PHOTOAFFINITY LABELING OF *ESCHERICHIA COLI* RIBOSOMES WITH AN ARYL AZIDE ANALOGUE OF PUROMYCIN

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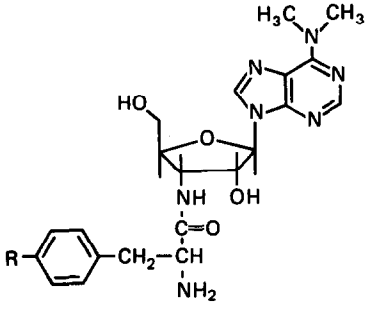
1. Introduction

Affinity labeling studies have contributed importantly in the indentification of several functional sites on the *E. coli* ribosome [1–3]. The puromycin binding site has been the subject of several such studies [4–9]. We have shown that native puromycin can itself be used as a specific photoaffinity label [4,5]. To investigate the question of to what extent the labeling results obtained in this work were dependent on the special photochemical reaction(s) leading to incorporation, we have begun a program to repeat the photoaffinity labeling experiment with several different photolabile derivatives of puromycin. We describe here the synthesis and characterization of a new photolabile derivative of puromycin, 6-dimethylamino-9-[3′-deoxy-3′-(*p*-azido-L-phenylalanylamino)-β-D-ribofuranosyl] purine, which we call '*p*-azidopuromycin' (fig.1), and present preliminary ribosomal photolabeling results obtained with this derivative.



p-Azidopuromycin: R = $-N_3$
Puromycin: R = $-OCH_3$

Fig.1. Structures of puromycin and *p*-azidopuromycin.

2. Materials and methods

Puromycin dihydrochloride and puromycin aminonucleoside were purchased from Unites States Biochemical Corporation. [G-^{3}H]Puromycin aminonucleoside (14 900 Ci/mol) and [8-^{3}H]puromycin (3500 Ci/mol) were purchased from Amersham. Ribosomes were prepared after the method in [10] as in [4,5]. Ribosomal and photolysis buffer contained 50 mM KCl, 50 mM Tris (pH 7.4, room temp.) and 10 mM $MgCl_2$. Syntheses and manipulations of all photolabile compounds were carried out under reduced light. The purity of compounds was verified by thin-layer chromatography (TLC) in at least two solvents. Analytical TLC was performed with Merck silica gel 60F-254 plastic-backed plates, and preparative TLC with Merck silica gel 60F-254 glass-backed plates, 0.25 mm or 0.5 mm thickness. Solvent system A (S_A): ethanol/chloroform, 1:9 v/v. Solvent system B (S_B): ethyl acetate/methanol/H_2O/acetic acid 25:10:1:1, by vol. Solvent system C (S_C): isopropanol/H_2O/NH_4OH, 6:3:1, by vol. Descending paper chromatography was performed using prewashed [11] Whatman 3MM paper. All reagents and solvents were of the highest quality commercially available and were used without purification.

N-*t*BOC–*p*NO_2–L-phenylalanine [12] was synthesized in 71% yield using 2-(*t*-Butoxycarbonyloxyimino)-2-phenylacetonitrile, 'BOC-ON' [13]. *N*-*t*BOC-*p*-azido-L-phenylalanine was prepared as in [12]. The EEDQ (*N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline)-mediated coupling reaction [14] was used to synthesize *N*-*t*BOC-*p*-azidopuromycin. EEDQ (138 μmol) was added to puromycin aminonucleoside (68 μmol) and *N*-*t*BOC-*p*-azido-L-phenyl-

alanine (132 μmol) in ethanol (1 ml). The solution was stirred for 1.5 h at room temperature during which time a precipitate formed. Upon evaporation of the solvent, the residue was dissolved in chloroform and subjected to preparative TLC (S_A). The product band was isolated and extracted with ethanol/chloroform (7:13, v/v). Centrifugation, filtration and evaporation of solvent afforded *N-t*BOC-*p*-azidopuromycin in 67% yield. [$R_F(S_A)$ 0.43; $R_F(S_B)$ 0.84; $R_F(S_C)$ 0.68; ultraviolet spectrum (ethanol): λ_{max} 262 nm (ϵ 24 800 M^{-1} cm^{-1}), λ_{min} 230 nm, λ_{sh} 275 nm; infrared spectrum (KBr): 2100 cm^{-1}, 1660 cm^{-1}, 1680 cm^{-1}, 1600 cm^{-1}. Deprotection was accomplished by treatment with anhydrous trifluoroacetic acid for 5 min at room temperature. The azido group is stable under these conditions. Most of the excess acid was removed by evaporation under reduced pressure. Full removal necessitated repeated evaporations of acetonitrile solutions of the residue. The final residue was dissolved in ethanol and purified by preparative TLC (S_B). Extraction of the product band with ethanol, followed by centrifugation, filtration, and evaporation of the solvent yielded pure *p*-azidopuromycin in 80% yield. A sample of *p*-azidopuromycin spotted on Whatman 3MM paper gave a positive ninhydrin test. [$R_F(S_A)$ 0.05 (puromycin, 0.03); $R_F(S_B)$ 0.17 (puromycin, 0.12); $R_F(S_C)$ 0.77 (puromycin, 0.76); ultraviolet spectrum (ethanol): λ_{max} 262 nm, λ_{min} 230 nm, λ_{sh} 275 nm; infrared spectrum (KBr): 2100 cm^{-1}, 1660 cm^{-1}, 1600 cm^{-1}; ^1H NMR (220 MHz, D_2O, ppm downfield from 3-(Trimethylsilyl)-tetradeutero sodium propionate): 3.34 (singlet: $-N(CH_3)_2$), 5.84 (doublet: anomeric H), 7.17 (quartet, A_2B_2 pattern: azidophenyl H), 8.03, 8.16 (singlets: purine H-2, H-8). *N-t*BOC-*p*-azido-[G-^{3}H]puromycin was synthesized in 51% yield when [G-^{3}H]puromycin aminonucleoside (4 mCi, 0.27 μmol) was treated with a 20-fold molar excess of EEDQ and *N-t*BOC-*p*-azido-L-phenylalanine in ethanol (0.05 ml) for 3 h at room temperature. The isolation and purification of radioactive *N-t*BOC-*p*-azidopuromycin was as described above. The BOC group was removed either by treatment with trifluoroacetic acid as above, or with 2 N HCl in ethyl acetate at 0°C for 0.5 h. The latter method led to a smaller amount of radioactive impurities. Preparative TLC was performed in S_A, and final purification accomplished by descending paper chromatography in S_C. The product band was eluted with ethanol. The radioactive compound had R_F values identical to those for *p*-azidopuromycin in solvent systems S_A, S_B, and S_C. It was stored at −20°C in ethanol with no apparent decomposition over several months.

Peptidyl transferase assays were performed using the method in [15]. Ribosome photoincorporation experiments were performed at 5 ± 1°C using 2 different sets of Rayonet lamps, RPR-2537 Å and RPR-3500 Å, as described, as were determinations of incorporation of the affinity label into RNA and protein and one-dimensional gel electrophoresis of ribosomal protein [4,5]. Two-dimensional polyacrylamide gel electrophoresis of ribosomal protein was performed using the modification [16] of the method in [17].

3. Results and discussion

3.1. *Chemical and biochemical properties of* p-*azidopuromycin*

The ultraviolet spectrum of *p*-azidopuromycin has an $A_{262\ nm}$ max and a shoulder at 275 nm (corresponding to the absorbance of the N^6, N^6-dimethyl-, adenosine moiety). Brief photolysis leads to a rapid loss of A_{262}, the appearance of a new λ_{max} at 275 nm and a broad shoulder centered at 310 nm. In our photolysis apparatus, *p*-azidopuromycin dissolved in ribosome buffer has a half-life of 7 s using the 2537 Å lamps and 4.2 min using the 3500 Å lamps, as determined by monitoring A_{262}. Although *p*-azidopuromycin is stable in ribosomal buffer at room temperature, the presence of dithiothreitol (5 mM) leads to a marked time-dependent spectral change. The A_{max} shifts from 262 nm to 275 nm with a half-life of conversion of 95 min (room temp.) [18]. Accordingly, solutions of *p*-azidopuromycin in DTT were kept cold and used soon after preparation.

The biochemical competence of *p*-azidopuromycin was demonstrated by its ability to accept [^{14}C]AcPhe from [^{14}C]AcPhe-tRNA^{Phe} bound in the ribosomal peptidyl site at a rate comparable to that obtained with puromycin (data not shown).

3.2. *Photoincorporation of* p-*azidopuromycin into ribosomes*

p-Azidopuromycin was found to efficiently co-

Table 1
Incorporation of *p*-azidopuromycin into ribosomes

No.	Radioactive ligand	Added ligand	Prephoto-lysis	Total incorp. (mol/mol ribosome)	Protein incorp.	RNA incorp.
1	p-Azidopuromycin 0.03 mM	–	–	0.77	0.56	0.21
2	p-Azidopuromycin 0.03 mM	–	+	0.14	0.12	0.02
3	p-Azidopuromycin 0.03 mM	Puromycin 1.9 mM	–	0.27	0.24	0.03
4	Puromycin 0.03 mM	–	–	0.03	0.02	0.01

Photolysis conditions: 70 S ribosomes, 1–2 μM; Photolysis for 4 min at 2537 Å. Results reported are corrected for background, defined as the radioactivity obtained in the ribosomal pellet when the photolysis step is omitted. Prephotolyzed *p*-azidopuromycin gave a higher background than *p*-azidopuromycin itself and this was taken into account in obtaining the numbers listed. This higher background was shown not to arise from a dark reaction with ribosomes and reflects incomplete removal of non-incorporated compound on ethanol precipitation of the ribosomes

valently label 70 S ribosomes on irradiation with either the 2537 Å or 3500 Å lamps. Some of the more salient features of the incorporation process are summarized in table 1. Overall incorporation is markedly reduced if *p*-azidopuromycin is first prephotolyzed to destroy the azido group and then rephotolyzed in the presence of ribosomes, providing strong evidence that incorporation is predominantly nitrene-dependent. This conclusion is supported by the finding that the amount of native puromycin incorporated into ribosomes is much less than that seen for *p*-azidopuromycin when both are irradiated under similar conditions. Photoincorporation of *p*-azidopuromycin proceeds mostly into protein and is extensively decreased by 1.9 mM puromycin which is evidence that much of the labeling is site-specific [5]. Labeling is also substantially decreased by 5 mM DTT (data not shown). The results in table 1 are for experiments using the 2537 Å lamps. Qualitatively similar results were obtained on irradiation with the 3500 Å lamps. One-dimensional polyacrylamide gel patterns of 70 S protein extracted from ribosomes labelled by *p*-azidopuromycin in the presence and absence of puromycin are shown in fig.2. Irradiation with the 3500 Å lamps yields a pattern showing a wide distribution of radioactivity, with no clear protection effects by puromycin. By contrast, irradiation with the 2537 Å lamps yields a pattern with 3 prominent peaks of radioactivity (I, II, III) of which two (I, II) are well-protected and one (III) is only partially protected by puromycin. Because of this difference, two-dimensional gel electrophoretic analysis of the incorporation products was carried out on proteins obtained from experiments using the 2537 Å lamps.

The results of this analysis are presented in fig.3, which represents the average of 4 photoincorporation experiments. The relative numbers shown refer to radioactivity comigrating with the proteins indicated, taking the radioactivity in S18 as 1.0. The acidic proteins (S1,L7,L9,L10,L12,S6) are not shown. Preliminary work had shown that none of these proteins was labeled to a major extent. Accordingly, subsequent two-dimensional gel electrophoreses were performed only on the basic proteins to maximize the resolution obtainable [5]. Analyzed in this manner there are two major labeled proteins, S18 and L11, and several proteins labeled to a secondary extent, most prominently L2, L3, L17, L18, L27, and S3, S4 and S5. The results are consistent with the one-dimensional pattern seen in fig.2B since proteins L2, L3, L11, S3, S4 and S5 fall in the region of the gel corresponding to peak I, L17 and L18 fall in peak II, and S18 falls in peak III [19,20]. From the two-dimensional results, S18 labeling should dominate peak III. Thus, the lack of protection by puromycin of peak III is evidence that S18 labeling is non-specific and does not proceed via an affinity labeling process. This recalls earlier work with *N*-bromoacetyl-Phe-tRNA, which was found to label S18 extensively but via a poly(U)-independent process [21], and may

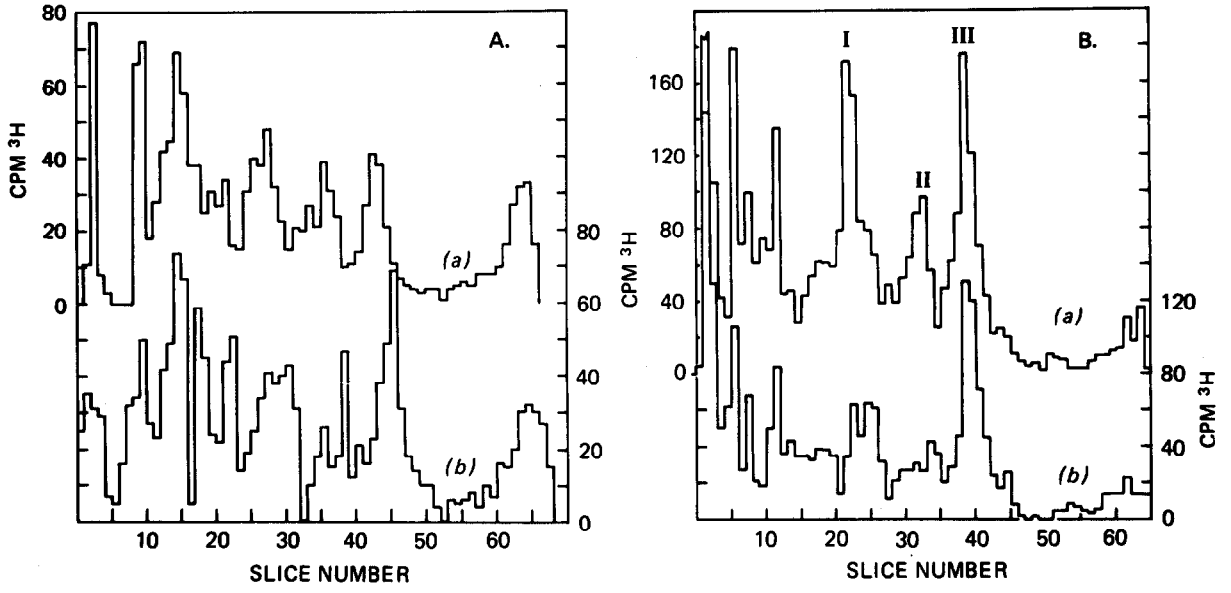


Fig.2. One-dimensional polyacrylamide gel electrophoretograms of labeled proteins from 70 S particles in the absence and presence of puromycin. (A) 54 A_{260} units/ml ribosomes; 0.033 mM *p*-azidopuromycin (1350 Ci/mol): a. no puromycin; b. plus 2.0 mM puromycin; photolysis was for 5 min with 3500 Å lamps. (B) 56 A_{260} units/ml ribosomes; 0.033 mM *p*-azidopuromycin (1350 Ci/mol): a. no puromycin; b. plus 1.9 mM puromycin; photolysis was for 4 min with 2537 Å lamps.

reflect the high intrinsic nucleophilicity of S18 [22].

From a quantitative point of view, the labeling pattern seen in fig.3 must be considered preliminary for 2 reasons:

1. There are several areas of the gel which were reproducibly found to contain appreciable amounts of radioactivity but which do not stain for protein. This may be due to an alteration of electrophoretic mobility on incorporation of *p*-azidopuromycin into a ribosomal protein in analogy to what has been seen in other ribosomal affinity labeling studies [5,23]. In the present case, the most prominent such areas are in the vicinities of S18 and L11, but there are other proteins sufficiently close by that the assignment of this radioactivity to a given protein could not be made unambiguously. For this reason, radioactivity in areas not staining for protein is not included in fig.3.

2. It is possible that some of the radioactivity incorporated into a given protein may show up in proteins which are near neighbors in the two-dimensional gel electrophoretogram. Thus the radioactivity seen in the group S3, S4 and S5 on the one hand, and the group L17 and L18 on the

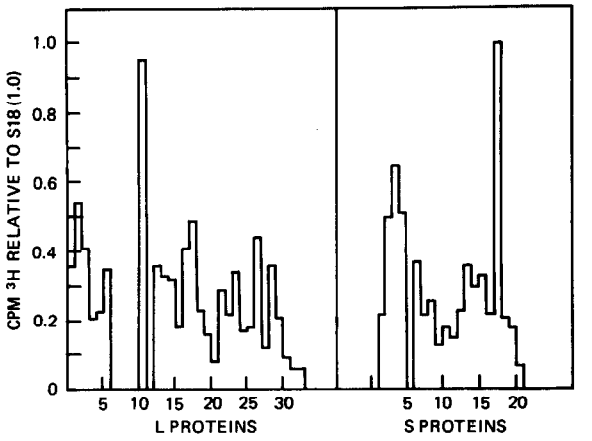


Fig.3. Two-dimensional gel electrophoresis pattern of 70 S ribosomal protein labeling. Experimental conditions were essentially as in fig.2B, except that higher specific radioactivity *p*-azidopuromycin was used (3000 Ci/mol).

other, might actually arise from the labeling of only a single protein in each of these groupings.

Immunoprecipitation studies, utilizing antibodies specific to ribosomal proteins, are now underway to resolve these uncertainties.

4. Conclusion

The peptidyl transferase center is known to be localized on the 50 S subunit [24] and by its inhibitory role puromycin should bind to this center. It is thus of interest to compare the present results with those obtained in other studies of the puromycin site and the peptidyl transferase center. The major 50 S protein photoaffinity labeled by native puromycin is L23 [4,5]. Although this protein is not a major incorporation site for *p*-azidopuromycin, it is important to note that immune electron microscopy studies place most of the 50 S proteins which are labeled by *p*-azidopuromycin, including L2, L11, L18 and L27, in the vicinity of L23 and within a limited area of the 50 S subunit [15]. Furthermore, L2, L11, L18 and L27 have been affinity labeled by both photolabile and electrophilic derivatives of *N*-acyl-aminoacyl-tRNA and chloramphenicol [21,26,28] and both L2 [29] and L11 [30] have been functionally implicated at the peptidyl transferase center. Thus, with respect to the 50 S subunit, photoaffinity labeling studies with both puromycin and *p*-azidopuromycin give consistent results in that both compounds incorporate predominantly into the same general area, and are in support of other results localizing the peptidyl transferase center to this area [1,25], although the particular proteins labeled by each compound appear to depend on the photochemistry utilized. A similar argument may apply to the 30 S proteins labeled by *p*-azidopuromycin, since parts of S3, S4, S5 and S18 have been shown by immune electron microscopy to be in the vicinity of S14 [25,31], the major 30 S protein photoaffinity labeled by puromycin [4,5].

Acknowledgements

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Note added in proof

The synthesis of the analogous compound, 2′(3′)-*O*-(4-azido-L-phenylalanyl) adenosine, has been reported [23].

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