Puromycin Photoaffinity Labels Small- and Large-Subunit Proteins at the A Site of the *Drosophila* Ribosome†

Michael Gilly and Maria Pellegrini*

Molecular Biology Section, University of Southern California, Los Angeles, California 90089

Received January 10, 1985

ABSTRACT: [^{3}H]Puromycin covalently incorporates into the protein and to a much lesser extent into the RNA components of *Drosophila* ribosomes in the presence of 254-nm light. The photoincorporation reaction takes place with a small number of large- (L2 and L17) and small- (S8 and S22) subunit proteins as determined by two-dimensional gel analysis. More quantitative one-dimensional gel results show that puromycin reacts with each of these proteins in a functional site specific manner. The small percentage of the total labeling that occurs with rRNA also appears to be site specific. The rRNA labeling arises from a puromycin-mediated cross-linking of ribosomal protein and rRNA. Ionic conditions shift the pattern of puromycin-labeled ribosomal proteins. These results suggest that puromycin can occupy two distinct sites on *Drosophila* 80S ribosomes. The pattern of ribosomal proteins labeled by puromycin is affected by the presence of other antibiotics such as emetine, anisomycin, and trichodermin.

Photoaffinity labeling is well established as an important method for identifying functional sites on the ribosome [reviewed by Cooperman (1980)]. In particular, the antibiotic puromycin has been used to localize protein and RNA components in both *Escherichia coli* and rat liver ribosomes (Cooperman et al., 1975; Jaynes et al., 1978; Grant et al., 1979a,b; Reboud et al., 1981). Since puromycin is a substrate for the ribosomal peptidyltransferase reaction, localization of its binding site can also provide valuable information concerning this active center. Here we present evidence that puromycin can function as a photoaffinity label for the A site of *Drosophila* ribosomes in a functional site specific manner. Particular ribosomal components can then be localized at or near the puromycin binding site.

Materials and Methods

Ribosome Isolation Procedure. Ribosomes were isolated from frozen, dechorionated, 0–24-h *Drosophila melanogaster* embryos essentially according to the method of Santon & Pellegrini (1980), except the ribosome pelleting step was accomplished by centrifugation at 22000 rpm for 18 h at 4 °C, in a Beckman SW27 rotor. The ribosome pellets were quick-frozen in liquid nitrogen and stored at −70 °C. In order to prepare ribosomes for use in either the fragment assay or affinity labeling experiments, frozen ribosome pellets were thawed, then stirred slowly on ice for 1.5 h, and centrifuged in an Eppendorf microfuge for 15 min, 4 °C, to sediment nondispersed material. These 80S ribosomes contain a few proteins that are removed during subunit separation in 100 mM KCl. As a result, the 80S ribosomes are referred to as crude and those proteins not present on separated subunits as crude 80S proteins.

Optimizing Ionic Conditions for Puromycin Reaction. In the fragment assay for peptide bond formation, *N*-acetyl-[^{3}H]leucine of the *N*-acetyl[^{3}H]leucyl pentanucleotide 3′ fragment of tRNA is enzymatically transferred to puromycin by ribosomes in the presence of alcohol, Mg^{2+}, and K^+. The synthesis and isolation of the 3′ fragment of tRNA and the fragment assay were carried out as described by Monro (1971). The ionic composition of the fragment reaction buffer was optimized for *Drosophila* ribosomes by a modified version of the method of incomplete factorial experiments (Carter & Carter, 1979). In the first part of the optimization procedure, several different salts were individually tested for their effects on the rate of the peptide-transfer reaction. In the second half of the optimization procedure, either three or four selected concentrations of the salts chosen during the initial screening (KCl, NaCl, and $MgCl_2$) were tested in all permutations for their combined effect on the rate of reaction. The optimizing conditions were 35 mM Tris-HCl,[1] pH 7.6, 200 mM KCl, 100 mM NaCl, 20 mM $MgCl_2$, 30% MeOH, and 12.5 A_{260}/mL of 80S crude ribosomes. The concentrations of puromycin (500 μM) and the fragment (9.4 nM) were held constant during the screening procedure.

For use in affinity-labeling experiments, we modified the buffer, which had been optimized for the fragment reaction by increasing the concentration of Tris buffer to 50 mM and omitting the fragment and methanol. The latter component was present only to facilitate binding of the fragment to ribosomes (Celma et al., 1970).

Photolytic Incorporation of Puromycin. Incorporation mixtures consisted of 80 A_{260} units/mL of 80S crude ribosomes, 200 mM KCl, 100 mM NaCl, 20 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.6 (referred to as buffer F), and a [8(N)-^{3}H]puromycin (8 Ci/mmol, Amersham) concentration as indicated. In order to study the effect of different ionic conditions on the labeling, some reaction mixtures contained 15 mM $MgCl_2$ instead of 20 mM and no KCl (referred to as buffer S). The ribosomes were added last. It should be noted that [^{3}H]puromycin may have to be purified by HPLC to prevent nonspecific incorporation of radioactivity. Prior to irradiation, the complete reaction mixtures were incubated 30 min at 4 °C. During the irradiation procedure, samples were contained in the wells of a plastic tissue culture multiwell dish

† This work was supported by a research grant from the National Institutes of Health (GM-30443) and a Dreyfus Teacher Scholar grant to M.P. M.P. is a fellow of the Alfred P. Sloan Foundation.

[1] Abbreviations: rRNA, ribosomal RNA; TCA, trichloroacetic acid; 1D, one dimensional; 2D, two dimensional; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.

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submersed in an ice/water bath. A mercury vapor lamp having peak energy output at 254 nm (Ultra-Violet Products, San Gabriel, CA) was positioned 13 cm above the samples to be irradiated. Incident radiation dosage was measured with a digital radiometer at 2.0 mW/cm^2. Following UV exposure, the incorporation mixtures were diluted into 3 volumes of 20 mM Tris-HCl, pH 7.4/100 mM KCl and immediately precipitated by the addition of 2 volumes of cold ethanol. After 3 h at −20 °C, the precipitation was complete. Ribosomal proteins were extracted from the ethanol pellets by the Mg^{2+}/acetic acid procedure (Sherton & Wool, 1974). The acetic acid insoluble rRNA was pelleted and stored at −20 °C until being prepared for analysis (see below). The ribosomal protein containing supernatant was extensively dialyzed against 10% acetic acid/1 mM DTT and lyophilized. The protein samples were then brought up in 3% acetic acid. The concentrations were determined with the Bio-Rad protein assay, and radioactivity was determined by liquid scintillation counting.

Analysis of Ribosomal Constituents. The acetic acid insoluble fraction containing the rRNAs was washed once with 67% acetic acid, then brought into solution, and neutralized with 1 M Bis-Tris at 4 °C. Following dialysis against 50 mM Bis-Tris, pH 7.0, each sample was split three ways. One-third was TCA-precipitated and analyzed for radioactivity. The two remaining portions were subjected to enzymatic digestion by the addition of either 400 μg/mL RNase A and 5 μg/mL T1 RNase or 400 μg/mL proteinase K. Digestions were carried out for 3 h at 37 °C. Under these conditions there is neither detectable hydrolysis of yeast tRNA by the protease treatment nor degradation of BSA by the RNase treatment. These procedures were carried out in order to determine whether radioactivity in the acetic acid insoluble fraction was due to labeled rRNA or to labeled protein that had cross-linked to rRNA during the photoincorporation. The TCA precipitates from each preparation were analyzed for radioactivity.

In order to quantitate the amount of radioactivity in ribosomal proteins, they were separated on acidic 1D polyacrylamide gels (Mets & Bogorad, 1974). These gels were cut into 1.7-mm segments with a hand-held gel slicer and the individual pieces digested by incubation with 0.5 mL of 30% hydrogen peroxide for 4 h, at 55 °C. The samples were counted for radioactivity following the addition of 10 mL of Beta-Phase liquid scintillation cocktail.

Ribosomal proteins were resolved by 2D polyacrylamide gel electrophoresis using the system of Mets & Bogorad (1974) as modified by Warner & Gorenstein (1977). Separation in the first dimension of this gel system is based on charge at pH 5, while the second dimension is an SDS sizing gel. Under the conditions used in the first dimension, all ribosomal proteins are positively charged and migrate toward the cathode. No protein was detected upon Coomassie staining of tube gels, which had been electrophoresed toward the anode. Two-dimensional gels were prepared for autoradiography by impregnation with En^{3}Hance (New England Nuclear) according to manufacturer's instructions. Dried gels were exposed to preflashed X-ray film at −70 °C.

Proteins are numbered according to the system of Chooi et al. (1980). This was accomplished by a four-corner analysis of the protein spot positions in four 2D gel systems as has been worked out for yeast (Michel et al., 1983) and rat liver ribosomal proteins (Madjar et al., 1979). In some cases we have identified proteins as belonging to either the large or small subunit where Chooi et al. did not designate a subunit origin. In these cases we use their numbers, but an L or S in parentheses follows the number to designate large- or small-subunit proteins, respectively. It should be noted that when ribosomal proteins are run in the acid/SDS gel system used here, they may show substantial mobility changes from the basic/acid gel system in which they were originally numbered.

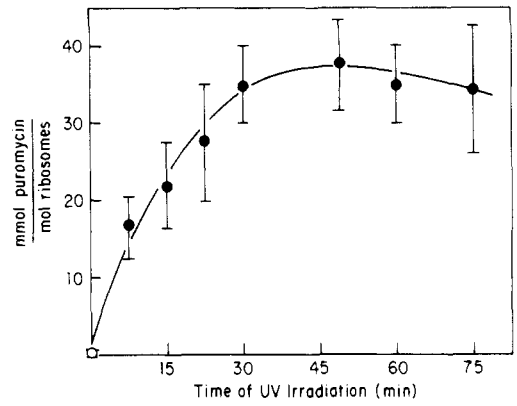

FIGURE 1: Light dependence of incorporation of puromycin into ribosomes. *Drosophila* 80S ribosomes were mixed with 20 μM [^{3}H]puromycin and irradiated at 254 nm for the times indicated. Both ribosomal protein and rRNA fractions from the labeled samples were assayed for incorporation of the antibiotic and the results pooled. Each point represents the average of nine samples.

Antibiotic Competition Experiments. [^{3}H]Puromycin (20 μM) and either trichodermin, anisomycin, or emetine (200 μM) were added to the optimized reaction buffer F; again, the ribosomes were added last. Following photolysis for 30 min, the mixture was ethanol precipitated, and proteins and RNA were isolated as described.

RESULTS

Light Dependence of Puromycin Photoincorporation. In view of the low yields that can result from photoactivated affinity-labeling experiments (Cooperman, 1980), we first attempted to optimize the specific interaction between the puromycin affinity label and the peptidyltransferase target binding site. Using the incomplete factorial method of Carter & Carter (1979) to vary the numerous reaction parameters, we attempted to maximize the rate of peptide bond formation as a means of optimizing the biologically relevant interaction between puromycin and the *Drosophila* ribosome. The set of ionic conditions giving optimal puromycin reactivity in the fragment reaction is provided in buffer F.

Using these conditions (buffer F), we then determined that [^{3}H]puromycin covalently photoincorporates into *Drosophila* ribosomes as a function of the amount of incident UV light. During the course of UV exposure, aliquots of the incorporation mixture were removed, diluted, and immediately ethanol precipitated. In order to avoid measuring noncovalently bound puromycin, the samples were then separated into acetic acid soluble (protein) and insoluble (RNA) fractions. These fractions were dialyzed (protein) or redissolved and then dialyzed (RNA) before being analyzed. Pooled results of the [^{3}H]puromycin found in the RNA and protein fractions as a consequence of UV irradiation are shown in Figure 1. Labeling of both protein and RNA fractions is apparently linear initially, but after prolonged UV exposure this labeling is saturated or possibly reversed to a small extent. The latter may be due to a photolytic breakdown of the puromycin–ribosome moiety. It is interesting to note that the same time dependence of the labeling pattern was observed whether the total puromycin concentration in the reaction was 20 μM or 2.5 mM, again indicating the light dependence of the reaction. In order to maximize the labeling signal, subsequent experi-

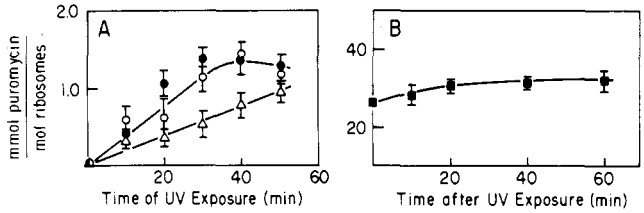

FIGURE 2: (A) Light dependence of labeling of acetic acid insoluble fraction. The acetic acid insoluble fraction of labeled ribosomes was assayed for incorporation of [^{3}H]puromycin (●) as a function of time of UV exposure. In addition, samples from this fraction were digested with RNase (○) or proteinase K (△), and the remaining TCA-insoluble [^{3}H]puromycin was determined. (B) Light-independent labeling of 80S ribosomes. Following 20 min of irradiation, samples of puromycin-labeled ribosomes were maintained in the dark under the same reaction conditions for various times, and the total amount of puromycin labeling in pooled protein and RNA fractions (■) was determined.

ments were carried out with a 30-min UV exposure. In the absence of light, no significant incorporation of [^{3}H]puromycin into ribosomes was observed. Nor did photolysis of [^{3}H]puromycin or ribosomes prior to mixing result in any labeling of the ribosomes.

Studies involving the photoincorporation of puromycin into prokaryotic and eukaryotic ribosomes have demonstrated that an unidentified photoproduct of the incorporation reaction can lead to continued ribosome labeling subsequent to the conclusion of UV irradiation (Cooperman et al., 1975; Reboud et al., 1981). Any long-lived reactive photoproduct can potentially label components of the ribosome other than those present at the substrate binding site. In order to assess the significance of such nonspecific labeling, we photolyzed standard reaction mixtures, containing 20 μM [^{3}H]puromycin, for 20 min. Following the irradiation procedure, an aliquot of the incorporation mixture was immediately diluted and ethanol precipitated. The remaining, unprecipitated stock was maintained in darkness at 4 °C. Aliquots from this mixture were diluted, and the reaction was quenched by ethanol precipitation at specified time points. Washed ethanol precipitates were then treated with acetic acid and dialyzed. Pooled results of the radioactivity found in the protein and RNA fractions are shown in Figure 2B. Some light-independent labeling of ribosomes is observed. However, in our standard affinity-labeling reactions the amount of labeling that takes place after the cessation of UV exposure and before the reaction is quenched by the addition of ethanol is small and was ignored in future experiments. UV irradiation of 80S ribosomes (without puromycin) for the times used in these experiments did not show any alteration in the pattern or staining intensity of any ribosomal proteins by 1D or 2D gel analysis. This makes it unlikely that the affinity-labeled protein spots we observed contained protein dimers or multimers.

Identification of the Puromycin-Linked Ribosomal Components. In several photoincorporation experiments such as those shown in Figure 1, the majority of [^{3}H]puromycin was found in the acetic acid soluble or ribosomal protein fraction (97%). When the acetic acid insoluble material from the time course in Figure 1 was analyzed for radioactivity, a labeling behavior identical with that of the protein fraction was found. Since the appearance of radioactivity in the RNA (acetic acid insoluble) fraction upon UV irradiation could result from either a true affinity-labeling process or from the UV-induced cross-linking of RNA to labeled proteins, both protease and RNase digests of the acetic acid insoluble material were analyzed for radioactivity. It should be noted that high doses of 254 nm light are known to cause cross-linking of ribosomal proteins and rRNA [reviewed by Zimmerman (1980)]. A labeled cross-linked RNA–protein moiety might be expected to be acid insoluble in our system.

Following proteinase K digestion of the acetic acid insoluble fraction, a nearly linear increase of [^{3}H]puromycin incorporation throughout the duration of UV exposure was observed (Figure 2A). Digestion of the acetic acid insoluble fraction by RNase A/T1 RNase treatment (Figure 2A) gave a plot identical in shape with that shown in Figure 1. These results indicate that a simple incorporation of [^{3}H]puromycin into rRNA does not occur. Such an RNA–puromycin molecule would be rendered TCA soluble by RNase treatment. Since all the label in the RNase-treated acetic acid insoluble fraction is still TCA precipitable, a simple RNA–puromycin linkage is ruled out. On the other hand, the acetic acid insoluble material, when treated with proteinase K, shows a decrease of TCA-precipitable radioactivity compared to the RNase-treated and control samples but not a total elimination of this labeling. The decrease in precipitable label with proteinase treatment is attributed to the direct linkage of [^{3}H]puromycin to protein. This protein in turn was either trapped by RNA in the acetic acid insoluble fraction or possibly cross-linked to that RNA as a puromycin–protein–RNA moiety. The remaining proteinase-resistant TCA-precipitable label can only be due to RNA linked to a [^{3}H]puromycin molecule, which in turn had been linked to protein. Only such a puromycin cross-linked moiety (RNA–puromycin–protein) would give a TCA-precipitable product following either RNase or proteinase digestion. Although this RNA–puromycin–protein moiety accounts for only a small percentage (~3%) of the total puromycin labeling of ribosomal components, it appears to occur from a functional puromycin binding site (as described later) and therefore can give added information as to the nature of this site.

Ribosomal Proteins at the Puromycin Binding Site. In order to identify the specific ribosomal proteins involved in the puromycin binding site(s), we separated the [^{3}H]puromycin photoaffinity-labeled ribosomal proteins first by 1D, then 2D, gel electrophoresis. One-dimensional tube-gel analysis provides nearly quantitative recovery (>95%) of the applied label, but does not allow complete resolution of the ribosomal proteins. Three major regions (I–III) of the 1D gels contain radioactivity. Each of these areas is known to correspond to one or two individual ribosomal proteins in our 2D gels (Figure 3A). Autoradiography of the 2D gel ensures detection of all labeled areas of the gel. Each labeled area corresponds to positions of ribosomal proteins perhaps shifted very slightly above and to the left of the major stained areas for these proteins. The labeled areas of companion gels were cut out and assayed for radioactivity by liquid scintillation counting. On the basis of these results, we calculate a total recovery of radioactivity in the 2D spots to be approximately 3% of that loaded onto the first dimension. However, the overall distribution of label in the 1D and 2D gels is very similar.

The 2D gel analysis of [^{3}H]puromycin-linked proteins, labeled under conditions selected to optimize puromycin reactivity, buffer F, shows two large L2 and L17 and two small S8 and S22 subunit proteins are the major labeled products. These results are shown in Figure 3A,C. It is important to note here that different salt conditions (buffer S) during the photolabeling can significantly alter the pattern of puromycin-linked ribosomal proteins. These salt conditions were chosen as a comparison because they are similar to those used in other puromycin-labeling experiments (Cooperman et al., 1975; Reboud et al., 1981). In this case, the major labeled

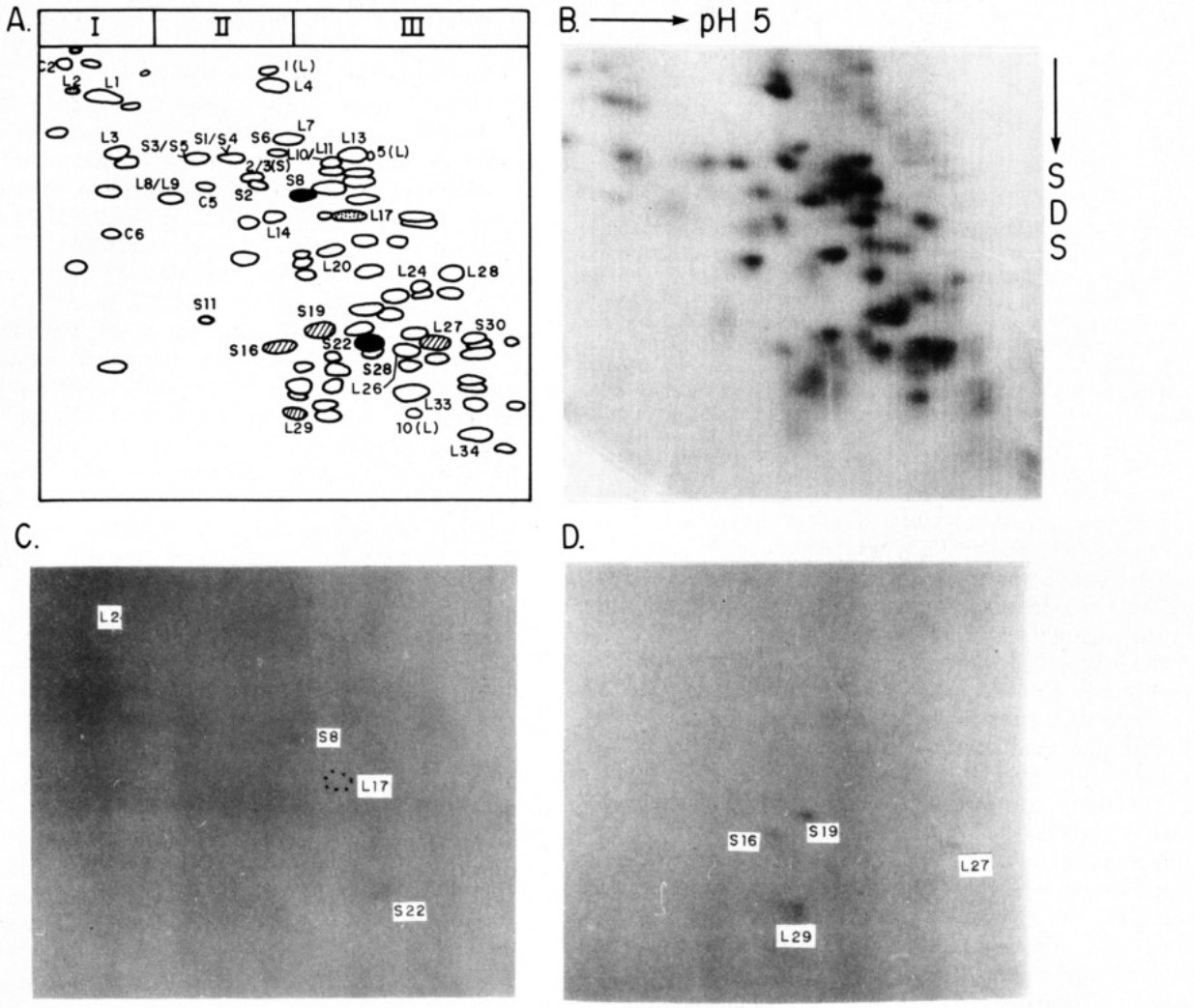

FIGURE 3: Two-dimensional gel analysis of [^{3}H]puromycin-labeled ribosomal protein. (A) Schematic drawing of the labeled protein pattern. The proteins labeled under optimized conditions, buffer F, are indicated by dark (more heavily labeled) or stipled (less heavily labeled) shading. Peaks (I–III) of radioactivity found in a 1D gel of this sample are indicated above the drawing. Proteins labeled under the less active but commonly used buffer S conditions are shown by cross-hatching. Labeled proteins are also designated as small-, S, or large-, L, subunit proteins or as found only on crude, C, 80S ribosomes. Numbering of affinity-labeled spots and several reference spots is according to Chooi et al. (1980). See Materials and Methods for details. (B) Photograph of Coomassie Blue stained pattern of ribosomal proteins from crude 80S ribosomes photoreacted with puromycin. (C) Autoradiograph of labeled ribosomal proteins from an optimized buffer F reaction. (D) Autoradiograph of labeled ribosomal proteins from a buffer S reaction.

proteins are two different small-subunit and two different large-subunit proteins as indicated in Figure 3A,D. These buffer S conditions were only 36% as efficient in allowing the formation of peptidylpuromycin in the fragment assay for *Drosophila* ribosomes. The extent of labeling of ribosomal components is also 3-fold less. We speculate that the proteins labeled in buffer S conditions may be near the functional puromycin binding site but do not represent the site of puromycin binding during maximal peptidyltransferase activity in *Drosophila* ribosomes.

Site Specificity of Puromycin Labeling. The quantitative recovery of labeled protein from the 1D gel analysis can be used to test for functional site specificity of labeling of each ribosomal protein. Within the initial phase of labeling (30 min of UV irradiation), our finding that the amount of incorporation into a given protein approaches a saturating value as a function of ligand concentration is evidence for site-specific labeling. Quantitation of label bound at different puromycin concentrations can also provide a measure of the dissociation constant (K_D). A K_D similar to a biologically relevant K_D is strong evidence for functional-site labeling. Analysis for saturability and determination of K_D for the 1D gel peak II protein is shown in Figure 4. All three peaks on the 1D gels showed similar saturation behavior, indicating that labeling occurs site-specifically. A summary of the K_D values obtained for all three peaks is presented in Table I. Each has a value comparable to that found for puromycin photoincorporation into *E. coli* (0.6 mM) (Cooperman et al., 1975) and rat liver ribosomes (0.7 mM) (Reboud et al., 1981) and similar to that found for puromycin in the peptidyltransferase assay (0.2 mM) (Pestka, 1970).

Table I: K_D Values for [^{3}H]Puromycin Linked to Ribosomal Components[a]

component	K_D (μM)
ribosomal protein(s)	
peak I	240
peak II	200
peak III	560
ribosomal RNA	720

[a] K_D values were obtained from 1D gel analysis of three puromycin-labeled ribosomal protein fractions (peaks I–III in Figure 3A). The RNA (acetic acid insoluble) fraction recovered for each labeling was also analyzed for puromycin incorporation (see Materials and Methods).

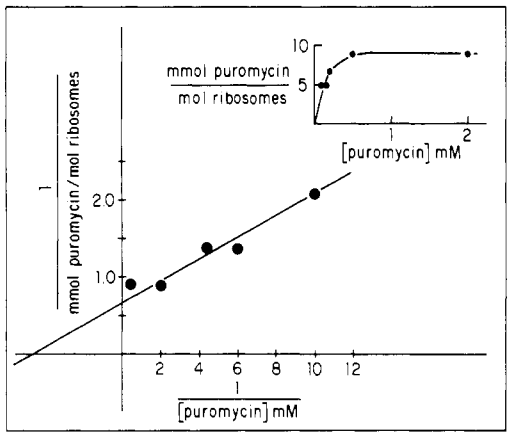

FIGURE 4: Saturation of puromycin photolabeling at high concentrations. The amount of [^{3}H]puromycin incorporated into specific ribosomal proteins after 30 min of UV irradiation was assayed by counting the radioactivity within single peaks of material following electrophoresis on a 1D gel. In each experiment, the concentration of puromycin in the labeling reaction was varied. The data for 1D gel peak II are graphed here.

In each of these experiments, the acetic acid insoluble (RNA) fraction was also assayed for [^{3}H]puromycin content and the K_D for the puromycin reaction determined. As seen in Table I, the value obtained indicates that this reaction, too, takes place from a functional binding site for puromycin.

Changes in Puromycin Binding due to the Binding of Other Peptidyltransferase Inhibitors. Several eukaryotic-specific inhibitors of protein synthesis are thought to act at the peptide bond forming step of protein synthesis [reviewed by Gale et al. (1981)]. Among these are emetine, trichodermin, and anisomycin. Each of these drugs when incubated with ribosomes in a 10-fold excess over puromycin in the standard photolabeling reaction effected a small but significant change in the pattern of puromycin labeling. Two-dimensional gel analysis of ribosomal proteins photolabeled by puromycin in the presence of any of these drugs showed a small overall decrease in labeling of the four proteins labeled under standard reaction conditions. Overall, the total radioactivity associated with [^{3}H]puromycin-labeled ribosomal components in the presence of the competing antibiotics on the basis of two experiments was 85%, 79%, and 73% of the control (no competition) for trichodermin, anisomycin, and emetine, respectively.

Discussion

Puromycin Photolabeling Is Covalent and Functional Site Specific. We have used photoaffinity-labeling techniques to identify the components of *Drosophila* ribosomes that interact with puromycin, an A-site substrate for peptidyltransferase. The advantages of photoaffinity labeling include the ability to control the timing of the reaction and the ability of the photolabel to react with a variety of protein and RNA moieties on the ribosomes. The photolysis of puromycin bound to 80S *Drosophila* ribosomes leads to incorporation of the antibiotic into both ribosomal proteins and rRNA. This photoaffinity labeling of 4 of the 75 *Drosophila* ribosomal proteins is covalent as judged by the stability of the puromycin–protein association through treatment with 67% acetic acid and subsequent analysis by 2D SDS gel electrophoresis. Although the mechanism of the photochemical reaction(s) is not understood, the photoincorporation of puromcyin into ribosomes is clearly dependent on light fluence (Figure 2). The small but detectable light-independent labeling of ribosomes that occurs after the conclusion of photolysis is not significant enough to affect our results. A site-specific photolabeling with puromycin is implied by the saturation of this reaction with increasing puromycin concentrations. This is shown in Figure 4. Each of the labeled protein and rRNA components shows a similarly saturable labeling profile.

A direct assay for the photoincorporation of puromycin from a functional A site location is not available. As Jaynes et al. (1978) have shown, photoreacted puromycin can no longer participate in peptide bond formation. Therefore, we have used the fact that the K_D for puromycin reactivity is similar to the K_m for puromycin in the A site to be indirect evidence for functional-site labeling. The K_D's we obtained for puromycin labeling of the ribosomal proteins fall between 0.2 and 0.6 mM (Table I) while the K_m for puromycin in the *E. coli* peptidylpuromycin fragment assay is 0.2 mM (Pestka, 1970). These K_D's are also similar to the K_D's observed for the puromycin photoaffinity labeling of *E. coli* (0.65 mM) and rat liver ribosomes (0.70 mM) (Cooperman et al., 1975; Reboud et al., 1981).

Ribosomal Proteins and Possibly rRNA Are Found at the Puromycin Binding Site. The specific ribosomal components found linked to puromycin at the binding site associated with optimal peptidyltransferase activity include four ribosomal proteins and possibly rRNA. Among the labeled ribosomal proteins are two small subunit proteins and one large subunit protein and a protein associated with crude ribosomes. These results suggest that puromycin occupies a site or sites at the interface of the two subunits. Such a location for the puromycin binding site and peptidyltransferase as a whole has been demonstrated for *E. coli* ribosomes (Olson et al., 1982). Likewise, puromycin photoincorporates into both small and large subunit proteins in rat liver ribosomes (Reboud et al., 1981).

In addition, we have shown that puromycin becomes attached to rRNA. Although this reaction accounts for only a small portion of the overall affinity labeling, it occurs in a functional site specific manner (Table I). The observation of a photoactivated puromycin link to rRNA has been made in *E. coli* and rat liver ribosomes (Cooperman et al., 1975; Reboud et al., 1981). The presence of 23S rRNA at the peptidyltransferase center of *E. coli* ribosomes is also well estabilhsed [e.g., Breitmeyer & Noller (1976) and Barta et al. (1984)]. Under different but commonly used ionic conditions, i.e., buffer S conditions, we find that [^{3}H]puromycin photoincorporates into four ribosomal proteins that differ from those labeled under conditions that optimize puromycin activity. Again, both small- and large-subunit proteins are labeled. These data suggest that puromycin may occupy two distinct binding sites at the subunit interface. Again, this has been seen for *E. coli* ribosomes (Olson et al., 1982).

Interaction of Other Eukaryotic-Specific Antibiotics with the Puromycin Binding Site. Several eukaryotic-specific antibiotics are thought to inhibit protein synthesis by acting on the peptide-transfer step. Among these are emetine, anisomycin, and trichodermin [reviewed by Gale et al. (1981)]. Emetine appears to inhibit peptidyltransferase indirectly by acting on the 40S subunit. Anisomycin and trichodermin act more directly on this active site but do not prevent substrate binding to the A or P site [reviewed by Gale et al. (1981)]. When any one of these drugs is present in excess during a puromycin photoaffinity-labeling reaction, the pattern of labeled proteins is altered, emetine being responsible for the greatest change in puromycin labeling. It is interesting that the small subunit binding antibiotic, emetine, most effects this puromycin switch to a new binding mode.

The results presented here provide data on the *Drosophila* ribosomal proteins present at the puromycin binding site, the A site of peptidyltransferase. In addition, the proteins of a second puromycin binding site have been identified, perhaps analogous to that seen in *E. coli* ribosomes. It is noteworthy that in both *E. coli*, *Drosophila*, and rat liver ribosomes one of the puromycin affinity-labeled proteins is among the smaller and more basic proteins. It is possible to speculate that such a small basic protein is necessary to construct the active A site. A schematic drawing showing the placement of these A-site proteins and proteins labeled by a trichodermin analogue is presented in Gilly et al. (1985). One of the A-site proteins, S8, is also labeled by this analogue of trichodermin, which suggests that the binding site of this drug partially overlaps that of puromycin.

ACKNOWLEDGMENTS

We thank Jeffrey Bell for helpful suggestions and Sarah Wright for the preparation of the manuscript.

Registry No. Puromycin, 53-79-2.

REFERENCES

Barta, A., Steiner, G., Brosius, J., Noller, H. F., & Kuechler, E. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3607–3611.

Breitmeyer, J. B., & Noller, H. F. (1976) *J. Mol. Biol. 101*, 297–306.

Carter, C. W., Jr., & Carter, C. W. (1979) *J. Biol. Chem. 284*, 12219–12223.

Celma, M. L, Monro, R. E., & Vazquez, D. (1970) *FEBS Lett. 6*, 273–277.

Chooi, W. Y., Sabatini, L. M., Macklin, M., & Fraser, D. (1980) *Biochemistry 19*, 1425–1433.

Cooperman, B. S. (1980) in *Ribosomes, Structure, Function and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 531–554, University Park Press, Baltimore.

Cooperman, B. S., Jaynes, E. N., Brunswick, D. J., & Luddy, M. A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2974–2978.

Fabijanski, S., & Pellegrini, M. (1979) *Biochemistry 18*, 5674–5679.

Gale, E. F., Cundliffe, E., Reynolds, P. E., Richmond, M. H., & Waring, M. J. (1981) *The Molecular Basis of Antibiotic Action*, pp 402–547, Wiley, New York.

Gilly, M., Benson, N. R., & Pellegrini, M. (1985) *Biochemistry* (following paper in this issue).

Grant, P. G., Strycharz, W. A., Jaynes, E. N., Jr., & Cooperman, B. S. (1979a) *Biochemistry 18*, 2149–2154.

Grant, P. G., Cooperman, B. S., & Strycharz, W. A. (1979b) *Biochemistry 18*, 2154–2160.

Jaynes, E. N., Jr., Grant, P. G., Giangrande, G., Weider, R., & Cooperman, B. S. (1978) *Biochemistry 17*, 561–569.

Madjar, J. J., Arpin, M., Buisson, M., & Reboud, J.-P. (1979) *Mol. Gen. Genet. 171*, 121–134.

Mets, L., & Bogorad, L. (1974) *Anal. Biochem. 57*, 200–209.

Michel, S., Traut, R. R., & Lee, J. C. (1983) *Mol. Gen. Genet. 191*, 251–256.

Munro, R. E. (1971) *Methods Enzymol. 20*, 472–481.

Olson, H. M., Grant, P. G., Cooperman, B. S., & Glitz, D. G. (1982) *J. Biol. Chem. 257*, 2649–2656.

Pestka, S. (1970) *Arch. Biochem. Biophys. 136*, 80–88.

Reboud, A. M., Dubost, S., Buisson, D., & Reboud, J. P. (1981) *Biochemistry 20*, 5281–5288.

Santon, J. B., & Pellegrini, M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5649–5653.

Sherton, C., & Wool, I. G. (1974) *Methods Enzymol. 30*, 506–525.

Warner, J. R., & Gorenstein, G. (1977) *Cell (Cambridge, Mass.) 11*, 201–212.

Zimmerman, R. A. (1980) in *Ribosomes, Structure, Function and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 531–554, University Park Press, Baltimore.