CHROMSYMP. 498

APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO THE RECONSTITUTION OF RIBOSOMAL SUBUNITS

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SUMMARY

We are currently utilizing reversed-phase high-performance liquid chromato-graphy (RP-HPLC) in reconstitution experiments designed to study the structure and function of *Escherichia coli* ribosomes. The applications of RP-HPLC in these experiments include: (a) preparation of individual proteins or groups of proteins on a milligram scale for reconstitution pools, (b) analysis of the protein stoichiometry of reconstituted subunits, (c) determination of the extent and specificity of modification of proteins extracted from ribosomal subunits which have been subjected to chemical modification, and (d) resolution of modified forms of proteins S14 and L23 from the corresponding unmodified proteins. Proteins prepared by RP-HPLC from 30S and 50S ribosomal subunits were found to reconstitute into 30S and 50S subunits respectively, as well as into slower sedimenting particles. The reconstituted subunits contain a full complement of proteins and are active in ribosomal function assays, whereas the slower sedimenting particles lack several proteins and have little or no activity.

INTRODUCTION

The *Escherichia coli* ribosome is a large ($M_r = 2.3 \cdot 10^6$) ribonucleoprotein complex containing 52 unique proteins and three molecules of rRNA in two dissimilar subunits[1,2]. The large subunit has a sedimentation coefficient of 50S and contains 32 unique proteins designated L1–L34 and one molecule each of 23S and 5S rRNA. The small subunit has a sedimentation coefficient of 30S and contains 21 unique proteins, designated S1–S21, and one molecule of 16S rRNA. Despite the numbering system, there are only 52 unique proteins: (a) L7 is the NH_2-terminal acylated form of L12[3], (b) L8 is a complex of L10 and L7/L12[4], and (c) L26 and S20 are identical[5]. Elsewhere we have established that reversed-phase high-performance liquid chromatography (RP-HPLC) is the method of choice for both the analysis and preparation of *E. coli* ribosomal proteins[6–8]. In this paper we report on the current status of our ongoing efforts to exploit the RP-HPLC methodology in carrying out experiments coupling the approaches of reconstitution and affinity labeling in defining protein topography and function in the *E. coli* ribosome.

The reconstitution of ribosomal subunits *in vitro* from their constituent com-

ponents has been an important tool in elucidating the structural and functional roles of various ribosomal components. Total reconstitution of the 30S subunit can be achieved in a single-step incubation of its components[9] while reconstitution of the 50S subunit requires a two-step incubation[10,11]. Many experiments have been carried out in which the 30S[9] or 50S[12] subunit is reconstituted with the omission of a single protein, and the effect of the omission on ribosomal function is measured.

Affinity labeling has led to identification of ribosomal proteins involved in the binding of mRNA, tRNA, antibiotics such as chloramphenicol, puromycin, tetracycline and streptomycin, and in the putative peptidyl transferase center[13,14]. In addition, proteins have been localized on the ribosome by a combination of photoaffinity labeling and immunoelectron microscopy. For example, antibodies to N^6,N^6-dimethyladenosine cross-react with puromycin and have been used to localize puromycin covalently bound to proteins S14[15,16] and L23[17]. However, a problem inherent in assessing the significance of these experiments is that the molar fraction of either S14 or L23 labeling is very low, making it difficult to detect an alteration in functional activity arising as a result of labeling.

The overall goal of the current work is to address the question of significance by carrying out functional assays on reconstituted subunits containing puromycin-labeled S14 or L23 in place of unmodified S14 or L23, which we may call a single replacement experiment. We report here on the application of RP-HPLC toward the successful completion of several key steps on the way toward that goal.

EXPERIMENTAL

Materials
       Sequanal grade trifluoroacetic acid was purchased from Pierce. HPLC grade acetonitrile (UV cutoff 190 nm) was purchased from Fisher Scientific. Ultra-pure sucrose was purchased from Schwarz/Mann. Coomassie brilliant blue G-250 was obtained from Sigma. [³H]Puromycin (1.44 Ci/mmole) was prepared from [³H]puromycin aminonucleoside (Amersham) by a method paralleling that described previously for *p*-azidopuromycin[18]. All other chemicals were reagent grade. Reagent grade urea (Sigma) was purified by treatment of 8 *M* solutions with 4 g/l Norit A and 10 g/l Amberlite MB-3 to decolorize and remove cyanate, respectively.

Isolation of ribosomal proteins
       E. coli ribosomes, 30S and 50S subunits, and ribosomal proteins were prepared as described previously[7]. Acetone precipitated proteins from 30S subunits were re-dissolved in buffer A [20 m*M* Tris–HCl (pH 7.4 at 23°C), 20 m*M* magnesium acetate, 500 m*M* ammonium chloride, 6 *M* urea, 6 m*M* β-mercaptoethanol] prior to HPLC. Peaks collected from HPLC in chilled (dry ice) polypropylene tubes were dried in a Savant Speed-Vac evaporator, redissolved in buffer A and stored in small aliquots at −80°C. Acetic acid-extracted 50S proteins were applied directly to the HPLC column in 67% acetic acid. Peaks collected from HPLC in chilled polypropylene tubes were dried as for the 30S proteins, redissolved in buffer B [20 m*M* Tris–HCl (pH 7.5 at 0°C), 4 m*M* magnesium acetate, 400 m*M* ammonium chloride, 6 *M* urea, 5 m*M* β-mercaptoethanol] and dialyzed overnight against the same buffer. Urea was removed by three 45-min dialyses against 200–500 volumes of buffer B lacking urea.

The protein solution was centrifuged 5 min at 5000 g and stored in small aliquots at $-80°C$.

High-performance liquid chromatography

Methods previously described[7] were adapted to an automated HPLC system consisting of a Perkin-Elmer Series 4 liquid chromatograph, 1SS-100 autosampler, LC-15B detector with 280-nm filter (10-μl cell, 1-cm path-length), LC-75 variable-wavelength detector (8-μl cell, 6-mm, path-length, electronically corrected to 1 cm equiv.), and 3600 data station. RP-HPLC on SynCropak RP-P silica columns (6.5 μm silica, 300A pore; 250 × 4.1 mm I.D.; SynChrom) was performed with the following modifications: solvent A, twice-filtered, deionized, reverse-osmosis purified water; solvent B, 1% (w/v) trifluoroacetic acid in water; solvent C, twice-filtered acetonitrile. Solvent B was delivered at a constant 10% of the total combined flow-rate of 0.7 ml/min while gradients in solvents A and C were run. Solvents were kept under helium pressure during use.

Determination of protein concentration

Protein concentrations were determined by the Coomassie brilliant blue G250 micro-assay of Bradford[19] using bovine serum albumin as a standard. Alternatively, the concentration of protein pools was estimated by comparing the integrated peak areas from the chromatogram of an aliquot of each pool to areas obtained from chromatograms of protein extracted from known amounts of subunits. Standard deviation of areas per pmol extracted was less than 12% for each peak from seven chromatograms.

Reconstitution

30S subunits. TP30 was reconstituted with 16S rRNA essentially as described by Held *et al.*[9] with the exception of the buffers used. Proteins that had been stored in buffer A were dialyzed against 200–500 volumes of buffer A without urea for 3 × 45 min just prior to reconstitution. Reconstituted particles were pelleted at 217,000 g for 3.5 h in a Beckman L-8 ultracentrifuge using a Ti50 rotor. The supernatants were removed, except for the bottom 1-ml, and the pellets were resuspended in this mother liquor by sonication. The resulting solutions were centrifuged for 90 min through linear 15–30% sucrose gradients made up in buffer C [50 mM Tris–HCl (pH 7.6 at 23°C), 50 mM potassium chloride, 1 mM magnesium chloride, 6 mM β-mercaptoethanol] in a Beckman VTi50 rotor at 4°C as described elsewhere[20]. Peaks with absorbance at 260 nm were pooled and precipitated with two volumes of ethanol. The pellets were redissolved in buffer D [50 mM Tris–HCl (pH 7.5 at 23°C), 100 mM magnesium chloride, 50 mM potassium chloride] and aliquots were either assayed for PhetRNA^{Phe} binding as described previously[21] or extracted for analysis by HPLC.

50S subunits. TP50 was reconstituted with 23S and 5S rRNAs as described by Nierhaus and Dohme[10] with the protein storage buffer suggested by Schulze and Nierhaus[22]. The storage buffer for rRNA [10 mM Tris–HCl (pH 7.6 at 0°C), 10 mM magnesium chloride, 50 mM ammonium chloride, 3 mM β-mercaptoethanol] was suggested by Dr. Michael Stark (personal communication). Reconstituted particles were centrifuged through sucrose as described above. Peaks were pooled and precip-

itated with two volumes ethanol and the pellet was redissolved in buffer B made 20 mM in magnesium acetate. Aliquots were assayed for peptidyltransferase activity as described[22] using 33% ethanol and the remainder was extracted, and the protein content was analyzed by HPLC.

Photoaffinity labeling

Photolyses were conducted in quartz tubes in a Rayonet RPR reactor using 3500-Å lamps essentially as described by Jaynes *et al.*[23]. Photolyzed subunits were precipitated with two volumes of 10% β-mercaptoethanol in ethanol, redissolved in buffer D and reprecipitated. Proteins were extracted with acetic acid and precipitated with acetone as described previously[20]. TP30 was redissolved in buffer A and TP50 was redissolved in 0.1% trifluoroacetic acid in water prior to HPLC. Eluted fractions (0.7 ml) were collected in 7-ml polypropylene vials (Wheaton). Following addition of 5.9 ml of Triton–toluene (25:75) cocktail[23], samples were counted in an Intertechnique SL30 liquid scintillation counter.

RESULTS AND DISCUSSION

Preparation of ribosomal proteins for reconstitution

A major advantage of RP-HPLC over classical protein purification methods is that it is a very rapid procedure affording a relatively high yield of proteins. These advantages are critical to reconstitution experiments since they eliminate protein preparation as the limiting step. We reported previously[8] that 1–3 mg of ribosomal protein could be chromatographed in a single run without significant loss of resolution. We have subsequently found that this quantity can be increased to as much as 5 mg of TP30 or TP50 (total proteins from 30S and 50S ribosomal subunits, respectively) when a slight loss of resolution is acceptable (see below). Recovery of total protein from such preparative runs is typically greater than 70%.

Chromatograms for TP30 and TP50 are shown in Figs. 1 and 2, respectively. The chromatogram of TP30 is very similar to that published previously[7]. Some variability in resolution has been observed which depends upon the age of the column. For example, the resolution of S15 and S18 decreased with column age. Protein S21 was observed to emerge as two peaks, the earlier-eluted one most likely being an oxidized form of the later-eluted peak. The first peak could be completely converted to the later-eluted peak by dialysis in buffer A that contains 6 mM 2-mercaptoethanol.

The chromatogram of TP50 shown in Fig. 2 was obtained by using a gradient specifically designed to yield maximum resolution in the L-18/22/23/29 region. Resolution in the remainder of the chromatogram has not been severely compromised by using this gradient. Increased resolution in the aforementioned region was accomplished by introduction of a region of low φ'* around 35–36% acetonitrile. Increased resolution in any region of the chromatogram could be obtained simply by decreasing φ' in that region.

Protein pools for reconstitution were prepared by one of two methods. In the first, individual RP-HPLC peaks were collected, lyophilyzed, redissolved, assayed for

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* φ' = Change in volume fraction solvent B per unit volume.

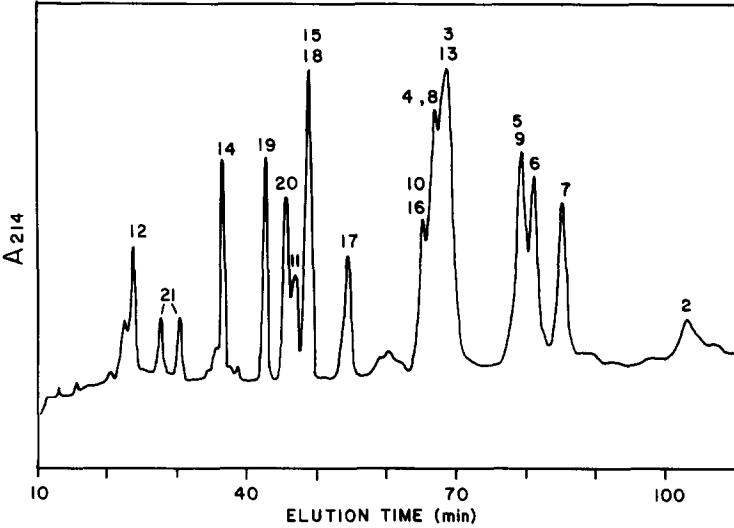Fig. 1. RP-HPLC of TP30. A solution of TP30 (264 pmol) in 10 μl buffer A was applied to a SynChropak RP-P column and eluted with a convex gradient (curve .2, Perkin-Elmer Series 4) of 15% to 45% aceto-nitrile in 120 min. Absorbance at 214 nm was measured at 0.075 a.u.f.s. Protein S21 was often eluted as two peaks, the earlier-eluted peak being an oxidized form of the later-eluted peak. Protein S1 was not present in the 30S subunit preparation from which these proteins were extracted. S1 was eluted at ap-proximately 120 min. Protein identifications are according to Kerlavage et al.[7].
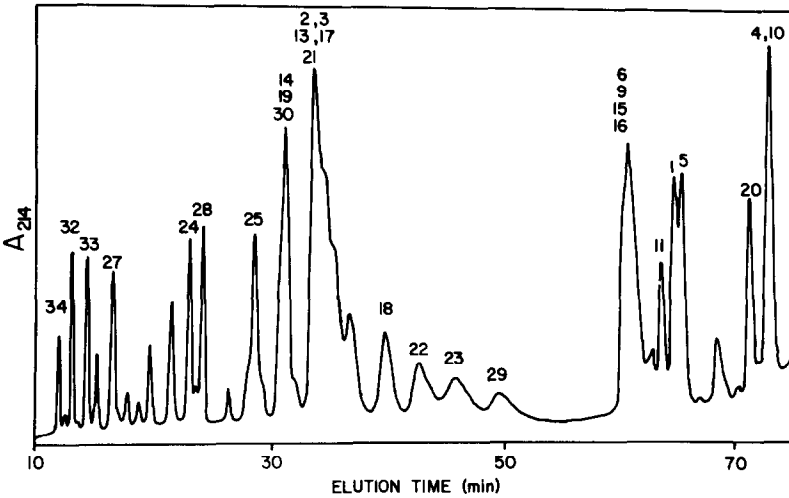

Fig. 2. RP-HPLC of TP50. A solution of TP50 (700 pmoles) in 60 μl 0.1% trifluoroacetic acid was applied to a SynChropak RP-P column and eluted with the following gradient: 17–34.9% acetonitrile in 20 min (curve .2); 34.9–36% acetonitrile in 10 min (linear); 36% for 10 min; 36–45% acetonitrile in 10 min (linear); 45–50% acetonitrile in 5 min (linear); 50–75% acetonitrile in 5 min (linear); 75% acetonitrile for 5 min. Absorbance at 214 nm was measured at 0.6 a.u.f.s. Proteins L7 and L12 were present in low amounts in the 50S subunits from which these proteins were extracted and are not shown. For their elution position see ref. 7.

protein concentration, and pooled as needed to give an equivalent number of moles of each protein. In cases where a peak contained more than one protein, the combined M_r values were used to determine an average protein concentration. Alternatively, pools containing several RP-HPLC peaks were collected directly from the column and an average concentration for all of the proteins in each of those pools was determined. Since there is a fair inverse correlation between retention time and percent recovery of the proteins[8], pools could be designed such that they contained roughly equivalent amounts of each protein. Both of these methods worked well in reconstitution experiments, the former being used routinely in 30S reconstitutions and the latter in 50S reconstitutions. It should be noted that care had to be taken when sample loads greater than 2 mg were used since under these conditions proteins were eluted earlier by as much as 1% acetonitrile.

Reconstitution

Once individual RP-HPLC protein peaks or pools of RP-HPLC protein peaks are available they can be reconstituted with rRNA according to standard procedures. The extent of reconstitution is monitored by sucrose gradient centrifugation as shown in Fig. 3. The upper panel shows the sucrose density gradient profile of particles
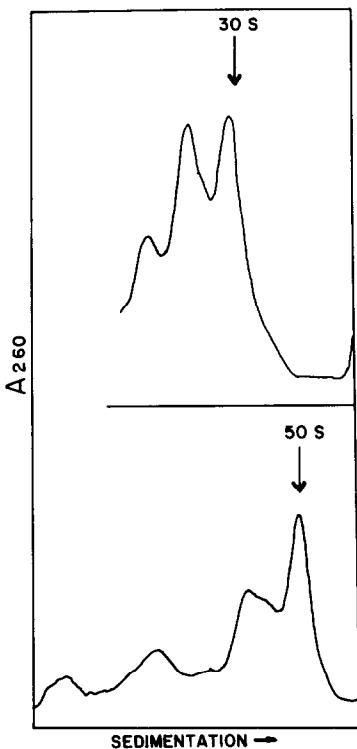

Fig. 3. Sucrose gradient centrifugation of reconstituted ribosomal subunits. Upper panel: fractionation profile for a 36-ml sucrose gradient centrifugation of particles formed in a 30S reconstitution (1560 pmol 16S RNA). Lower panel: fractionation profile for a 36-ml sucrose gradient centrifugation of particles formed in a 50S reconstitution (1600 pmol 23S and 5S RNA). Both gradients were run for 90 min. For both gradients absorbance at 260 nm was measured at 0.5 a.u.f.s. The sedimentation positions of native 30S and 50S subunits from identical runs are indicated.

formed from the reconstitution of HPLC-prepared TP30 and 16S rRNA. Two major peaks are observed, one with a sedimentation coefficient of 30S and one with a slightly lower sedimentation coefficient. In such experiments, the percent of total A_{260}-absorbing material sedimenting at 30S ranged from 42% to 100%. The overall yield of 30S particles from starting 16S RNA varied from 23% to 53%*. The protein profiles from the 30S and lighter particles (Fig. 3, upper panel) are shown in Fig. 4. Reconstituted 30S particles typically contained all of the 30S proteins in nearly stoichiometric amounts with the exception of S1, S2 and S21 which were variable (Fig. 4, upper panel). The protein content of the lighter particles varied considerably with respect to several proteins; in addition to S1, S2 and S21, various combinations of S21, S19, S15 or S18, S3 or S13, S5 or S6 or S9, and S7 were present in reduced amount. Only the peak containing S3 and S13 (arrow, Fig. 4) was consistently lower in the lighter particles.
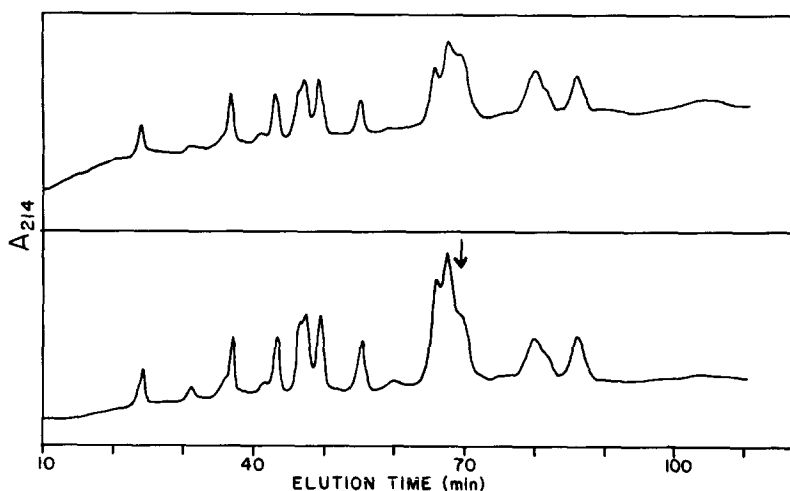

Fig. 4. RP-HPLC analysis of reconstituted 30S and lighter particles. Total protein was extracted from the particles shown in Fig. 3 (upper panel), dissolved in buffer A, applied to a SynChropak RP-P column and eluted with the gradient described in the legend to Fig. 1. Upper panel: protein from 273 pmol 30S subunits. Absorbance at 214 nm was measured at 0.043 a.u.f.s. Lower panel: protein from 296 pmol lighter particles. Absorbance at 214 nm was measured at 0.090 a.u.f.s. Arrow indicates position of proteins S3 and S13.

The lower panel of Fig. 3 shows the sucrose density gradient profile of particles formed from the reconstitution of HPLC-prepared TP50 and 23S plus 5S rRNA. Again, two major peaks are observed, the larger of which has a sedimentation coefficient of 50S. In 50S reconstitutions, the percent of total A_{260} absorbing material sedimenting at 50S ranged from 20% to 50%. The overall yield of 50S particles from starting 23S rRNA was 10% to 35%. The protein profiles from the 50S and lighter particles in Fig. 3, lower panel, are shown in Fig. 5. Reconstituted 50S particles

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* In sucrose gradient centrifugation of native ribosomal 30S or 50S subunits the maximum recovery of A_{260} absorbing material is *ca.* 60%.
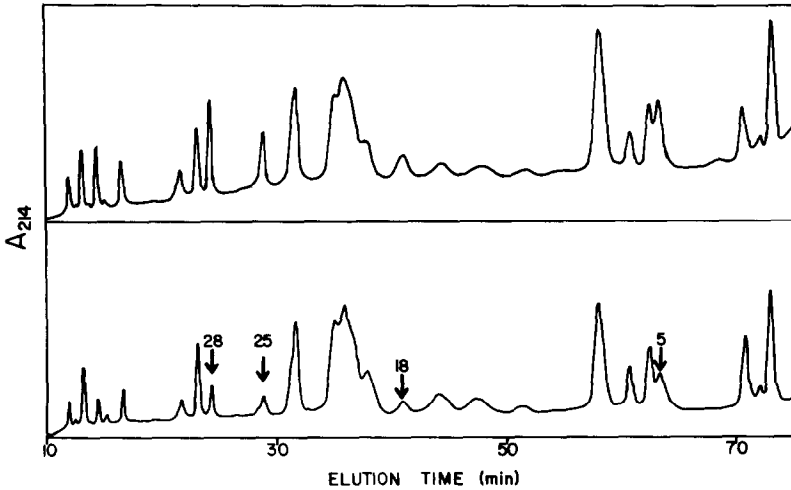
Fig. 5. RP-HPLC analysis of reconstituted 50S and lighter particles. Total protein was extracted from the particles shown in Fig. 3 (lower panel), dissolved in 0.1% trifluoroacetic acid, applied to a SynChropak RP-P column and eluted with the gradient described in the legend to Fig. 2. Absorbance at 214 nm was measured at 0.15 a.u.f.s. Upper panel: protein from 180 pmol 50S subunits. Lower panel: protein from 200 pmol lighter particles. Proteins present in low stoichiometry are indicated.

typically contained all of the 50S proteins* in nearly stoichiometric amounts (compare Fig. 5, upper panel, and Fig. 2). The lighter particles consistently contained less than stoichiometric amounts of L5, L18, L25 and L28 (Fig. 5, lower panel).

Activity of reconstituted particles

An essential requirement for the attainment of the overall goal of this work is that ribosomal subunits reconstituted by using HPLC-prepared proteins be functionally active. This requirement has been met for reconstituted 30S and 50S subunits.

We have used messenger RNA-dependent PhetRNA^{Phe} binding to monitor 30S subunit activity. Non-HPLC treated TP30 was reconstituted with 16S rRNA into 30S subunits with 100% or greater activity in this assay compared to native 30S subunits at a protein to RNA ratio of about 1.8 (ref. 9, this laboratory). For HPLC-prepared proteins the protein to RNA ratio at which maximal activity was achieved varied with the preparation. The highest activity we have obtained was 84% of native 30S subunits at a protein to RNA ratio of 3.6; however, experiments could routinely be performed at a protein to RNA ratio of 1.8 with a relative activity of approximately 30%. The reason that a higher HPLC-prepared protein to RNA ratio was required for high activity may be due to partial or full denaturation of one or more proteins. Activity was not diminished when proteins used in reconstitution experiments were stored in buffer A at −80°C for up to six months. The reconstituted particles which sedimented at less than 30S (Fig. 3, upper panel) showed no activity in this assay implying either the loss of an essential component of the PhetRNA^{Phe}

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* L7 and L12 were not tested.

binding site or an improper conformation for PhetRNA^{Phe} binding due to the missing proteins.

We monitored 50S subunit activity with a peptidyltransferase assay[22]. Non-HPLC treated and HPLC-treated TP50 were reconstituted with 23S + 5S rRNA into 50S subunits with roughly the same activity. The maximum activity obtained for HPLC-treated TP50 was 51.6% of the activity of native salt-washed 50S subunits at a protein to RNA ratio of 1.2. Proteins stored for more than approximately two weeks had to be used at a higher protein to RNA ratio and gave a lower maximal activity. The reconstituted particles which sedimented at less than 50S (Fig. 3, lower panel) showed less than 8% activity in this assay, again implying loss of the active site for peptidyltransferase in these particles due either to a conformational change or to the absence of some proteins. It is interesting to note that three of the four proteins found in reduced amounts of these particles, *i.e.*, L5, L18 and L25, bind directly to 5S rRNA[24], and that this protein–RNA complex is essential for peptidyltransferase activity.

Separation of modified from unmodified proteins

Above we have described the utility of RP-HPLC both in preparing ribosomal proteins for reconstitution and for monitoring the results of reconstitution. A third application is in the preparation of modified protein for use in single replacement reconstitution experiments. In this work it is of critical importance that the modified protein be fully separated from the corresponding unmodified protein. It is not, however, as important that the modified protein be fully separated from other unlabeled proteins, except that the determination of the stoichiometry of labeling is made more difficult. The technique of lowering φ' in the region of elution of the protein of interest (see Fig. 2) has been successfully applied to this problem for two modified proteins, S14 and L23.

S14 has previously been shown[23] to be the major 30S protein covalently modified by the antibiotic puromycin upon photolysis at 3500Å, and this incorporation is stimulated by prior photolysis in the presence of a second antibiotic, chloramphenicol[25]. Puromycin is a structural analog of the 3′-end of aminoacyl tRNA and we are interested in studying the possibility that S14 is a part of a tRNA binding site. Separation of puromycin-modified S14 from unmodified S14 is especially important for reconstitution experiments since in typical photolysis experiments the extent of photoincorporation of puromycin is low (approximately 0.01 mol/mol for puromycin alone, 0.06 mol/mol for puromycin in the presence of chloramphenicol). We previously reported[8] that when TP30 from [³H]puromycin-labeled 30S subunits was analyzed by RP-HPLC, radioactivity was eluted just prior to S14. In order to exploit this apparent selectivity of the column, φ' was greatly reduced in the region of S14 elution. Fig. 6 shows the same time segment of chromatograms from three labeling experiments. With this gradient S14 was eluted at *ca.* 29 min and S19 was eluted at *ca.* 41 min. The upper tracing is from an analysis of proteins from 30S subunits photolyzed for 15 min in the presence of 0.5 mM chloramphenicol. When compared with profiles of proteins from 30S subunits that either were not photolyzed or were photolyzed in the absence of chloramphenicol, the only changes apparent in the A_{214} profile are in the region of S14 elution. The A_{214} peak of native S14 is diminished by *ca.* 50% accompanied by the appearance of a new doublet which was eluted
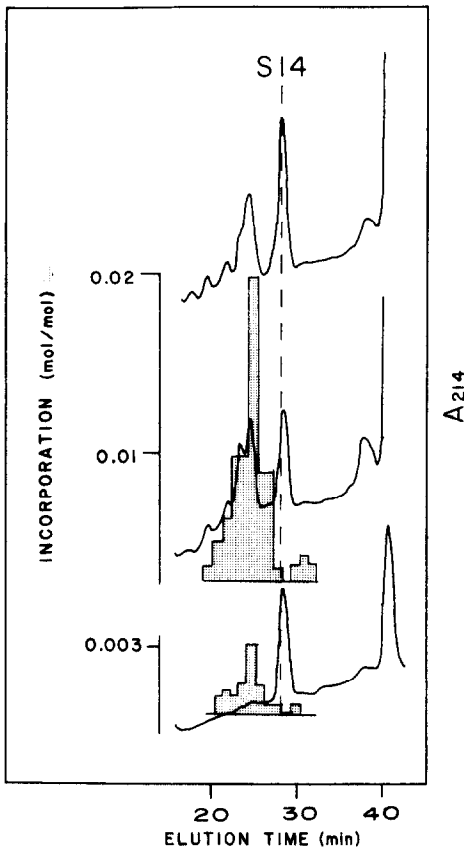
Fig. 6. RP-HPLC analysis of modified S14. Upper tracing: TP30 from 30S subunits photolyzed in the presence of chloramphenicol. Middle tracing: TP30 subunits photolyzed in the presence of chloramphenicol. Middle tracing: TP30 from 30S subunits photolyzed in the presence of chloramphenicol and [³H]puromycin. Lower tracing: TP30 from 30S subunits photolyzed in the presence of [³H]puromycin. All samples (each 390 pmol) were dissolved in buffer A, applied to a SynChropak RP-P column and eluted with the following gradient: 26% to 33% acetonitrile in 60 min, 33% to 45% acetonitrile in 30 min, 45% to 55% acetonitrile in 10 min, 55% acetonitrile for 10 min. All steps were linear. Absorbance at 214 nm was measured at 0.1 a.u.f.s. The elution position of unmodified S14 is indicated by the dashed line. The peak to the far right is S19.

between 23 min and 26 min. The A_{214} of this new doublet is approximately equal to the A_{214} loss in the native S14 peak, and on this basis we assume that it corresponds to a chloramphenicol-plus-light-induced modification of S14. Labeling experiments with [¹⁴C]chloramphenicol showed very little covalent labeling in this general region of the chromatogram, thus demonstrating that the amount of protein in the new doublet cannot be accounted for by invoking covalent attachment of chloramphenicol[26]. The middle tracing is from an analysis of 30S subunits photolyzed for 15 min in the presence of 0.5 mM chloramphenicol and subsequently photolyzed for 30 min in the presence of 0.13 mM [³H]puromycin. In this case an even further reduction in the A_{214} of the S14 peak was observed with a concomitant increase in the A_{214} of

the earlier-eluted doublet. The ^{3}H counts eluted with this doublet peak at *ca.* 25 min. The extent of covalent [^{3}H]puromycin incorporation in this experiment was 0.06 mol puromycin/mol S14 (native and modified). The lower tracing is from an analysis of 30S subunits photolyzed for 120 min in the presence of 0.13 mM [^{3}H]puromycin alone. In this case the A_{214} profile resembles that of an unphotolyzed sample. The ^{3}H counts again peak at 25 min. The extent of covalent [^{3}H]puromycin incorporation in this experiment was 0.006 mol puromycin/mol S14 (native and modified). The latter two experiments demonstrate clearly the increased efficiency with which puromycin is photoincorporated into S14 that has been photo-modified in the presence of chloramphenicol. Assuming, from the altered S14 migration in the lower trace, that puromycin incorporation proceeds into a modified S14, what remains to be determined is whether the presence of chloramphenicol merely leads to an increased rate of S14 photomodification or rather to the formation of different modified species having higher photoreactivity. In any event, Fig. 6 clearly shows that it is possible to obtain several modified species of S14 fully separated from native S14. This will allow us, if these modified proteins are used to replace native S14 in reconstitution experiments, to study the effects of these modifications upon ribosomal functions.

L23 is the major 50S protein covalently modified by puromycin upon photolysis at 3500 Å (ref. 23). The study of this modification is of interest since puromycin is a substrate for the peptidyl-transferase site on the 50S subunit. The extent of covalent modification of L23 by puromycin is also low (*ca.* 0.01 mol/mol) and, unlike S14, L23 is eluted in a very crowded region of the chromatogram, making isolation of pure modified L23 difficult. However, Fig. 7 demonstrates that puromycin-labeled L23 can be separated from unlabeled L23 through use of an isocratic elution step at 35.2% acetonitrile. Such resolution should allow us to prepare a supply of stoichiometrically puromycin-labeled L23 for use in reconstitution experiments.
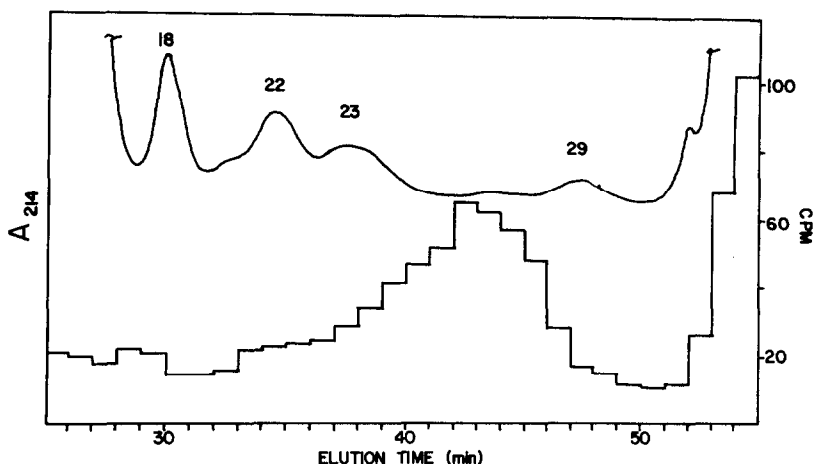

Fig. 7. RP-HPLC analysis of [^{3}H]puromycin-modified L23. TP50 (160 pmol) from 50S subunits photolyzed in the presence of [^{3}H]puromycin were dissolved in 0.1% trifluoroacetic acid, applied to a SynChropak RP-P column and eluted with the following gradient: 17% to 35.2% acetonitrile in 10 min (curve .2); 35.2% for 30 min; 35.2% to 75% acetonitrile in 15 min (linear). Absorbance at 214 nm was measured at 0.01 a.u.f.s.

CONCLUSIONS AND PROGNOSIS

We have demonstrated that RP-HPLC is a rapid, high-yield method for the preparation of ribosomal proteins and that proteins so prepared can be reconstituted into ribosomal subunits which are active in specific functional assays. We have also shown that alteration of gradient elution conditions allows RP-HPLC to be tailored to the analysis and preparation of specific modified proteins of interest. It remains now to exploit these successes by preparing and functionally testing single-replacement reconstituted subunits containing either protein S14 modified by photolysis in the presence of chloramphenicol or puromycin-labeled S14 or L23. Such experiments are currently underway and will be reported on in detail elsewhere.

ACKNOWLEDGEMENTS

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