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THERMODYNAMICS OF ASSOCIATION AND DISSOCIATION OF TIGHT COUPLE AND LOOSE COUPLE RIBOSOMES

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ABSTRACT: Association of ribosomal subunits is a prerequisite for the initiation of the protein synthesis. *In vivo,* this process is mediated by different protein factors and this phenomenon can be mimicked by Mg^{++}*in vitro.* Monovalent cations such as K^+ and NH_4^+ are known to affect the association and has been suggested to be involved in the fine tuning of the conformation of a particular region of 23S rRNA. Tight Couple(TC) and Loose Couple(LC) ribosomes require different concentration of Mg^{++} for their association and the two populations use different rRNA sites for their association. Magnesium dependent association of TC and LC ribosomes was studied using light scattering. These studies demonstrate that the association of TC and LC is reversible and in true equilibrium. The number of binding sites and association constants are different in these two populations and a decrease in temperature favors the association of subunits to a significant extent in case of LC. Effect of K^+ and NH_4^+ on the magnesium dependent association of TC and LC ribosomes was studied. In combination, K^+ and NH_4^+ increase the association of LC ribosomes to a significant extent to TC ribosomes in a temperature dependent manner. These studies strongly suggest that TC and LC ribosomes are distinct conformational states of elongating bacterial ribosome during protein synthesis.

KEYWORDS: Protein Synthesis, Ribosome, Translocation, Interconversion, TC and LC Ribosomes, Light scattering, Thermodynamics, Association-Dissociation.

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1.INTRODUCTION

Initiation of translation is the rate limiting step in protein synthesis involving the association of ribosomal subunits to form 70S couple. The two subunits of ribosome associate at the initiation step and dissociate during the termination. Association of the two subunits may be the result of the interactions between RNA-RNA, RNA-protein, or protein-protein or a combination of all the three. Of all these, RNA-RNA interaction has been strongly implicated [1] *In vivo*, 70S initiation complex forms through an intermediate 30S initiation complex which includes mRNA, initiator tRNA along with other initiation factors. Among the initiation factors, IF3 is known as anti-association factor as it inhibits the association of 30S and 50S subunits. The 30S initiation complex associates with 50S subunit resulting the formation of 70S initiation complex. Association of subunits lead to conformational changes in the structure of ribosome which is best exemplified by the creation of the aminoacyl site (A-site) upon association of 30S and 50S subunits [2]. *E. coli* 70S ribosomes can be dissociated into two subunits at low magnesium concentrations [3]. Ribosomes are isolated normally at high magnesium concentration (10mM) where the two ribosomal subunits remain associated. This magnesium dependent association of the subunits and their equilibrium *in vitro* depends on the ratio of the divalent to monovalent cations in the medium [4]. Under physiological conditions, 70S ribosomes are in dynamic equilibrium with their subunits and the equilibrium is towards 70S formation [5]. The initiation factors modulate this equilibrium [6]. The interface between the subunits plays a central role in the association [7]. In this study, thermodynamics of association of TC and LC ribosomes have been carried out using light scattering. These studies demonstrate that TC and LC ribosomes represent two distinct conformational states of ribosomes in *E.coli*.

2. MATERIALS AND METHODS

All chemicals used were of analytical grade. All stock solutions, except the ribosomal suspensions were filtered through nitrocellulose filters (Millipore, pore size, 0.45 micron) to eliminate dust particles which interfere with light scattering measurements.

2.1 Preparation of ribosomes**:** Ribosomes and then fractiontio of ribosomal population into TC and LC ribosomes were carried out as described earlier.[8]

2.2 Light Scattering Measurements**:** Light scattering measurements were carried with some modifications using Perkin Elmer LS-5B spectrofluorimeter equipped with thermostated cell holder. Maximum scattering was taken as 100% 70S formation. All measurements were made at room temperature. In case of measurements at fixed temperatures, the desired temperature was regulated to $\pm$ 0.5^0C by means of water circulating bath and the exact temperature in the cell was measured with a Digital Multimeter. The pH was held constant at 7.5.The excitation and emission monochromators were set at 400nm, with slit widths of 5nm respectively. A total volume of 0.5ml sample was placed in a cuvette (5 x 5 mm).

2.3 Measurement of 70S dissociation**:** Magnesium dependent dissociation 70S TC or 70S LC, was carried as described. Ribosome from a stock (250-300 A_{260}/ml) was diluted into 1ml of 20mM Tris-HCl, pH 7.5, containing 30mM ammonium chloride and 5mM β-mercaptoethanol and the final concentration of magnesium was varied from 1 to 30mM. Final concentration of ribosome in each reaction mixture was $4A_{260}$/ml. The reaction mixtures were allowed to equilibrate for 10min at the given temperature and the relative light scattering was determined. In case of the measurements in presence of monovalent cations, ribosome was diluted from the stock in 20mM Tris-HCl, pH 7.5 containing 5mM β-mercaptoethanol along with the desired concentration of the monovalent cation.

2.4Measurement of ribosomal subunit re-association: A stock of ribosome (250-300 A_{260}/ml) was diluted into 15ml of 20mM Tris-HCl pH 7.5, containing 30mM NH_4Cl and 5mM β-mercaptoethanol with 1mM magnesium acetate. In 1 ml aliquots, magnesium acetate was added in the range of 1mM to 30mM. Samples were allowed to equilibrate at the experimental temperature for 10min and the relative scattering was recorded. The final concentration of ribosome was $4A_{260}$/ml. Maximum scattering was taken as 100% 70S formation. . Ribosome concentration was determined by the absorbance at 260 nm with the value of 1 A_{260} corresponding to 67μg of 70S ribosome.

2.5 Calculation of association constant (K_{assoc}):Association constant, K was calculated as described by [11,Wyman (1964]. Equilibrium scheme for the magnesium dependent association of ribosomal subunits *in vitro* is described as:

$$30S + 50S + nMg^{++} \rightleftharpoons 70S \cdot Mg^{++}n \qquad (1)$$

$$K_{assoc} = \frac{[70S \cdot Mg^{++}n]}{[30S]\,[50S][Mg^{++}]^n} \qquad (2)$$

$$\log \frac{[70S \cdot Mg^{++}n]}{[30S]\,[50S]} = n \log [Mg^{++}] + \log K_{asso} \qquad (3)$$

where, n denotes the difference between the number of magnesium ions bound to the 70S ribosome as compared to that bound to the free subunits.. The distribution of 30S, 50S and 70S●$Mg^{++}{}_n$ is determined assuming that these three to be the only species present. Concentration of each species was calculated from the known total amount of all species present as determined from A_{260}. The free magnesium concentration is essentially equal to the concentration of magnesium ions added.

2.6 Calculation of Thermodynamic parameters: The standard free energyΔG) change at a given temperature is calculated from

$$\Delta G = -RT \ln K_{assoc}. \qquad (4)$$

where R = Gas constant (1.987 cal/mol/deg.K) and T= Temperature in Kelvin.

Standard enthalpy change (ΔHis calculated from vant Hoff's Plot as

$$\Delta H = -(\text{Slope value of vant Hoff's Plot}) \times R \qquad (5)$$

Standard entropy change,ΔS) was calculated as

$$\Delta S = \frac{\Delta H - \Delta G}{T} \qquad (6)$$

3. RESULTS AND DISCUSSION

A linear correlation between light scattering and ribosome concentration was observed(Fig.1)

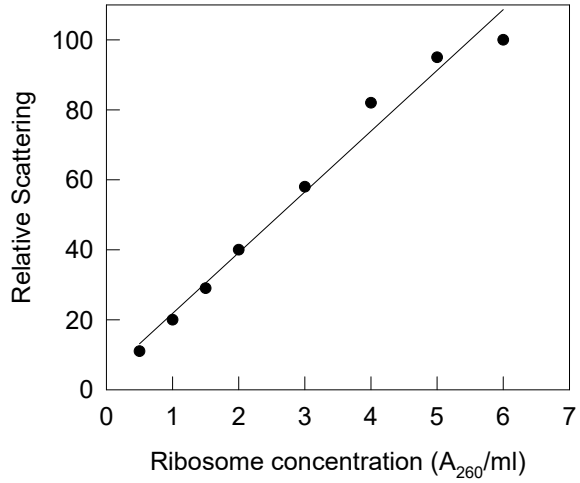

Fig. 1. Light scattering as a function of ribosome concentration.

3.1 Reversible association of the ribosomal subunits **:** Dissociation and re-association of 70S ribosomal subunits in case of TC and LC ribosomes was carried. LC and Tc ribosomes display true reversible association of their subunits.ribosomes (Fig.2) The transition in case of TC was sharp as compared to LC ribosomes. The concentration of magnesium where half of the population exists as 70S, was found to be 2.6mM and 8.4mM in case of TC and LC respectively indicating their distinct and different association dependence on magnesium ion.

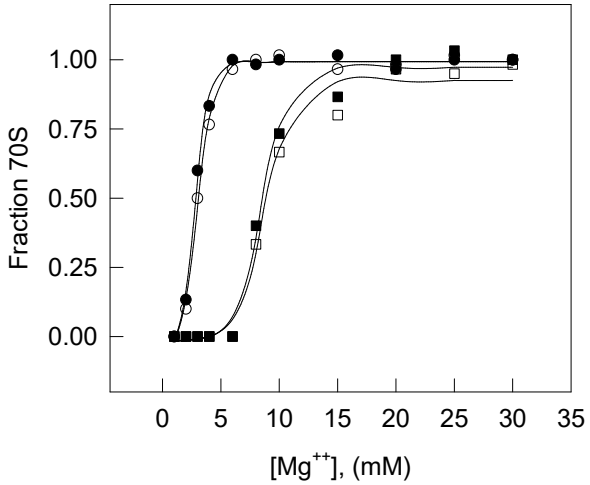

Fig. 2. Reversibility of subunit association in TC and LC ribosomes.

●TC Dissociation ○ TC Association

■LC Dissociation □LC Association

3.2 Concentration dependence of association equilibrium**:** Dissociation of 70S in case of TC and LC as a function of Mg^{++} at different concentration of the reacting species showed that an increase in the concentration of ribosome shifted $[Mg^{++}]_{50\%}$ to lower side (Fig.3). The shift of $[Mg^{++}]_{50\%}$ was from 3.0mM to 2.8mM in case of TC while 8.0mM to 7.8mM in case of LC.

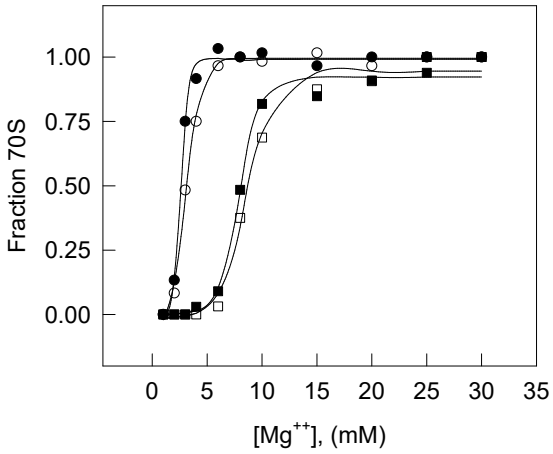

Fig. 3. Concentration dependence of association equilibrium.

●TC $4A_{260}$/ml □TC $2A_{260}$/ml

■LC $4A_{260}$/ml ■LC $2A_{260}$/ml

3.3 Effect of temperature on the association equilibrium of TC and LC: As tight couple and loose couple ribosomes are assumed to be different conformational states, the extent of their magnesium dependent association was expected to be different and temperature dependent. The magnesium dependent association of TC and LC ribosomes was thus studied as a function of temperature (Fig. 4). An increase in temperature favoured dissociation in both the populations of ribosomes. The titration curve broadens at both the ends significantly in case of loose couple while tight couple showed marginal broadening. However, loose couple seems to be significantly affected as compared to tight couple ribosomes. An increase in the steepness of the titration curve was observed, with the lowering of temperature in case of LC ribosomes. This was reflected in the values of $[Mg^{++}]_{50\%}$, which shifted to lower concentrations significantly in LC ribosomes, as the temperature decreased..

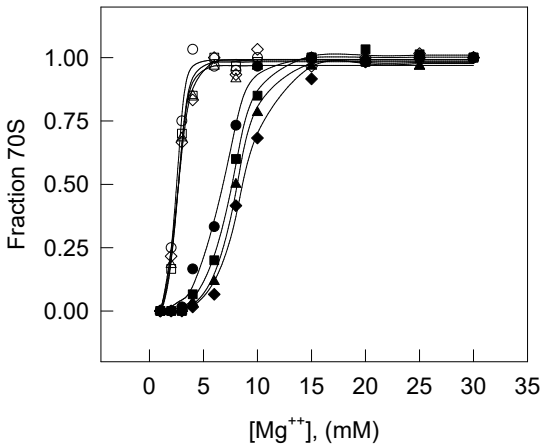

Fig. 4. Effect of temperature on the association equilibrium of TC and LC ribosomes. Dissociation of 70S TC and 70S LC was carried out as described in legend to Fig. 3. Fixed temperature measurements were carried out as described under methods.

●LC 10°C ■LC 15°C ▲LC 25°C ♦LC 37°C

○TC 10°C □TC 15°C ΔTC 25°C ◊TC 37°C

Hill Plot for TC and LC is shown in fig.5

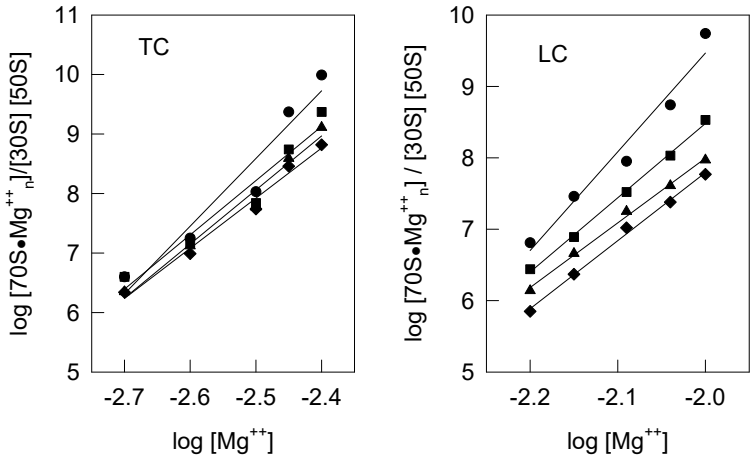

Fig. 5. **Hill Plot of association equilibrium of TC and LC ribosomes at different temperatures.**

●10°C ■15°C ▲25°C ♦37°C

From Hill plot, n, $K_{assoc.}$and transition midpoints were calculated (Table 1).

Table 1. Effect of temperature on association of TC and LC ribosomes

	$[Mg^{++}]_{50\%}$ (mM)		n		K_{assoc}	
	TC	LC	TC	LC	TC	LC
10	2.6	6.6	11.3	13.8	1058×10^{34}	1307×10^{34}
15	2.7	7.6	9.1	10.4	100×10^{29}	2×10^{29}
25	2.7	8.2	9.0	9.5	579×10^{28}	0.01×10^{28}
37	2.9	8.7	8.4	9.0	9.5×10^{28}	0.002×10^{28}

Value of K_{assoc} increased with decrease in temperature indicating that association of subunits is

favoured at lower temperature as opposed to an increase in the temperature, which favours dissociation. LC ribosomes displayed more increae in association constant value with decrease in temperature as compared to TC ribosomes. Number of binding sites for magnesisum (n**)** also increased with decrease in temperature. At lower temperatures, the association constants of tight and loose couple ribosomes are comparable but the number of binding sites **(n)** and requirement of magnesium ions for association are different. It may be pointed out that n, is an empirical value which denotes the difference between the number of magnesium ions bound to the 70S as compared to that bound to the free subunits. The difference in n suggest that loose couple and tight couple ribosomes undergo temperature dependent differential changes in their conformation. Thermodynamic parameters of association were also calculated using vantHoff 's Plot (Fig. 6) and summarized in Table 2.

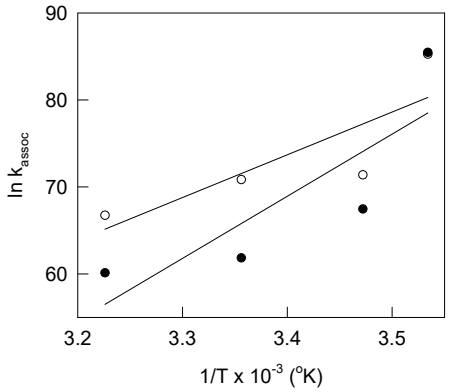

Fig. 6. vant Hoff's plot for the association equilibrium data of TC and LC ribosomes.

●LC ○TC

These parameters include change in standard free energy (ΔG), standard enthalpy (ΔH and entropy change (ΔS). Change in standard enthalpy was -97.6 kcal/mole in TC against a value of -141.9 kcal/mole for LC ribosome. The association seems to be significantly exothermic in case of loose couple ribosomes as compared to tight couple ribosomes which indicated substantial conformational change on association in loose couple than tight couple ribosomes. The standard entropy changes were -0.186 kcal/K/mole and -0.353 kcal/K/mole for TC and LC respectively. Lowering of temperature resulted in substantial decrease in ΔG in the case of loose couple ribosomes, although loose couple seems to have some sort of loose organization and appears to be more disordered than tight couple ribosomes. This disorder seems to be temperature dependent (Table 2).

Table 2. Standard free energy changes at different temperatures

Temp (^{o}C)	ΔG (kcal/mole)	
	TC	LC
10	-47.9	-48.0
15	-42.8	-38.6
25	-41.9	-36.6
37	-41.1	-35.6

Pre- and post translocational states of ribosomes have been reported to be separated by an activation energy barrier (Schilling-Bartezko et al., 1992).

3.4 Effect of Potassium and Ammonium ions on the association of TC and LC ribosomes: The monovalent ions, potassium and ammonium are known to stabilize tertiary structure of ribosomal RNA[9]. The *in vivo* concentration of these ions is about 150mM [10]. These two ions are equivalent due to their similar ionic radii. Effect of these ions on TC and LC ribosomes should be different if there is a difference in the conformation of these populations. Magnesium dependent association of TC and LC ribosome at three different concentrations of potassium chloride was studied[Fig. 7].

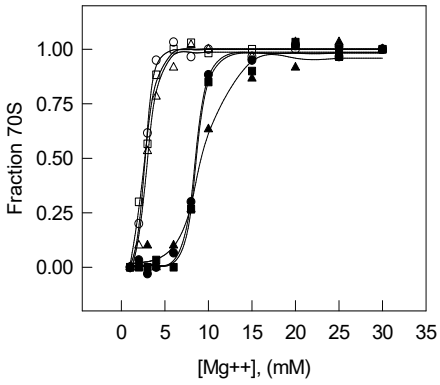

Fig. 7. Effect of potassium chloride on the association equilibrium TC and LC ribosomes.

●LC 60mM KCl ■LC 90mM KCl ▲LC 150mM KCl

○TC 60mM KCl □TC 90mM KCl ΔTC 150mM KCl

Decrease in **n** with increase in potassium chloride concentration may be due to the possibility of a competition between potassium and magnesium ions for binding sites on the ribosome (Table 3).

Table 3. Effect of Potassium Chloride on association of TC and LC ribosomes

KCl (mM)	$[Mg^{++}]_{50\%}$ (mM)		N	
	TC	LC	TC	LC
60	2.8	8.8	9.8	12.6
90	2.8	9.2	8.4	11.2

150	3.0	9.4	7.9	9.0

3.4 Effect of ammonium chloride on the association of TC and LC ribosomes was also studied. (Fig. 8). The titration curve broadens more in case of LC ribosomes. Table 4 presents the values of **n** and $[Mg^{++}]_{50\%}$ at different ammonium chloride concentrations. Behaviour of potassium and ammonium ions were found to be quite similar. Magnesium ion has different ionic radius than these two ions. Therefore potassium and ammonium ions seems to bind at their specific sites or they might be competing with magnesium ions at unspecific binding sites, instead of the sites specific for magnesium.

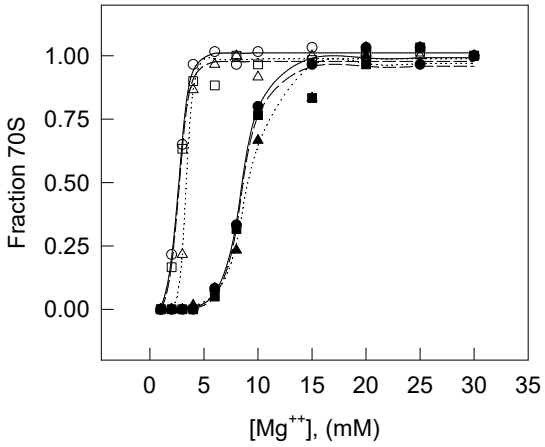

Fig. 8. **Effect of ammonium chloride on the association equilibrium of TC and LC ribosomes.**

●LC 60mM NH_4Cl ■LC 90mM NH_4Cl ▲LC 150mM NH_4Cl

○TC 60mM NH_4Cl □TC 90mM NH_4Cl ΔTC 150mM NH_4Cl

Table 4.Effect of Ammonium Chloride on association of TC and LC ribosomes

NH_4Cl (mM)	**$[Mg^{++}]_{50\%}$ (mM)**		**n**	
	TC	**LC**	**TC**	**LC**
60	2.8	8.7	10.1	11.9
90	2.8	9.0	8.8	10.4
150	2.9	9.4	8.0	8.6

It has been proposed that a concentration of 100mM potassium and ammonium ions along with magnesium is required for the proper conformation of a part of 23S rRNA [11- 12]. Thie part of 23S rRNA (1058-1108) has been proposed to be involved in the interaction of EF-G to ribosome and can adopt more than one conformation [13]. The conformational difference between TC and LC ribosomes has been assumed to be at the base of the stalk region of ribosomes, which encompasses the region where EF-G binds [14-15]. Magnesium ion titration in presence of 100mM potassium chloride and 100mM ammonium chloride were carried out in case of TC and LC ribosomes as a function of temperature (Fig. 9). Lowering of the temperature leads to an increase in the association of TC and LC ribosomes.

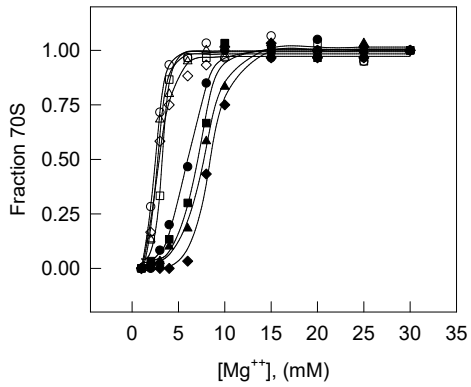

Fig. 9. Effect of 100mM ammonium chloride and 100mM potassium chloride on the association equilibrium of TC and LC ribosomes at different temperatures.

●LC 10°C ■LC 15°C ▲LC 25°C ♦LC 37°C

○TC 10°C □TC 15°C• ΔTC 25°C ◊TC 37°C

Double reciprocal plot for TC and LC ribosomes is shown in Fig. 10. and **n** as well association constant, $\mathbf{K_{assoc}}$were calculated [Table 5].

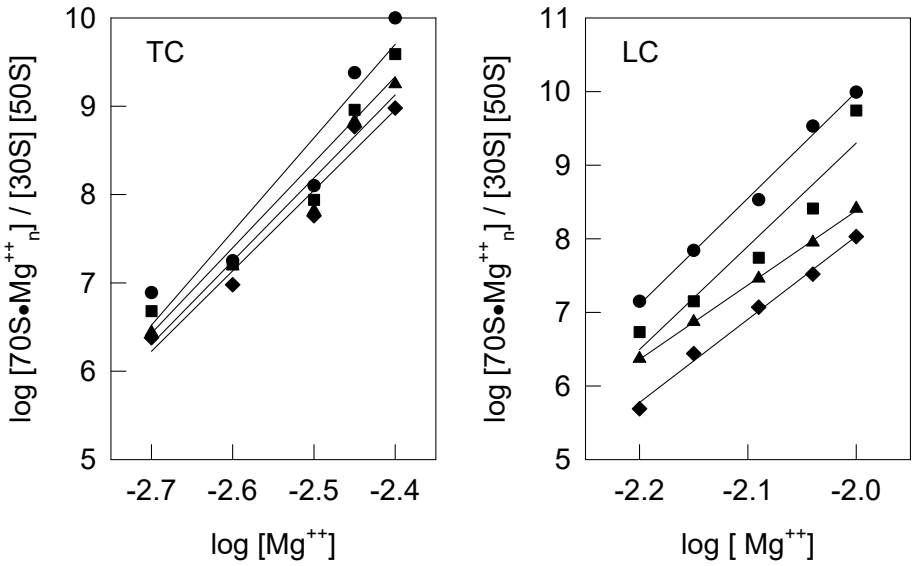

Fig.10. Lograthmic plot of the ratio of determined species versus magnesium concentration for the association equilibrium in presence of 100mM potassium chloride and 100mM ammonium chloride at different temperatures.

●10°C ■15°C ▲25°C ♦37°C

Table 5. Temperature dependent association of TC and LC ribosome in presence of 100 mMKCl and 100 mM NH_4Cl

Temp (^oC)	$[Mg^{++}]_{50\%}$ (mM)		n		K_{assoc}	
	TC	LC	TC	LC	TC	LC
10	2.5	6.0	10.6	14.4	1.36×10^{35}	700×10^{37}
15	2.7	7.1	9.7	14.0	3.67×10^{32}	2.2×10^{36}
25	2.7	7.8	9.4	11.3	4.9×10^{31}	0.4×10^{31}
37	2.8	8.5	9.1	10.1	6.79×10^{30}	0.03×10^{30}

The tight couple and loose couple ribosomes have comparable K_{assoc} constant but there is difference in **n** values. Standard enthalpy changes and standard entropy values of TC and LC ribosomes in the presence of potassium and ammonium were calculated as described. The standard enthalpy changes were -167.3 kcal/mole and -57.0 kcal/mole for LC and TC ribosomes as calculated from the vantHoff 's plot (Fig.11) respectively. Free energy changes at different temperatures[Table 6] showed that substantial lowering of free energy takes place with decrease in temperature in loose couple as compared to tight couple ribosomes.

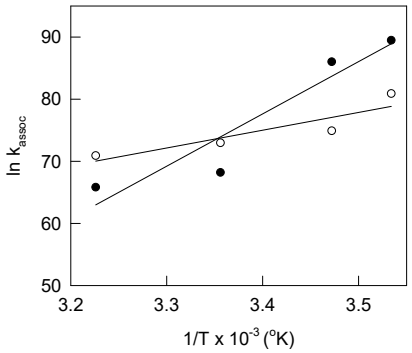

Fig. 11.vant Hoff's plot for the association equilibrium data [Table.5]

●LC ○TC

These difference in ΔH, ΔS suggest a conformational rearrangement or remodelling in LC ribosomes

Table 6. Standard free energy changes at different temperature in presence of 100 mMKCl and 100 mM NH_4Cl

Temp. (^oC)	ΔG (kcal/mol)	
	TC	LC
10	-45.4	-50.3
15	-43.9	-49.2
25	-43.7	-40.3
37	-43.2	-40.5

DISCUSSION

Ribosomes are molecular devices which synthesize proteins in the cell. The association of the two subunits during the initiation of translation is an important step. As ribosome is a multi-component nanomachine conformational flexibility is an essential requirement. The presence of magnesium ion is necessary for the structural integrity of ribosomes in case of *E. coli* and the actual role of magnesium is still not clear. Ribosomes are highly charged polyanions and the difference in the electrostatic free energies of 70S, 50S and 30S may contribute a large repulsive term to the free energy of the particle. Divalent cation is strongly bound most probably by anionic phosphodiester groups of the nucleic acid. Magnesium is several orders of magnitude more effective than monovalent ions in assisting the formation of specific secondary structure in nucleic acids which in turn determine the tertiary structure of RNA [16]. Loose couple ribosome requires at least 10mM Mg^{++} for association *in vitro* whereas tight couple ribosome require 4mM Mg^{++} to associate As ribosomal RNA has secondary and tertiary structures, all phosphate groups are not identical in their ability to interact with magnesium ions. Since **n** is greater in the case of LC ribosomes as compared to TC , LC ribosomes thus undergo differential conformational change in nature and degree relative to TC ribosomes. Besides, LC and TC ribosomes have been reported to have different conformations and use different sites of association [17-18, 8], The distinct and different association behaviour of tight couple and loose couple ribosomes in presence of 100mM potassium chloride and 100mM ammonium chloride suggest different degree of conformational rearrangement in TC and LC ribosomes. These observations suggests a possibility that there is some sort of conformational rearrangement in case of LC ribosomes as the temperature is decreased in presence of 100mM KCl and 100mM NH_4Cl. The pre- and post-translocational states of ribosomes are reported to be separated by high activation energy barrier and elongation factors reduce this activation energy barrier [19]. The double reciprocal plots are linear only in the range of magnesium concentrations, of 2-4mM in the case of TC and 6-9mM in case of LC ribosomes. The observed decrease in the $[Mg^{++}]_{50\%}$ with temperature may be due to the effect of temperature on hydrogen bonding and hydrophobic interactions which play a significant role at short distances. The notion that translating ribosomes undergo reversible conformational transitions between pre- and post- translocation states promoted by EF-G with GTP is known [20-22]. There are reports also suggesting that TC and LC ribosomes have distinct conformations[23-25] In conclusion, this paper clearly demonstrates that TC and LC ribosomes represent different discrete conformational states of bacterial ribosomes during protein synthesis.

4. CONCLUSION

In conclusion, this paper clearly demonstrates that TC and LC ribosomes represent different discrete conformational states of bacterial ribosomes during protein synthesis.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

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