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CHARACTERIZATION OF RIBOSOMES FROM THE SEED OF *PINUS LAMBERTIANA*

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Key Word Index—*Pinus lambertiana*; Pinaceae; ribosomes; subunits; sedimentation.

Abstract—Ribosomes isolated from seeds of the sugar pine, *Pinus lambertiana*, have been characterized: The ribosome has a sedimentation coefficient ($s^0_{20,w}$) of 78·2 *S* and contains 41% RNA and 58% protein. On dialysis against buffer containing 0·5–1 mM $MgCl_2$, the ribosome was reversibly transformed into an intermediate form (60 *S*). Further removal of Mg^{2+} causes the intermediate ribosome to dissociate into subunits (30 *S* and 40 *S*). Treatment of the intermediate ribosome with *p*-chloromercuribenzoic acid caused the dissociation of the particle into subunits. Incubating the 80 *S* ribosome with the sulfhydryl reagent caused a rapid transformation of the particle into an intermediate type particle. These results suggest that sulfhydryl groups are involved not only in associating the subunits but also in maintaining the compact structure of the ribosomes. The ribosome contains three ribosomal RNA components of 28 *S*, 18 *S* and 5 *S*. The base compositions of the three ribosomal RNA components are different.

INTRODUCTION

Pinus lambertiana Dougl. is a member of a fairly large group of commercially important gymnosperms. Seeds from this tree are dormant when shed and must undergo afterripening before germination. Since *de novo* synthesis of protein appears to be an important part of germination[1–3] characterization of the ribosomes may be helpful in studying development of protein-synthetic capacity as dormancy is broken. Little information is available in the literature about the protein synthesizing system of gymnosperm seeds.

RESULTS

General Properties of the Pine Seed Ribosomes

On the analytical ultracentrafuge the ribosomes in Tris-KCl buffer (50 mM Tris-HCl, pH 7·4, 50 mM KCl) containing 10 mM $MgCl_2$ sedimented predominantly as monoribosomes with a sedimentation coefficient at infinite dilution ($s^0_{20,w}$) of 78·2 *S*. (This will be referred to as the 80 *S* ribosome in this paper.) The ribosome preparation contained 41% RNA and 58% protein and had an absorptivity ($a^{1\%}_{1cm}$) of 99 at 260 nm in the same buffer.

Dissociation of the Ribosome by Dialysis

When the pine seed ribosomes were dialyzed against Tris-KCl buffer at low Mg^{2+} ion concentrations, they did not dissociate directly into subunits, but rather proceeded through

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an 'intermediate' form before the dissociation. Fig. 1 demonstrates the transition of the monoribosome into the intermediate form as the concentration of the Mg^{2+} in the dialysis buffer was lowered from 3 to 0·5 mM. In the presence of 3 mM $MgCl_2$ (Fig. 1a), most of the ribosomes existed in the 80 *S* form (71 *S*) whereas at a $MgCl_2$ concentration of 0·5 mM (Fig. 1c), almost all of the ribosomes were in the intermediate form (51 *S*). Dialysis of the intermediate ribosome at 0·5 mM Mg^{2+} for 2 days did not change the sedimentation property of the particle. The intermediate form usually had a sedimentation coefficient between 50 *S* and 60 *S* depending upon the concentration of the sample, and will be referred to as the 60 *S* ribosome. Figure 1d is a sedimentation pattern of reassociated 80 *S* ribosomes, obtained by dialyzing the intermediate ribosomes against 10 mM $MgCl_2$ in Tris-KCl buffer. This result indicates that the transformation between 80 *S* ribosomes and the intermediate ribosomes is reversible.

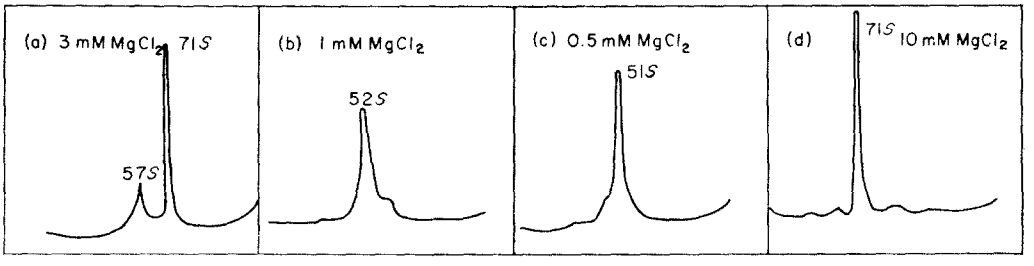

FIG. 1. THE TRANSITION BETWEEN THE 80 *S* AND 60 *S* PARTICLES.
Washed ribosomes were dialyzed for 12 hr at 4° against Tris-KCl buffer containing (a) 3 mM $MgCl_2$ (b) 1 mM $MgCl_2$ (c) 0·5 mM $MgCl_2$. For (d), a portion of the sample from (c) was dialyzed against 10 mM $MgCl_2$ in the same Tris-KCl buffer for 12 hr. The sample concentration was 0·7% for all the samples. Schlieren patterns for (a), (b) and (c) were taken 22 min after the centrifuge reached 39 460 rpm at a phase angle of 65°. Pattern (d) was taken 15 min after the centrifuge reached 39 460 rpm at a phase angle of 55°.

The 60 *S* ribosome could also be obtained by centrifuging 80 *S* ribosome preparation through a 7·5–30% sucrose density gradient containing 0·5 or 1 mM $MgCl_2$. Particles recovered from the gradient could also be converted to the 80 *S* form upon dialysis against 10 mM $MgCl_2$.

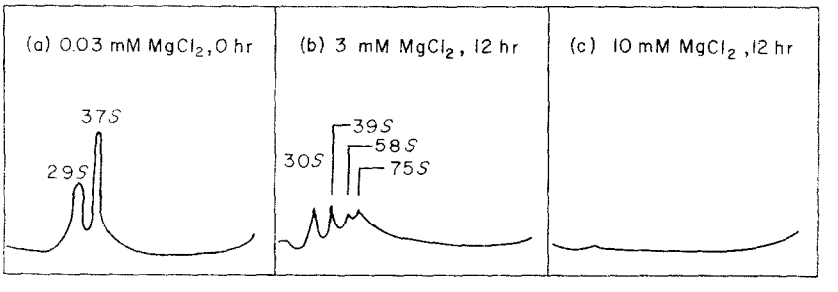

FIG. 2. PARTIAL REASSOCIATION OF RIBOSOME SUBUNITS INTO 60 *S* AND 80 *S* PARTICLES.
Ribosomes were dissociated by dialyzing against Tris-KCl buffer containing 0·03 mM $MgCl_2$ for 18 hr. The completely dissociated sample (a) was then dialyzed (b) for 12 hr against 3 mM $MgCl_2$ or (c) for 12 hr against 10 mM $MgCl_2$ in Tris-KCl buffer. All the patterns were taken 10 min after reaching 39 460 rpm at a phase angle of 55°.

When the ribosome preparation was dialyzed against buffer with a Mg^{2+} concentration lower than 0·5 mM, the ribosomes dissociated into subunits with sedimentation coefficients of about 30 *S* and 40 *S*. A sedimentation pattern of the ribosome preparation dialyzed at 0·03 mM $MgCl_2$ for 18 hr is shown in Fig. 2a. These dissociated ribosome subunits could not be reassociated to the 80 *S* form upon dialysis against 10 mM $MgCl_2$. At this Mg^{2+} concentration aggregation occurred when the two subunits were dialyzed either alone or together in Tris-KCl buffer. Neither the addition of sulfhydryl compounds such as β-mercaptoethanol or dithiothreitol nor the reduction of the ionic strength of the buffer by lowering the KCl concentration prevented this aggregation. However, dialysis of the subunits against 3 mM $MgCl_2$ resulted in a portion of the subunits being reassociated to form particles sedimenting in the 80 *S* and 60 *S* regions. This is shown in Fig. 2b,c. In this experiment, the ribosome sample was first dialyzed against 0·03 mM $MgCl_2$ to allow complete dissociation of the ribosomes into subunits (Fig. 2a). The dissociated sample was then dialyzed against 3 or 10 mM $MgCl_2$ for reassociation. Figure 2c shows the complete loss of ribosome particles in 10 mM $MgCl_2$ solution, whereas in the case of 3 mM $MgCl_2$ (Fig. 2b), particles sedimented at rates corresponding to 60 *S* and 80 *S*. This partial reassociation was found to occur only in a narrow magnesium ion concentration range. Subunits aggregated when the Mg^{2+} concentration was 5 mM or higher and remained unchanged when the ion concentration was 1 mM or lower.

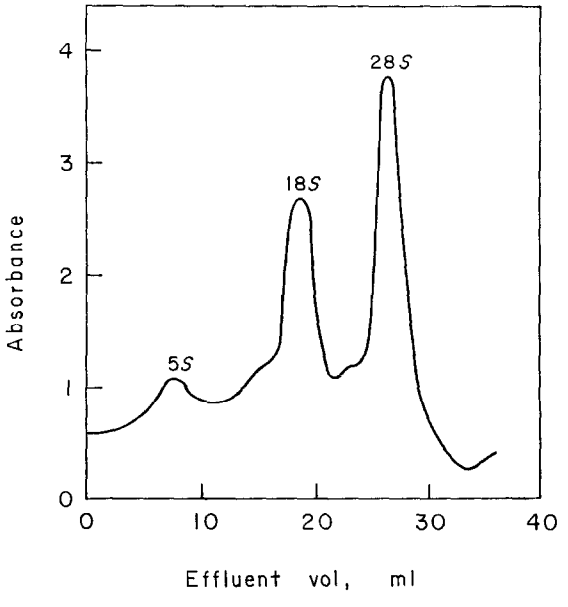

FIG. 3. CO-SEDIMENTATION PATTERN OF *r*RNAs ISOLATED FROM DISSOCIATED AND UNDISSOCIATED RIBOSOMES.

Ribosomes were completely dissociated into subunits by dialyzing against 0·03 mM $MgCl_2$ in Tris-KCl buffer for 18 hr. Ribosomal RNA was isolated from the dissociated ribosome sample with the same procedure as described in Experimental for undissociated ribosomes except that Tris-KCl buffer (containing 0·03 mM $MgCl_2$) was used in this case. Equal amounts of RNA from both dissociated and undissociated ribosomes were mixed and 1·5 mg of RNA was layered on a 2–20% linear sucrose density gradient. The gradient was centrifuged at 25 000 rpm at 4° for 14 hr in a SW27 rotor. Absorbance is at 254 nm.

In order to investigate the possibility that the lack of complete reassociation of the subunit was caused by RNA degradation during the dissociation process, RNA was isolated from undissociated 80 *S* ribosomes and from the subunits. These RNA preparations were co-sedimented through a sucrose density gradient. The co-sedimentation pattern (shown in Fig. 3) was identical to that from the 80 *S* ribosome alone and indicated that there was no significant degradation of RNA during the dissociation process.

Effect of p-*Chloromercuribenzoic Acid on the Dissociation Behavior of the Ribosome*

The effect of *p*-chloromercuribenzoic acid (PCMB) on pine seed ribosomes was studied by treating the ribosome with PCMB at 25° in Tris-KCl buffer containing different concentrations of $MgCl_2$. In 10 mM $MgCl_2$ solution, incubation of the 80 *S* ribosomes with 0·5 mM PCMB for 30 min had no apparent effect on the sedimentation behavior of the particles. However, prolonged incubation or incubation at higher PCMB concentrations caused aggregation of the 80 *S* particles. The effect of PCMB on intermediate ribosomes (60 *S*) was studied in 1 mM $MgCl_2$ solution. Ribosomes were first dialyzed against 1 mM $MgCl_2$ to convert the 80 *S* ribosome to the intermediate form and then treated with 1 mM PCMB. Such treatment for 2 hr caused the dissociation of most of the intermediate ribosomes into subunits.

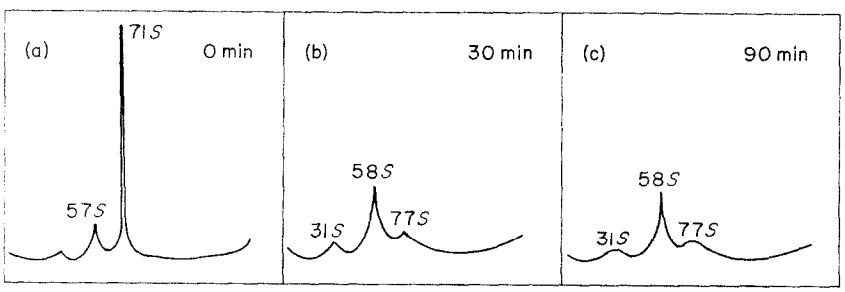

FIG. 4. THE EFFECT OF *p*-CHLOROMERCURIBENZOIC ACID ON 80 *S* RIBOSOMES.
Ribosomes (0·6% solution) were first dialyzed for 9 hr against Tris-KCl buffer containing 3 mM $MgCl_2$ and then treated with 1 mM PCMB at 25° for (a) 0 min, (b) 30 min, and (c) 90 min. All the patterns were taken 14 min after reaching 42 040 rpm at a phase angle of 55°.

It was noted in the above studies that a 30 min incubation with PCMB removed the 80 *S* peak in 1 mM $MgCl_2$ (see Fig. 1b). This seemed to suggest that PCMB could also cause the transformation of 80 *S* ribosomes into the intermediate form under certain conditions. To test this possibility, ribosomes at 3 mM $MgCl_2$ were treated with 1 mM PCMB. The results of this experiment are shown in Fig. 4, which clearly demonstrates that PCMB can cause a rapid shift of the 80 *S* ribosome into an intermediate type particle (58 *S*).

Properties of the Ribosomal RNA

Ribosomal RNA components were separated in sucrose density gradients and the sedimentation pattern of the RNA was identical to Fig. 3. The two major RNA components appeared in a ratio very close to 2:1. Sedimentation coefficients of the two components at infinite dilution were found to be 28·0 *S* and 18·2 *S*, determined in 0·1 M glycine, pH 9·5, 0·1 M KCl and 0·01 M EDTA. The sedimentation coefficients for the low MW component is about 5 *S* (estimated from its position in the sucrose gradient). The shoulder components

appearing between the 28 *S* and 18 *S* peaks and at the light side of the 18 *S* peak may have originated from plastids or mitochondria. The absorption of a 1% solution of the RNA at 260 nm was 203.

Base compositions of the RNA components showed a significant difference (Table 1). Unlike bacterial RNA, the base composition of the two major RNA components of most plant *r*RNA are usually different.[4] The base composition of the pine seed 28 *S* *r*RNA is very similar to other plant 28 *S* RNAs which are usually richest in guanylic acid, whereas the uridylic acid content is usually lowest.[4]

TABLE 1. NUCLEOTIDE COMPOSITION OF PINE SEED RIBOSOMAL RNA

RNA	C*	G	A	U
28 *S*	25·4 ± 0·7†	30·6 ± 0·9	22·9 ± 0·1	21·1 ± 0·5
18 *S*	23·5 ± 0·3	28·5 ± 0·8	23·1 ± 0·4	24·9 ± 1·2
5 *S*	24·1 ± 0·4	27·9 ± 0·2	20·1 ± 0·4	27·3 ± 0·3

* A = 2′ (3′) Adenylic acid; G = 2′ (3′) guanylic acid; U = 2′ (3′) uridylic acid; C = 2′ (3′) cytidylic acid.

† Mean ± standard deviation.

DISCUSSION

In the isolation of ribosomes from the seeds of *Pinus lambertiana*, both the pH and ionic strength of the homogenization medium were found to be important. Also, it was necessary to include deoxycholate in the medium. Hsiao isolated ribosomes from corn roots with a medium containing about 0·4% sodium deoxycholate, and found no apparent difference in properties of the ribosomes isolated with and without the detergent.[5]

Like other plant cytoplasmic ribosomes, the pine seed ribosome has a sedimentation coefficient of the 80 *S* class. The RNA to protein ratio of this ribosome is similar to that of most plant ribosomes which usually contain about 40% RNA and 60% protein.[6] Biological activity of the whole seed ribosome preparation was not examined; however, ribosomes isolated from embryos were active in incorporation of radioactive amino acids into hot-trichloroacetic acid insoluble materials.[7]

The dissociation behavior of the pine seed ribosome is distinct from that of most other plant ribosomes. We believe that the intermediate ribosomes (60 *S*) are unfolded 80 *S* ribosomes. Thermal denaturation studies[8] show that the intermediate ribosome has a lower melting temperature than the 80 *S* ribosome, indicating that the intermediate particle may have a more unfolded structure than the 80 *S* particle. It is unlikely that the intermediate particles are ribosomes deficient in some ribosomal proteins. The complete conversion of the intermediate ribosomes isolated from sucrose gradient to the 80 *S* form upon restoring the Mg^{2+} concentration argues against this possibility.

The intermediate type ribosome has also been observed to occur in ribosome prepara-

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tions from Jensen rat sarcoma,[9] Novikoff hepatoma,[10] rat liver[11] and *Chlamydomonas reinhardii*.[12] The dissociation pattern of ribosomes from *C. reinhardii* are very similar to pine seed ribosome. Both the cytoplasmic and chloroplastic ribosomes of this alga dissociate into 30 *S* and 40 *S* subunits through intermediate forms.

It is not known whether the intermediate ribosome has any biological significance. However, since this particle occurs at a Mg^{2+} concentration close to the physiological value, it may be important in view of some of the recent reports on *E. coli* ribosome. Bosch *et al.* have reported that electrophoretic analysis of *E. coli* ribosome preparation with polyacrylamide gel revealed a heterogeneity which could not be detected by conventional sucrose density gradient centrifugation.[13] They found a second monoribosome component (70 *S*1) which had a sedimentation coefficient 2–4 *S* smaller than the regular 70 *S* component. The treatment of the ribosome preparation with a ribosomal dissociation factor increased the proportion of this 70 *S*1 component. Further increase of the concentration of the dissociation factor caused the dissociation of the component into subunits. Another intermediate type ribosome has also been reported by Bosch's group.[14] They found that 'native' ribosome subunits reassociated to form particles of sedimentation coefficient about 61 *S* while 'derived' subunits associated to form 70 *S* ribosomes. An even more fascinating finding was reported by Schreier and Noll who showed that in a purified system containing polyuridylic acid and transfer factor *T* and factor *G*, the active ribosomal complex passed through a cycle of contraction and expansion with the addition of each amino acid.[15] They found that binding of aminoacyl-*t*RNA preceded the stable compact state, which corresponds to the 70 *S* conformation, whereas translocation with the *G* factor caused the expansion of the ribosome to a less stable 60 *S* form.

It has been shown by investigators from many laboratories (e.g. Refs. 16 and 17) that both the *E. coli* 30 *S* and 50 *S* ribosomes undergo stepwise unfolding upon removal of Mg^{2+}. Some of the unfolding steps were shown to be irreversible. A similar unfolding process may also occur during dissociation of the pine seed intermediate ribosomes. It is conceivable that some ribosomal proteins may split off during the process. However, RNA degradation is unlikely because of the co-sedimentation results (Fig. 4) and the fact that the sedimentation pattern of RNA from the 60 *S* particle showed no extraneous peaks.

The sulfhydryl reagent, *p*-chloromercuribenzoic acid (PCMB) has been reported to cause the dissociation of *E. coli*,[18–20] *B. subtilis*,[21] and rat liver ribosomes.[22] Parish *et al.*[23] studied the effect of PCMB on ribosomes from several sources and observed that the dissociation of ribosomes occurred only in those which contained 4-thiouridylic acid. Therefore, because of the effect of PCMB on dissociating pine seed ribosomes, one would expect

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that these ribosomes contain 4-thiouridylic acid. In addition to the effect of PCMB on the dissociation of the intermediate ribosome into subunits, PCMB can also cause the transformation of the 80 *S* ribosome into the 60 *S* intermediate type particle (Fig. 4). Tamaoki and Miyazawa[24] observed a similar transformation with *E. coli* ribosomes. This finding indicates that sulfhydryl groups on ribosomes may be involved in the maintenance of the conformational structure of these ribosomes as well as in the association of the subunits.

EXPERIMENTAL

Isolation of ribosomes. Seeds (50 g) (Forest Seeds of California, Davis, California) were washed and soaked in distilled H_2O overnight in a refrigerator. Seeds were then homogenized in a Sorvall Omni-mixer at maximum speed for 2 min with 64 ml of homogenization buffer (50 mM Tris-HCl, pH 7·4, 50 mM KCl, 10 mM $MgCl_2$, 6 mM mercaptoethanol, and 400 mM sucrose) and with 16 ml of 2% sodium deoxycholate. This and all following steps were performed in the cold. The homogenate was squeezed through four layers of cheesecloth and the filtrate was centrifuged for 30 min at 27 000 *g*. The resulting supernatant was centrifuged for 3 hr at 108 000 *g*. The ribosome pellets were resuspended in 20 ml of homogenization buffer without sucrose and the resulting suspension was subjected to a second high speed centrifugation. Ribosomes from this step were used for *r*RNA isolation whereas ribosomes used for other experiments were prepared by two additional washes with the same buffer (no sucrose).

Isolation and separation of ribosomal RNA. RNA was isolated from ribosomes following the phenol-deoxycholate method of Click and Hackett[25] except that ribosomes rather than frozen tissue were used as the starting material. Ribosomal RNA components were fractionated in a 2–20% linear sucrose density gradient in a glycine buffer (100 mM glycine, pH 9·5, 100 mM KCl and 10 mM EDTA). One ml samples containing 1·5 mg RNA were applied to the gradients and the gradients were centrifuged at 25 000 rpm for 22 hr in a Spinco SW27 rotor. The gradients were analyzed and fractionated with an ISCO gradient fractionator. Fractions were pooled and the RNA was precipitated by the addition of 2 vol. isopropanol at 0°.

Analytical ultracentrifugation. All sedimentation analyses were performed from 3 to 6° with a Spinco Model E analytical ultracentrifuge using Schlieren optics. Photographs were analyzed with a comparator. All sedimentation coefficients were corrected to the viscosity and density of H_2O at 20°.

Thermal denaturation of rRNA. The effect of temp, on a *r*RNA solution was followed with a spectrophotometer which contained a thermostatically regulated chamber. The changes in absorbance were measured at 260 nm. No correction was made for heat expansion of the solution.

Base composition determination and chemical analysis. Base compositions of the RNA components were determined using a PC procedure described by Lane.[26] Protein content of the ribosome preparation was determined by the method of Lowry *et al.*[27] Dische's orcinol method[28] was used for the RNA determination.

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