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On the Structural Specificity of Puromycin Binding to *Escherichia coli* Ribosomes†

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ABSTRACT: We have examined the structural specificity of the puromycin binding sites on the *Escherichia coli* ribosome that we have previously identified [Nicholson, A. W., Hall, C. C., Strycharz, W. A., & Cooperman, B. S. (1982) *Biochemistry 19*, 3809–3817, and references cited therein] by examining the interactions of a series of adenine-containing compounds with these sites. We have used as measures of such interactions (a) the inhibition of [^{3}H]puromycin photoincorporation into ribosomal proteins from these sites, (b) the site-specific photoincorporation of the ^{3}H-labeled compounds themselves, and (c) the inhibition of peptidyl transferase activity. For the first two of these measures we have made extensive use of a recently developed high-performance liquid chromatography (HPLC) method for ribosomal protein separation [Kerlavage, A. R., Weitzmann, C., Hasan, T., & Cooperman, B. S. (1983) *J. Chromatogr. 266*, 225–237]. We find that puromycin aminonucleoside (PANS) contains all of the structural elements necessary for specific binding to the three major puromycin binding sites, those of higher affinity leading to photoincorporation into L23 and S14 and that of lower affinity leading to photoincorporation into S7. Although tight binding to the L23 and S7 sites requires both the N^6,N^6-dimethyl and 3′-amino groups within PANS, only the N^6,N^6-dimethyl group and not the 3′-amino group is required for binding to the S14 site. Our current results reinforce our previous conclusion that photoincorporation into L23 takes place from the A′ site within the peptidyl transferase center and lead us to speculate that the S14 site might be specific for the binding of modified nucleosides. They also force the conclusion that puromycin photoincorporation proceeds through its adenosyl moiety.

Photoaffinity labeling has proven itself to be an effective tool for identifying components at binding and functional sites of complex biological receptors. The antibiotic puromycin is a substrate for the peptidyl transferase center of the ribosome, and in previous work we have utilized both puromycin (Cooperman et al., 1975; Jaynes et al., 1978; Grant et al., 1979a,b) and an aryl azide derivative of puromycin, *p*-azidopuromycin[1] (Nicholson et al., 1982a,b), as photoaffinity labels for the peptidyl transferase center of the *Escherichia coli* ribosome. The major results of this work have been the following.

(1) Protein L23 is the major 50S protein labeled by both puromycin and *p*-azidopuromycin. The labeling of L23 proceeds from a site on the 50S subunit. There is a striking parallelism between the effects of added ligands on inhibiting labeling of L23 and on inhibiting peptidyl transferase, which we have taken as evidence that the labeling of L23 proceeds from the A′ site, defined as the site of binding of the 3′-end of aminoacyl-tRNA within the peptidyl transferase center.

(2) Other L proteins, particularly L18/22 and L15, also appear to be labeled by *p*-azidopuromycin from this site.

(3) Small subunit proteins are also labeled from sites on the 30S subunit, most notably S14 by puromycin and S7 and S18 by *p*-azidopuromycin.

(4) Puromycin aminonucleoside (PANS), which lacks the *O*-methyltyrosine moiety of puromycin, is almost as effective as puromycin in inhibiting L protein labeling by *p*-azidopuromycin and is a good competitive inhibitor of peptidyl transferase. It is, however, somewhat less effective in inhibiting *p*-azidopuromycin labeling of S7 and S18.

The finding that puromycin binds specifically to sites on the 30S and 50S subunits and that the full puromycin structure is not always essential for such binding raises the question of

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[1] Abbreviations: *p*-azidopuromycin, 6-(dimethylamino)-9-[3-deoxy-3-[(*p*-azido-L-phenylalanyl)amino]-D-ribofuranosyl]purine; N-AcPhe, *N*-acetylphenylalanine; PAGE, polyacrylamide gel electrophoresis; PANS, puromycin aminonucleoside; RP-HPLC, reverse-phase high-performance liquid chromatography; TP30, TP50, and TP70, total protein from 30S and 50S subunits and 70S ribosomes, respectively; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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the structural specificity of these sites. We address this question below by examining the binding to these sites of a series of adenine-containing compounds, using, as measures of binding, (a) the inhibition of [^{3}H]puromycin photoincorporation, (b) the site-specific photoincorporation of the ^{3}H-labeled compounds themselves, and (c) the inhibition of peptidyl transferase activity. The first two of these measures require precise determination of the radioactivity incorporated into specific ribosomal proteins in a large number of samples. For this purpose we have made extensive use of the HPLC method for ribosomal protein separation recently developed in this laboratory (Kerlavage et al., 1982, 1983a,b).

Our results reinforce the notion that L23 labeling takes place from the A′ site, demonstrate that PANS is the minimal structure for tight binding to this site, show that both S7 and S14 are significantly labeled by puromycin from separate sites on the 30S subunit, with the S14 site having considerably higher affinity, and reveal a striking disparity in the structural requirements for binding to the S14 and L23 sites. We conclude with a brief discussion of the possible functional significance of puromycin binding sites on the 30S subunit.

Experimental Procedures

Materials. The following buffers were used: TMK, 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 50 mM KCl; TMKNa, TMK plus 400 mM NaCl.

Trifluoroacetic acid (Pierce), HPLC-grade acetonitrile (Fisher), tRNA^{Phe} (Boehringer-Mannheim), puromycin, PANS, N^6,N^6-dimethyladenosine, adenosine, and adenine (all Sigma), and [^{14}C]phenylalanine (500 Ci/mol, New England Nuclear) were used without further purification. 3′-Amino-3′-deoxyadenosine was a gift from Dr. Robert Suhadolnik of Temple University. [8-^{3}H]Puromycin was prepared from [8-^{3}H]PANS (Amersham) essentially as described for the preparation of [8-^{3}H]-*p*-azidopuromycin (Nicholson & Cooperman, 1978), except that all products were ethanol soluble and were purified by HPLC. [8-^{3}H]Puromycin, as well as the other radioactive adenine-containing compounds used in photoincorporation experiments, [2-^{3}H]- and [8-^{3}H]adenine (Amersham), [8-^{3}H]adenosine (Amersham), and [8-^{3}H]-N^6,N^6-dimethyladenosine (Moravek Biochemicals) were all purified by HPLC (Kerlavage et al., 1985) no more than 30 days prior to use. It is important to note that failure to remove contaminating radioactivity from commercially supplied radioactive compounds in some cases led to artifactual photoincorporation results.

[^{14}C]-*N*-Acetyl-Phe-tRNA^{Phe} was prepared as described (Nicholson et al., 1982b). The 70S ribosomes were prepared from *E. coli* Q 13 bacteria as described in Jaynes et al. (1978) as method T. Ribosomal subunits were isolated by ultracentrifugation through a 15%–30% sucrose density gradient made up in TMKNa buffer containing 1% β-mercaptoethanol, with a VTi 50 rotor, as previously described (Goldman et al., 1983).

Methods. Photolytic incoporation of radioactive puromycin or of other radioactive adenine-containing compounds into 70S ribosomes (100 A_{260}/mL) was performed in TMK buffer at 4 °C with Rayonet RPR 3500-Å lamps as described (Jaynes et al., 1978). A 2-h irradiation period was used. Immediately following photolysis, ribosomes were precipitated with 2 volumes of ethanol–β-mercaptoethanol (9:1). The pellets were resuspended in TMK and reprecipitated with two volumes of the ethanol–β-mercaptoethanol solution. This procedure was repeated a total of 3 times. The final pellet was either dissolved in TMK buffer containing 6 mM β-mercaptoethanol and extracted directly, with the Mg^{2+}–acetic acid procedure (Hardy et al., 1969), to prepare photolabeled TP70, or dissolved in TMKNa buffer containing 6 mM β-mercaptoethanol for eventual subunit separation. Subunits were precipitated from sucrose density gradients by addition of 2 volumes of ethanol and incubation at −20 °C overnight and extracted to prepare photolabeled TP50 and photolabeled TP30.

One-dimensional polyacrylamide gel electrophoresis (PAGE) analysis of ribosomal proteins was performed as described (Jaynes et al., 1978). Two-dimensional PAGE analysis of ribosomal was performed either with a modification (Grant et al., 1979a) of the Howard & Traut (1974) method or with the procedure of Kenny et al. (1979).

HPLC analysis of labeled ribosomal proteins was performed essentially as described (Kerlavage et al., 1983a) except that a Waters μBondapak C_{18} column was used instead of a Synchropak RP-P column. Radioactivity in eluted fractions was measured as previously described (Kerlavage et al., 1983b). Recoveries were typically 80%–90% for both applied protein and radioactivity. The peptidyl transferase assay, measuring N-AcPhe transfer from N-AcPhe-tRNA^{Phe} to puromycin, was performed as described (Nicholson et al., 1982b).

Results

RP-HPLC Analysis of Ribosomal Proteins Photolabeled by Puromycin. In our previous studies of puromycin photoincorporation into ribosomes we used a combination of one- and two-dimensional PAGE analyses and specific immunoprecipitation analysis to identify labeled proteins. The results presented in this paper represent the first application of reverse-phase high-performance liquid chromatography (RP-HPLC) analysis to a full photoaffinity labeling study of *E. coli* ribosomal proteins. We prefer this method to PAGE analysis because it is rapid, affords high yields of protein and of radioactivity, and is quite reproducible. It should, however, be emphasized that PAGE analysis was used quite extensively in the early phases of this work and, where comparisons were made, gave qualitatively and even semiquantitatively similar results to those obtained by RP-HPLC.

Typical HPLC analyses of TP50 and TP30 extracted from 50S subunits and 30S subunits derived from 70S ribosomes photoaffinity labeled with [^{3}H]puromycin and of TP70 extracted from photoaffinity-labeled 70S ribosomes are shown in panels A–C of Figures 1, respectively. Figure 1A shows a single major radioactive peak, eluting with protein L29 and just after proteins L22/L23. On the basis of our previous results, this peak can be assigned to labeled protein L23, demonstrating that in the highly resolving HPLC system used in this work modified L23, containing photoincorporated puromycin, has a slightly altered retention time compared with that of native protein. Figure 1B shows two major labeled peaks, one eluting just ahead of protein S14 and one coeluting with S7. On the basis of our previous results and the fact that S14 is well resolved from other ribosomal proteins, it is clear that the first peak is due to labeled S14. Protein S7 is also very well resolved, so we may confidently assign the second peak to labeled S7. This result recalls our finding that, in the presence of β-mercaptoethanol, S7 is the major 30S protein labeled by *p*-azidopuromycin (Nicholson et al., 1982a). Our previous failure to identify S7 as a major puromycin-labeled 30S protein (Jaynes et al., 1978; Grant et al., 1979) is attributable to the poor resolution we obtained for proteins S4, S5, and S7 in the two-dimensional PAGE analysis we employed. Thus, we interpreted the radioactivity in the area containing these proteins as representing labeling of all three proteins, whereas it is clear from Figure 1B that of these three proteins only S7 is appreciably labeled. Figure 1C shows four major peaks of radioactivity. The first three represent labeled

Table I: Photoincorporation of [^{3}H]Puromycin into Ribosomal Proteins: Effects of Added Ligands[a]

added nonradioactive ligand[b]	L23		S14		S7	
	TP70	TP50	TP70	TP30	TP70	TP30
	Incorporation (% mol/mol)					
	0.84 ± 0.28	0.63 ± 0.05	0.26 ± 0.07	0.12 ± 0.005	0.29 ± 0.07	0.18
	Relative Incorporation[c]					
puromycin	0.12	nd[d]	0.10	nd	0.53	nd
PANS	0.25 ± 0.04	0.15 ± 0.01	0.23 ± 0.01	0.21	0.76 ± 0.01	0.65
3′-amino-3′-deoxyadenosine	0.63 ± 0.08	0.45 ± 0.02	1.57 ± 0.02	1.82 ± 0.11	1.17 ± 0.13	1.01
N^6,N^6-dimethyladenosine	0.97 ± 0.04	0.71 ± 0.08	0.32 ± 0.01	0.21 ± 0.07	0.97 ± 0.01	0.68
adenosine	1.08	1.05	1.21	0.91	1.06	nd
adenine	1.38	1.04	2.14	nd	1.87	nd

[a] Incorporation levels were determined from ^{3}H radioactivity coeluting with the indicated proteins in RP-HPLC chromatograms of TP70 from labeled 70S ribosomes or of TP50 and TP30 derived from labeled 70S ribosomes. Photolysis and RP-HPLC analysis were performed as described in Figure 1; [^{3}H]puromycin (1 Ci/mmol) concentration was 0.1 mM. [b] All present at 2.0 mM. [c] Relative incorporation values were calculated as the ratio of incorporated radioactivity in the presence of added nonradioactive ligand to incorporated radioactivity in the absence of nonradioactive ligand. [d] nd, not determined.

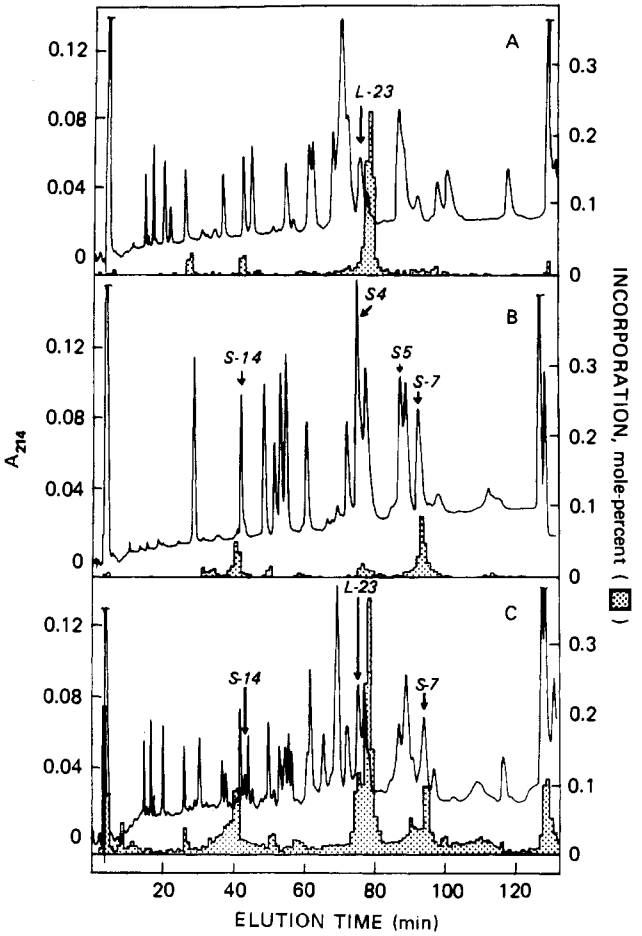

FIGURE 1: RP-HPLC analyses of TP50, TP30, and TP70 derived from *E. coli* ribosomes photoaffinity labeled with [^{3}H]puromycin: (A) TP50; (B) TP30; (C) TP70. Continuous solid line records A_{214}, and shaded histogram shows radioactivity in sample. Heavy arrows indicate proteins. Elution positions of ribosomal proteins were determined previously (Kerlavage et al., 1982, 1983a). The photoincorporation experiment was conducted as described under Methods, at 0.1 mM [^{3}H]puromycin, specific radioactivity 1.0 Ci/mmol. The samples analyzed were extracted from 4.6, 3.6, and 5.9 A_{260} units of 50S, 30S, and 70S particles, respectively.

S14, L23, and S7 and confirm our earlier results that L23 is the major labeled protein. The fourth peak, at 130 min, occurs in the region of the chromatogram in which the acetonitrile content of the eluting solvent is increasing rapidly and coelutes with an aggregate of denatured protein (Kerlavage et al., 1983b). The radioactivity in this peak was found to be highly variable, not subject to inhibition in the presence of other

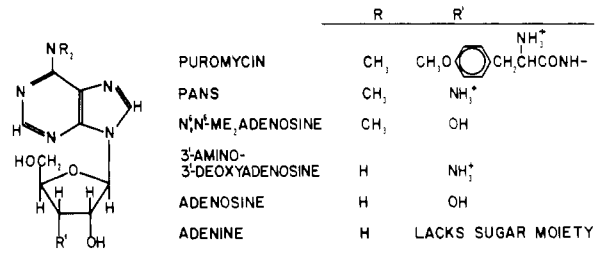

FIGURE 2: Adenine-containing compounds used in this study.

ligands (see below), and essentially absent from TP30 and TP50 chromatograms. It probably represents the sum of small amounts of nonspecific incorporation into several proteins and was not investigated further.

From these results it is clear that while HPLC analysis at the subunit level is important for the identification of a radioactively labeled peak, the radioactivity in a known peak is readily quantitated by HPLC analysis of TP70. Because S7, S14, and L23 are all well resolved from one another, many of the analyses presented below could be performed on TP70. This not only eliminates the large sample losses we incur (~50%) during subunit preparation by sucrose density gradient ultracentrifugation but also reduces the number of HPLC analyses required by half.

Effect of Nonradioactive Puromycin and Puromycin Analogues on [^{3}H]Puromycin Photoincorporation into Ribosomal Proteins. Unlabeled puromycin, PANS, 3′-amino-3′-deoxyadenosine, N^6,N^6-dimethyladenosine, adenosine, and adenine (Figure 2) were added to photolysis mixtures to test their effects on puromycin photoincorporation as measured by HPLC analysis. In all cases, the only major peaks seen were those shown in Figure 1, so that the results may be accurately and conveniently summarized in tabular form (Table I). Although the qualitative effects are the same regardless of whether TP70 or subunit proteins are analyzed, there is a clear tendency for apparent competitive effects to be greater for analyses at the subunit level. This is almost certainly due to greater contamination of the major peaks seen on TP70 analysis with proteins labeled nonspecifically to small extents. Making the assumption that the extent to which an added ligand depresses puromycin photoincorporation into a protein reflects the competitive binding affinity of that ligand for the site from which photoincorporation occurs allows the following conclusions to be drawn from the data presented in Table I.

(1) Puromycin labels L23 and S14 from relatively high-affinity sites (K_D < 1 mM), in accord with our earlier results (Jaynes et al., 1978) but labels S7 from a site of considerably lower affinity (K_D ~ 2 mM). *p*-Azidopuromycin also labels

Table II: Photoincorporation of ^{3}H-Labeled Puromycin and Puromycin Analogues into Ribosomal Proteins: Effects of Added Nonradioactive Puromycin[a]

	incorporation (% mol/mol)					
	L23		S14		S7	
radioactive ligand	TP70	TP50	TP70	TP30	TP70	TP30
puromycin, 0.1 mM[b]	0.84 (0.10)	0.63	0.27 (0.028)	0.12	0.29 (0.15)	0.18
PANS, 0.1 mM	0.70 (0.045)	nd[c]	0.13 (0.009)	nd	0.15 (0.036)	nd
N^6,N^6-dimethyladenosine, 0.25 mM	nd	0.05 (0.01)	nd	0.48 (0.10)	nd	0.06 (0.01)

[a] Incorporation levels were determined as described in Table I. Values in parentheses were obtained for photolyses conducted in the presence of nonradioactive puromycin (2.0 mM). [b] In the case of puromycin incorporation, values in parentheses were calculated by assuming no change in the specific radioactivity puromycin. [c] nd, not determined.

S7 from a site of lower affinity than the site from which it labels L23 (Nicholson et al., 1982b).

(2) PANS binds somewhat more weakly than puromycin to each of these sites but shows the same pattern of specificity; binding to the sites leading to L23 and S14 labeling is comparable and tighter than binding to the site leading to S7 labeling.

(3) N^6,N^6-Dimethyladenosine binds to the S14 site about as well as does PANS but shows much weaker binding to both the L23 and S7 sites. By contrast, 3′-amino-3′-deoxyadenosine, though binding more weakly than PANS, apparently binds to the L23 site more tightly than to either the S14 site or the S7 site [although the observed stimulation into these latter sites complicates our interpretation of the photoincorporation effects (see Discussion)].

(4) Adenosine shows no apparent binding to any of the sites. Adenine stimulates puromycin labeling of all of the sites, especially the two on the 30S subunit.

Photoincorporation of Tritiated PANS and Related Compounds. On irradiation at 350 nm, [^{3}H]PANS photoincoporates into ribosomes, predominantly into ribosomal proteins. A typical RP-HPLC analysis of the labeled proteins is shown in Figure 3A. From a comparison of Figure 3A with Figure 1C, it is clear that the overall labeling pattern seen with [^{3}H]PANS is virtually identical with that seen for [^{3}H]puromycin: i.e., a dominant peak eluting near protein L23 and two secondary peaks eluting near protein S14 and with protein S7, respectively. In addition, a third secondary peak elutes in the vicinity of proteins S10, S16, and L18. Two-dimensional PAGE analysis (Figure 4) of labeled TP70 shows the major peak of radioactivity migrating just slightly retarded (in the second dimension) with respect to protein L23. This is identical with the location of the major peak of radioactivity found for proteins derived from ribosomes labeled with [^{3}H]puromycin (Jaynes et al., 1978) and confirms the identity of L23 as the major labeled peak. Less intense areas of radioactivity are seen near proteins S14, S7, L18, and S16, thus providing supporting evidence for the identification of these proteins as labeled to secondary extents.

In addition to the protein labeling pattern, photoincorporation of PANS shows two other important similarities to photoincorporation of puromycin. First, added unlabeled puromycin decreases photoincorporation of [^{3}H]PANS with the effect being larger for incorporation into proteins L23 and S14 and smaller for incorporation into protein S7. These results are summarized in Table II. Second, photolysis in the presence of chloramphenicol leads to a marked stimulation of [^{3}H]PANS incorporation into protein S14 (as it does for [^{3}H]puromycin; Grant et al., 1979) so much so that protein S14 becomes the major labeled protein (Figure 4B). The similarity of PANS and puromycin photoincorporation results leads to the conclusion that puromycin photoincorporation proceeds through its N^6,N^6-dimethyladenosine moiety and not through its tyrosine methyl ether moiety as was previously speculated (Jaynes et al., 1978).

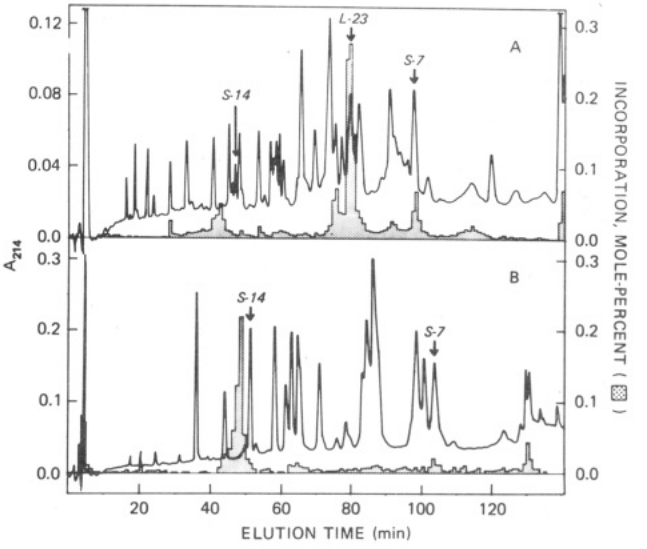

FIGURE 3: RP-HPLC analyses of ribosomal proteins photoaffinity labeled with [^{3}H]PANS or N^6,N^6-dimethyladenosine: (A) TP70 from ribosomes labeled with [^{3}H]PANS, specific radioactivity 2.7 Ci/mmol; (B) TP30 from 70S ribosomes photoaffinity labeled with [^{3}H]-N^6,N^6-dimethyladenosine, specific activity 0.5 Ci/mmol. The samples analyzed were extracted from 5.7 and 6.8 A_{260} units of 70S and 30S particles, respectively. Continuous solid line records A_{214}, and shaded histogram shows radioactivity in sample.

[^{3}H]-N^6,N^6-Dimethyladenosine (0.25 mM) also photoincorporates into ribosomal protein on irradiation at 350 nm. A single peak of radioactivity appears in the elution profile of TP30, eluting just ahead of protein S14 (Figure 3B). This labeling is markedly decreased by 2.0 mM puromycin. No major peak of radioactivity is observed in the 50S protein profile. In particular, L23 is not labeled (Table II).

Photolysis of ribosomes with [^{3}H]adenosine (0.25 mM) does not result in any appreciable amount of labeling at the subunit level, and no labeling of any individual protein is detectable. Even at light doses nearly 6 times higher than those in the standard procedure used to generate the data in Tables I and II, incorporation was negligible.

Adenine (0.25 mM), labeled with tritium at the 2-position or at the 8-position, photoincorporates into 70S ribosomes with virtually all the label extracting with proteins. The extent of labeling is >10-fold reduced as compared with that of puromycin labeling. HPLC analyses of both TP30 and TP50 for photoincorporations conducted in the presence and absence of puromycin show little evidence of site-specific labeling.

Effect of Adenine and 3′-Amino-3′-deoxyadenosine on Peptidyl Transferase Activity. We previously have shown that PANS is a competitive inhibitor, vs. puromycin, of ribosome-catalyzed N-AcPhe transfer from N-AcPhe-RNA to puromycin, with a K_I of 0.5 mM, whereas N^6,N^6-dimethyladenosine and adenosine show lesser inhibition of this reaction (Nicholson et al., 1982b). These studies are here extended

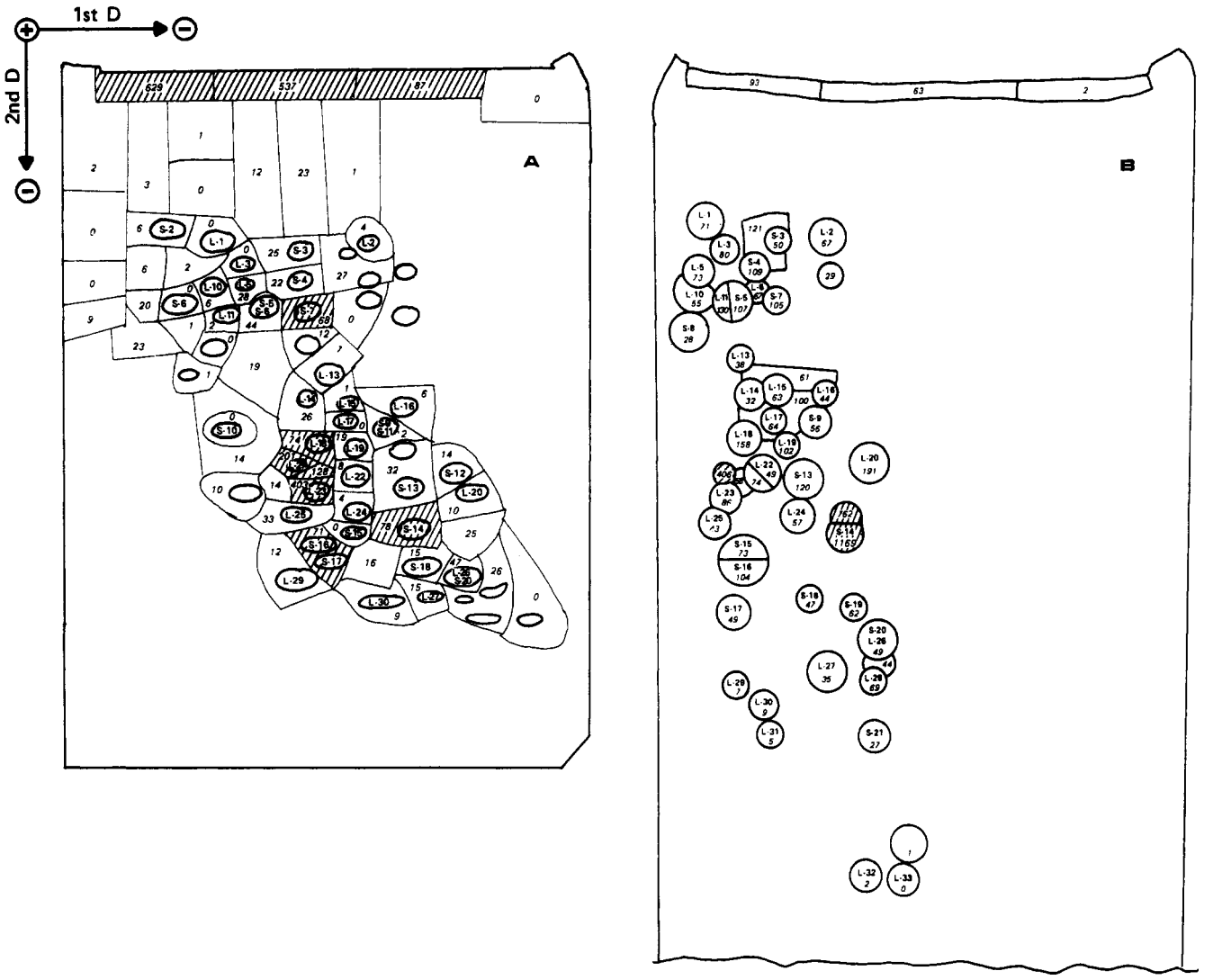

FIGURE 4: Two-dimensional urea–PAGE analysis of TP70 from ribosomes photoaffinity labeled with [^{3}H]PANS: (A) in the absence of chloramphenicol; (B) in the presence of 0.5 mM chloramphenicol. Boldface numbers refer to proteins, and italicized numbers correspond to radioactivity (cpm). The areas of highest radioactivity are shaded. In (A), 20 A_{260} units was extracted, and the gel was run according to Kenny et al. (1979). Heavy lines represent stained proteins. Light lines indicate gel regions containing the indicated radioactivity. In (B), 44 A_{260} units was extracted, and the gel was run with a modification (Grant et al., 1979a) of the Howard & Traut (1974) method. Stained proteins and some surrounding gel regions, as indicated by heavy lines, were cut out and counted.

to 3′-amino-3′-deoxyadenosine and adenine. Peptidyl transferase reactions display first-order kinetics as previously described (Nicholson et al., 1982b). The observed rate constants in the presence of different concentrations of these two compounds, when plotted in Eadie–Hofstee form (Figure 5), yield results that preclude a simple competitive inhibition mechanism for either of these compounds, unlike what was observed previously with PANS. Attempts to fit our data to a simple noncompetitive scheme (eq 1) were also unsuccessful, in that

$$k_{obsd} = \frac{k_2}{1 + \frac{[I]}{K_I'}} - \frac{k_{obsd}K_D\left[1 + \frac{[I]}{K_I}\right]}{[Puro]\left[1 + \frac{[I]}{K_I'}\right]} \quad (1)$$

$$V = k_{obsd}[C]_T = k_2[C\cdot Puro]$$

C = the ribosome–poly(U)–N-AcPhe-tRNA complex

$$K_D = \frac{[C][Puro]}{[C\cdot Puro]} \qquad K_I = \frac{[C][I]}{[C\cdot I]} \qquad K_I' = \frac{[C\cdot Puro][I]}{[C\cdot Puro\cdot I]}$$

$$[C]_T = [C] + [C\cdot Puro] + [C\cdot I] + [C\cdot Puro\cdot I]$$

nonconstant values of K_I and K_I' were obtained. For adenine, K_I' decreases from 1.9 to 0.6 mM as adenine concentration increases from 0.36 to 1.5 mM; similarly, K_I' for 3′-amino-3′-deoxyadenosine decreases from 3.5 to 3.0 mM as 3′-amino-3′-deoxyadenosine concentration is raised from 1.0 to 1.5 mM. Our calculated K_I values are less reliable and do not show a clear trend, but average about 0.3 mM for adenine and 1 mM for 3′-amino-3′-deoxyadenosine.

Discussion

The work reported in this paper was prompted by two earlier results obtained by this group: first, that puromycin photoincorporated into the *E. coli* ribosome from at least two different binding sites, one on the 50S subunit leading to incorporation into L23 and believed to be the peptidyl transferase center and one on the 30S subunit (Jaynes et al., 1978; Grant et al., 1979) leading to incorporation into S14; second, that PANS binds to the 50S site almost as well as puromycin, as evidenced by its ability to inhibit photoincorporation of *p*-azidopuromycin from this site as well as to function as a competitive inhibitor of puromycin in a peptidyl transferase assay (Nicholson et al., 1982b). These two effects of PANS showed clearly that the *O*-methyltyrosine moiety of

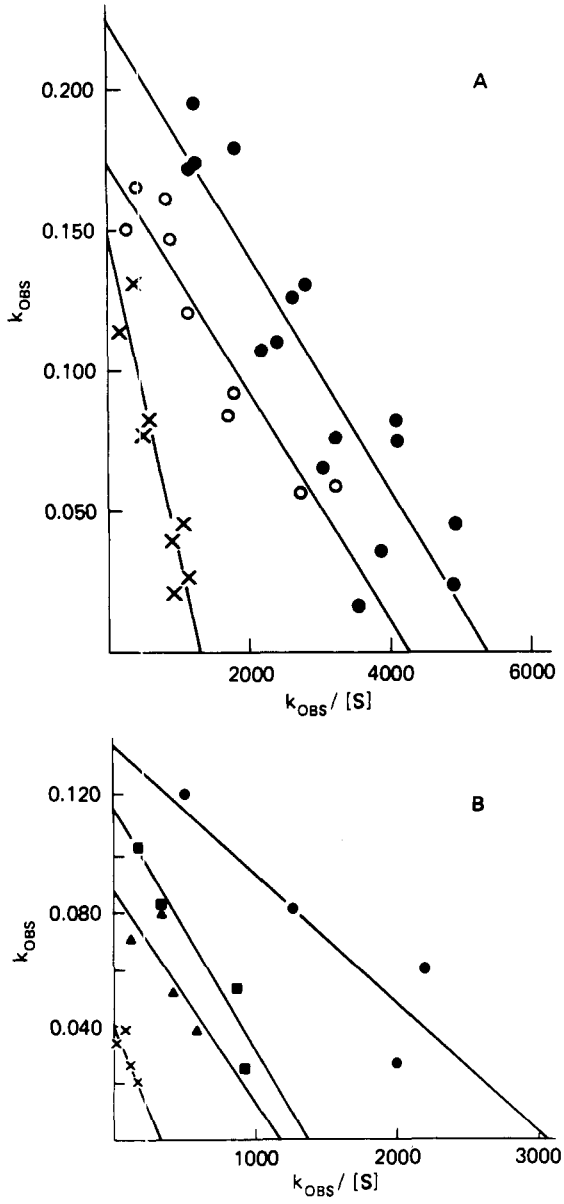

FIGURE 5: Eadie–Hofstee plots demonstrating inhibition of peptidyl transferase activity. (A) By 3′-amino-3′-deoxyadenosine: (●) no inhibitor present; (○) 1.0 mM inhibitor; (×) 1.5 mM inhibitor. (B) By adenine: (●) no inhibitor present; (■) 0.36 mM inhibitor; (▲) 0.72 mM inhibitor; (×) 1.46 mM inhibitor.

puromycin was not essential either for binding to the A′ site of the peptidyl transferase center or for binding to the 50S site leading to photoincorporation into L23 and provided addition evidence that these two sites are, in fact, the same (Nicholson et al., 1982b). Having established that PANS contained all the necessary structural determinants for binding to the L23 site, the questions we address in this paper are, first, can even simpler adenosine derivatives than PANS bind strongly to the L23 site, second, is the parallelism between binding to the L23 site and effects on peptidyl transferase maintained for the new derivatives examined, and, third, is the structural specificity for binding to the S14 site the same as for binding to the L23 site? Our current finding that S7 was also a major 30S protein labeled by puromycin led us to broaden our inquiry to the 30S site leading to photoincorporation into S7 as well. We examine these questions in turn below.

PANS appears to be in the minimal structure necessary for tight binding to the L23 site. As measured by inhibition of puromycin photoincorporation, the order of affinities is PANS > 3′-amino-3′-deoxyadenosine > N^6,N^6-dimethyladenosine > adenosine. The lack of appreciable photoincorporation of N^6,N^6-dimethyladenosine into L23 provides an independent measure of the poor affinity of this ligand for the L23 site. Adenosine also shows no photoincorporation into L23, a result that is consistent with the lack of adenosine inhibition of puromycin photoincorporation into L23. However, the interpretation of this result is ambiguous, since it depends on a knowledge of the mechanism of photoincorporation that we do not currently have. If photoincorporation depends exclusively on the generation of an electronically excited state of the adenosyl moiety in puromycin or the puromycin analogues examined in this work, then the lack of adenosine photoincorporation may be due to the poor overlap between the adenosine chromophore (as compared with the N^6,N^6-dimethyladenosine chromophore) and the output of the RPR 3500-Å lamps. This conclusion is based on two considerations. First, we have determined that the yield of puromycin photoincorporation is similar whether the sample undergoing irradiation is contained in a quartz tube that is transparent above 240 nm or in a flint glass tube that is essentially opaque below 305 nm, so that for the RPR 3500-Å lamps used it is light of wavelength > 305 nm that is responsible for puromycin photoincorporation. Second, the adenosine chromophore (λ_{max} 260 nm) is blue-shifted with respect to the N^6,N^6-dimethyladenosine chromophore (λ_{max} 275 nm) and as a consequence has much lower absorbance at wavelengths > 300 nm. For example, we estimate ϵ_{305} for N^6,N^6-dimethyladenosine to be approximately 500 whereas it is <50 for adenosine. Alternatively, photoincorporation may proceed exclusively via an electronically excited state of, for example, the tyrosine side chain in L23 (L23 contains no tryptophan) (Giri et al., 1984), in which case the differences in the adenosine and N^6,N^6-dimethyladenosine chromophores would be largely irrelevant and the lack of adenosine photoincorporation would be likely to directly reflect weak adenosine binding to the L23 site. A third measure of binding is provided by studies of peptidyl transferase. Thus, N^6,N^6-dimethyladenosine and adenosine (Nicholson et al., 1982b) and 3′-amino-3′-deoxyadenosine (this work) are weaker inhibitors of this activity than is PANS (Nicholson et al., 1982b), providing yet another example of the parallelism between effects on puromycin labeling of L23 and effects on peptidyl tranferase.[2] To summarize, both the 3′-amino and the N^6,N^6-dimethyl groups are important for binding to the L23 site, with the 3′-amino group being perhaps more important.

The specificity for binding to the S14 site is different. Here it is clear that N^6,N^6-dimethyladenosine includes all the necessary determinants for tight binding and that the 3′-amino group is unimportant. Thus, N^6,N^6-dimethyladenosine blocks puromycin incorporation into S14 almost as well as does PANS and photoincorporates into S14 even better than does PANS. On the other hand, the dimethyl group is critical for binding, since adenosine does not inhibit puromycin photoincorporation. In addition, adenosine does not itself photoincorporate, although the interpretation of this result is subject to the same ambiguity as discussed above for L23 labeling.

[2] It is true that adenine, which strongly inhibits peptidyl transferase as measured by its apparent K_I value of approximately 0.3 mM, does not inhibit puromycin photoincorporation into L23, nor does it itself photoincorporate. However, the effect of adenine on peptidyl transferase is complex, and there is no compelling reason for interpreting its apparent K_I as being due to binding to the A′ site. Indeed, our results would suggest that it is *not* due to such binding.

Here it should be noted that S14 contains both a tyrosine and a tryptophan. 3′-Amino-3′-deoxyadenosine actually stimulates rather than inhibits puromycin photoincorporation into S14, which might be an indication of a conformational change of the ribosome induced by this compound, leading to increased access of S14 to puromycin. A similar effect might explain the stimulation of S14 labeling induced by adenine. Such effects would be consistent with the observed decrease of apparent K_I' as a function of inhibitor concentration in the peptidyl transferase assay, since this trend is indicative of inhibitor binding to a multiplicity of sites, which could well give rise to conformational changes. The structural specificity for the S7 site would appear from our data to more closely resemble that of the L23 site, with both the dimethylamino and 3′-amino groups required, but the binding to this site is so weak even with PANS or puromycin that this conclusion must be considered tentative.

Zemlicka et al. (1975) have obtained strong evidence that the charged α-ammonium group in puromycin is important for its functional binding to the A′ subsite. Our demonstration of the importance of the 3′-amino group for binding to both the L23 site and the peptidyl transferase center leads us to conclude that a 3′-amine is a reasonable substitute for an α-amine for such binding. The importance of the N^6,N^6-dimethyl group for binding to the 50S subunit is somewhat unexpected, given the lack of methyl groups at the 3′-terminus of tRNA. However, the A′ site is known to have a hydrophobic character (Symons et al., 1978; Krayevsky & Kukhanova, 1979), so that the increased affinity of PANS vs. 3′-amino-3′-deoxyadenosine could be due to a hydrophobic interaction.

A similar explanation could account for the much higher affinity of N^6,N^6-dimethyladenosine than adenosine for the S14 site on the 30S subunit. Although the functional significance of an adenosine site on the 30S subunit is not established, we previously (Nicholson et al., 1982b) have discussed the possibility that portions of S14 (and of S7) may fall within the tRNA binding locus on the 30S subunit and bind to adenine or purine-invariant positions in the family of tRNA structures (Singhal & Fallis, 1979; Sprinzl & Gauss, 1984), and Ivanov & Saminsky (1984) have recently shown that puromycin inhibits poly(U)-dependent N-AcPhe-tRNA^{Phe} binding to 30S subunits. An alternative though related possibility is that S14 is part of a site specific for the binding of N^6-modified adenosines. N^6,N^6-Dimethyladenosine is unlikely to be the natural ligand since its only known occurrence in *E. coli* is in the 3′-terminal region of 16S RNA, which has been demonstrated by immuno-electron-microscopy studies to be quite far from S14 (Politz & Glitz, 1977; Lake et al., 1974; Tischendorf et al., 1974; Grant et al., 1983). However, other N^6-modified adenosines, such as N^6-methyladenosine and N^6-isopentenyladenosine, occur frequently in tRNAs. The specificity of these modified nucleosides for binding to the S14 site is currently under investigation.[3]

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Registry No. PANS, 58-60-6; puromycin, 53-79-2; N^6,N^6-dimethyladenosine, 2620-62-4; 3′-amino-3′-deoxyadenosine, 2504-55-4; adenosine, 58-61-7; adenine, 73-24-5; peptidyltransferase, 9059-29-4.

[3] Added in proof: Recent experiments have shown that neither N^6-methyladenosine nor N^6-isopentenyladenosine blocks [^{3}H]puromycin photoincorporation into S14. Thus, the functional significance of the observed site specificity for N^6,N^6-dimethyladenosine remains unclear.

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