Reconstitution of *Escherichia coli* Ribosomes Containing Puromycin-Modified S14: Functional Effects of the Photoaffinity Labeling of a Protein Essential for tRNA Binding†

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ABSTRACT: In previous work we have shown that puromycin photoaffinity labels two proteins, L23 and S14, from separate sites of high affinity on *Escherichia coli* ribosomes [Jaynes, E. N., Jr., Grant, P. G., Giangrande, G., Wieder, R., & Cooperman, B. S. (1978) *Biochemistry 17*, 561–569; Weitzmann, C. J., & Cooperman, B. S. (1985) *Biochemistry 24*, 2268–2274], that puromycin-modified S14 is separable from native S14 by reverse-phase high-performance liquid chromatography (RP-HPLC), and that ribosomal proteins prepared by RP-HPLC can be reconstituted into active 30S subunits [Kerlavage, A. R., Weitzmann, C. J., & Cooperman, B. S. (1984) *J. Chromatogr. 317*, 201–212]. In this work we definitively identify puromycin-modified S14 by tryptic fingerprinting, an analysis that also provides evidence that the single tryptophan-containing peptide in S14 is the site of puromycin photoincorporation. We show that reconstituted 30S subunits, in which all of the S14 present is stoichiometrically modified with puromycin and all other ribosomal components are present in unmodified form, lack Phe-tRNA^{Phe} binding activity and further that 70S ribosomes containing such reconstituted 30S subunits have substantially diminished binding activity to both the A and P sites, as differentiated through use of tetracycline. Suitable control experiments strongly indicate that this loss of activity is a direct consequence of puromycin photoincorporation.

Photoaffinity labeling is an invaluable tool for the identification of structurally and functionally important sites of ribosomes as well as of other complex biological systems. In spite of the wealth of information obtained from such experiments, it is usually quite difficult to determine the functional consequences of photoaffinity labeling. In the case of ribosomal ligands that have been extensively studied (Cooperman, 1978, 1980; Ofengand, 1980), photoincorporation frequently occurs at very low stoichiometry so that functional assays generally reflect the properties of the dominant, unmodified ribosomes. Even when labeling occurs at high stoichiometry, if more than one ribosomal component is labeled, it can be difficult to sort out the contribution of the labeling of each component to overall effects upon function (Nicholson et al., 1982b).

In previous work we have utilized the antibiotic puromycin, an analogue of the 3′-end of aminoacylated tRNA and a substrate for the peptidyl transferase activity of the *Escherichia coli* ribosome, as a photoaffinity probe of the peptidyl transferase center of the ribosome (Cooperman et al., 1975; Jaynes et al., 1978; Grant et al., 1979a,b; Weitzmann & Cooperman, 1985). We found that the major proteins labeled by puromycin are L23, which is labeled from a site on the 50S subunit, and S14 and S7, which are labeled from different sites on the 30S subunit. We have also determined the structural specificity and relative affinity of puromycin binding at each of these sites (Weitzmann & Cooperman, 1985). This and related work (Nicholson et al., 1982a,b) have led us to conclude that photoincorporation into L23 takes place from the A site within the peptidyl transferase center and to speculate on a role for S14 as part of the tRNA binding locus (Olson et al., 1982; Grant et al., 1983).

We have previously demonstrated the ability to preparatively isolate individual ribosomal proteins by reverse-phase high-performance liquid chromatography (RP-HPLC)[1] (Kerlavage et al., 1982, 1983a,b) and have shown further that proteins prepared in this manner reconstitute into functional ribosomal subunits (Kerlavage et al., 1983a, 1984). We have also demonstrated that RP-HPLC can be used to separate puromycin-modified S14 from native S14 (Kerlavage et al., 1984). In this work we combine these approaches to study the effects of puromycin modification of S14 on the function of the *E. coli* ribosome.

Nomura et al. (1969) have shown that the single omission of S14 from reconstitution mixtures leads to substantial loss of Phe-tRNA^{Phe} binding activity in both reconstituted 30S subunits and 70S ribosomes. In this work we reproduce this earlier result and show further that addition to a reconstitution mixture of puromycin-modified S14 in place of native S14 fails to restore tRNA binding. Our results strengthen the argument for a specific role of S14 in tRNA binding and demonstrate that photoincorporation of puromycin has taken place at a functionally important site. They also provide a specific structural rationale for the observed inhibition of tRNA binding to 30S subunits by puromycin (Ivanov & Saminsky, 1984).

Experimental Procedures

Materials

The following buffers were used: (TKM10) 50 mM Tris-HCl (pH 7.6 at 23 °C), 50 mM KCl, 10 mM $MgCl_2$; (TKM1)

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[1] Abbreviations: poly(U), poly(uridylic acid); RP-HPLC, reverse-phase high-performance liquid chromatography; TC, tetracycline; TPCK, *N*-(*p*-toluenesulfonyl)-L-phenylalanine chloromethyl ketone; TP70 and TP30, total protein from 70S ribosomes and 30S subunits, respectively; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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as above except 1 mM $MgCl_2$; (TKM20) 50 mM Tris-HCl (pH 7.6 at 23 °C), 100 mM KCl, 20 mM $MgCl_2$, 6 mM 2-mercaptoethanol; (A) 20 mM Tris-HCl (pH 7.4 at 23 °C), 20 mM magnesium acetate, 500 mM ammonium chloride, 6 M urea, 6 mM 2-mercaptoethanol; (B) buffer A without urea; (C) 25 mM Tris-HCl (pH 7.6 at 23 °C), 25 mM KCl, 100 mM $MgCl_2$, 3.6 M LiCl, 3 mM 2-mercaptoethanol.

HPLC-grade trifluoroacetic acid (Pierce), HPLC-grade acetonitrile (Fisher), puromycin and TPCK-trypsin (Sigma), $tRNA^{Phe}$ (Boehringer-Mannheim), [^{14}C]phenylalanine (450 Ci/mol, Amersham), and [^{3}H]phenylalanine (38 600 Ci/mol, Amersham) were used without further purification. Reagent-grade urea (Sigma) was purified by treatment of 8 M solutions with 4 g/L Norit A (Fisher) and 10 g/L Amberlite MB-3 (Thomas Scientific) to decolorize and remove cyanate, respectively. All other chemicals were reagent-grade. Spectropor 3 dialysis tubing (Thomas Scientific) was soaked in distilled water (15 min) prior to use.

[8-^{3}H]Puromycin was prepared from [8-^{3}H]puromycin aminonucleoside (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 RP-HPLC (Kerlavage et al., 1985). [8-^{3}H]Puromycin was repurified by RP-HPLC to remove decomposition products no more than 30 days prior to use. [^{3}H]Phe-$tRNA^{Phe}$ and [^{14}C]Phe-$tRNA^{Phe}$ were prepared as described previously (Goldman et al., 1980). The 70S ribosomes were prepared from *E. coli* Q13 as described by Jaynes et al. (1978) as method T. Ribosomal subunits were isolated as previously described (Goldman et al., 1983). The 16S rRNA was prepared from 30S subunits essentially as described by Hall et al. (1985) except that bentonite was omitted and rRNA was precipitated 3 times by incubation with ethanol for 1 h to remove all traces of phenol.

Methods

Photoincorporation of [^{3}H]Puromycin into 70S Ribosomes. Photolytic incorporation of [^{3}H]puromycin (0.5 mM, 65.3 Ci/mol) into 70S ribosomes (100 A_{260} units/mL) was performed in TKM10 buffer at 4 °C for 20 h with Rayonet RPR 3500-Å lamps as previously described (Jaynes et al., 1978). Photolyses were routinely performed on stirred solutions with 3000–5000 A_{260} units of 70S ribosomes in 13 × 100 mm glass tubes placed at a distance of 2–5 mm from each lamp inside a Rayonet reactor. Immediately following photolysis, ribosomes were precipitated with 2 volumes of ethanol–2-mercaptoethanol (9:1). The pellets were resuspended in TKM10 buffer containing 6 mM 2-mercaptoethanol and reprecipitated with 2 volumes of ethanol, a procedure that removes essentially all unbound puromycin. The final pellet was redissolved in TKM10 buffer at a concentration of approximately 700 A_{260} equiv/mL and stored at −80 °C. The extent of labeling was typically 1.6 [^{3}H]puromycin/70S ribosome, as determined by measuring the ^{3}H cpm/A_{260} ratio.

Extraction of Proteins from [^{3}H]Puromycin-Labeled Ribosomes. Two methods were employed to extract proteins from [^{3}H]puromycin-labeled ribosomes. Method 1 is based on the procedure of Hardy et al. (1969). Because of the lability of [^{3}H]puromycin-labeled S14 toward acid, we altered the Hardy et al. conditions by decreasing the acetic acid from 67% to 50% and decreasing the incubation time (at 0 °C) from 45 to 5 min. This resulted in a doubling of the yield of [^{3}H]puromycin-labeled S14, as determined by RP-HPLC analysis (Kerlavage et al., 1984). Interestingly, the modified Hardy et al. procedure gave as good a yield of ribosomal proteins in general, as measured by RP-HPLC analysis, as did the original procedure. Following incubation, samples were centrifuged for 10 min at 12 000 rpm in an SM-24 rotor at 4 °C. Supernatants were next immediately injected onto an RP-HPLC column.

In method 2, a modification of the procedure of Hindennach et al. (1971), ribosomes were suspended in buffer C for 8 h at 0 °C and then centrifuged for 16 h at 25 000 rpm in a Ti50 rotor at 0 °C. Supernatants were either frozen at −80 °C for later use or injected directly onto an RP-HPLC column. Both acetic acid (method 1) and LiCl (method 2) elute in the break-through volume and pose no complications for purification of [^{3}H]puromycin-modified S14.

Method 2 serves as a partial purification of [^{3}H]puromycin-modified S14 since all forms of S14 are present in the LiCl supernatant, while some other ribosomal proteins are present in whole or part in the core particle. In addition, [^{3}H]puromycin-modified S14 is stable under conditions of LiCl extraction. The greater rapidity with which method 1 could be carried out made it the method of choice for certain applications.

Preparation and Purification of Ribosomal Proteins. The preparation and purification of individual ribosomal proteins or groups of proteins were carried out by RP-HPLC, using a Perkin-Elmer Series 4 system and SynChropak RP-P (C_{18}) column (250 × 4.1 mm i.d., 300-Å pore; SynChrom, Inc.) as described previously (Kerlavage et al., 1984). The flow rate in all runs was 0.7 mL/min. The storage and assay for protein content of purified ribosomal proteins was also carried out as previously described (Kerlavage et al., 1984).

[^{3}H]Puromycin-modified S14 was prepared either by extraction of ^{3}H-labeled 70S ribosomes, for use in reconstitution studies, or by extraction of 30S subunits derived from such 70S ribosomes, for use in tryptic digestion studies. In the latter case, 30S subunits were prepared from 70S ribosomes as previously described (Goldman et al., 1983). Extracts from either 30S subunits or 70S ribosomes were injected into the RP-P column described above. Since resolution was decreased by overloading, samples were applied in a batchwise fashion, with the amount per batch never exceeding the protein either from an acetic extract of ~110 A_{260} units of 30S subunits or from a LiCl extract of ~300 A_{260} units of 70S ribosomes. Accordingly, preparation of puromycin-modified S14 from large quantities of labeled 70S ribosomes or 30S subunits typically required that each RP-HPLC step had to be repeated several times in order to process the entire sample. Eluted fractions were frozen on collection and dried under vacuum in a Savant evaporator as soon as possible, in order to minimize decomposition of [^{3}H]puromycin-modified S14 due to the trifluoroacetic acid present in the eluting solvent.

Peptide Mapping. Ribosomes (1220 A_{260} units, 100 A_{260} units/mL) were photolyzed in the presence of [^{3}H]puromycin (65.3 Ci/mol, 0.5 mM) for 20 h at 4 °C and twice precipitated with ethanol as described above. The final pellet was redissolved in 8 mL of TKM1, divided into eight equal fractions, and separated into 30S and 50S subunits by centrifugation on eight 15%–30% sucrose density gradients made in TKM1 buffer as described in Goldman et al. (1983). The 30S subunits from all runs were pooled in a manner minimizing contamination by 50S subunits, precipitated by overnight incubation at −20 °C with 2 volumes of ethanol, collected by centrifugation for 15 min (Sorvall SM-24 rotor, 12 000 rpm), and redissolved in 2 mL of TKM10. The recovery of [^{3}H]-puromycin-labeled 30S subunits was 220 A_{260} units containing 0.5 [^{3}H]puromycin/30S subunit. The labeled 30S subunits were extracted in two equal batches for 5 min with 50% acetic

acid. Extracts were immediately chromatographed on RP-HPLC. The vacuum-dried S14(A), S14(B), and *hν*-S14 peaks from both RP-HPLC runs (see Results, Figure 2D) were redissolved in NH_4HCO_3 (50 mM, pH 8.3) and pooled, giving a final volume of 100 μL. Unmodified proteins were isolated as described previously (Kerlavage et al., 1984) and redissolved in NH_4HCO_3 as above. TPCK-trypsin was added at a 1:50 weight ratio to the sample, and the mixture was incubated at 37 °C for 4 h. The same amount of trypsin was then added again, and the sample was incubated at 37 °C for an additional 4 h. Samples were applied directly to a RP-HPLC column and eluted with a linear gradient of 0%–35% CH_3CN in 60 min with the trifluoroacetic acid–CH_3CN solvent system previously described (Kerlavage et al., 1984).

Reconstitution. Protein pools were constructed comprising all 30S subunit proteins in stoichiometric amounts except S14 and S1. The absence of S1 did not affect the formation of 30S subunits. S14 was either omitted, added in a stoichiometric amount, or replaced by another protein or a modified form of S14. Protein pools (1.5–3 nmol; 4.5 μM) were dialyzed overnight in Spectropor 3 dialysis tubing against 200 volumes of buffer A and subsequently against three changes of 200–500 volumes of buffer B, each for 45 min. The dialyzed protein was removed from the dialysis bags, the bags were washed with 1 volume of buffer B, and the wash was added to the protein. The total protein solution was combined with 16S rRNA (10–20 A_{260} units) at a molar ratio of 1.8:1 in a total volume of 5 mL and incubated for 45 min at 40 °C, as described by Held et al. (1973). Modified S14 was added at a variable molar ratio to 16S RNA, as indicated in Table I. The samples were cooled on ice for 10 min and reconstituted particles were pelleted at 217000*g* for 3.5 h in a Beckman L-8 ultracentrifuge using a Ti50 rotor. The entire supernatant was removed so that the reconstituted subunits would not be contaminated with unbound proteins. Pellets were redissolved in TKM1 if they were to be applied to sucrose gradients or in TKM20 if they were to be assayed directly for tRNA binding.

tRNA Binding. [^{3}H]Phe-tRNA^{Phe} binding to 30S subunits was assayed as described previously (Goldman et al., 1980). Concentrations in the binding assay made up in TKM20 buffer were as follows: 30S subunits, 0.13 μM; [^{3}H]Phe-tRNA^{Phe}, 0.07 μM; poly(U), 0.24 mg/mL. Suitable control experiments demonstrated that measured binding activity is strictly proportional to 30S concentration (0.08–1.0 μM) under these conditions. Reconstituted 30S subunits were typically assayed without prior centrifugation through sucrose density gradients, although some reconstituted subunits were assayed after sucrose density gradient purification.

[^{14}C]Phe-tRNA^{Phe} binding to 30S subunits was also assayed in the presence of a 2.5-fold molar excess of 50S subunits. These assays were performed in TKM10 buffer containing 10% ethanol. The ethanol had little effect on the assay and permitted added tetracycline to remain in solution. 50S subunits, isolated according to Goldman et al. (1983), were rerun on sucrose density gradients to avoid contamination by 30S subunits. Purified 50S subunits had <1% of the poly(U)-dependent Phe-tRNA^{Phe} binding of 30S subunits.

Results

Outline of Method. In this paper we determine the functional consequence of the photoaffinity labeling by puromycin of protein S14 within an intact 70S ribosome by preparing and functionally testing both a 30S subunit and a 70S ribosome in which [^{3}H]puromycin-modified S14 (S14*) essentially completely replaces S14 and all other ribosomal components

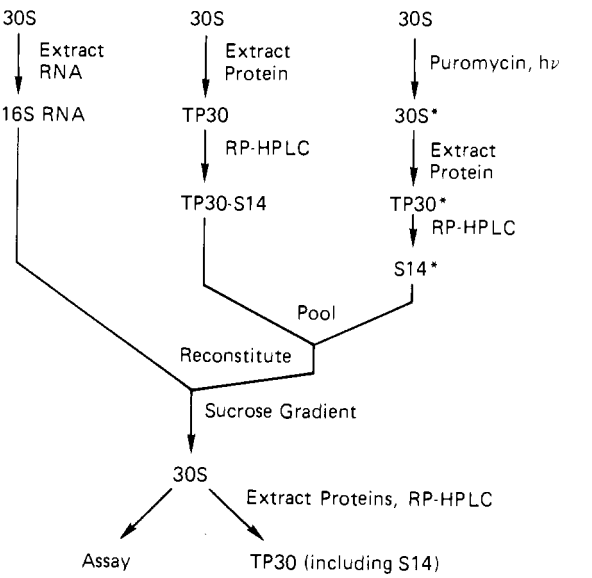

FIGURE 1: 30S subunit reconstitution with [^{3}H]puromycin-modified S14. See text for explanation. An (*) indicates the presence of covalently attached label.

are unmodified. Our approach is outlined in Figure 1.

*Purification and Identification of S14**. (*a*) *RP-HPLC Purification.* Direct extraction (method 2) of [^{3}H]puromycin-modified 70S ribosomes afforded the highest yield of [^{3}H]puromycin-modified S14 because it eliminated the need for subunit separation by sucrose gradient centrifugation, a step that proceeds in rather low yield (50%–60%). RP-HPLC purification of 70S extracts was used to prepare both [^{3}H]-puromycin-modified S14 and *hν*-S14 (defined as S14 isolated from [^{3}H]puromycin-modified 70S ribosomes that has the same retention time on RP-HPLC as does native S14 and that contains no incorporated puromycin) for reconstitution experiments. A typical purification of a 70S extract is shown in Figure 2A, in which a very shallow gradient is used to give high resolution in the region of S14 elution. The nonradioactive peak eluting at 52 min is the source of the *hν*-S14. A major area of ^{3}H radioactivity eluted near or with L24. Fractions corresponding to elution times of ca. 36–40 min were combined, vacuum-dried, reapplied to the RP-HPLC column, and reeluted with a still shallower gradient in the region of interest. Four distinct protein peaks were observed (Figure 2B) of which one is L24 [identified by its lack of absorbance at 280 nm: L24 contains neither tryptophan nor tyrosine (Wittmann-Liebold, 1979)] and three (A, A′, and B) correspond to proteins containing photoincorporated radioactivity.[2] The most highly radioactive, peak A (ca. 41–44 min, in Figure 2B), was pooled separately. It had 0.79 mol of puromycin photoincorporated per mole of protein, as estimated from the known specific radioactivity of [^{3}H]puromycin employed and the protein content of the sample measured by a Bradford (1976) assay. This value is clearly much higher than the corresponding incorporation into protein eluting as peaks A and B, implying the presence in these latter peaks of photomodified protein having no [^{3}H]puromycin incorporation.

The 41–44-min fractions from Figure 2B and corresponding fractions from similar RP-HPLC runs were pooled to provide

[2] In a separate paper (Cooperman et al., 1986) we described three different forms of puromycin-modified S14 (A, B, and C). Peak C in that paper corresponds to the small peak of radioactivity eluting between 47 and 50 min in Figure 2A. The HPLC gradient utilized in the current study (Figure 2B) has revealed a peak not observed in the prior study, which we have designated A′.

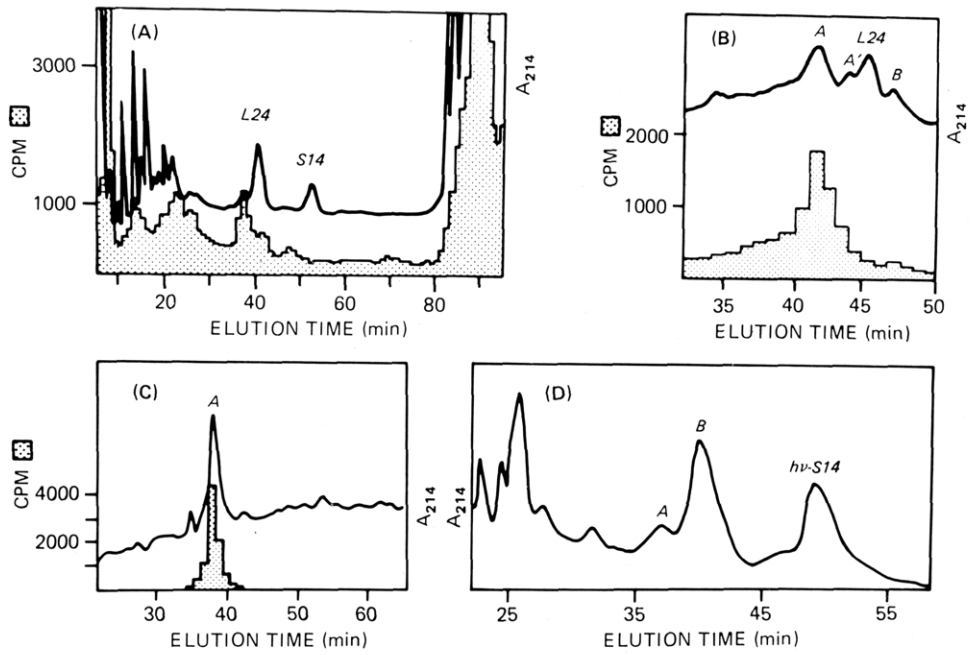

FIGURE 2: Purification of [^{3}H]puromycin-modified S14 from labeled 70S. (Panel A) The supernatant (250 μL) from a 3.6 M LiCl extract of 10 A_{260} units of [^{3}H]puromycin-labeled 70S ribosomes was applied to a Synchropak RP-P column and eluted with the following gradient: 15%–27.5% CH_3CN in 10 min (convex curve 0.2, Perkin-Elmer Series 4), 27.5%–29% CH_3CN in 45 min, and 29%–65% CH_3CN in 15 min. An essentially identical pattern but with broader peaks was obtained when RP-HPLC was carried out on a sample extracted from up to 300 A_{260} units of [^{3}H]puromycin-labeled ribosomes. (Panel B) Fractions corresponding to elution times 36–40 min in panel A, containing a major radioactive peak as well as part of L24, were pooled, lyophilyzed, redissolved in 0.1% CF_3COOH, and reapplied to the RP-P column. In the chromatogram shown, protein from 78 A_{260} units was applied. A similar pattern was obtained when protein from up to 300 A_{260} units of a similar sample was applied except that A′ was not resolved from L24. The following gradient was employed: 15%–27.5% CH_3CN in 10 min (curve 0.2) and 27.5%–29% CH_3CN in 60 min. Peak A (41–44 min) was pooled and lyophilyzed. (Panel C) An aliquot of peak A from panel B was redissolved in 0.1% CF_3COOH and chromatographed on an RP-P column with the following gradient: 15%–45% CH_3CN in 120 min (curve 0.2). (Panel D) The supernatant (1.85 mL) from a 50% acetic acid extract of 108 A_{260} units of 30S subunits obtained from [^{3}H]-puromycin-labeled 70S ribosomes was applied to the RP-P column and eluted with the same gradient as that used in panel A. Peak A (34.75–37.75 min) and peak B (37.75–43 min) contained 7900 and 13 600 cpm, respectively. The *hν*-S14 peak (47.75–53 min) contained no radioactivity. Shaded areas represent radioactivity from [^{3}H]puromycin.

the quantity of peak A needed for the reconstitution experiments described below. The purity of this material is demonstrated by the analytical HPLC analysis shown in Figure 2C.

[^{3}H]Puromycin-modified S14 could also be purified by first separating the puromycin-modified 70S ribosomes into 30S and 50S subunits and then subjecting a TP30 extract to RP-HPLC. A typical chromatogram is shown in Figure 2D. This procedure, though affording lower yields, was used to prepare [^{3}H]puromycin-modified S14 and *hν*-S14 for tryptic digestion analysis that would be free from contamination with L24 or any other 50S protein. The pooled fractions corresponding to elution times 34.75–37.75 min, 37.75–43 min, and 47.75–53 min are the sources of peaks A, B, and *hν*-S14, respectively, in the tryptic digestion experiments reported below.

(*b*) *Tryptic Digestion.* RP-HPLC analyses of tryptic digests of native S14, *hν*-S14, peak A, peak B, and native L24 are shown in Figure 3.

These analyses permit the following conclusions to be drawn:

(1) Peak A and peak B are S14 derivatives, as demonstrated by the similarity in the digestion patterns of A, B, and native S14 (note, particularly, peptide peaks 1–7). By contrast, the patterns for peaks A and B at 214 nm differ markedly from that seen for L24. Moreover, the tryptic peptides for L24 have little or no absorbance at 280 nm. Similarly, in results not presented, the trypsin digestion patterns for other proteins (S19, L28, and L31) having retention times on RP-HPLC similar to that of S14 (Kerlavage et al., 1983a) were quite different from those observed for peaks A and B.

(2) Both peaks A and B yield the same three radioactive tryptic peptide peaks, a large, broad peak (9, 9a) centered at 50 min and two smaller, sharper peaks at 29 min (3a) and at 32 min (5a). It is worth noting that peaks 9 and 9a have approximately 3-fold higher A_{280}/A_{214} ratios than does the corresponding peak 9 derived from native S14, as would be expected for incorporation of the puromycin chromophore. Similarly, peptides 3a and 5a have relatively high A_{280}/A_{214} ratios.

(3) The photolysis process itself does give rise to changes in the tryptic digestion pattern even in the absence of puromycin incorporation. Thus, tryptic peptide peak 8a, which absorbs at 280 nm, arises from digestion of *hν*-S14, of peak A, and of peak B, but it is not seen on digestion of native S14.

(4) Peaks A and B are significantly different from one another, as probed by tryptic digestion patterns. Thus, peak 8, having absorbance at 214 nm only, is absent in the digest of peak A but present in the digest of peak B, and there are a number of peaks eluting between 42 and 48 min in the digest of peak B that are not present in the digest of peak A.

Reconstitution of 30S Subunits Containing S14 (Peak A).* Reconstitution of 30S subunits were carried out as described (see Methods) with 16S RNA and the following protein samples: (a) TP30 – S14, (b) TP30 – S14 + S14, (c) TP30 – S14 + S14* (peak A), and (d) TP30 – S14 + *hν*-S14. Peak A was used as the source of S14* in these studies rather than peak B because it both was obtained in pure form in higher yield and was less labile toward loss of incorporated radioactivity on handling. Each reconstitution led to formation of 30S subunits, with yields, on the basis of A_{260} recovered in the initial high speed pelleting step, of 26%–42%.

A typical gradient profile is shown in Figure 4 for a reconstitution of type c and demonstrates not only the formation

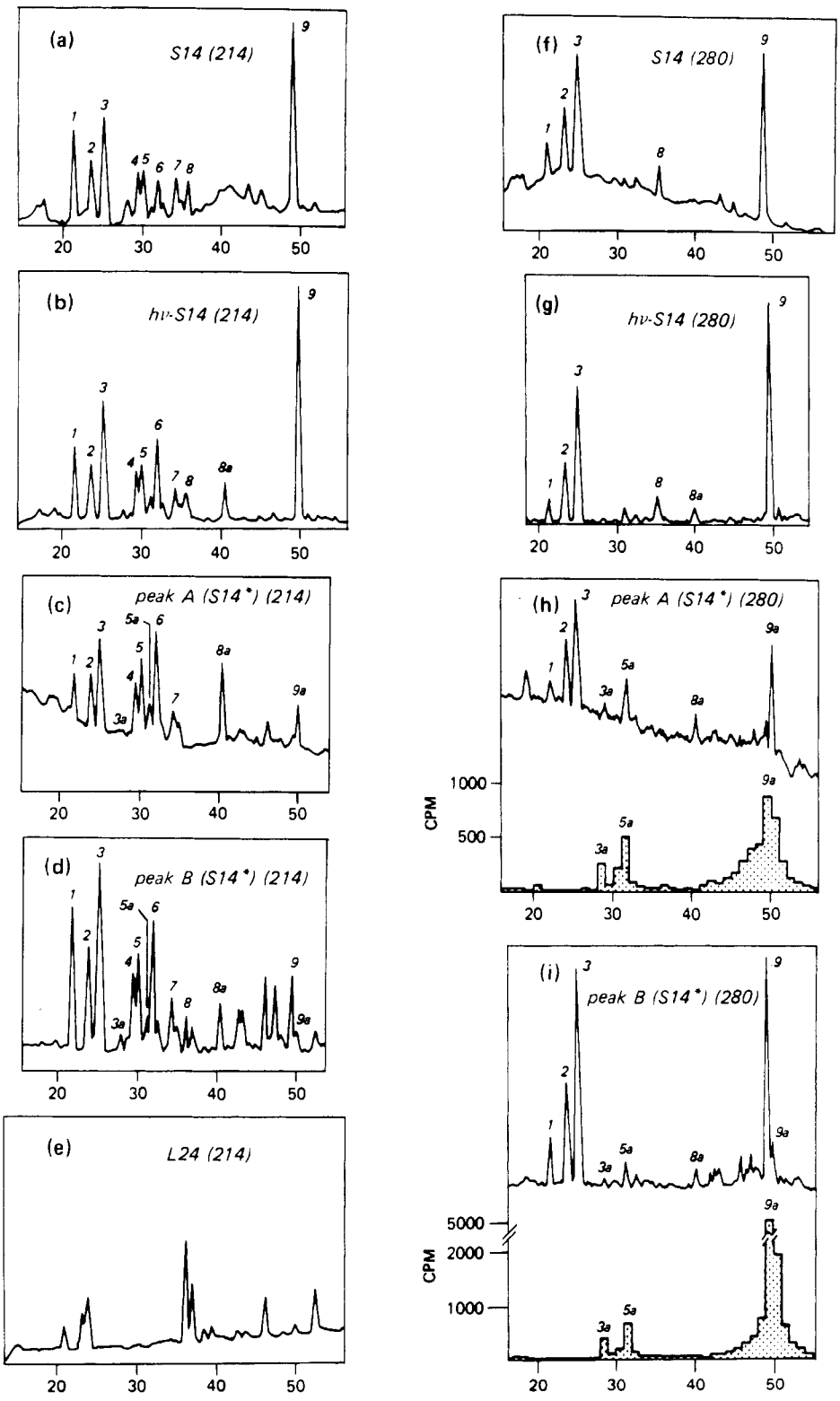

FIGURE 3: Tryptic digest RP-HPLC maps: (a and f) native S14, monitored at 214 and 280 nm, respectively; (b and g) *hν*-S14, monitored at 214 and 280 nm, respectively; (c and h) peak A (S14*), monitored at 214 and 280 nm, respectively; (d and i) peak B (S14*), monitored at 214 and 280 nm, respectively; (e) native L24, monitored at 214 nm. Panels h and i also show the radioactivity of covalently attached [^{3}H]puromycin. Samples were chromatographed with a gradient of 0%–35% CH_3CN in 60 min. Each X axis is elution time in minutes.

of a 30S subunit but also the uptake of S14* into the subunit. Table I lists the stoichiometries of S14* uptake into 30S subunits as a function of the amount of S14* added to the reconstitution mix. From the results obtained, it is clear that uptake of S14* tends toward a saturation value of one S14*/30S subunit. It should be noted that S14* uptake into 30S subunits requires larger amounts of added protein than does the corresponding uptake of S14.

RP-HPLC analyses of proteins extracted from 30S subunits derived from reconstitutions a–c are shown in Figure 5. These elution profiles demonstrate that the full complement of 30S proteins is incorporated into the 30S subunits in the absence (upper trace) or presence (middle trace) of S14 or when S14* replaces S14 (lower trace). Note that the radioactivity and A_{214} peaks at 38 min in the lower trace of Figure 5 correspond exactly to peak A in Figure 2C. In control experiments not

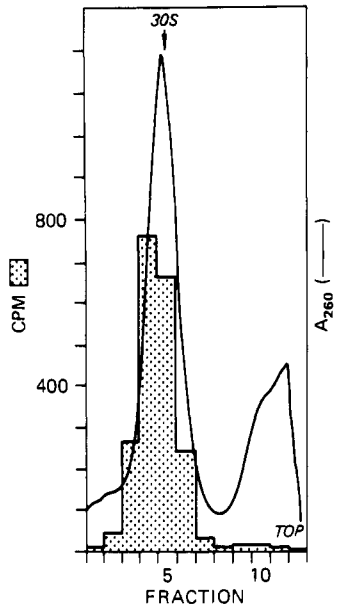

FIGURE 4: Sucrose gradient analysis of 30S subunits reconstituted with S14* (peak A). The fractional profile for a 5-mL sucrose gradient of 30S subunits reconstituted in the presence of an 8-fold molar excess of S14* over 16S rRNA. The peak contains 2.1 A_{260} units and 0.85 mol of [^{3}H]puromycin/mol of 30S subunit. The direction of sedimentation is from right to left. The sedimentation position of native 30S subunits is indicated by the arrow. The shaded area represents S14* radioactivity. Samples were applied to a 15%–30% sucrose gradient made up in buffer TKM1 containing 6 mM 2-mercaptoethanol. Centrifugation was carried out in a VTi50 rotor at 50 000 rpm for 90 min.

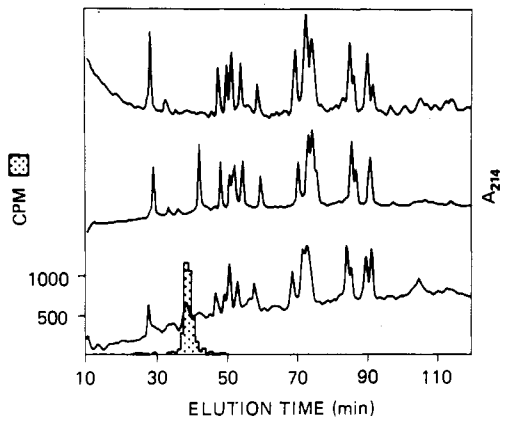

FIGURE 5: RP-HPLC analysis of TP30 extracted from reconstituted 30S subunits. Total protein was extracted by method 1 from pelleted reconstituted 30S subunits (2.0 A_{260} units) and chromatographed with the gradient described for Figure 2C. Particles were reconstituted from TP30 – S14 (2.0 A_{260} units, upper trace), TP30 (3.5 A_{260} units, middle trace), and TP30 – S14 + S14* (1.6 A_{260} units, lower trace). Fractions from the lower chromatogram were collected and counted for radioactivity to identify S14* (shaded).

shown, addition of L24 to either TP30 or TP30 – S14 followed by reconstitution led to no L24 uptake in the reconstituted 30S particle.

Phe-tRNA^{Phe} Binding Activities of Reconstituted 30S Subunits. The 30S subunits prepared from reconstitutions a–d above were tested for their ability to bind [^{14}C]Phe-tRNA^{Phe} in a poly(U)-dependent manner. Three points are worth noting. First, the absolute value of Phe-tRNA^{Phe} bound per reconstituted 30S particle was subject to considerable day-to-day variation, falling in the range of 30%–90% of that observed with native 30S particles. This variability reflects a composite of potential experimental problems, including the

Table I: Phe-tRNA^{Phe} Binding Activity of Reconstituted 30S Subunits

protein in reconstitution mix	S14 (or derivatized S14)/30S[a]	puromycin/30S[b]	relative Phe-tRNA^{Phe} binding activity[c]
(1) TP30 – S14 + S14 (1.8 equiv)	1.0	0.0	1.00
(2) TP30 – S14 (1.8 equiv)	0.0	0.0	0.01 ± 0.01 (n = 3)
(3) TP30 – S14 + $h\nu$-S14[e]	1.0	0.0	0.85 ± 0.06 (n = 2)
(4) TP30 – S14 + peak A (puromycin–S14) (1 equiv)	ND[f]	0.24	ND
(5) TP30 – S14 + peak A (puromycin–S14) (4 equiv)	0.6	0.68	0.03 ± 0.02 (n = 2)
(6) TP30 – S14 + peak A (puromycin–S14) (8 equiv)	0.9	0.85	0.05

[a] Approximate values determined from S14 peak area at 214 nm in HPLC analysis of TP30 from reconstituted 30S subunits. [b] Determined from cpm per A_{260} in reconstituted 30S subunits. [c] tRNA binding measurements on a given sample were measured in duplicate and gave average deviations of ±5%–10%. n refers to the number of independent sets of experiments for which relative binding activity was determined. The activity of 30S particles reconstituted from HPLC-prepared TP30 is taken as 1.0 and varied from 0.013 to 0.053 Phe-tRNA^{Phe}/30S for different sets of experiments. All error ranges are average deviations. [d] Relative to 16S rRNA. TP30 – S14 was present in 1.8 equiv in all experiments. [e] $h\nu$-S14 is obtained from ribosomes photolyzed in the presence of puromycin but has neither an altered retention time on HPLC analysis nor any incorporated puromycin. [f] Not determined.

preparation of intact 16S RNA, the stability of HPLC-purified proteins toward storage, and the difficulty of adding different pools of proteins to the reconstitution mix in the optimal stoichiometry. Second, such variability can be factored out by comparing one reconstitution experiment with another, resulting in relative Phe-tRNA^{Phe} binding activities that are rather reproducible between independently performed sets of experiments (Table I). Third, the relative Phe-tRNA^{Phe} binding activity of reconstituted particles was essentially unchanged whether measured before or after sucrose density gradient purification, although absolute values did increase in some cases.

The results collected in Table I allow the following conclusions to be drawn: (a) subunits reconstituted with TP30-S14 have essentially no Phe-tRNA^{Phe} binding activity (no. 2); (b) subunits reconstituted with $h\nu$-S14 have almost full binding activity (no. 3); (c) addition of S14* to the reconstitution mix in place of S14 does not restore Phe-tRNA^{Phe} binding activity (nos. 5 and 6).

Reconstituted 30S subunits were similarly assayed to test the effect upon Phe-tRNA^{Phe} binding activity of added 50S subunits under conditions that favor formation of 70S ribosomes (Table II). Addition of 50S subunits to reconstituted 30S subunits containing native S14 caused a 3.2-fold increase in poly(U)-dependent Phe-tRNA^{Phe} binding (no. 1). Most of this increased binding was inhibited by the addition of 30 μM tetracycline (TC) to the assay mix. As shown in Table II, 30S subunits reconstituted without S14 or with S14* have negligible Phe-tRNA^{Phe} binding. Addition of 50S subunits to these subunits does lead to Phe-tRNA^{Phe} binding activity. Although this 50S-induced activity is 2-fold greater for 30S subunits containing S14* (no. 3) than for 30S subunits lacking S14 (no. 2), the binding activity in both of these experiments is reduced

Table II: Effect of Added 50S Subunits upon Phe-tRNA^{Phe} Binding Activity of Reconstituted 30S Subunits

protein in reconstitution[a]	relative Phe-tRNA^{Phe} binding activity[b] 30S	30S + 50S	30S + 50S + 30 μM tetracycline	calcd A-site binding[c]
(1) TP30 – S14 + S14	1.0	3.2	1.3	1.3
(2) TP30 – S14	0 (0.16)[d]	0.4 (0.9)	0.2 (0.4)	0.2
(3) TP30 – S14 + peak A	0 (0.16)	0.8 (1.3)	0.2 (0.4)	0.6

[a] TP30 was present in 1.8-fold molar excess over 16S rRNA. S14* was present in 4-fold molar excess over 16S rRNA and was present in 0.7 copy in the reconstituted 30S particle. [b] Poly(U)-dependent binding. Activity for 30S particles reconstituted from HPLC-prepared TP30 is taken as 1.0 (see Table I, footnote *c*). Lines 1 and 2 report average values from two independent sets of experiments and are reproducible within ±5%. Line 3 reports the average value (±5%) for duplicates from a single experimental set. [c] Assuming that to a first approximation 30 μM tetracycline inhibits virtually all A-site binding and no P-site binding (Kaji & Ryoji, 1979; Gale et al., 1981). [d] The preparation of TP30 – S14 used in these reconstitutions was contaminated with ca. 20% native S14. The numbers in parentheses are the observed ratios while the other ratios are corrected for the activity due to the presence of S14.

to the same level by addition of TC.

Discussion

Affinity labeling is now a widely used technique for attempting to identify specific functional sites within biological macromolecules or macromolecular assemblies (Jakoby & Wilchek, 1977). In general, covalent labeling is achieved either by photolytic means or through the use of electrophilic reagents. Because the reactive intermediates generated photolytically are often highly reactive chemically, photoaffinity labeling presents the important advantage that the distribution of covalent bond formation within the target macromolecule has a high probability of paralleling the distribution of noncovalent ligand binding, with, under appropriate condition, the highest affinity site labeled to the highest extent. By contrast, electrophilic affinity labels often show high selectivity toward particular nucleophiles, raising the possibility that major labeling may occur at a weak binding site having a reactive nucleophile. On the other hand, electrophilic affinity labeling often proceeds in high yield. In favorable cases it is possible to fully derivatize the native macromolecule. Such high yields are very seldom found in photoaffinity labeling reactions. As a result, it is unusual to be able to determine the functional consequences of photoaffinity labeling,[3] so that this strong test that photoaffinity labeling has occurred at a functionally important site is most often lacking.

In this work we describe an approach for overcoming this limitation, at least for the case of photoaffinity-labeled ribosomal proteins. Thus, we have prepared and tested the function of a ribosome in which one protein, S14, is photoaffinity labeled to a stochiometric extent and all other ribosomal components are unmodified. Previous workers have reconstituted 30S or 50S subunits in which a single protein has been modified, either to test functional consequences (Kahan et al., 1974; Shimizu & Craven, 1976; Hernandez et al., 1977; Baxter & Zahid, 1978; Auron et al., 1978; Baxter et al., 1980) or to introduce a probe for electron microscopy or fluorescence energy transfer studies (Stöffler-Meilicke et al., 1983, 1984; Maasen et al., 1984; Odom et al., 1984), but this earlier work involved non-site-specific modification of ribosomal proteins to high stoichiometries with group-specific reagents. The advantage of our approach is that because modification proceeds from a specific site with a functionally important ligand, there is a much clearer rationale for interpreting changes in function of the reconstituted particle as reflecting specific modification of a functional site. The special challenge posed in the current work is that photoaffinity labeling of S14 by puromycin proceeds to only a minor (<2%—see Table I) extent, necessitating the clean separation of puromycin-labeled S14 from unlabeled S14 in order for the experiment to be carried out. The high resolution, high yield, and rapidity (important because of the lability of puromycin-labeled S14) available with RP-HPLC makes this technique ideally suited for this purpose.

There is a great deal of published evidence for the involvement of S14 in tRNA binding [Cooperman (1980) and references cited therein]. Indeed, our control reconstitution experiments are similar to those of Nomura et al. (1969) in showing that reconstituted particles have virtually full activity toward poly(U)-dependent Phe-tRNA^{Phe} binding and that the single omission of S14 leads to substantial loss of such activity. Our experiments also show that a greater fraction of binding activity is retained (12%) in the single omission 70S ribosomes than in the single omission 30S subunits (<1%, Table I, no. 2), something that was not apparent from the earlier study. The major new results from our work are that both 30S subunits and 70S ribosomes that have been reconstituted with puromycin-modified S14 (S14*) in place of S14 show much weaker poly(U)-dependent Phe-tRNA^{Phe} binding. The effect is more pronounced for 30S subunits (S14* restores 3%–5% of the binding restored by native S14—Table I) than for 70S ribosomes (S14* restores 15% of the binding restored by native S14—Table II). Furthermore, assuming to a first approximation that 30 μM tetracycline blocks almost all tRNA binding to the A site of 70S ribosomes but has little effect on binding to the P site (Kaji & Ryoji, 1979; Gale et al., 1981; Hasan et al., 1985) S14* restores 25% of the A-site binding restored by native S14 but restores none of the P-site binding (Table II).

These effects of S14* are almost certainly a direct result of puromycin photoincorporation. The alternative, that they are due to some uncharacterized photochemical damage to S14, is rendered unlikely by the great similarity in the tryptic digestion patterns of peak A and *hν*-S14 (with the exception of those tryptic peaks derived from peak A that incorporate radioactivity) and the fact that reconstitution with *hν*-S14 virtually completely restores tRNA binding activity. Ivanov and Saminsky (1984) have noted that puromycin inhibits poly(U)-dependent *N*-AcPhe-tRNA^{Phe} binding to 30S subunits, and Abdurashidova et al. (1985) have obtained photoinduced cross-linking results providing strong evidence that *N*-AcPhe-tRNA^{Phe} and Phe-tRNA^{Phe} bind to the 30S subunit in virtually identical manners. Our results suggest that puromycin inhibition of tRNA binding to 30S subunits results from puromycin binding in the vicinity of S14.

Despite the strong structural similarity between puromycin and the 3′-terminus of aminoacyl-tRNA, a similarity that accounts for the activity of puromycin as a peptidyl acceptor in peptidyl transferase (Allen & Zamecnik, 1962; Nathans, 1964), our results indicate that the perturbation caused by reconstituting 30S subunits with S14* is not restricted to the

[3] A special case where such a function can be tested occurs when a new function is conferred by virtue of the photoaffinity labeling process—see, for example, Hsiung et al. (1974).

region of 3′-terminus binding. Had it been, S14* would have restored full tRNA binding to 70S ribosomes, since both the A-site and the P-site 3′-termini are bound to the peptidyl transferase center on the 50S subunit, where they would be expected to be unaffected by small changes in S14 structure. Rather, it appears that the perturbing effect of the incorporated puromycin is propagated more widely in the tRNA binding locus, weakening binding to both the A and the P sites. This conclusion is disappointing in a sense, since, for the purpose of constructing a detailed structure–function map of the ribosome, it would have been preferable to be able to associate a loss of a more precise function than overall tRNA binding with the incorporation of puromycin into S14. However, by analogy with the pleiotropic effects that have been found to result from either point-specific mutations in ribosomal proteins (Kreider & Brownstein, 1974; Olsson et al., 1974) or chemical modification of a single amino acid residue in a single proton (Auron et al., 1978), it can hardly be considered surprising.

We conclude with some speculative assignments of the identities of the tryptic peptides corresponding to peak 9 in the digest of S14 and peak 9a in the digest of peak A. S14 has been sequenced by Yaguchi et al. (1983). It contains a single tryptophan, at position 41. Although Arg-40 precedes Trp-41, the peptide bond between them is apparently poorly susceptible to trypsin cleavage. As a result, Trp-41 falls within the largest peptide produced by tryptic digestion, stretching from Ala-28 to Lys-46 and having the structure:

...Ala^{28}-Ile-Ile^{30}-Ser-Asp-Val-Asn-Ala-Ser-Asp-Glu-Asp-Arg^{40}-Trp-Asn-Ala-Val-Leu-Lys^{46}...

Of all the peaks present in the tryptic digestion of S14 (Figure 4), peak 9 has both the highest A_{280}, consistent with it containing a tryptophan, and the highest A_{214}, consistent with it being the largest peptide. We thus believe that peak 9 corresponds to peptide 28–46 in S14. Speculating further, we believe it likely that peak 9a in the tryptic digest of peak A (Figure 4) corresponds to peptide 41–46 containing puromycin photoincorporated into Trp-41, i.e., that the incorporation of puromycin into Trp-41 renders the 40–41 peptide bond more susceptible to trypsin cleavage. This assignment is based on the following considerations: (1) Peak 9a has a much lower A_{214} than does peak 9, so that it must correspond to a smaller peptide. (2) Peak 9a has a slightly higher retention time than does peak 9. The calculated retention time for residues 41–46 (33 min; Meek, 1980) is slightly longer than that for residues 28–46 (30.3 min).[4] (3) Tryptophan is considerably more photolabile than is puromycin (Jaynes et al., 1978), so that photoinduced tryptophan incorporation into puromycin has a higher probability than photoinduced puromycin incorporation into a photochemically inert side chain. Clearly, further work will be required to test the validity of our assignments for peaks 9 and 9a.

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[4] Meek's conditions are sufficiently different from ours (column, Bio-Rad ODS C_{18}; linear gradient, 0–100% CH_3CN, 1 mL/min, 0.75%/min; ion-pairing agent, 0.1% phosphoric acid) that we would expect to measure different absolute R_t values. However, the order of peptide elution should be conserved.

Registry No. Puromycin, 53-79-2.

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Comparison of the Folding of β-Globin and Ovalbumin Gene Containing Chromatin Isolated from Chicken Oviduct and Erythrocytes

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ABSTRACT: The dependence of chromatin conformation upon salt concentration has been studied for chicken ovalbumin and β-globin genes isolated from oviduct and adult erythrocytes. At NaCl concentrations of 25 or 50 mM, the sedimentation properties, as a function of DNA size, of ovalbumin and globin chromatin are similar regardless of the source of the chromatin. In 100 mM NaCl, however, β-globin chromatin isolated from erythrocytes sediments more slowly than an ovalbumin chromatin fraction from erythrocytes containing DNA of the same size. When the same experiment is carried out with material isolated from oviduct nuclei, the relative sedimentation rates are reversed, so that the ovalbumin chromatin sediments more slowly. This behavior cannot be accounted for by differences in binding of RNA polymerase or other molecules associated with transcription, or by partial aggregation of the chromatin. The most reasonable explanation is that transcriptionally active chromatin with a history of transcriptional activity, although largely covered with histones and capable of considerable compaction, is not able to form a fully compact structure as the ionic strength is raised. This behavior is consistent with a slight depletion in active chromatin of core histones or histone H1/H5 or both.

The chromatin of genes that are expressed is distinguished from bulk chromatin by an unusually sensitivity to nucleases. On the other hand, at least in the case of genes that are transcribed at only moderate rates, a considerable fraction of the transcribed sequence can be found packaged in nucleosomes. It is possible that these facts could be explained by the partial disruption of the 30-nm-thick chromatin fiber in the neighborhood of transcriptionally active genes, presumably resulting from partial loss of histone H1 or of core histone octamers, or from the unfolding of the octamers after some modification [see, for example, Karpov et al. (1984)].

In earlier studies, the folding of chromatin into 30-nm fibers was examined by using sequence-specific probes that permitted comparison of expressed with inactive genes. Felsenfeld et al. (1982) found that at concentrations of NaCl up to 50 mM, the sedimentation properties of chromatin fibers from chicken erythrocytes containing the adult β (β^A) globin gene were indistinguishable from those of bulk chromatin. On the other hand, Kimura et al. (1983), using a chromatin fraction excised

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