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

(Received for publication, December 18, 1984)

Debi P. Burma, Anand K. Srivastava, Suman Srivastava, and Debabrata Dash

From the Molecular Biology Unit, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221 005, U. P., India

On incubation of 50 S ribosomes, isolated from either tight couple (TC) or loose couple (LC) 70 S ribosomes, with elongation factor G (EF-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(\(\text{CH}_2\))P) or guanyl-5'-yl imidodiphosphate (GMP-P(NH)P) and including a polymer of 5'-uridylid 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 L17/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 some time, 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 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 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., 1972). 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(CH\(_2\))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

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 (6–30%) centrifugation in the presence of

* The abbreviations used are: TC, tight couple; LC, loose couple; GMP-P(CH\(_2\))P, guanyl-5'-yl methylenediphosphate; GMP-P(NH)P, guanyl-5'-yl imidodiphosphate; EF, elongation factor.

1 "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 Mg²⁺. It is well known that TC 50 S ribosomes associate with 30 S ribosomes at this Mg²⁺ 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 (³²P-labeled) were subjected to sucrose gradient (5–30%) centrifugation in the presence of 4 mM Mg²⁺ 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 Mg²⁺ (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 Mg²⁺) 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). ³²P-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 their association capacity (at 10 mM Mg²⁺) 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 Mg²⁺. 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 NH₄Cl 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 Mg²⁺ (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 NH₄Cl wash as they interfered with the enzyme action (Bochkareva and Girschovich, 1984). The 1 M NH₄Cl 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 Mg²⁺, 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 NH₄Cl. Without this wash, the preparation had no protein-synthesizing capacity.

**Association Capacity of Isolated 23 S RNAs**—The Mg²⁺-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](image1.png) **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 NH₄Cl, 10 mM magnesium acetate, and 6 mM β-mercaptoethanol, and the Mg²⁺ concentration was 4 mM. A, TC 50 S ribosomes: untreated () and treated (O). B, LC 50 S ribosomes: untreated () and treated (O).

![Fig. 2](image2.png) **Fig. 2.** Conversion of LC to TC 50 S ribosomes, measured by association capacity before and after kethoxal treatment. The treatment of ³²P-labeled LC 50 S ribosomes (40 pmol; 1.2 × 10⁶ 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 × 10⁶ counts/min) was subjected to density gradient (5–30%) centrifugation as described in the miniprint. The gradient contained TMA, and the Mf%+ concentration was 10 Mf%. B, after kethoxal treatment (gradient at 10 mM Mg²⁺): LC 50 S ribosomes (A), treated LC 50 S ribosomes (O), and treated 50 S ribosomes as control (in B only) (L).
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 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)₃P 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)₃P, 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
tRNA is bound to 70 S ribosomes before treatment. The TC to LC 50 S ribosomes can be further increased if Phe-been reached, unconverted 70 S ribosomes were isolated from again partial conversion took place (results not presented). The gradient and treated with EF-G and GMP-P(NH)P, and conversion, indicating thereby that some sort of equilibrium was attained.

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.

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 11 A), 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 Mg²⁺ 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 Mg²⁺ 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. 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 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. 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 (3 $A_{260}$ 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.

(1) LC 505 RIBOSOMES
KETHOXAL
K EFGTTP
NC DISE
ASSOCIATION
CAPACITY
CAPACITY
CAPACITY

(2) TC 505 RIBOSOMES
KETHOXAL
K EFGMPPNP
POLY(U)
ASSOCIATION
CAPACITY
ASSOCIATION
CAPACITY

ASSOCIATION
CAPACITY

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 11B), 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. 11B). 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. 11B) as both TC and LC 50 S ribosomes associate at this Mg$^{2+}$ concentration. At 4 mM Mg$^{2+}$, LC 50 S ribosomes do
Ribosomes and Translocation

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 50 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 NH₄Cl, 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) (180 μg), and GMP-P(NH)p (18 nmol) in 0.7 ml of Tris-HCl, pH 7.5, containing 160 mM NH₄Cl, 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 NH₄Cl. 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 Mg²⁺ 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 Mg²⁺ (●)) and as above but after removal of kethoxal (gradient in TMA at 10 mM Mg²⁺ (●) and 4 mM Mg²⁺ (●)) and as above but after removal of kethoxal (gradient in TMA at 10 mM Mg²⁺ (●) and 4 mM Mg²⁺ (●)). Not associate, while dekethoxalated TC 50 S ribosomes asso-

not associate, while dekethoxalated TC 50 S ribosomes associate under this condition (Fig. 11B). 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(CH₃)₂P 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 Mg²⁺ concentration (Razuddin 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(CH₃)₂P, 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 (Byassmuni 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 Mg²⁺ (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) RNA in the L7/L12 stalk region of TC 50 S ribosomes is unfolded, whereas RNA 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 unfolding 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 GDP 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 LC 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 RNA 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 and peptidyl-tRNA.

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.

Acknowledgments—The gift of fusidic acid, GMP-P(CH2)P, and GMP-P(NH2)P from Wim Moller of the University of Leiden, The Netherlands, is gratefully acknowledged. Kethoxal was gift from The Upjohn Co.

REFERENCES


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.
Tight and Loose Couple 50 S Ribosomes and Translocation

SUGGESTIONS TO

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

Debi P. Burma, Anand K. Srivastava, Daman Srivastava and Debraj Uash

EXPERIMENTAL PROCEDURES

Materials

GTP was purchased from Sigma Chemical Company, U.S.A. Carboxylic acid, EMG(IP)(OH)P and EMG(1NH)P were obtained as gift from Dr. W. K. Miller 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, Upsala Sweden. Ammonium sulfate was obtained as a gift from Upjohn Company, U.S.A. L-Ah-ribofuranose was used as a substrate company, U.S.A. A 1% solution was prepared according to the method of Chapman and Miller (1977). The purity of ribosomes was determined by electrophoresis. They had no significant protein contamination as judged from the radioactivity of the preparations from 35S-labeled ribosomes.

Preparation of EF-G and EF-T

Elongation factors T and G were prepared from P-100 fraction of the extract of E. coli (NH4)2SO4 cells according to the method of Gerson et al. (1971). To 22.3 ml of P-100 fraction (7.56 g of N2H4Cl were added (amount at 1 ml). Then 15 ml polyethylene glycol 300 (50% w/v) were added drop by drop followed by 5 ml of dextran 500 (25% w/v). 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 1M NaOH. Sodium sulfite (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-G and EF-T) were precipitated by the addition of 13.9 g of ammonium sulfate to saturation. EF-G and EF-T were successively eluted from the precipitate by extraction with solutions containing decreasing concentration of ammonium sulfate. It was extracted first with 27.8% (w/v) ammonium sulfate (three times), then 24% (w/v) ammonium sulfate (three times) and finally with 19.6% (w/v) ammonium sulfate (three times). The ammonium sulfate solutions contained 1 M dithiothreitol and their pH was adjusted to 7.8 with NaOH. The 24% ammonium sulfate eluate was used as the source of EF-T and 19.6% eluate as that of EF-G. The combined 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 dialyzed against the same buffer solution was separately placed on the top of a DEAE-cellulose column, equilibrated with buffer B and 0.4 M KCl and the column was washed with the same buffer. Elution factor was eluted with a linear gradient of KC1 (0.1-0.3 M) in buffer B. The fractions containing EF-G (or EF-G) activity were pooled and concentrated by the addition of ammonium sulfate (4.5 g per 10 ml pooled material). The mixture was stirred for 1 h and the precipitate was collected by centrifugation and dissolved and dialyzed against buffer B. The purity of the preparation was checked by polyacrylamide gel electrophoresis and both were found to be homogeneous.

Interconversion of LC and LC 50 s Ribosomes

LC 50 s ribosomes or LC 50 s ribosomes (200 pmol) along with equivalent amount of 35S-labeled ribosomes were treated at 25°C for 30 min with 1 nmol of EF-G and 13 nmol of GTP in total volume of 0.3 ml containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl (40 mM MgCl2 if only 35S-labeled ribosomes were treated). After 1 h reaction ribosomes were pooled separately and precipitated with ethanol, as described earlier. The purity of both the 35S-labeled ribosomes and subsequent analysis by sucrose gradient centrifugation at 4 °C was determined by radioactivity of the preparations from 35S-labeled ribosomes.
To study the conversion of LC to TC 50s ribosomes in the presence of EF-G, GTP and fusidic acid, either nonradioactive or 32P-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 that used in the incubation mixture. In case of radioactive ribosomes 40 pmol of LC 50s ribosomes (1.2x10^6 counts/min) were treated with 200 pmol of EF-G, 2.5 pmol of GTP and 2 pmol of fusidic acid, when the treatment with the mixture was carried out with GTP analogues (GMP(1P) or GMP(2P)) in a total volume of 0.2 ml of Tris-HCl pH 7.5 containing 100 mM HC1, 10 mM MgCl, 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 (10,000 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 KC1 and then slowly cooled to 25°C.

Assay methods for the conversion of LC 50s ribosomes to TC 50s ribosomes or 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 60) for the association. The solution containing LC or LC 50S ribosomes, was dialysed against Tris containing either 4 or 10 mM Mg++ and then mixed with equivalent 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 125 min at 120,000 g in 5 ml cups of SW 50.1 rotor of 50-S preparative ultracentrifuge of Beckman Instrument Co. Fractions (0.23 ml) were collected and absorbencies were measured at 260 nm in Zeiss UV spectrophotometer. When 32P-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 a Beckman Liquid scintillation Counter of LBK, Sweden.

Assay of 30S ribosomes as well as 23S RNA following heat treatment

50s ribosomes were dialysed against 100 mM triethanolamine-HCl, pH 7.5 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 scheme of Stahl and Marzluf (1976) by replacing 100 mM triethanolamine-HCl, pH 7.5 containing 10 mM Mg++ and then dissolved and dialysed against Tris containing 10 mM Mg++. Each preparation was mixed with equivalent amount of 30S ribosomes and subjected to sucrose density (5-30%) gradient centrifugation at 120,000 g for 135 min at 4°C in TCA containing 10 mM Mg++. Removal of kethoxal (if needed) was done by dialyzing the kethoxal-treated ribosomes at 37°C for 3 h against a solution pH 8.0 containing 0.013 M Tris, 10 mM Mg++ and 0.003 M HC1. For kethoxal treatment of heated LC 23S RNA it was first dialyzed against 100 mM triethanolamine-HCl, pH 7.5 containing 400 mM HC1 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 binding properties of 23S RNA isolated from kethoxal-treated 50S ribosomes or 23S RNA, directly treated with kethoxal, 4 mg/ml of radioactive RNA containing 2x10^6 counts/min of 200 pmol of nonradioactive RNA were mixed with equivalent amount of 16S RNA under reconstitution condition and subjected to sucrose density (5-25%) gradient centrifugation under reconstitution condition for 6 h at 96,000 rpm. Fractions were collected and counted or their absorbancies at 260 nm were measured as above.

Nuclease l 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 175,000 g for 4 h, dissolved and dialysed against Tris containing 10 mM Mg++. Then HC1 concentration was raised to 16 M and after keeping for 4 h at 4°C ribosomes were again pelleted, dissolved and dialysed against Tris. There was no loss of biological activity of untreated ribosomes indicating thereby that the ribosomes were not damaged by this treatment. Nuclease I degradation assay was carried out as described by Dalbo and Sveden (1972). A260 (4 units) were added to 16 x10^6 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 A260 was followed in PCR II Zeiss spectrophotometer.

Polyphenylalanine synthesis - Before carrying out polyphenylalanine synthesis, treated 30S ribosomes were freed from treating reagents as described above. For measuring the polyphenylalanine synthesizing activity according to the method of Jamba and Miller (1976) the incubation was carried out at 37°C for 30 min in a total volume of 0.17 ml of 20 mM Tris-HCl, pH 8.0, 20 mM HC1, 14 mM glycylglycine and 16 mM Mg++ which also contained 1.3 x10^9 units of 30S ribosomes along with equal amounts of 30S ribosomes, 10 μg of poly(U), 10 μg of EF-1, 8 μg of EF-G, 0.25 mM GTP and 100 pmol of [3H]-glutamic acid-2-UCA (100 counts/min/μg). The reaction was terminated by the addition of 3% 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 RNA - Thermal denaturation profiles of 23S RNA 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 holder. 23S RNA (0.5 μg/μl) unit per ml) was taken in 0.2 ml of 20 mM Tris-HCl, pH 7.5 containing 400 mM HC1 and heated from 35°C to 90°C (with a rate of 0.25°C/min) and the increase in absorbancy was followed with the help of an automatic recorder.

Circular dichroic spectroscopy of 23S RNA - Circular dichroic measurements of 23S RNA, isolated from LC 50S or TC 50S ribosomes and treated LC 23S RNA (2 μg/ml units/ml) were obtained in 20 mM Tris-HCl, pH 7.5 and 400 mM HC1 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).