SPINACH CHLOROPLAST RIBOSOMES ACTIVE IN PROTEIN SYNTHESIS

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1. Introduction

Chloroplasts possess ribosomes resembling those of bacteria in sedimentation behaviour, in the size of their RNA components and in response to antibiotics [1–4]. Further characterization has not been possible on account of the failure of several groups to isolate ribosomal particles retaining more than a minimal biological activity *in vitro* [5–7].

We now describe a procedure for the isolation of spinach chloroplast ribosomes capable of catalyzing the synthesis of polyphenylalanine in response to poly (U) at rates approaching those given by *Escherichia coli* ribosomes. Some properties of these ribosomes are also described. This procedure should be applicable to chloroplasts from other sources and so may facilitate further analysis of mutations presumed to alter chloroplast ribosomes [8, 9].

2. Methods and materials

500 g de-veined leaves from fresh, commercially obtained spinach were washed in distilled water at 0°. 250-g portions were suspended in 250 ml medium I and blended in a fruit liquidiser (Braun) for 45 sec. After filtration through Perlon cloth, the extract was centrifuged 360 *g* for 5 min at 2°. The supernatant was decanted and centrifuged 1200 *g* for 15 min. The crude chloroplast pellet was then washed once by resuspension and centrifugation in the same medium.

Lysis of chloroplasts was accomplished by addition of either sodium deoxycholate or Triton X-100 to final concentrations of 0.5% or 1.0%, respectively, to the suspension in 50 ml AMT-50. After clarification at 26 000 *g* for 20 min, the lysate was layered over 1 M sucrose containing AMT-500. Ribosomes were pelleted by centrifugation for either 12 hr at 30 000 rpm (Spino-30 rotor) or 5 hr at 40 000 rpm (MSE 8 × 50 superspeed). Pellets were rinsed with AMT-150 and resuspended in the same medium. After a clarifying spin (10 000 *g* 10 min), the suspension was stored in liquid N_2. Preparations could be frozen and thawed several times without noticeable loss of activity. Methods used for preparation of *E. coli* ribosomes supernatant enzymes, assay of poly (U)-directed polyphenylalanine synthesis and peptidyl transferase activity have been described [10, 11].

Buffers used: AMT-50: 50 mM NH_4Cl, 10 mM Mg-acetate, 10 mM Tris-HCl (pH 7.5), 6 mM 2-mercaptoethanol. AMT-150 or 500: as AMT-50, but with NH_4Cl concentrations of 150 and 500 mM, respectively. Medium I: 0.7 M sucrose, 0.1 M Tris-HCl (pH 7.5) 5 mM $MgCl_2$, 50 mM KCl, 5 mM 2-mercaptoethanol

Bacterial contamination of chloroplast preparations was estimated by plating serial dilutions of suspensions on either Bacto-tryptone–yeast extract agar [12] or yeast-extract–peptone–glucose agar. Colonies were counted after 3 days incubation at 30°. Both media gave essentially identical results.

3. Results

The yield of chloroplast ribosomes from the above method was 40–80 A_{260} units per 500 g leaves. Absorbance ratios 260/280 and 260/230 were 1.8 and

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1.2, respectively (mean of three preparations). Table 1 shows that such ribosomes are active in poly (U)-directed polyphenylalanine synthesis in combination with supernatant enzymes from *E. coli*. This activity is about 50 times higher than previously observed in related systems [5–7]. Dependence on poly (U) addition is high and requirement for supernatant enzymes is absolute.

Since chloroplast preparation from commercial spinach are heavily contaminated with bacteria (approx. 8×10^8/500 g leaves), it could be argued that activity seen is due to the presence of bacterial ribosomes in chloroplast ribosome preparations. We consider this unlikely for three reasons:

i) On the basis of a maximal RNA content of 2.5×10^{-10} mg per bacterium (see [12]) 10^9 bacteria would maximally yield 0.25 mg RNA, i.e only 8% of the actual yield of 3 mg RNA obtained from 500 g leaves.

ii) Triton X-100 is reported not to lyse bacteria [13]; nevertheless, yield and activity are unaltered by the use of Triton in place of deoxycholate in lysis of chloroplasts.

iii) Leaves briefly rinsed in 0.5% sodium hypochlorite at 0° yield chloroplasts with only 1% of the bacterial contamination of untreated leaves; yield of ribosomes was again unaffected and activity (2.7 nmoles Phe/mg RNA/30 min) was within the range of activity given by normal preparations (see table 1).

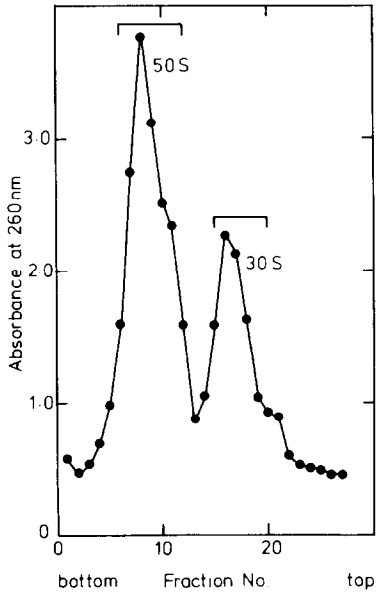

Fig. 1. Separation of chloroplast subunits. Ribosomes were layered on 15–30% isokinetic sucrose gradients containing 50 mM NH_4Cl, 10 mM Tris-HCl (pH 7.5), 0.1 mM Mg-acetate, 6 mM 2-mercaptoethanol. Centrifugation was for 15¼ hr at 24 000 rpm and 4° in a Spinco SW-25.1 rotor. Fractions were collected drop-wise from a hole pricked in the bottom of the tube. Appropriate fractions (indicated by brackets) were pooled and concentrated by polyethylene glycol precipitation after adjustment of Mg^{2+} concentration to 10 mM (A. Bollen, personal communication).

Table 1
Poly (U)-directed synthesis of polyphenylalanine by chloroplast and *E. coli* ribosomes

Source of ribosomes	Addition or omission	[^{14}C]Phe incorporation (nmoles/mg RNA/30 min)	
Chloroplast	None	3.1	(1.8–4.9)
	Minus poly (U)	0.15	(0.11–0.27)
	Minus *E. coli* supernatant	<0.004	
	30 S subunit alone	0.19	(0.18–0.20)
	50 S subunit alone	0.24	
	30 S + 50 S	0.72	(0.65–0.78)
E. coli	None	21.6	

Figures represent the mean of three determinations with two preparations (range in parentheses). They have been corrected for zero time values (circa 150 cpm). Conditions of incubation were those of [10] using [^{14}C] labelled (U)-phenylalanine, specific activity 103 mCi/mmole. Ribosome concentration was between 0.4 and 0.8 A_{260} per assay. Activity of *E. coli* supernatant in the absence of ribosomes was never greater than 0.004 nmoles Phe/30 min. Ribosomal subunits were separated as described in the legend to fig. 1.

The possibility that these chloroplast preparations contain mitochondria is remote, since sensitivity of ATPase activity to oligomycin, a characteristic unique to the mitochondrial enzyme, was less than 3% (see [14]).

Both subunits are required for ribosomal activity. Complete dissociation of ribosomes into subunits sedimenting at 50 S and 30 S in sucrose gradients is achieved by use of ionic conditions similar to those dissociating *E. coli* ribosomes (fig. 1). Table 1 shows that these subunits apart are poorly active in protein synthesis; in combination they possess between 20–25% of the activity of the original, undissociated 70 S ribosome. In view of this and of results obtained in subunit exchange experiments [15], it is likely that

Table 2
Effects of antibiotics on chloroplast ribosomal peptidyl transferase activity.

Addition or omission	Acetyl-[^{3}H]leucyl-puromycin formed (% control)	
None	100	
Puromycin (–)	4	
Chloramphenicol (+) (66 μg/ml)	35	(28 – 41)
Erythromycin (+) (13 μg/ml)	101	(100–102)
Erythromycin + chloramphenicol	98	(97 – 99)
Lincomycin (+) (10^{-5} M)	33	(23 – 42)
Lincomycin (+) (10^{-4} M)	4	
Anisomycin (+) (40 μg/ml)	86	

Peptidyl tranferase activity was measured exactly as described in [11]. 100% corresponds to approx. 5200 cpm. Values have been corrected for a minus ribosome blank (about 200 cpm) and are the mean of two determinations with different ribosomal preparations (range given in parentheses).

the chloroplast 30 S subunit is highly susceptible to damage during isolation. A similar phenomenon was noticed previously for the 30 S subunit of *Bacillus subtilis* [16].

3.1. *Response to antibiotics*

Peptidyl transferase activity of chloroplast ribosomes, as measured by a modified fragment reaction with acetyl leucyl-tRNA as substrate, is sensitive to inhibition by both chloramphenicol and lincomycin (table 2). Chloramphenicol inhibition is released by erythromycin, indicating sensitivity to this antibiotic also. Activity is only slightly affected by anisomycin, an inhibitor specific for eukaryotic 80 S ribosomes.

4. Discussion

We have described in this report a method for the isolation of spinach chloroplast ribosomes highly active in protein synthesis *in vitro*. The method was originally devised for the purification of mitochondrial ribosomes from yeast [10] and may owe its effectiveness to the removal of ribosome-bound ribonuclease, membrane fragments or endogenous mRNA. We suggest that it may be generally applicable to the preparation of organelle ribosomes from other sources.

The availability of these ribosomes has allowed us to confirm directly that chloroplast ribosomes of higher plants have features in common with both bacterial and mitochondrial ribosomes in being able to use bacterial peptide chain elongation factors, in their sensitivity to a range of antibiotics and in their dissociation into subunits. Subunit exchange experiments suggest, however, that homologies between the two sorts of organelle ribosomes may be more limited than was previously thought: while chloroplast ribosomal subunits form active hybrid ribosomes in combination with *E. coli* ribosomal subunits [15, 17] mitochondrial subunits do not and subunit exchange between chloroplast and mitochondrial ribosomal subunits cannot be detected [15].

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