonance.

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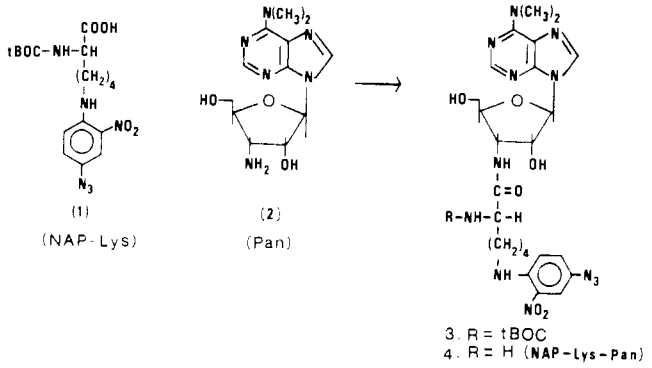
SCHEME I



changes of buffer containing 0.01 M Tris-Cl (pH 7.5), 0.05 M KCl, 0.01 M $Mg(OAc)_2$, and 0.003 M β-mercaptoethanol for 3 days at 4 °C. A red polymerization product remaining after dialysis was removed by centrifugation at 17 000 rpm for 30 min. The photolyzed ribosome solution was stored in liquid nitrogen until further use.

Incorporation of Ac[^{14}C]-L-Phenylalanine into Photolyzed Ribosomes. A reaction mixture containing 0.1 M Tris-Cl (pH 7.5), 0.1 M NH_4Cl (pH 7.6), 0.015 M $Mg(OAc)_2$, 0.65 mM dithiothreitol, 118 μg of poly(U), 1.2 mM GTP, 15 μL FWR (63 μg of protein), 37.5 A_{260} units of photolyzed ribosomes, and 14.8 A_{260} units of Ac[^{14}C]-L-Phe-tRNA (6.9 pmol of [^{14}C]Phe per A_{260}) in a total volume of 1 mL was incubated at 28 °C for 45 min. The amount of radioactivity incorporated was determined by either ribonuclease or sodium hydroxide digestion of the ribosomal mixture.

Measurement of Radioactivity in Protein Fraction of Ribosomes. Ribonuclease digestion (Czernilofsky & Kuechler, 1972) consisted of adjusting the above ribosome mixture to 3 and 0.05 M concentrations of urea and EDTA, respectively, followed by incubation with 10 μg/mL of ribonuclease A and 2.8 μg/mL of ribonuclease T_1 at 37 °C for 2 h. Trichloroacetic acid was added to a final concentration of 10%. The mixtures were placed in ice for 30 min and filtered through Millipore filters (type HA, 0.45 μm) and washed three times with cold 5% trichloroacetic acid. The filters were dried and placed in counting vials containing 8 mL of toluene–permablend liquid scintillator and counted in a Beckmann LS-150. Counting efficiency was approximately 90%.

Alkaline hydrolysis was carried out by adjusting the radiolabeled ribosome mixtures to a final concentration of 0.5 N NaOH. The alkaline mixtures were incubated at 37 °C for 30 min and neutralized with an equal equivalent of HCl. The incubation mixtures were placed in ice and trichloroacetic acid was added to a final concentration of 10%. The mixtures were filtered, washed, and counted as described in the preceding paragraph.

N^{α} - tert - Butyloxycarbonyl - N^{ϵ} - (2-nitro-4-azidophenyl)-L-lysine (**1**). All reactions were performed in the dark. To a solution of *N^{ϵ}-tert*-butyloxycarbonyl-L-lysine (493 mg, 2.0 mmol) in a mixture of 1 N NaOH (4 mL) and ethanol (10 mL) was added 4-azido-2-nitrofluorobenzene(364 mg, 2.0 mmol). The solution was heated under reflux for 4.5 h and then concentrated to approximately 1 mL under reduced pressure. Water was added (30 mL) and the pH was adjusted to 1.5 with cold 1 N HCl. A gummy red precipitate was extracted into ethyl acetate (4 × 50 mL) and the combined extracts were washed with half-saturated NaCl (50 mL), dried (Na_2SO_4), and evaporated to a deep red solid (450 mg). Separation of the major product on 20 × 20 cm silica gel 254 (E. Merck, Darmstadt) plates (2-mm thickness) developed with chloroform/methanol/glacial acetic acid (90:5:5) gave the pure product (R_f 0.43) as a deep red oil (232 mg, 37.8%): IR (neat) 3400 (OH, NH), 2050 (arylazide), 1725 (COOH), 1690 (carbamate), 1520, 1340 (NO_2).

Anal. Calcd for $C_{17}H_{24}N_6O_6 \cdot H_2O$: C, 47.88; H, 6.14; N, 19.71. Found: C, 47.69; H, 6.02; N, 19.76.

6-Dimethylamino-9-[3'-(N^{α}-tert-butyloxycarbonyl-N^{ϵ}-(2-nitroazidophenyl)-L-lysinylamino)-3'-deoxy-β-D-ribofuranosyl]purine (**3**). A mixture of **1** (408 mg, 1.0 mmol), puromycin aminonucleoside (294 mg, 1.0 mmol), *N*-hydroxysuccinimide (117 mg, 1.0 mmol), and dicyclohexylcarbodiimide (207 mg, 1.0 mmol) in dry dioxane (6 mL) and dry dimethylformamide (2 mL) was stirred at ambient temperature for 24 hr. The precipitated dicyclohexylurea was removed by filtration and washed with ethyl acetate (20 mL). The combined filtrate and wash were evaporated in vacuo, dissolved in hot ethyl acetate, cooled, and filtered. The filtrate was evaporated to a brown solid: 620 mg (90.5%), mp 130 °C (dec); IR (KBr) 3420 (br, OH, NH), 2120 (arylazide), 1690 (carbamate), 1660 (amide), 1600 (purine), 1520 cm^{-1} (NO_2); ^{1}H NMR (MeOH-d_4) δ 1.0–2.02 (m, 6, Cα$H(CH_2)_3$CHNH-), 1.43 (s, 9, $C(CH_3)_3$), 5.88 (s, 1, H-1'), 6.72–7.48 (m, 3, aromatic H), 7.97 and 8.15 (2 s, 2 × 1, H-2 and H-8), 3.35 (s, 6, $N(CH_3)_2$).

Anal. Calcd for $C_{29}H_{40}N_{12}O_8$: C, 50.87; H, 5.89. Found: C, 50.48; H, 5.96.

6-Dimethylamino-9-[3'-(N^{ϵ}-(2-nitro-4-azidophenyl)-L-lysinylamino-3'-deoxy-β-D-ribofuranosyl]purine (**4**). A mixture of **3** (150 mg, 0.22 mmol), methylene chloride (1.5 mL, distilled from $CaCl_2$), and anhydrous trifluoroacetic acid (1.5 mL) was allowed to stand at ambient temperature for 5 min while protected from light and moisture. The excess trifluoroacetic acid was removed immediately in vacuo (1 mm, 25 °C) by azeotroping with dried acetonitrile (10 × 3/mL). TLC on silica gel (developed with chloroform–methanol, 4:1) revealed an orange spot at R_f 0.24 (desired product) and a second UV absorbing spot at R_f 0.44 (6-dimethylaminopurine) with the remaining material at the origin. The deep red product was dissolved in methanol (7 mL) and stirred with Dowex 1 (OH^-) resin (prewashed with methanol) for 5 min. The resin was removed by filtration and washed with methanol. The combined filtrate and wash were evaporated to dryness and yielded a deep red solid, 107 mg. Preparative TLC (developed with chloroform–methanol, 4:1) gave pure **4** as an orange red solid: 25.3 mg; mp 87 °C (softened), 130 °C (dec); IR (KBr) 3420 (br, OH, NH, NH_2), 2100 (arylazide), 1660 (amide), 1520, 1340 cm^{-1} (NO_2); UV_{max} (EtOH) 270 (ε 31 000) and 464 nm (ε 3200).

Anal. Calcd for $C_{24}H_{32}N_{16}O_6 \cdot 0.5CHCl_3$: C, 45.57; H, 5.08. Found: C, 45.51; H, 5.24.

Results and Discussion

The synthesis of NAP-Lys-Pan (**4**) was carried out in the absence of direct light because of the photosensitivity of the 2-nitro-4-azidophenyl group. Condensation of *N^{α}-tert*-butyloxycarbonyllysine and 4-fluoro-2-nitrophenylazide in refluxing aqueous ethanol gave *N^{α}*-Boc-*N^{ϵ}*-(2-nitro-4-azidophenyl)lysine (**1**). Subsequent coupling of **1** with Pan (**2**) in the presence of DCC and NHS yielded the corresponding *N^{α}*-Boc-*N^{ϵ}*-NAP-Lys-Pan (**3**) (Scheme I). When **3** was deblocked in anhydrous trifluoroacetic acid, the desired NAP-Lys-Pan (**4**) was obtained.

Since recognition by the peptidyl transferase center is prerequisite to selective affinity labeling of the A site, the substrate

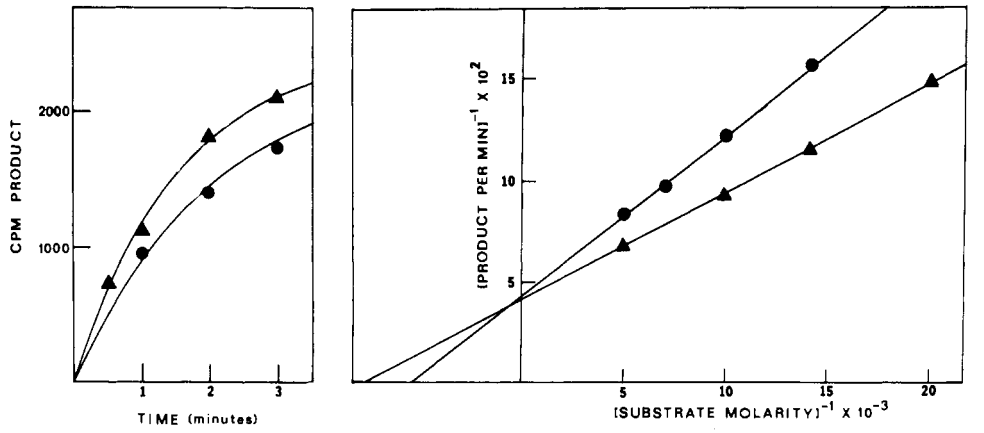


FIGURE 1: The course of reaction at 28 °C of Ac[^{14}C]-L-Phe-tRNA as a donor substrate with puromycin (▲) and with NAP-Lys-Pan (●) with *E. coli* ribosomes is illustrated in the left panel. Both substrates were tested at 2 × 10^{-4} M concentration. The Ac[^{14}C]-L-Phe-tRNA was bound to the ribosomes in the presence of poly(U) as described previously (Vince & Fong, 1978). The binding mixture was incubated at 28 °C for 8 min to allow maximum charging of the ribosomes. The peptidyl transferase reaction was initiated by the addition of puromycin or NAP-Lys-Pan to the incubation mixture. Reactions were incubated at 28 °C for specified times and product formation was measured as cpm extracted into ethyl acetate. The right panel illustrates a double-reciprocal plot for the transpeptidation reaction with puromycin (▲) and with NAP-Lys-Pan (●).

efficiency of NAP-Lys-Pan was investigated. The course of reaction of Ac[^{14}C]Phe-tRNA as a donor substrate with puromycin and with NAP-Lys-Pan is presented in Figure 1. The rate of product formation indicates that the analogue exhibits excellent acceptor activity in the peptidyl transferase reaction with respect to puromycin. A double-reciprocal plot for the transpeptidation reaction indicates that NAP-Lys-Pan has an affinity for the A site only slightly less than puromycin, judging from the Michaelis constants for the two substrates (Figure 1).

The visible spectrum of NAP-Lys-Pan exhibited a λ_{max} at 465 nm corresponding to the absorption of the arylazide. The disappearance of this absorption band upon irradiation with visible light indicated that the arylazide was decomposed photolytically (Figure 2). The photolysis was followed to completion within 30 min when the light source was located 3 in. from the reaction mixture. Therefore, in a separate experiment the photolysis of NAP-Lys-Pan in the presence of *E. coli* ribosomes was continued for 45 min to ensure complete photolysis. The photoaffinity labeling of 70S ribosomes was performed in a two-step process as illustrated in Figure 3. In the first step, an incubation mixture of washed ribosomes and NAP-Lys-Pan was photolyzed as described in the Experimental Section. Upon irradiation, the photosensitive arylazide generates an active arylnitrene which is capable of forming a covalent linkage with the ribosome (Figure 3-b). The unbound analogue molecules were removed from the ribosome mixture by dialysis. Since the α-amino group remains intact, a bound analogue molecule that is correctly oriented within the A site should be capable of accepting a donor substrate from the P site. Thus, in the second step poly(U) was employed to direct Ac[^{14}C]Phe-tRNA to the P sites of the photolabeled ribosomes (Figure 3-c). Transpeptidation subsequently occurs resulting in ^{14}C labeling of the A site as illustrated in Figure 3-d. The ribosome complex was dissociated with urea and EDTA, and incubated with ribonuclease A and ribonuclease T_1 to destroy the excess Ac[^{14}C]Phe-tRNA and ribosomal RNA (Czernilofsky & Kuechler, 1972). The amount of Ac[^{14}C]Phe transferred to the ribosome-bound ligand was estimated by the radioactivity associated with the precipitated protein fraction. The results presented in Table I indicate that the labeling efficiency was approximately 2.6% for the two-step process. In control experiments in which step one was carried out in the absence of visible light it was noted that NAP-Lys-Pan gave

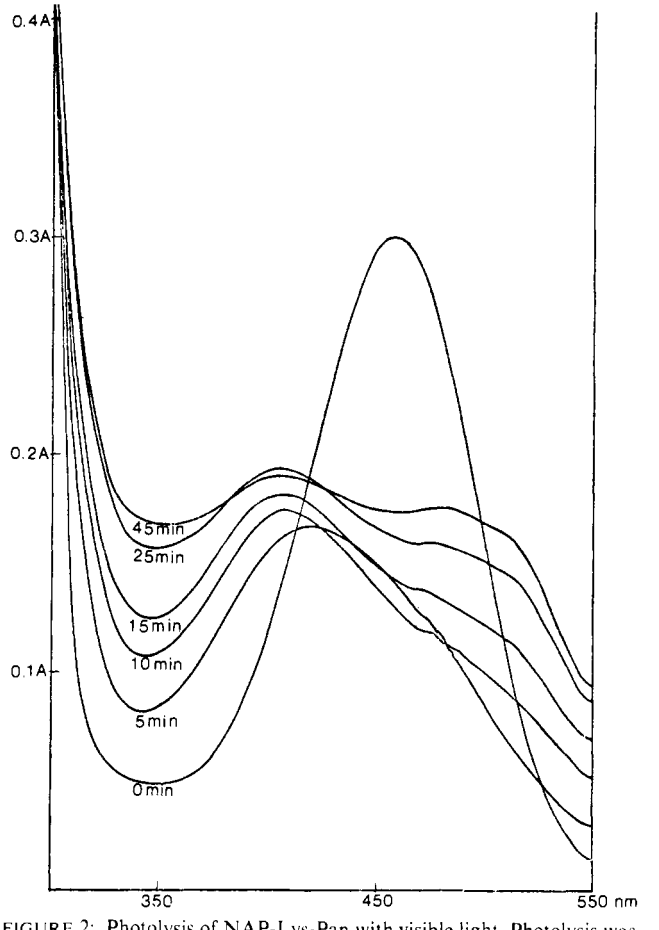


FIGURE 2: Photolysis of NAP-Lys-Pan with visible light. Photolysis was carried out at 4 °C as described in the Experimental Section, and the reaction was followed by measuring the visible absorbance of aliquots with a Beckman 25 recording spectrophotometer.

a small amount of linking (Table I). A similar observation of irradiation-independent binding during the photoaffinity labeling of ribosomes with *S*-(*p*-azidophenacyl)valyl-tRNA has been reported (Schwartz et al., 1975). This observation was explained with the suggestion that the unirradiated compound acts as a chemical affinity probe because it is known that the arylazide is readily displaced by nucleophiles.

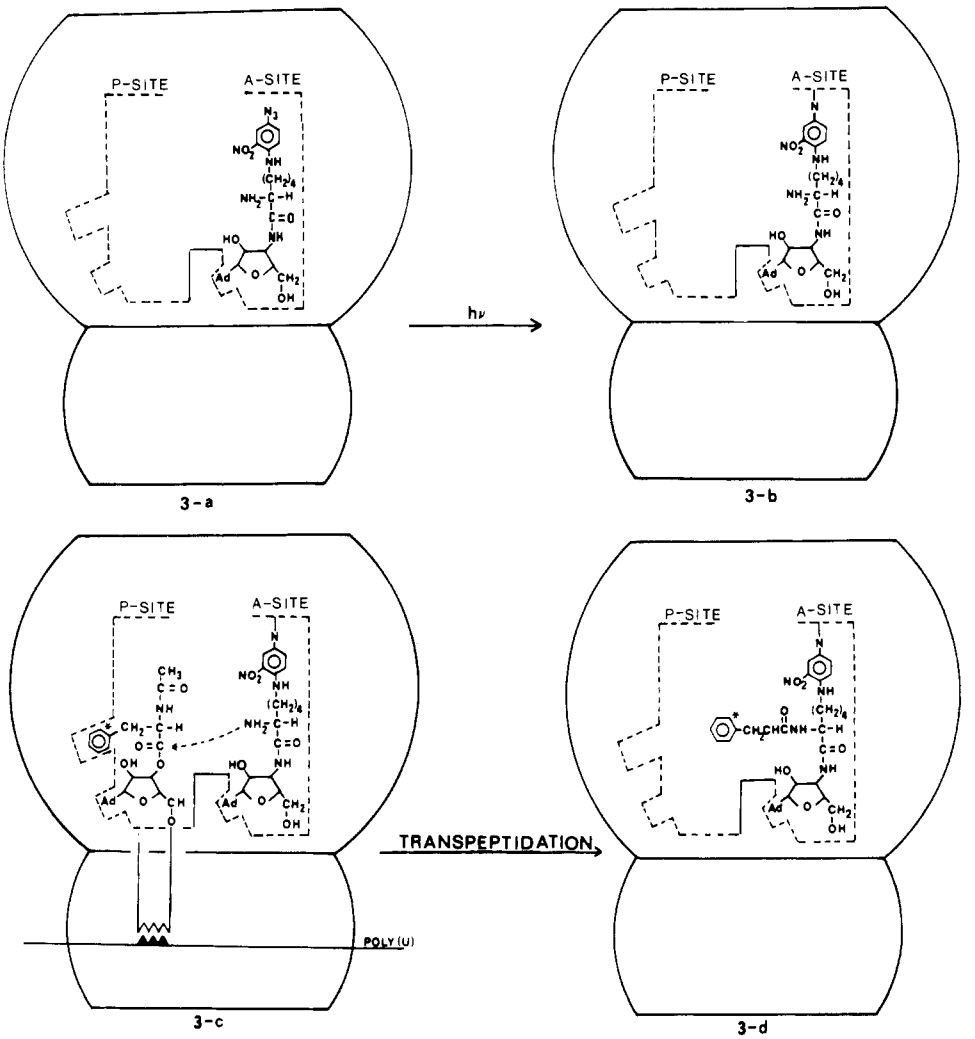


FIGURE 3: Two-step photoaffinity labeling process of *E. coli* ribosomes with NAP-Lys-Pan. The conversion of a reversible complex (3-a) to an irreversible complex (3-b) is catalyzed by irradiation with visible light. Photolyzed ribosomes are charged with Ac[^{14}C]Phe-tRNA in the presence of poly(U) as illustrated in 3-c. Peptide bond formation may proceed via direct attack of the α-amino group of bound NAP-Lys-Pan upon the ester linkage of the peptidyl-tRNA (transpeptidation) yielding a labeled A-site (3-d).

TABLE I: Incorporation of Ac[^{14}C]phenylalanine into Photolyzed Ribosomes.[a]

NAP-Lys-Pan	photolysis time (min)	radioact. incorp (cpm)[b]	% of labeling
none	45	142	
1 mM	45	7476	2.60
1 mM	0	1728	0.61

[a] The photolysis reaction of **4** with 70S *E. coli* ribosomes and the incorporation of Ac[^{14}C]phenylalanine into the photolyzed ribosomes were as described in the Experimental Section. [b] Aliquots containing 11.5 A_{260} units of ribosomes were treated with urea, EDTA, and ribonucleases A and T_1 as described. The ribosomal proteins were precipitated with 10% trichloroacetic acid and the radioactivity associated with the precipitated proteins was determined by scintillation counting.

TABLE II: Poly(U)-Dependent Incorporation of Ac[^{14}C]-phenylalanine into Photolyzed Ribosomes.[a]

NAP-Lys-Pan	photolysis time (min)	radioact.[b] incorp.		% of labeling	
		+poly(U)	−poly(U)	+poly(U)	−poly(U)
1 mM	45	7708	726	2.74	0.26
1 mM	0	1774	220	0.62	

[a] Photolyzed ribosomes were incubated with Ac[^{14}C]Phe-tRNA in the presence or absence of poly(U) as described in the Experimental Section. [b] cpm of Ac[^{14}C]phenylalanine incorporated into the protein fraction of 11.5 A_{260} of ribosomes was determined as described in the Experimental Section.

Specific labeling of the target site is a primary concern in affinity labeling studies. In the present case, the transfer of Ac[^{14}C]Phe to the ribosome-linked puromycin analogue must occur codon-specifically because mRNA is required for the binding of AcPhe-tRNA to the ribosome. The data presented in Table II clearly demonstrate that only in the presence of poly(U) is significant radioactivity incorporated into the photolyzed ribosomes. These experiments imply that the radioactive labeling process must occur through peptide bond formation catalyzed by peptidyl transferase.

In a separate experiment, the distribution of radioactive label between the 50S and 30S ribosomal subunits was investigated. The labeled ribosomes were dissociated into subunits by dialysis against low magnesium buffer and subsequently

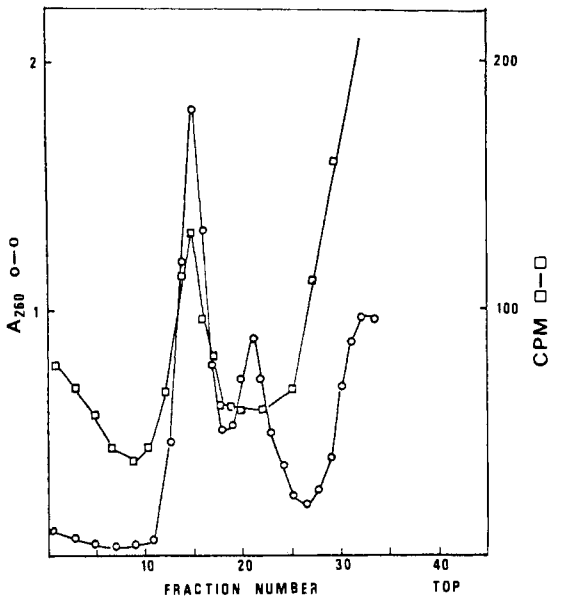


FIGURE 4: Sucrose gradient analysis of dissociated photoaffinity labeled ribosomes. The radiolabeled ribosomal mixture was dialyzed against a buffer containing 15 mM NH_4Cl (pH 7.6), 1 mM $MgCl_2$, 20 mM Tris-Cl (pH 7.5), and 3 mM β-mercaptoethanol at 4 °C for 20 h. The dialyzed ribosome mixture (4.4 A_{260} units) was layered onto 11.5 mL of 5–30% (w/v) linear sucrose gradient in dialysis buffer and the gradient was centrifuged at 40 000 rpm for 4 h in a Beckman SW40 rotor. The gradient was analyzed from the bottom and UV absorbance at 260 nm was monitored by a Beckman 25 recording spectrophotometer equipped with a 60-μL flow cell (path length 2 mm). Fractions (300 μL) were collected and the radioactivity was measured by placing each fraction in 10 mL of toluene–Triton X (2:1) and counting in a Beckman LS-150.

separated by centrifugation through a sucrose density gradient Scintillation counting of the gradient fractions indicated that the radioactivity was concentrated in the 50S subunit fraction (Figure 4). Since the peptidyl transferase center is an integral part of the 50S subunit, this experiment confirms the specificity of labeling.

The results from the above experiments clearly indicate that the puromycin analogue, NAP-Lys-Pan, interacts with the A site and is capable of acceptor substrate activity in the peptidyl transferase reaction. Some of the covalently bound analogue molecules are presumably oriented in a position analogous to the aminoacyl terminus of tRNA as evidenced by the transpeptidation of Ac[^{14}C]Phe. Fractionation of ribosomal components indicated that the irreversibly bound Ac[^{14}C]Phe was associated with the protein fraction of the ribosome. The results suggest that the affinity labeling moiety of NAP-Lys-Pan binds to a protein component of the A site which is normally occupied by at least the amino acid moiety of aminoacyl-tRNA or puromycin. Recently, puromycin itself has been used as a photoinduced affinity label, and the major labeled ribosomal proteins were L23 and S14 (Cooperman et al., 1975; Jaynes et al., 1978). However, it was suggested that the failure to detect AcPhe transfer to the incorporated puromycin may indicate that incorporation is not at a functional site (Jaynes et al., 1978). Affinity labeling of 23S RNA has also been accomplished with analogues of L-Phe-tRNA containing reactive ligands at the α-amino group of L-phenylalanine (Pellegrini & Cantor, 1977). The derivatization of the α-amine in these analogues most probably directed them to the P site as previously suggested (Harris & Symons, 1973). More recently, the specific labeling of 23S RNA at the A site has been accomplished with the puromycin derivative, BAP-Pan-Phe, in which the α-amine is underivatized (Eckerman & Symons, 1978). No specific labeling of a protein fraction with this analogue was reported. The present report provides preliminary evidence that NAP-Lys-Pan specifically enters the A site and binds to a protein component while retaining its substrate activity in the peptidyl transferase reaction. These studies encourage the continued use of puromycin analogues equipped with photosensitive amino acid moieties for the study of ribosomal structure. Work is in progress with NAP-Lys-Pan and related analogues to obtain higher efficiencies of labeling and to characterize the labeled protein component(s).

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