Interconversion of Tight and Loose Couple 50 S Ribosomes and Translocation in Protein Synthesis*

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On incubation of 50 S ribosomes, isolated from either tight couple (TC) or loose couple (LC) 70 S ribosomes, with elongation factor G (EG-G) and guanosine 5'-triphosphate, a mixture of TC and LC 50 S ribosomes is formed. There is almost complete conversion of LC 50 S ribosomes to TC 50 S ribosomes on treatment with EF-G, GTP, and fusidic acid. Similarly, TC 50 S ribosomes are converted to LC 50 S ribosomes, although partially, by treatment with EF-G and a GTP analogue like guanyl-5'-yl methylenediphosphate (GMP-P(CH₂)P) or guanyl-5'-yl imidodiphosphate (GMP-P(NH)P) and including a polymer of 5'-uridylic acid (poly(U)) in the incubation mixture. Furthermore, LC 23 S RNA isolated from LC 50 S ribosomes is converted to TC 23 S RNA on heat treatment, but similar treatment does not affect TC 23 S RNA. The interconversion was followed by several physical and biological characteristics of TC and LC 50 S ribosomes, like association capacities with 30 S ribosomes before and after kethoxal treatment, susceptibility to RNase I and polyphenylalanine-synthesizing capacity in association with 30 S ribosomes, as well as thermal denaturation profiles, circular dichroic spectra, and association capacity of isolated 23 S RNAs. These data strongly support the proposition that TC and LC 50 S ribosomes are the products of translocation during protein synthesis. The conformational change of 23 S RNA induced by EF-G and GTP is most probably responsible for the interconversion, and L7/L12 proteins play an important role in the process. A two-site model based on kethoxal data has also been proposed to explain the tightness and looseness of 70 S couples.

The translocation of peptidyl-tRNA from the aminoacyl (A) site to the peptidyl (P) site is one of the important steps in protein synthesis. The phenomenon has been known for quite sometime, and models have been proposed from time to time to explain its mechanism (Spirin, 1969; Hardesty et al., 1969; Leder, 1973; Nierhaus, 1982). Unfortunately, however, no concrete evidence is still available for any one of them. The evidences presented here are strong enough to support a more specific model proposed recently from this laboratory (Burma, 1984).

The model is based on a very important observation made

in this laboratory that 23 S RNAs present in TC[1] and LC 50 S ribosomes derived from tight and loose couple 70 S ribosomes have different conformations (Burma et al., 1984). That the difference between TC and LC 70 S ribosomes lies in 50 S ribosomes was realized quite early (van Diggelen and Bosch, 1973; van Diggelen et al., 1973). However, no difference could be found in protein composition. The difference in RNA composition was ruled out without any direct evidence. Employing various physical and biological criteria, it has been unequivocally shown in this laboratory that the difference lies in 23 S RNA (Burma et al., 1984).

Due to the low biological activity of LC ribosomes, they were thought to be damaged ones although the nature of the damage was not known. That this is not true has been unequivocally shown here by the demonstration that LC 50 S ribosomes can be converted to TC 50 S ribosomes by treatment with EF-G, GTP, and fusidic acid. That this conversion takes place has been shown by various physical as well as biological criteria. Similarly, it has been shown that TC 50 S ribosomes can be converted to LC 50 S ribosomes, although less efficiently (50–60%), by treatment with EF-G and a nonhydrolyzable GTP analogue, GMP-P(CH₂)P or GMP-P(NH)P. Finally, data will be presented to demonstrate that isolated LC 23 S RNA can be converted to TC 23 S RNA by heat treatment. The proposed model of translocation (Burma, 1984) will be discussed on the basis of these data, and an attempt will be made to explain the different association capacities of TC and LC 50 S ribosomes on the basis of a "two-site" model for the association.

EXPERIMENTAL PROCEDURES[2]

RESULTS

Interconversion of TC and LC 50 S Ribosomes on Treatment with EF-G and GTP

Since the properties of TC and LC 50 S ribosomes and isolated 23 S RNAs indicated that the two populations of 50 S ribosomes might be the products of translocation (Burma et al., 1984), each type of 50 S ribosomes were treated with the translocating agents, EF-G and GTP, and then subjected to sucrose gradient (5–30%) centrifugation in the presence of

[1] The abbreviations used are: TC, tight couple; LC, loose couple; GMP-P(CH₂)P, guanyl-5'-yl methylenediphosphonate; GMP-P(NH)P, guanyl-5'-yl imidodiphosphate; EF, elongation factor.

[2] "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84 M-3796, cite the authors, and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

4 mM Mg^{2+}. It is well known that TC 50 S ribosomes associate with 30 S ribosomes at this Mg^{2+} concentration, while LC 50 S ribosomes fail to do so. When TC 50 S ribosomes are treated with EF-G and GTP, a small fraction of those (20% or so) fail to associate and thus appear to be converted to LC 50 S ribosomes (Fig. 1A). The conversion is somewhat better in the case of LC 50 S ribosomes (Fig. 1B). It should be mentioned here that no such conversion is effected by omitting either EF-G or GTP (results not presented).

Conversion of LC 50 S Ribosomes to TC 50 S Ribosomes

Almost complete conversion of LC 50 S ribosomes to TC 50 S ribosomes was achieved by treatment with EF-G, GTP, and fusidic acid. Fusidic acid is known to inhibit protein synthesis by not allowing EF-G·GDP complex to come off the 50 S ribosomes following the hydrolysis of GTP (Kuriki et al., 1970; Bodley et al., 1970). That the conversion takes place is shown by the following methods. It should be mentioned here that similar treatment had no effect on the TC 50 S ribosomes.

Association Capacity—LC 50 S ribosomes (^{32}P-labeled) were subjected to sucrose gradient (5–30%) centrifugation in the presence of 4 mM Mg^{2+} before and after treatment with EF-G, GTP, and fusidic acid. As expected, LC 50 S ribosomes do not associate with 30 S ribosomes at 4 mM Mg^{2+} (Fig. 2A, *solid line*). After treatment, the most of the product does associate (Fig. 2A, *dashed line*), indicating thereby that the conversion has taken place.

Kethoxal Treatment—It was shown earlier that TC 50 S ribosomes lost their association capacity (even at 10 mM Mg^{2+}) on treatment with kethoxal, a guanine-specific reagent, whereas the association capacity of LC 50 S ribosomes was not affected due to such treatment (Burma et al., 1984). ^{32}P-labeled LC 50 S ribosomes were therefore modified with kethoxal before and after treatment with EF-G, GTP, and fusidic acid and then subjected to sucrose gradient centrifugation (Fig. 2B). As expected, LC 50 S ribosomes do not lose
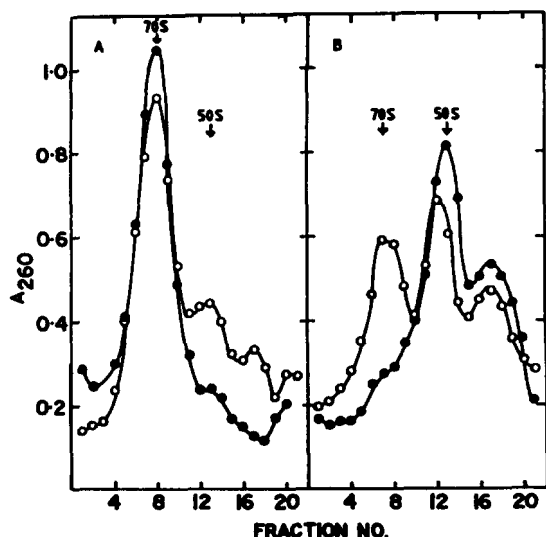

FIG. 2. **Conversion of LC to TC 50 S ribosomes, measured by association capacity before and after kethoxal treatment.** The treatment of ^{32}P-labeled LC 50 S ribosomes (40 pmol; 1.2×10^6 counts/min) with EF-G (200 pmol), GTP (2.5 nmol), and fusidic acid (5 mM) and the subsequent treatment with kethoxal are described under "Experimental Procedures." Equimolar amount of TC 50 S ribosomes were treated with kethoxal as a control. A fraction containing 7.4 pmol of treated or untreated 50 S ribosomes (2×10^4 counts/min) was subjected to density gradient (5–30%) centrifugation as described in the miniprint. The gradient contained TMA, and the Mg^{2+} concentration was 4 or 10 mM as indicated below. A, before kethoxal treatment (gradient at 4 mM Mg^{2+}); B, after kethoxal treatment (gradient at 10 mM Mg^{2+}): LC 50 S ribosomes (●), treated LC 50 S ribosomes (▲), and treated 50 S ribosomes as control (in B only) (△).

their association capacity (at 10 mM Mg^{2+}) on treatment with kethoxal (Fig. 2B, *solid line*), whereas the converted product does not associate with 30 S ribosomes (Fig. 2B) even at 10 mM Mg^{2+}. That TC 50 S ribosomes lose their association capacity on treatment with kethoxal is also shown in Fig. 2B. These results clearly show that LC 50 S ribosomes have been converted to TC 50 S ribosomes. Similar results were obtained following the removal of EF-G and GTP by a 1 M NH_4Cl wash of 50 S ribosomes.

Action of RNase I—TC and LC 50 S ribosomes have been shown, respectively, to be highly sensitive and quite resistant to RNase I at 2.5 mM Mg^{2+} (Burma et al., 1984). Therefore, the sensitivity of LC 50 S ribosomes to this enzyme before and after conversion was tested (Fig. 3). In this case, however, EF-G, GDP, and fusidic acid had to be removed from the treated 50 S ribosomes by a 1 M NH_4Cl wash as they interfered with the enzyme action (Bochkareva and Girschovich, 1984). The 1 M NH_4Cl wash did not affect the biological activity of the ribosomes. As shown earlier, TC 50 S ribosomes are degraded at a very fast rate by RNase I in presence of 2.5 mM Mg^{2+}, whereas LC 50 S ribosomes are fairly resistant (Fig. 3). On conversion of LC 50 S ribosomes to TC 50 S ribosomes, about 75–80% of the population become sensitive to RNase I.

Polyphenylalanine Synthesis—That LC 50 S ribosomes are much less biologically active than TC 50 S ribosomes is well known. However, LC 50 S ribosomes can be converted to active ribosomes by treatment with EF-G, GTP, and fusidic acid (Table I), showing that LC 50 S ribosomes are not damaged ones. It should be mentioned here that the biological activity was measured after washing off the treating reagents with 1 M NH_4Cl. Without this wash, the preparation had no protein-synthesizing capacity.

Association Capacity of Isolated 23 S RNAs—The Mg^{2+} dependence of the association of 23 S RNAs isolated from TC and LC 50 S ribosomes is somewhat different (Burma et al., 1984). However, 23 S RNAs (TC and LC) isolated from

FIG. 1. **Interconversion of TC and LC 50 S ribosomes.** TC and LC 50 S ribosomes (200 pmol each) were individually treated with EF-G (1 nmol) and GTP (13 nmol) as described under "Experimental Procedures." After treatment, the association capacity of the preparation with 30 S ribosomes was checked by 5–30% sucrose gradient centrifugation. The gradient contained (20 mM Tris-HCl, pH 7.6, 30 mM NH_4Cl, 10 mM magnesium acetate, and 6 mM β-mercaptoethanol, and the Mg^{2+} concentration was 4 mM. A, TC 50 S ribosomes: untreated (●) and treated (○). B, LC 50 S ribosomes: untreated (●) and treated (○).
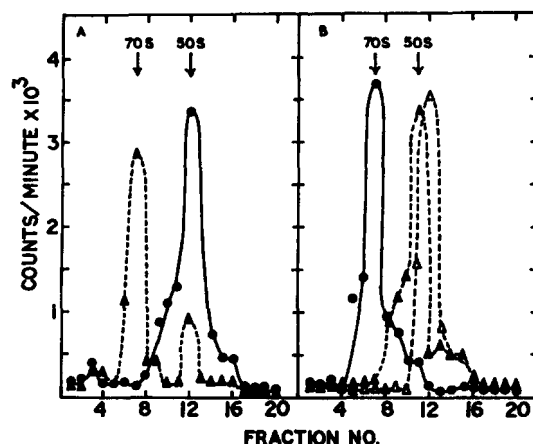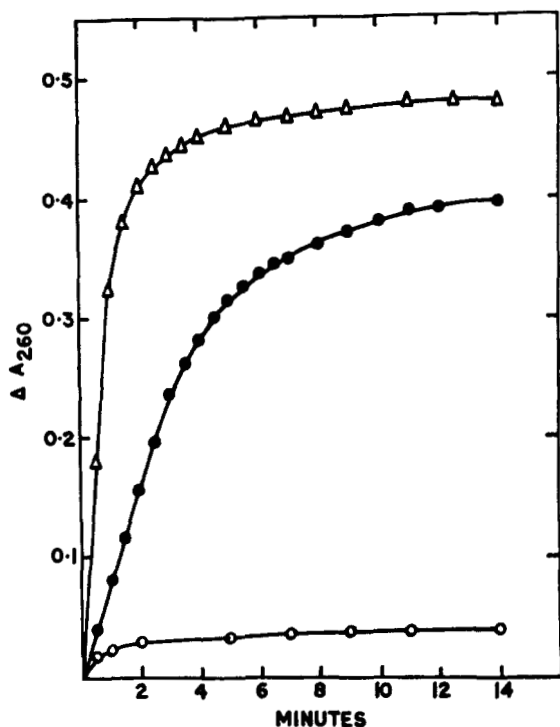
FIG. 3. **Conversion of LC to TC 50 S ribosomes, measured by RNase I action.** The method of treatment of LC 50 S ribosomes (1200 pmol) with EF-G (6 nmol), GTP (78 nmol), and fusidic acid (5 mM) is described in the Miniprint. After treatment, the ribosomes were pelleted and then washed with 1 M NH₄Cl to remove the treating reagents. They were dissolved and dialyzed against TMA containing 2.5 mM Mg²⁺. The RNase I degradation assay was carried out at 2.5 mM Mg²⁺ as described by Datta and Burma (1972). O, LC 50 S ribosomes; △, TC 50 S ribosomes; ●, treated LC 50 S ribosomes.

TABLE I

Polyphenylalanine synthesis by treated LC 50 S ribosomes

The incubation and the method of assay are described under "Experimental Procedures."

Type of 50 S ribosomes	Phenylalanine polymerized
	pmol
LC 50 S ribosomes	6.4
TC 50 S ribosomes	19.3
LC 50 S ribosomes (treated with EF-G, GTP, and fusidic acid)	15.6

kethoxal-treated TC and LC 50 S ribosomes can be more clearly distinguished. TC 23 S RNA isolated from kethoxal-treated 50 S ribosomes completely lose their association capacity with 16 S RNA, even in presence of 20 mM Mg²⁺ under reconstitution conditions, as described by Burma et al. (1983) for the complex formation. LC 23 S RNA isolated from the kethoxal-treated LC 50 S ribosomes fully retains its association capacity as shown earlier (Burma et al., 1984). This is also evident from the results presented in Fig. 4. LC 23 S RNA isolated from kethoxal-treated LC 50 S ribosomes (³²P-labeled) which were earlier treated with EF-G, GTP, and fusidic acid does not have association capacity (Fig. 4, *solid line*). 23 S RNA (isolated from untreated LC 50 S ribosomes) following treatment with kethoxal remains unchanged so far as its association capacity with 16 S RNA is concerned (Fig. 4, *dashed line*). The results obtained with intact 50 S ribosomes are thus reflected in isolated 23 S RNAs.

Thermal Melting Profiles of Isolated 23 S RNAs—Finally, the thermal melting profiles of isolated 23 S RNAs were
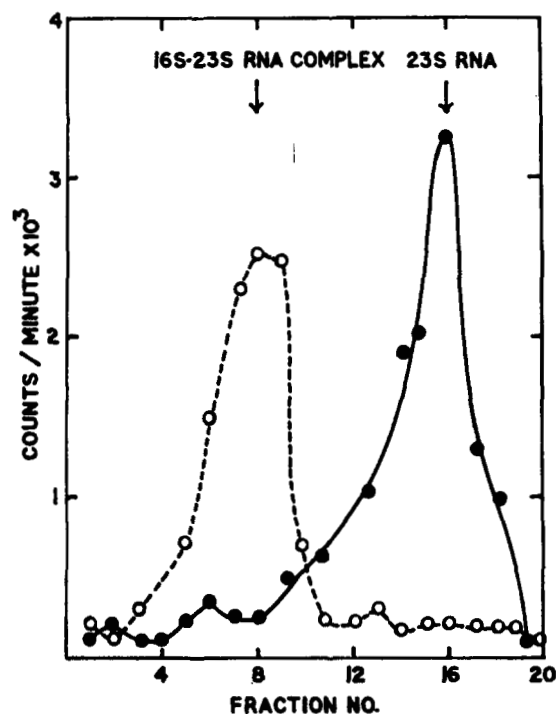

FIG. 4. **Conversion of LC to TC 50 S ribosomes, measured by the association capacity of 23 S RNAs isolated from kethoxal-treated 50 S ribosomes.** ³²P-labeled LC 50 S ribosomes were treated with EF-G, GTP, and fusidic acid as described in the legend to Fig. 2. Both untreated and treated LC 50 S ribosomes were subsequently treated with kethoxal, and 23 S RNAs were isolated as described in the Miniprint. The association capacity of isolated 23 S RNAs with nonradioactive 16 S RNA at 20 mM Mg²⁺ was measured by sucrose gradient (5–20%) centrifugation. The gradient contained reconstitution buffer (20 mM Tris-HCl, pH 7.5, 400 mM KCl, and 20 mM Mg²⁺). Both the samples (each 4 pmol; 2 × 10⁴ counts/min) were subjected to centrifugation. O, 23 S RNA (from LC 50 S ribosomes); ●, 23 S RNA (from treated LC 50 S ribosomes).

studied to establish the conversion of LC 50 S ribosomes to TC 50 S ribosomes. RNA isolated from TC 50 S ribosomes has a slightly different melting profile than that from LC 50 S ribosomes (Burma et al., 1984). About 2% more hyperchromicity was observed in the latter case as shown in Fig. 5. 23 S RNA isolated from LC 50 S ribosomes treated with EF-G, GTP, and fusidic acid behaves like TC 23 S RNA and not like LC 23 S RNA. So this is more strong evidence in favor of the conversion of LC 50 S ribosomes to TC 50 S ribosomes. It should be mentioned here that no conversion takes place if EF-G or GTP is omitted from the incubation mixture.

Partial Conversion of TC 70 S Ribosomes to LC 70 S Ribosomes

Since EF-G, GTP, and fusidic acid are expected to lock the 50 S ribosomes in the GDP-dependent conformation or TC conformation (Burma, 1984), it was assumed that EF-G and nonhydrolyzable GTP analogues (like GMP-P(CH₂)P or GMP-P(NH)P) might lock the 50 S ribosomes in the other conformation (GTP-dependent conformation or LC conformation). It is also known that the hydrolysis of GTP is not necessary for translocation (Kaziro, 1978). Therefore, TC 50 S ribosomes were treated with EF-G and GMP-P(CH₂)P or GMP-P(NH)P, but the conversion was found to be 30–40% only. It was somewhat increased (50–60% conversion) on treatment of TC 70 S ribosomes, instead of TC 50 S ribosomes, with either analogue in presence of poly(U). A typical result is shown in Fig. 6. Approximately 60% conversion is
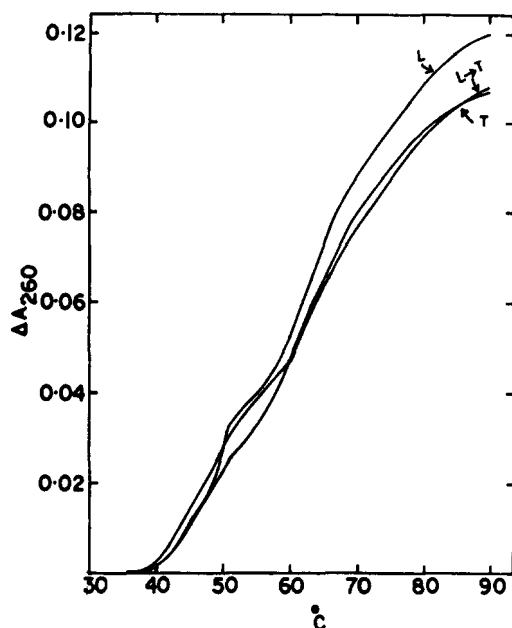
FIG. 5. **Conversion of LC to TC 50 S ribosomes determined by the thermal melting profiles of isolated 23 S RNAs.** 23 S RNAs were isolated by the sodium dodecyl sulfate-phenol method from TC 50 S ribosomes as well as untreated and treated (with EF-G, GTP, and fusidic acid) LC 50 S ribosomes. Their melting profiles were studied simultaneously using 0.1 A_{260} unit in 0.2 ml of 20 mM Tris-HCl, pH 7.5, containing 400 mM KCl and heating at a rate of 0.5 °C/min as described under "Experimental Procedures." L, 23 S RNA from LC 50 S ribosomes; T, 23 S RNA from TC 50 S ribosomes; L→T 23 S RNA from treated LC 50 S ribosomes.

observed. In order to find out whether an equilibrium has been reached, unconverted 70 S ribosomes were isolated from the gradient and treated with EF-G and GMP-P(NH)P, and again partial conversion took place (results not presented). On repeating the similar treatment, there was again partial conversion, indicating thereby that some sort of equilibrium was attained.

It has been very recently observed that the conversion of TC to LC 50 S ribosomes can be further increased if Phe-tRNA is bound to 70 S ribosomes before treatment. The situation is, however, complicated as Phe-tRNA binding itself leads to partial conversion.[3]

Conversion of LC 23 S RNA to TC 23 S RNA by Heat Treatment

It was suspected from the earlier data, especially the action of RNase I on TC and LC 50 S ribosomes and the thermal melting profiles of isolated TC and LC 23 S RNAs, that LC 23 S RNA may have somewhat more folded structure than TC 23 S RNA (Burma *et al.*, 1984). Therefore, an attempt was made with success to convert LC 23 S RNA to TC 23 S RNA by heat treatment. As shown in Fig. 7, LC 23 S RNA after heating at 90 °C followed by slow cooling behaves as TC 23 S RNA in its melting profile. That TC 23 S RNA is produced by heat treatment was also shown by kethoxal treatment (Fig. 8). LC 23 S RNA on kethoxal treatment does not lose its capacity to associate with 16 S RNA, whereas heat-treated LC 23 S RNA, like untreated TC 23 S RNA, loses this capacity. It should be mentioned here that, on heating LC 23 S RNA, no degradation took place as checked by urea-polyacrylamide gel electrophoresis.
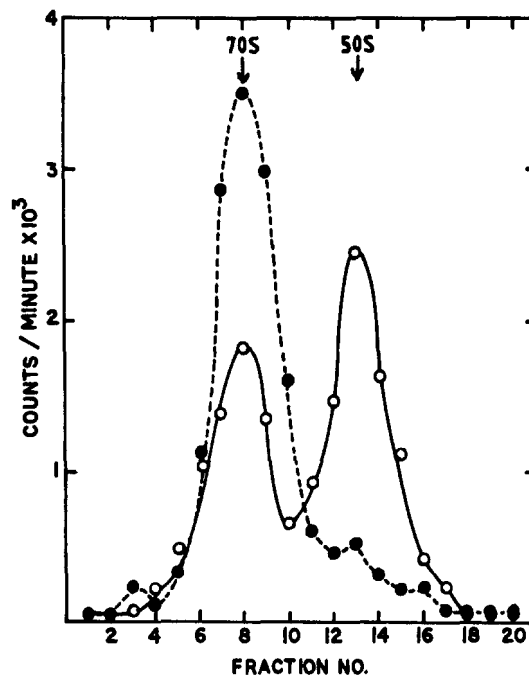
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[3] S. Srivastava and D. P. Burma, personal communication.

FIG. 6. **Partial conversion of TC to LC 70 S ribosomes, measured by association capacity.** ^{32}P-labeled TC 50 S ribosomes (40 pmol; 2 × 10^4 counts/min) in association with an equivalent amount of nonradioactive 30 S ribosomes were treated with EF-G (200 pmol) and GMP-P(NH)P (0.8 nmol) in presence of poly(U) (8 μg) in 0.3 ml of Tris-HCl, pH 7.5, containing 160 mM NH₄Cl, 10 mM magnesium acetate, and 1 mM dithiothreitol as described under "Experimental Procedures." Sucrose gradient (5–30%) centrifugation was carried out in presence of TMA containing 4 mM Mg^{2+}. ●, untreated TC 50 S ribosomes; ○, treated TC 50 S ribosomes.

A more direct evidence for the conformational change of 23 S RNA based on CD spectra is presented in Fig. 9. LC 23 S RNA has slightly more ellipticity than TC 23 S RNA (Burma *et al.*, 1984). On heating LC 23 S RNA, its ellipticity becomes identical with that of TC 23 S RNA. It is well known that ellipticity increases with the increasing molecular order. LC 23 S RNA must have slightly more ordered structure than TC 23 S RNA, and this is lost on heat treatment.

Evidence for Different Sites of Association of TC and LC 50 S Ribosomes with 30 S Ribosomes

It was suspected from the kethoxal treatment data that the sites for association of TC and LC 50 S ribosomes with 30 S ribosomes may be different. To test this, the following experiments (described in the flow sheet in Fig. 10) was designed. In the first set of experiments (Figs. 10 and 11A), LC 50 S ribosomes were first treated with kethoxal and then converted to TC 50 S ribosomes by treatment with EF-G, GTP, and fusidic acid. As expected, LC 50 S ribosomes associate with 30 S ribosomes at 10 mM Mg^{2+} even after kethoxal treatment (data not presented). However, after treatment with EF-G, GTP, and fusidic acid, they lose their association capacity due to their conversion to TC 50 S ribosomes. Subsequently, kethoxal was removed from the converted product by dialysis at alkaline pH, and it was subjected to sucrose gradient centrifugation at 4 mM Mg^{2+} to show that the product was TC 50 S ribosomes. These results clearly indicate that (i) the site of association of LC 50 S ribosomes is not affected by kethoxal treatment, and (ii) the site of association of TC 50 S ribosomes is affected by kethoxal treatment even when it is done at the LC 50 S ribosome level (this is reflected when LC 50 S ribosomes are converted to TC 50 S ribosomes).
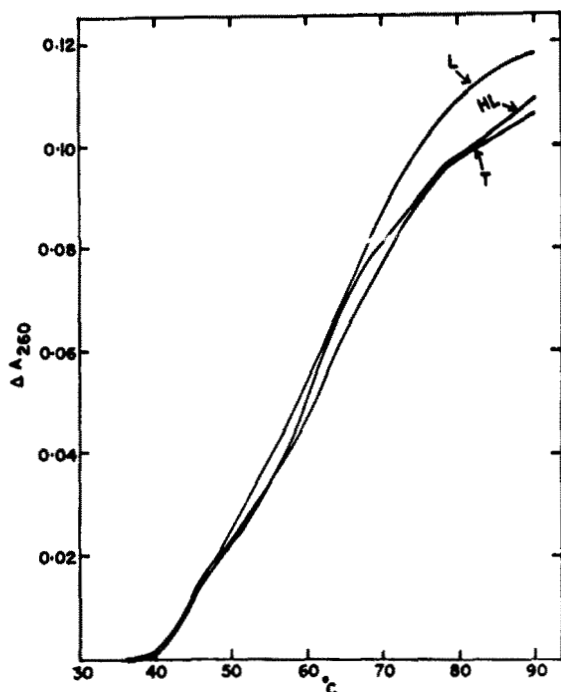
FIG. 7. **Conversion of LC 23 S RNA to TC 23 S RNA by heating, determined by thermal melting profile.** 23 S RNA (10 A_{260} units) isolated from LC 50 S ribosomes by the sodium dodecyl sulfate-phenol method was heated at 90 °C for 5 min in 0.5 ml of 20 mM Tris-HCl, pH 7.5, and 400 mM KCl and then slowly cooled to 25 °C. Its melting profile along with those of untreated LC and TC 23 S RNAs were determined simultaneously as described in the legend to Fig. 5. *L*, LC 23 S RNA; *T*, TC 23 S RNA; *HL*, heated LC 23 S RNA.
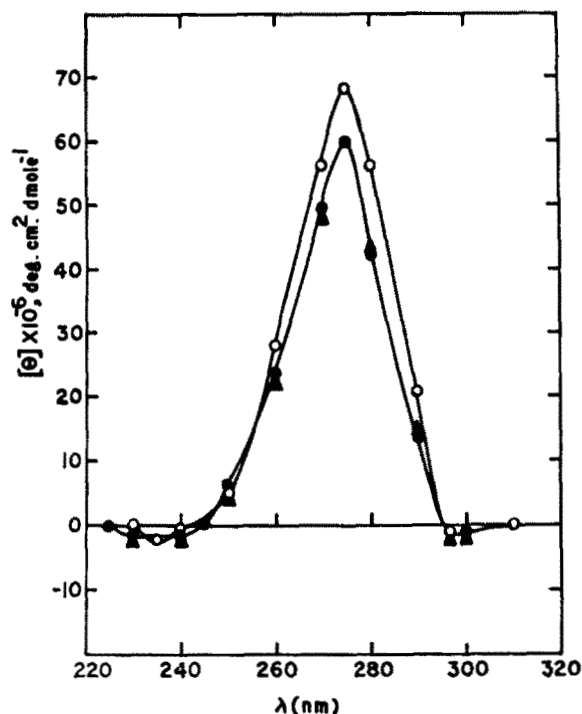

FIG. 9. **Conversion of LC 23 S RNA to TC 23 S RNA by heat treatment, measured by circular dichroism.** LC 23 S RNA was heated at 90 °C for 5 min as described in the legend to Fig. 7. Circular dichroic measurements of heated LC 23 S RNA as well as unheated samples of LC and TC 23 S RNAs (2 A_{260} units of each/ml) were carried out as described under "Experimental Procedures." O, LC 23 S RNA; ●, TC 23 S RNA; ▲, heat-treated LC 23 S RNA.
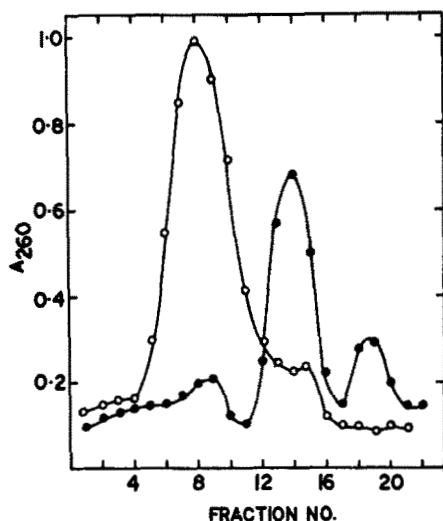

FIG. 8. **Conversion of LC 23 S RNA to TC 23 S RNA by heat treatment, measured by its association capacity with 16 S RNA following kethoxal treatment.** LC 23 S RNA (10 A_{260} units) was heated at 90 °C for 5 min as described in the legend to Fig. 7 and then treated with kethoxal (4 mg/ml) in 100 mM triethanolamine HCl, pH 7.5, 400 mM KCl, and 20 mM Mg^{2+} as described under "Experimental Procedures." Untreated LC 23 S RNA was also treated with kethoxal. Both treated and untreated LC 23 S RNAs were precipitated with ethanol (−20 °C) and dissolved and dialyzed against reconstitution buffer. The association capacity of both (5 A_{260} units of each) with an equivalent amount of 16 S RNA was determined by sucrose gradient (5–20%) centrifugation as described earlier. The gradient contained reconstitution buffer. O, LC 23 S RNA; ●, heat-treated LC 23 S RNA.
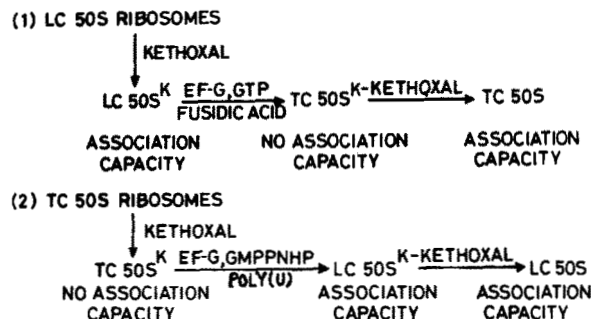

FIG. 10. **Design of experiments and summary of the results demonstrating that TC and LC 50 S ribosomes have different sites of association with 30 S ribosomes.**

In the second set of experiments (Figs. 10 and 11*B*), TC 50 S ribosomes were first treated with kethoxal and then converted (partially) to LC 50 S ribosomes by treatment with EF-G and GMP-P(NH)P. As expected, TC 50 S ribosomes lost their association capacity on treatment with kethoxal (results not presented). If treatment with EF-G and GMP-P(NH)P followed and gradient centrifugation was done at 10 mM Mg^{2+}, three peaks corresponding to 70 S, 50 S, and 30 S ribosomes were obtained (Fig. 11*B*). This is due to the partial conversion of TC 50 S ribosomes to LC 50 S ribosomes. LC 50 S ribosomes (produced as a result of conversion) associate with 30 S ribosomes at 10 mM Mg^{2+}, and TC 50 S ribosomes (unconverted) have no association capacity due to kethoxal treatment. At 4 mM Mg^{2+}, no 70 S peak was observed as LC 50 S ribosomes do not associate at 4 mM Mg^{2+}. After removal of kethoxal, only 70 S peak was observed at 10 mM Mg^{2+} (Fig. 11*B*) as both TC and LC 50 S ribosomes associate at this Mg^{2+} concentration. At 4 mM Mg^{2+}, LC 50 S ribosomes do
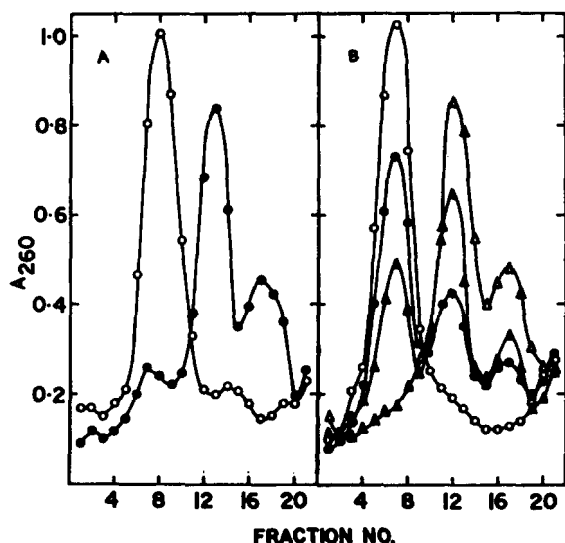
FIG. 11. Demonstration of two different sites of association of 50 S ribosomes with 30 S ribosomes. LC and TC 50 S ribosomes (1.5 nmol of each) were separately treated with kethoxal, and excess kethoxal was removed as described in the legend to Fig. 2. After addition of equivalent amount of 30 S ribosomes to each, the conversion was effected by treatment with EF-G, GTP, and fusidic acid (in the former case) and EF-G, poly(U), and GMP-P(NH)P (in the latter case). In the first case (*A*), LC 50 S ribosomes (0.6 nmol) with an equivalent amount of 30 S ribosomes were treated with EF-G (3 nmol), GTP (37.5 nmol), and fusidic acid (5 mM) in 0.5 ml of 50 mM Tris-HCl, pH 7.5, 160 mM NH_4Cl, 10 mM magnesium acetate, and 12 mM β-mercaptoethanol. In the second case (*B*), TC 50 S ribosomes (0.8 nmol) along with an equivalent amount of 30 S ribosomes were treated with EF-G (4 nmol), poly(U) (160 μg), and GMP-P(NH)P (16 nmol) in 0.7 ml of Tris-HCl, pH 7.5, containing 160 mM NH_4Cl, 1 mM dithiothreitol, and 10 mM magnesium acetate. Kethoxal was removed from a portion of the treated ribosomes by dialyzing against 0.013 M Tris, pH 8.0, containing 10 mM magnesium acetate and 30 mM NH_4Cl. Association capacities of the aliquots containing 180 pmol of treated ribosomes before and after removal of kethoxal were checked by sucrose gradient (5–30%) centrifugation in TMA at Mg^{2+} concentrations indicated below. *A*, LC 50 S ribosomes → TC 50 S ribosomes: kethoxal-treated LC 50 S ribosomes following conversion (gradient in TMA at 10 mM Mg^{2+}) (●) and as above but after removal of kethoxal (gradient in TMA at 4 mM Mg^{2+}) (○). *B*, TC 50 S ribosomes → LC 50 S ribosomes: kethoxal-treated TC 50 S ribosomes following conversion (gradient in TMA at 10 mM Mg^{2+} (▲) and 4 mM Mg^{2+} (△)) and as above but after removal of kethoxal (gradient in TMA at 10 mM Mg^{2+} (○) and 4 mM Mg^{2+} (●).

not associate, while dekethoxalated TC 50 S ribosomes associate under this condition (Fig. 11*B*). These data show that the site of association of TC 50 S ribosomes is modified by kethoxal, leading to the loss of their association capacity. When kethoxal-treated TC 50 S ribosomes are converted to LC 50 S ribosomes, the latter, however, associate, indicating thereby that the site of association of TC 50 S ribosomes is not utilized by LC 50 S ribosomes.

DISCUSSION

While studying the mechanism of specific bimolecular complex formation between 16 S and 23 S RNAs (Nag and Burma, 1982; Burma *et al.*, 1983; Nag *et al.*, 1983; Tewari and Burma, 1983), it was realized that 50 S ribosomes occur in two distinct forms and these are derived from TC and LC 70 S ribosomes (Burma *et al.*, 1984). Not only do the TC and LC 50 S ribosomes differ in many physical and biological properties, but TC and LC 23 S RNAs (derived from TC and LC 50 S ribosomes) also differ.

The most interesting observation as recorded here is the interconversion of TC and LC 50 S ribosomes. LC 50 S

ribosomes can be converted to TC 50 S ribosomes on treatment with EF-G, GTP, and fusidic acid, and the reverse can be done with the help of EF-G and a GTP analogue (either GMP-P(CH₂)P or GMP-P(NH)P). In the former case, the conversion is almost 100%, whereas in the latter case, maximally 60% conversion takes place and also when the treatment is done at the 70 S ribosome level as well as in presence of poly(U). However, it is possible to convert the whole population of TC 50 S ribosomes to LC 50 S ribosomes provided the product (LC 50 S ribosomes) is removed, the remaining TC 50 S ribosomes are retreated, and this step is repeated a number of times. Apparently the partial conversion is due to the attainment of some sort of equilibrium. The reason for this is not known at present. According to the proposed model (Burma, 1984), based on the experimental evidence presented here, the conversion of TC 50 S ribosomes to LC 50 S ribosomes requires the folding of 23 S RNA in the L7/L12 stalk region. This folding may not be energetically favorable. Other explanations are also possible. For example, GTP analogues were used for this conversion. These may not be as effective as GTP in inducing the conformational change. The interconversion can also be effected with the help of EF-G and GTP in absence of an inhibitor or GTP analogue, but in both the cases a mixture of the two is obtained. The conversion from TC to LC 50 S ribosomes is, however, comparatively much less than the reverse one. It has been further demonstrated that isolated 23 S RNA of LC 50 S ribosomes on heat treatment is converted to TC 23 S RNA (of TC 50 S ribosomes).

It was shown in this laboratory quite sometime ago that only L7/L12, L10, and L4 proteins are released and 23 S RNA is split into two fragments on treatment of 50 S ribosomes with RNase I in presence of high Mg^{2+} concentration (Raziuddin *et al.*, 1979). Later it was confirmed that the split is at the one-third region from the 5'-end where L10 (along with L7/L12) is expected to bind. Naturally 23 S RNA in the stalk region is susceptible to the attack of RNase I. Bochkareva and Girshovich (1984) subsequently showed that this region is protected against the attack of RNase I by EF-G which is known to bind at the base of the stalk region (Girshovich *et al.*, 1981). Very recently, Gudkov and Gongadze (1984) have shown that L7/L12 proteins become resistant to trypsin on treatment of 50 S ribosomes with EF-G, GTP, and fusidic acid, while on treatment with EF-G and GMP-P(CH₂)P, they become sensitive. It was shown in our laboratory earlier that, on removal of L7/L12 proteins from 50 S ribosomes, the core particles become more resistant to the attack of RNase I (Byasmuni and Burma, 1982). On adding back L7/L12, they become susceptible again. The dramatic difference between TC and LC 50 S ribosomes in sensitivity to RNase I at 2.5 mM Mg^{2+} (Burma *et al.*, 1984) points out that the difference lies in the conformations of 23 S RNA and also in the stalk region. These data coupled with earlier observations strengthen the belief that the switch over from TC to LC 50 S ribosomes and vice versa is due to the conformational change of 23 S RNA.

That EF-G-dependent GTPase activity catalyzed by 50 S ribosomes is intimately connected with the translocation in protein synthesis has been known for quite sometime, although it was realized that the hydrolysis of GTP is not directly concerned with the translocation process (Kaziro, 1978). It was argued upon that there are GTP- and GDP-dependent conformations of EF-G. It is clear from the present data that the TC 50 S ribosome conformation is most likely induced by EF-G and GDP and that of LC 50 S ribosomes is due to EF-G and GTP. Moreover, L7/L12 proteins are known

to be directly involved in translocation (Brot and Weissbach, 1981). Their location in the stalk region and the data obtained with RNase I treatment make us strongly believe that the conformational switch lies in this or a neighboring region. The conversion induced at the RNA level on heat treatment is also indicative of an unfolding process.

As mentioned already, a model of translocation has already been proposed on the basis of the results obtained in this laboratory and other laboratories (Burma, 1984). A schematic diagram of a slightly modified model is shown in Fig. 12. The basic features of the model are the following: (i) rRNA in the L7/L12 stalk region of TC 50 S ribosomes is unfolded, whereas rRNA in the L7/L12 stalk region of LC 50 S ribosomes is folded; (ii) the mobility of the L7/L12 stalk region is responsible for the folding and unfolding; (iii) the mobility is induced by EF-G and GTP which associate with 23 S RNA (Girshovich *et al.*, 1982; Skold, 1983) and act, most likely, through L10; (iv) the conformational change of TC to LC 50 S ribosomes is assumed to be induced by GTP (prior to its hydrolysis), and thus the LC 50 S ribosomes are supposed to be in GTP conformation; and (v) the interaction with EF-G and GTP leads to the hydrolysis of GTP to GDP, and the tight couple structure (unfolding in the stalk region) is again formed and this is retained even when EF-G and GDP are dissociated from 50 S ribosomes, thus the TC 50 S ribosomes are assumed to be in GDP conformation.

It is possible to explain the interconversion data with the help of this model. When EF-G and GMP-P(CH₂)P are added to TC 50 S ribosomes, they are locked in LC conformation (or GTP conformation) as the analogues cannot be hydrolyzed. When LC 50 S ribosomes are treated with EF-G, GTP, and fusidic acid, the binding of EF-G and GTP takes place, GTP is hydrolyzed and leads to TC conformation (GDP conformation), but the 50 S ribosomes are locked in that form as EF-G and GDP are not released due to the presence of fusidic acid. On heating LC 23 S RNA, its conversion to TC

23 S RNA is understood as unfolding of rRNA in the stalk region is necessary for this conversion. A basic question may be raised here why LC 50 S ribosomes are less biologically active. This conformation (free of EF-G and GTP) does not occur under physiological conditions and has not the requisite features for optimum binding of aminoacyl-tRNA, etc.

Another basic question may be raised here. Why is the association capacity of TC 50 S ribosomes as well as TC 23 S RNA lost on kethoxal treatment, while that of LC 50 S ribosomes and LC 23 S RNA is not affected by such treatment? This can be explained if we assume that there are two different sites of association of 23 S RNAs (in 50 S ribosomes) with 16 S RNA in 30 S ribosomes (Fig. 13). The site responsible for the association of TC 23 S RNA appears to be a strong binding site (site 1) and modifiable by kethoxal treatment, whereas the site responsible for the association of the loose couples is in all probability a weak binding site (site 2) and cannot be modified by kethoxal. Either guanine bases are absent in this site or, even if present, are not available for modification. When LC 50 S ribosomes are modified with kethoxal, their association with 30 S ribosomes is not affected; this is in accordance with the above suggestion. Under this condition, site 1 is modified with kethoxal as evident from the subsequent conversion data (Figs. 10 and 11). When kethoxal-treated LC 50 S ribosomes are converted to TC 50 S ribosomes, the latter do not associate with 30 S ribosomes, indicating that the site (site 1) was modified by kethoxal prior to conversion. This is also further supported by the fact that when kethoxal is removed from the converted LC 50 S ribosomes (newly produced TC 50 S ribosomes), they were capable of associating with 30 S ribosomes. This site (site 1) was definitely modified in LC 50 S ribosomes, yet the modification did not affect their association capacity. In case of TC 50 S ribosomes, kethoxal treatment modifies site 1 and thus affects their association capacity. Site 2 is not modifiable (supported by earlier data), yet it is not available for association (even weak) of TC 50 S ribosomes. When TC 50 S ribosomes are converted to LC 50 S ribosomes, the association capacity (weak) is observed. Naturally site 2 and not site 1 is being used for this association which is not affected even on removal of kethoxal. The two-site model thus explains all the data so far recorded.

FIG. 12. **A schematic diagram (revised) of the model for translocation proposed by Burma (1984).**
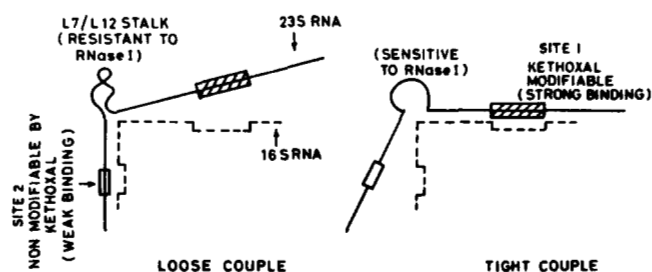
FIG. 13. **A two-site model of association of 30 S and 50 S ribosomes based on RNA-RNA interaction.**

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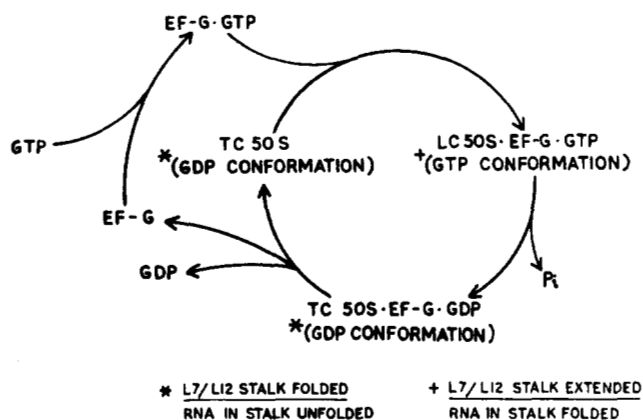
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SUPPLEMENTAL MATERIAL TO

Interconversion of tight and loose couple 50S ribosomes and translocation in protein synthesis.

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EXPERIMENTAL PROCEDURES

Materials

GTP was obtained from Sigma Chemical Company, U.S.A.. Fusidic acid, GMPP(CH$_2$)P and GMPP(NH)P were obtained as gift from Wim Moller of the University of Leiden, The Netherlands. Poly(U) was the product of Miles Laboratory, U.S.A.. Polyethylene glycol was purchased from Union Carbide Corporation, New York, U.S.A.. Dextran 500 was supplied by Pharmacia Fine Chemicals, Uppsala Sweden. Kethoxal was obtained as a gift from Upjohn Company, U.S.A.. DEAE-cellulose was of Whatman Company, U.S.A.. RNase I was prepared according to the method of Datta and Burma (1972). The mixture of tRNAs was prepared from 100,000xg supernatant of the extract of E.coli MRE600 according to the method of Zubay (1966). This mixture of tRNAs was acylated with ^{14}C-phenylalanine according to the method of Scott (1968). Other reagents were of analar quality.

All operations were carried out at 4°C until and unless otherwise stated.

Preparations of TC and LC 50S ribosomes

70S ribosomes (mixture of TC and LC) were prepared by ultracentrifugation of the alumina-extract of E.coli MRE600 cells in TMA (20 mM Tris-HCl, pH 7.6, 30 mM NH$_4$Cl, 10 mM magnesium acetate and 6 mM β-mercaptoethanol) as described by Raziuddin et al. (1979). TC and LC 50S ribosomes were isolated from the final preparation according to the method of Chapman and Noller (1977). The preparation of 70S ribosomes was dissolved in 20 mM Tris-HCl pH 7.5, 100 mM NH$_4$Cl, 6 mM β-mercaptoethanol and 4 mM magnesium acetate and dialysed for 36 h against the same buffer. The dialysed preparation was subjected to sucrose gradient (5-30%) centrifugation containing 20 mM Tris-HCl, pH 7.5, 100 mM NH$_4$Cl, 6 mM β-mercaptoethanol and 4 mM Mg^{++} at 100,000xg for 4 h at 4°C. Three distinct peaks corresponding to TC 70S, LC 50S and LC 30S ribosomes were obtained. TC 70S and LC 50S ribosomes were pooled separately avoiding cross contamination and precipitated with 0.7 vol. ethanol (-20°C) in presence of 100 mM Mg^{++}. TC 50S and 30S ribosomes were isolated from TC 70S ribosomes after dialysing the preparation against the dissociation buffer containing 20 mM Tris-HCl, pH 7.4, 30 mM NH$_4$Cl, 6 mM β-mercaptoethanol and 1 mM Mg^{++} and separated by ultracentrifugation at 100,000xg for 5 h through sucrose gradient (5-30%) in the same dissociation buffer. TC 50S and 30S ribosomes were pooled separately and precipitated with ethanol, as described earlier. Finally the purity of both TC 50S and LC 50S ribosomes was checked by adding equivalent amount of 30S ribosomes and subsequent analysis by sucrose gradient centrifugation at 4 mM Mg^{++}.

Preparation of LC 23S RNA from LC 50S ribosomes

LC 23S RNA was isolated from LC 50S ribosomes by treat-

ment with phenol in presence of sodium dodecyl sulfate and bentonite, followed by precipitation with ethanol as described by Amils et al. (1978). 23S RNA was separated from 5S RNA by gel filtration through Sephadex G-100 (Erdman et al., 1971). The purity of the preparations was checked by polyacrylamide gel electrophoresis. They had no significant protein contamination as judged from the radioactivity of the preparations from ^{35}S-labeled ribosomes.

Preparations of EF-G and EF-T

Elongation factors T and G were prepared from S-100 fraction of the extract of E.coli MRE600 cells according to the method of Gordon et al. (1971). To 53.3 ml of S-100 fraction 7.56 g of NH$_4$Cl were added (small amount at a time). Then 15 ml polyethylene glycol (30% w/w) were added drop by drop followed by 5 ml of dextran 500 (20% w/w). After stirring for 10 min the mixture was centrifuged. The volume of the upper polyethylene glycol phase was made up to 75 ml with buffer A (10 mM Tris-HCl pH 7.8, 10 mM magnesium acetate and 1 mM dithiothreitol) and the pH was adjusted to 7.8 with NH$_4$OH. Ammonium sulfate (12.6 g) was added to it with stirring for 10 min. After centrifugation the lower phase was taken out and its volume was adjusted to 62.5 ml with buffer A. The factors (EF-T and EF-G) were precipitated by the addition of 13.3 g of ammonium sulfate to saturation. EF-T and EF-G were successively eluted from the precipitate by extractions of the precipitate with solutions containing decreasing concentration of ammonium sulfate. It was extracted first with 27.8% (w/w) ammonium sulfate (three times), then 24% (w/w) ammonium sulfate (three times) and finally with 19.6% (w/w) ammonium sulfate (three times). The ammonium sulfate solutions contained 1 mM dithiothreitol and their pH was adjusted to 7.8 with NH$_4$OH . The 24% ammonium sulfate eluate was used as the source of EF-T and 19.6% eluate as that of EF-G. The combined three eluates (in each case) were treated with ammonium sulfate to saturation and the precipitate was dissolved in 2 ml of buffer B (10 mM Tris-HCl, pH 7.8 and 1 mM dithiothreitol) and extensively dialysed against the same. Each solution was separately placed on the top of a DEAE-cellulose column, equilibrated with buffer B and 0.1M KCl and the column was washed with the same buffer. Each factor was eluted with a linear gradient of KCl (0.1 - 0.3M) in buffer B. The fractions containing EF-T (or EF-G) activity were pooled and concentrated by the addition of ammonium sulfate (4.55 g per 10 ml pooled material). The mixture was stirred for 1 h and the precipitate was collected by centrifugation and dissolved and dialysed against buffer B. The purity of the preparations was checked by polyacrylamide gel electrophoresis and both were found to be homogeneous.

Interconversion of LC and TC 50S ribosomes

LC 50S ribosomes or TC 50S ribosomes (200 pmol) along with equivalent amount of 30S ribosomes were treated at 30°C for 30 min with 1 nmol of EF-G and 13 nmol of GTP in a total volume of 0.3 ml containing 50 mM Tris-HCl, pH 7.5, 160 mM NH$_4$Cl (80 mM NH$_4$Cl if only 50S ribosomes were treated), 10 mM Mg^{++} and 12 mM β-mercaptoethanol. The incubation mixture was subsequently cooled to 4°C and dialysed at 0°C against TMA but containing 4 mM Mg^{++}.

To study the conversion of LC to TC 50S ribosomes in
presence of EF-G, GTP and fusidic acid, either nonradioactive
or ^{32}P-labeled 50S ribosomes were used. When nonradioactive
ribosomes were used the incubation mixture was the same as
described above except that the incubation volume was 6 times
in size and fusidic acid (5 mM) was included in the incubation
mixture. In case of radioactive ribosomes 40 pmol of LC 50S
ribosomes (1.2x10^5 counts/min) were treated with 200 pmol of
EF-G, 2.5 nmol of GTP and 5 mM fusidic acid. When the treatment
of TC 50S ribosomes was carried out with GTP analogues (GMPP(CH$_2$)P
or GMPP(NH)P) the incubation mixture contained 40 pmol of
^{32}P-labeled TC 50S ribosomes (2x10^4 counts/min), equivalent
amount of 30S ribosomes, 8 µg of poly(U), 200 pmol of EF-G and
0.8 nmol of GMPP(CH$_2$)P or GMPP(NH)P in a total volume of 0.3 ml
of 50 mM Tris-HCl pH 7.5 containing 160 mM NH$_4$Cl, 10 mM Mg^{++}
and 1 mM dithiothreitol. The incubation was carried out at 37°C
for 30 min. The remaining procedure was the same as described
above.

Conversion of LC 23S RNA to TC 23S RNA by heat treatment

    LC 23S RNA (10 A$_{260}$ units) isolated from LC 50S ribosomes
was heated at 90°C for 5 min in a total volume of 0.5 ml of
20 mM Tris-HCl, pH 7.5 containing 400 mM KCl and then slowly
cooled to 25°C.

Assay methods for the conversion of LC 50S ribosomes to TC
50S ribosomes and vice versa as well as conversion of LC 23S
RNA to TC 23S RNA

Mg^{++}-dependent association of 50S ribosomes - The method is
based on the observation that TC 50S ribosomes associate with
30S ribosomes at 4 mM Mg^{++} whereas LC 50S ribosomes require
higher Mg^{++} concentration (10 mM or so) for the association. The
solution containing TC or LC 50S ribosomes, was dialysed against
TMA containing either 4 or 10 mM Mg^{++} and then mixed with equi-
valent amount of 30S ribosomes (if not added to the incubation
mixture during conversion). The mixture was subjected to
sucrose density (5-30%) gradient centrifugation at 4°C for 135 min
at 120,000xg in 5 ml cups of SW 50.1 rotor of L5-50B preparative
ultracentrifuge of Beckman Instrument Co. Fractions (0.23 ml)
were collected and absorbancies were measured at 260 nm in
Zeiss UV Spectrophotometer. When ^{32}P-labeled 50S ribosomes
(mixed with nonradioactive 30S ribosomes) were used fractions
were collected and dried on Whatman No. 3MM filter paper pieces
which were counted in scintillation fluid in Rack Beta Liquid
Scintillation Counter of LKB, Sweden.

Association capacities of 50S ribosomes as well as 23S RNAs
following kethoxal treatment

    50S ribosomes were dialysed against 100 mM triethanolamine-
HCl, pH 7.0 containing 10 mM Mg^{++} and then treated with kethoxal
(4 mg/ml) at 37°C for 60 min and subsequently cooled to 4°C
according to the procedure of Herr and Noller(1979).
Ribosomes were precipitated with 0.7 vol. of ethanol (-20°C) and
then dissolved and dialysed against TMA containing 10 mM Mg^{++}.
Each preparation was mixed with equimolar amount of 30S ribosomes
and subjected to sucrose density (5-30%) gradient centrifugation
at 120,000xg for 135 min at 4°C in TMA containing 10 mM Mg^{++}.
Removal of kethoxal (if needed) was done by dialysing the kethoxal-
treated ribosomes at 37°C for 3 h against a solution (pH 8.0)
containing 0.013M Tris, 10 mM Mg^{++} and 0.03M NH$_4$Cl. For kethoxal
treatment of heated LC 23S RNA it was first dialysed against 100 mM
triethanolamine-HCl, pH 7.5 containing 400 mM KCl and 20 mM Mg^{++}

and then treated with kethoxal as above. After treatment RNA was
precipitated with double volume of ethanol (-20°C), dissolved and
dialysed against the reconstitution buffer (20 mM Tris-HCl, pH 7.5,
400 mM KCl and 20 mM Mg^{++}).

    For testing the association capacities of 23S RNAs isolated
from kethoxal-treated 50S ribosomes or 23S RNAs,directly treated
with kethoxal, 4 pmol of radioactive RNA containing 2x10^4 counts/min
or 200 pmol of nonradioactive RNA were mixed with equimolar amount
of 16S RNA under reconstitution condition and subjected to sucrose
density (5-20%) gradient centrifugation under reconstitution
condition for 6 h at 96,000xg. Fractions were collected and counted
or their absorbancies at 260 nm were measured as above.

RNase I degradation assay - Before carrying out this assay ribosomes
had to be freed from treating reagents (EF-G, GTP and fusidic acid).
This was done in the following way. After treatment (as described
above) 50S ribosomes were pelleted by centrifugation at 175,000xg
for 4 h, dissolved in and dialysed against TMA containing 10 mM Mg^{++}.
Then NH$_4$Cl concentration was raised to 1M and after keeping for
4 h at 4°C ribosomes were again pelleted, dissolved in and
dialysed against TMA. There was no loss of biological activity
of untreated ribosomes indicating thereby that the ribosomes
were not damaged by this treatment. RNase I degradation assay was
carried out as described by Datta and Burma (1972). RNase I
(4 units) were added to 1 A$_{260}$ unit of untreated or treated
50S ribosomes in 1 ml of 100 mM Tris-HCl, pH 7.0 containing
2.5 mM Mg^{++}. Increase in A$_{260}$ was followed in PMQ II Zeiss
spectrophotometer.

Polyphenylalanine synthesis - Before carrying out polyphenyl-
alanine synthesis, treated 50S ribosomes were freed from treating
reagents as described above. For measuring the polyphenylalanine
synthesising activity according to the method of Wahba and Miller
(1974) the incubation was carried out at 37°C for 30 min in a
total volume of 0.17 ml of 50 mM Tris-HCl, pH 7.6, 80 mM NH$_4$Cl,
14 mM β-mercaptoethanol and 16 mM Mg^{++} which also contained
1.3 A$_{260}$ units of 50S ribosomes along with equimolar amount of
30S ribosomes, 10 µg of poly(U), 10 µg of EF-T, 8 µg of EF-G,
0.25 mM GTP and 100 pmol of ^{14}C-phenylalanyl-tRNA (100 counts/min/
pmol). The reaction was terminated by the addition of 3 ml of 10%
trichloroacetic acid. Then the mixture was heated for 15 min at
90°C. After cooling to 0°C for 1 h the mixture was filtered
through nitrocellulose filter which was counted in scintillation
fluid in liquid scintillation counter.

Thermal denaturation of 23S RNAs - Thermal denaturation profiles
of 23S RNAs isolated from LC 50S or TC 50S or LC 50S ribosomes
treated with EF-G, GTP and fusidic acid or heated LC 23S RNA
were obtained by using Gilford Spectrophotometer equipped with
temperature-regulated cell holders. 23S RNA (0.5 A$_{260}$ unit per ml)
was taken in 0.2 ml of 20 mM Tris-HCl, pH 7.5 containing 400 mM
KCl and heated from 36°C to 90°C (with a rate of 0.5°C/min) and
the increase in absorbancy was followed with the help of an
automatic recorder.

Circular dichroic spectra of 23S RNAs - Circular dichroic
measurements of 23S RNAs, isolated from LC 50S or TC 50S ribosomes
and heated LC 23S RNAs (2 A$_{260}$ units/ml) were obtained in 20 mM
Tris-HCl, pH 7.5 and 400 mM KCl in a Jobin-Yvon Dichrograph III
using strain-free quartz cells with optical path length of 10 mm
for the spectral region of 320 to 220 nm. The results are
expressed in molar ellipticity (θ).