Turbidimetric and potentiometric studies of ribosomal subunits from an erythromycin resistant mutant of *Escherichia coli*

J.S. Kliber, *G. Hui Bon Hoa, **M. Grunberg-Manago, [†]D. Pardo and [†]R. Rosset

Institut de Biologie Physico-chimique, Groupe U128, INSERM, 75005 Paris, France

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ABSTRACT

Turbidimetric and potentiometric techniques were applied to the analysis of an Ery^R mutant. Results show that in the mutant, the 30S subunits are drastically altered, as indicated by a higher Mg²⁺ requirement for subunit association and by an important difference in the titratable groups. Replacement of parental 50S by mutant 50S subunits does not decrease the association capacity with 30S parental subunits, but a structural difference is detected in the mutant 50S with potentiometric measurements. The mutation results in decreased ribosomal *in vitro* activities at 22°C including lowered polyphenyl-alanine synthesis, drastic altered initiation step and the loss of erythromycin binding to the ribosomes.

The results extend previous observation of a gene eryC part in the maturation of both subunits.

INTRODUCTION

Ribosomal mutants analysis has provided important additions to present knowledge of the ribosomes (1). Mutants selected or characterized by far altered function or defecting assembly have enabled the location of "ribosomal genes", and the definition of the precise function of several proteins (2) (3).

A great number of ribosomal mutants were first isolated as resistant to the antibiotics (erythromycin, spectinomycin, kasugamycin, streptomycin) ; some were found to be also "temperature sensitive" (4) (5) (6). Only rarely have the ribosomal mutants been used in studies of conformational alterations of the ribosomes (7).

The arrangement of molecules in the ribosomal particles have been best studied by a variety of chemical and physical methods (8) (9) (10) (11) (12). It seemed to us that a comparison of different ribosomal mutants and wild type ribosomes could provide important informations on the structure of the organelle.

Our present view is that ribosomes can adopt different conformations at every step of the translational process and we should thus be able to iso-

late temperature sensitive mutants defective in the performance of ribosomal functions and to correlate the altered activity with a specific structural defect.

In the present study, we have applied two physico-chemical parameters and found them convenient for the analysis of ribosomal mutants :

1) Light scattering is found to be a sensitive test to detect small structural changes in ribosomal subunit interaction (13).

2) The analysis of ribosomal particles as polyanions according to the method described by Kliber et al. (14), validate application of polyelectrolyte model to ribosomal subunits. In this model polyphosphate backbone of RNA can be considered in a rough approximation as cylindrical polyanions developing a strongly negative electrostatic potential Ψ . According to the polyelectrolyte theory (15) (16), this negative potential Ψ promoted within the polyanionic environment modifies the local proton concentrations as :

$$H^+ (local) = H^+_0 \exp (- \epsilon \Psi / kT) \quad (1)$$

where ϵ is the unit charge and where kT has its usual significance.

Then, any ionisable group located within this environment has a pK given by :

$$pK (\Psi) = pK_0 - 0.43 \epsilon \Psi / kT \quad (2)$$

Equation (2) allows to understand the titrimetric behavior of ribosomal subunits observed by Kliber et al. and to use this method to differentiate ribosomal particles presenting structural modifications at the level of the RNA and (or) on proteins.

The present study was performed with mutant 345-08 which is resistant to erythromycin and cold-sensitive. The mutation ery-08 affects eryC gene. In a previous paper (6), Pardo and Rosset found that the product of this gene appears to function in the maturation process of 23S and 16S RNA.

EXPERIMENTAL

1. Strains used

Strain 345 was isolated from E. coli K12 (AT2472) as spontaneous spectinomycin-resistant.

Strain 345-08 is a spontaneous selection of erythromycin-resistant mutant from strain 345 and shown to be cold-sensitive in vitro and in vivo. The mutational locus is in gene eryC which is linked neither to streptomycin region nor to any region in which ribosomal proteins genes have been located. Further characterization of strains 345 and 345-08 has been reported by Pardo and Rosset (17) (6) (18).

In this paper, the 30S and 50S ribosomal subunits are referred to as wt-30S, wt-50S for the parental strain 345 and m-30S, m-50S for the ribosomal subunits from the mutant 345-08.

2. Preparation of ribosomes

Ribosomes and S150 extract for in vitro assays were prepared according to the technique of Pardo and Rosset (18). For potentiometric titrations and turbidimetric measurements, the ribosomes were prepared by the method of Kurland (19) and kept in TSM buffer (Tris HCl, pH 7.8 ; 10^{-2} M ; $MgCl_2$ 10^{-2} M ; succinic acid 10^{-3} M). Subunits were prepared according to the technique of Staehelin and Maglott (20) and preactivated by heating 15 min at + 37°C in TSM buffer prior to use.

3. Ribosomal activities in vitro

a) The binding of ^{14}C -erythromycin in vitro was as described by S. Petska (21) with 50 mM KCl in all assays. Specific activity was 20,000 cpm per assay.

b) Poly U directed polypeptide synthesis employed the technique of Traub et al. (22) with the same S150 extract from the parental strain 345 as reference. Specific activity of ^{14}C -Phe was 129 m Curie/m mole, for each assay 0.5 μ Curie was used.

c) Binding of fMet-tRNA was according to Leder and Burzryn (23) with 3H -fMet-tRNA instead of ^{14}C -fMet-tRNA. Specific activity was 3,300 cpm/p mole.

4. Potentiometric titrations

The titration method has been described by Kliber et al. (14) using Tacussel instruments and a modified auto-burette system with a 0.25 μ l resolution of the reagent delivery. The sample cell was a 2 ml thermostated Pyrex vial containing a magnetic stirrer and was continuously flushed with nitrogen to exclude atmospheric CO_2 .

Radiometer GK 2321C semi-micro-combined electrode was used and the pH-meter was calibrated using Radiometer standard buffers with pH of 7.01, 4.00, and 9.21 at + 22°C ; and 6.97, 4.04, and 9.06 at + 42°C.

Titrant was 0.1 N to 0.2 N HCl and was obtained from Merck.

All titrations were obtained over the pH range 8.5 to 6 at + 22°C and + 42°C and were normalized at high pH ; the origin of bound H^+ was arbitrarily chosen as pH 8.5 and the curves plotted in terms of relative number of HCl or bound H^+ per mole of subunit as a function of pH for each magnesium concentration. Ribosomes suspensions and blanks were diluted in the following buffer : $1.5 \cdot 10^{-3}$ M Tris pH 7.8 ; $42.5 \cdot 10^{-3}$ M KCl ; $0.15 \cdot 10^{-3}$ M succinic acid ; $6 \cdot 10^{-2}$ M β -mercapto-ethanol. Concentration of ribosomal subunits varied from $9.3 \cdot 10^{-7}$ M to $3 \cdot 10^{-6}$ M. The extinction coefficients used were $\epsilon_{70S} = 4.15 \cdot 10^7$; $\epsilon_{30S} = 1.4 \cdot 10^7$; $\epsilon_{50S} = 2.75 \cdot 10^7$. The dilution of riboso-

mal solution after each titration was a negligible one. The accuracy of titration curves was about 10 %.

5. Turbidimetry

Equilibrium association curves were followed by turbidimetric measurements as described by Debey et al. (13) using an Aminco DW 2 spectrophotometer in dual mode with reference beam at 500 nm and measuring beam at 340 nm.

Ribosomal solutions were incubated 15 mn at 37°C in the assay buffer before measurements were performed. Reassociation of subunits from parental as well as mutant strains, was measured in 50 mM cacodylate pH 7, NH_4Cl 60 mM, by step-wise increases of magnesium concentration from 2 mM to 30 mM at a ribosomal concentration 30S + 50S of $7 A_{260}$ units/ml ($1.69 \cdot 10^{-7}$ M). The relative turbidity change of the parental strain 345 at 20 mM magnesium was taken as reference of fully associated 70S subunits ($\frac{I_{100} - I_0}{I_0} \approx 0.8$). The percentage of associated 70S particles formed at different magnesium concentrations (using the parental as well as the mutant strains) was determined by "r", the ratio of relative turbidity changes under the given conditions to the parental strain at 20 mM Mg^{2+} . The concentrations of magnesium yielding $r = 50\%$ association is defined as $(\text{Mg}^{2+})^{1/2}$.

6. Material used

^{14}C -erythromycin was a gift of Dr S. Pestka (21). f-Met-tRNA was prepared as previously described (24) by charging *E. coli* tRNA (General Biochemicals) with ^3H -methionine in the presence of formyl donor, and enzymatically discharging the non-formylated Met-tRNA in the presence of crude supernatant and pyrophosphate. Contaminating nucleotides were eliminated by gel filtration through a Sephadex G50 column.

The crude initiation factors were prepared as previously described (25) by dialysing the supernatant solution from the high salt-washed ribosomes against the following buffer : 0.01 M-Tris-HCl pH 7.5, 0.06 M- NH_4Cl ; 0.01 M MgCl_2 and 0.006 M- β -mercapto-ethanol). For titrimetry, magnesium chloride from Merck, was prepared in bidistilled water free of CO_2 . Potassium chloride and ammonium chloride were purchased from Merck and Prolabo.

RESULTS

I - Associations

a) Association of parental and mutant subunits

The equilibrium association between parental (wt-30S + wt-50S) and mutant (m-30S + m-50S) subunits were measured at 22°C and 42°C as function of magnesium concentration. The results (Fig. 1a) clearly demonstrate at

Figure 1 : Association equilibria curves of parental and mutant subunits in 50 mM cacodylate buffer pH 7, NH_4Cl 60 mM. The final concentration of stoichiometric mixture of isolated ^{14}C 30S and 50S was $1.69 \cdot 10^{-7}\text{M}$.

Figure 1a = association curves at non permissive temperature + 22°C.

Figure 1b = Association curves at permissive temperature + 42°C.

- mixture of wild type 30S and 50S.
- mixture of mutant 30S and 50S
- ▲-▲ mixture of wild type 30S and mutant 50S
- mixture of mutant 30S and wild type 50S.

22°C a displacement to higher Mg^{2+} concentration of the association curve of the mutant subunits as compared to the parental strain. At + 42°C (Fig.1b) the differences are partly compensated.

The magnesium concentrations yielding half association are for parental subunits (Mg^{2+}) $1/2 = 10$ mM at both temperatures and for mutant subunits, (Mg^{2+}) $1/2 = 20$ mM at + 22°C and 16 mM at + 42°C. It should be noted that the parental strain 345 is spectinomycin resistant which probably explains why its subunits require higher Mg^{2+} concentration for association than subunits from type A ribosomes as previously described by P. Debey et al. (13). Ribosomes from several antibiotic resistant mutants have been reported to show the association curves for their subunits displaced to high Mg^{2+} concentrations (27).

On the other hand we must note that ribosomes used in this work are prepared from a derivative of E. coli K12 and from E. coli MRE600 in the work of P. Debey et al. (13).

b) Subunits cross reactions

Replacement of mutant m-50S subunit by the parental wt-50S, did not correct the defect of associativity of the m-30S in figure 1a ; the dependance on Mg^{2+} of both association curves is similar. In contrast replacement of m-30S subunit by the parental wt-30S not only restored the associativity with m-50S at + 22°C, (Fig. 1a) but the (Mg^{2+}) 1/2 is displaced towards lower value, i.e (Mg^{2+}) 1/2 = 7 mM, compared to 20 mM with both mutant subunits and 10 mM with both parental subunits. The phenomenon is similar at + 42°C (Fig. 1b).

These experiments clearly indicate a defect in the mutant m-30S subunit. The associative result of the defect is slightly more pronounced at + 22°C than at + 42°C. This defect is probably structural as shown by the titration curves. In contrast, the m-50S subunit interacts more strongly with wt-30S than does the wt-50S, giving a value of (Mg^{2+}) 1/2 smaller than that obtained from parental wt-50S subunit. These associative differences might suggest structural differences which are best defined by other analytical procedures.

II - Titrations

Titration data at two temperatures are shown in figure 2 (+ 22°C) and figure 3 (+ 42°C). Two important differences are observed : first the shape of the curves and secondly the relative number of bound H^+ titrated over the whole pH range. The analysis of these data makes even more appear the difference by distinguishing 2 pH ranges : pH 8.5 to 7 and pH 7 to 6 (Table 1). All these aspects can be modulated by magnesium concentration, temperature and source of subunits.

a) Shape of the titration curves

The titration curves for the parental strain are quite flat over the whole pH range as previously observed by Kliber et al. (14) for ribosomes from E. coli MRE 600. In our data the pK of individual ionizable groups are not clearly resolved, except for the wt-50S subunit at + 42°C (Fig. 3b) where the pK can be estimated as around 8.2. The shapes of the titration curves for wild type and mutant subunits are markedly different. More titratable groups both below pH 7 and between pH 8.5 and 7 are shown for the m-30S than for the wt-30S subunits (Fig. 2a, 3a). On the contrary, the m-50S subunits show less titratable groups between pH 8.5 and 7 than the wt-50S sub-

Figure 2 : Titration curves of isolated parental and mutant subunits at + 22°C expressed in number of bound H^+ per mole of subunit in function of pH.

Figure 2 a = titration curves of isolated 30S subunits ; final concentration of wild type 30S was $1.8 \cdot 10^{-6}$ M and that of mutant 30S was $9.3 \cdot 10^{-7}$ M.

Figure 2 b = titration curves of isolated 50S subunits ; final concentration of wild type 50S was $4.73 \cdot 10^{-6}$ M and that of mutant 50S was $1.3 \cdot 10^{-6}$ M.

Ribosomes suspensions and blanks were diluted in the following buffer : $1.5 \cdot 10^{-3}$ M Tris pH 7.8 ; $42.5 \cdot 10^{-3}$ M KCl ; $0.15 \cdot 10^{-3}$ M succinic acid ; $6 \cdot 10^{-3}$ M β -mercapto-ethanol.

Ribosomes		22°C			42°C		
Subunit	Origin	pH ranges					
		8.5 - 7	7 - 6	8.5 - 6	8.5 - 7	7 - 6	8.5 - 6
30S	wt	100	92	192	160	104	264
	m	160	140	300	260	110	370
50S	wt	100	84	184	320	102	422
	m	60	120	180	250	120	370

Table 1 : Relative number of titratable groups over different pH range at 2 mM Mg^{2+} .

The results are expressed in number of bound H^+ per mole of subunit and are obtained from curves of Figures 2 and 3.

Figure 3 : Titration curves of isolated parental and mutant subunits at + 42°C expressed in number of bound H^+ per mole of subunit in function of pH.

Figure 3 a = titration curves of isolated 30S subunits ; final concentration of wild type 30S was $9.3 \cdot 10^{-7}$ M and that of mutant 30S was $8.6 \cdot 10^{-7}$ M. Figure 3 b = titration curves of isolated 50S subunits ; final concentration of wild type 50S was $3 \cdot 10^{-6}$ M and that of mutant 50S was 10^{-6} M.

Ribosomes suspensions and blanks were diluted in the following buffer : $1.5 \cdot 10^{-3}$ M Tris pH 7.8 ; $42.5 \cdot 10^{-3}$ M KCl ; $0.15 \cdot 10^{-3}$ M succinic acid ; $6 \cdot 10^{-3}$ M β -mercaptoethanol.

units (Fig. 2b, 3b) ; at + 42°C additional groups are found to titrate.

b) Relative number of titratable groups

The relative numbers of titratable groups over the whole pH range (8.5 to 6) as well as over the individual pH range (8.5 to 7) as given in table 1, at low magnesium concentration and both temperatures, show the greater difference of the m-30S subunits. Table 1 shows a 60 % increase at 22°C and 2 mM Mg^{2+} of groups titrated between 8.5 to 6, i.e 300 for m-30S and only 192 for wt-30S ; the contrast is smaller at + 42°C, only 40 % increase of titratable groups. These differences are appreciable in both individual

pH range at + 22°C, but are mostly found in the groups titrated between pH 8.5 to 7 at + 42°C.

In the case of 50S subunits, the data are less striking over the whole pH range (8.5 to 6) at + 22°C, and show only 12 % decrease of titratable groups at + 42°C for m-50S compared to wt-50S subunits. Differences are mostly found in the individual pH ranges where there are less titratable groups (60 instead of 100) between pH 8.5 to 7 and more (120 instead of 84) between pH 7 to 6 for m-50S (Table 1). At + 42°C the decrease of titratable groups between pH 8.5 to 7 is more appreciable. These differences are outside the experimental error range.

An increase of Mg^{2+} concentration to 15 mM at + 22°C does not change the number of titratable groups between the whole pH range from 8.5 to 6 neither in 30S nor in the 50S of parental strain, except at + 42°C for the 50S subunit (Table 1). These data are different with the earlier results obtained from *E. coli* MRE 600 ribosomes (28) ; in these previous experiments, the relative numbers of titratable groups per mole of subunit between pH 8.5 to 6, at 1 mM Mg^{2+} , 50 mM KCl and + 25°C are 97 for 30S and 76 for 50S subunits ; an increase of Mg^{2+} concentration to 10 mM increases these numbers of respectively 37 % and 117 %.

Activity cpm per 1 unit A_{260} of 70S	22°C		42°C	
	wt 345	mut. 345-08	wt 345	mut. 345-08
a) Binding of ^{14}C -erythromycin	1800	200	1800	1400
b) Polypeptide synthesis directed by poly U	500	100	1300	1300
c) Binding of (3H)-fMet-tRNA (initiation step)	2000	200	1000	200

Table 2 : *In vitro* ribosomal activities.

The results are expressed in cpm per 1 unit A_{260} of 70S ribosomes ($2.4 \cdot 10^{-8}$ M). Five experiments were performed for each assay, each with the same result. In each experiment we used 5 to 7 A_{260} of ribosomes. The results are expressed as activity for 1 A_{260} of ribosomes. In all experiments blanks, without ribosome, were subtracted.

a) cpm of ^{14}C -Erythromycin bound per 1 A_{260} of ribosomes

b) cpm of ^{14}C -poly Phe incorporated in TCA in soluble per 1 A_{260} of ribosome

c) *In vitro* assay of initiation step, cpm of (3H)-f-Met-tRNA bound per 1 A_{260} of ribosome.

III - Ribosomal activity in vitro

Three ribosomal activities were tested in vitro : binding of ^{14}C -erythromycin, poly U-directed polyphenylalanine synthesis and initiation complex formation. As can be seen in Table 2, at + 22°C the erythromycin binding is reduced 9 fold, the poly U dependent phenylalanine incorporated in insoluble TCA product decreased 5 fold and the poly AUG dependent fMet-tRNA binding is only 1/10 of what is observed with wild type ribosomes. At 42°C only the fMet-tRNA binding is affected, i.e is 1/5 of the parental activity. Binding of f-Met-tRNA to 70S ribosomes is more efficient at 22°C than at 42°C for the parental strain 345. We observed the same result with ribosomes from AT 2472 and D10. Under the conditions used in this work, initiation in vitro is maximum between 22°C and 32°C and decreases from 32°C to 42°C. Erythromycin binding and polyphenylalanine synthesis are both fully restored. Like some other classes of Ery^R mutants, ribosomes from 345-08 can bind the drug at permissive temperatures (33). However the initiation is not fully restored. Since initiation is dependent on the 30S subunit integrity, the results confirm that this subunit is altered.

DISCUSSION AND CONCLUSION

The results presented here demonstrate that light scattering and potentiometric titrations constitute sensitive tests to investigate ribosomal structural differences.

The ribosomes of the parental strain E. coli K12 345 used in this study are quite different from the strain E. coli MRE 600 previously investigated with similar techniques (13) (29) (14) (28). E. coli 345 is characterized by a higher Mg^{2+} requirement for the association of their subunits and more titratable groups between pH 6 and pH 8.5 (28). While increase of temperature from 22°C to 42°C affects the association and the number of titratable groups in MRE 600, it has no appreciable effect on the parental strain chosen to this study. It has already been reported that the subunit associativity is highly dependent on the strain and that some antibiotic resistant strains are characterized by ribosome equilibrium shifting to dissociation (27).

The mutation ery-08 drastically alters the 30S subunit. At low temperature (22°C), the m-30S poorly associates with 50S subunits of either mutant or wild type unless high Mg^{2+} concentration are present.

On the contrary, 50S subunits of this mutant do not show any decrease of associativity, they even appeared to be somewhat more active in this respect.

The structural differences between the mutant and wild type 30S are clearly demonstrated by titration groups. The difference is observed in the whole pH range from 8.5 to 6 suggesting that many titratable groups are involved. The mutant 30S have more titratable groups at either 22°C or 42°C (60 % increases or 100 groups in excess per ribosome). This probably is due to the existence of more unmasked groups on the surface of the protein molecules of the mutant 30S. In that pH range (pH 8.5 to 6) as shown by Tanford (30), imidazol and $\alpha\text{-NH}_2$ groups are the ones essentially titrated, suggesting that the difference lies in the titratable histidine groups of the mutant 30S subunit. This clearly indicates that the conformations of proteins are drastically changed in the mutant. Such changes might result from conformational changes of the ribosomal RNA altering the local electrostatic potential values (Ψ) and then, via equation (2) giving the pK of any ionizing group under the influence of Ψ as a foreseeable implication of the polyelectrolyte theory. These groups are positively charged at pH 7 (31) and therefore tend to decrease the net proton charge $\bar{Z}$ of m-30S subunit and as a consequence the electrostatic free energy ΔG_{el} . On the other hand, according to the conclusions of Wishnia et al. (26), increasing the concentration of Mg^{2+} cations will decrease the contribution of electrostatic repulsion to the free energy of activation which, in principle, ought to have promoted the association between ribosomal subunits. In the case of 30S from the mutant 345-08, these modulations are not sufficient to decrease the free energy of association ΔG_{ass} of the reaction $m\text{-}30S + m\text{-}50S \rightarrow m\text{-}70S$, suggesting that the non-electrostatic term ΔG_{other} (< 0) in the free energy of association $\Delta G_{ass} = \Delta G_{el} + \Delta G_{other}$ is dominant compared to the electrostatic term ΔG_{el} . This non-electrostatic free energy ΔG_{other} probably represents some intrasubunit modification, according to Wishnia et al. (26). The "non-associativity" of m-30S can tentatively be explained by an increase of the non-electrostatic term ΔG_{other} increasing the total free energy of association at pH 7 and as a consequence decreases the associativity of m-30S subunit. Activity test also confirms a structural defect in the 30S subunits.

For the 50S ribosomal subunits while at + 22°C there is not an appreciable difference in the total number of the titratable groups between pH 8.5 and 6, there is a notable difference for the titratable groups between pH 8.5 and 7. The mutant shows a decrease in the number of titratable groups between pH 8.5 to 7 which probably indicates less titratable histidine groups for m-50S subunit and a conformational difference between the two subunits. On the other hand, the increase of efficiency of interaction between

m-50S and the 30S subunits suggests that the conformational change of m-50S subunit possibly changes the favorable part of ΔG_{ass} , decreasing ΔG_{other} and shifting the association curve into a region of low Mg^{2+} concentration. But the exact relationship between associativity and conformation is probably more complicated and needs some direct and structural experiments.

These results obtained in vitro are in agreement with some previous observations in vivo (6) which indicate that the mutation affecting the eryC gene in the mutant 345-08 affects both ribosomal subunits. The present in vitro investigation further demonstrate that the main defect lies in the small subunit. Such a defect was unexpected in view of previous analysis in vivo (18) showing the mutant as resistant to erythromycin ; but recently Saltzman and Apirion (32) have shown specific interaction between 50S from an Ery^R mutant and 30S with altered S5 and S12 proteins. The 345-08 mutant was shown to be cold sensitive in vivo. At permissive temperature, 345-08 grows as well as the parent 345, either in liquid medium or on plate. In vitro however a raise in temperature only partially bridges the difference between the mutant and the wild type 30S.

The advantage of considering the ribosomal particles as polyanions consists in making it possible to explain the associative properties of both mutant 30S and 50S subunits and to demonstrate where the main conformational defect lies.

A question remains : what is the product of the eryC gene ? We retain two hypothesis : 1) The product of this gene is a ribosomal protein playing an important part in the assembly process of both subunits especially in the maturation of the 23S and 16S RNA. 2) The component altered is an external factor involved in the biosynthesis of both subunits. Further experiments are in progress to choose between the two hypothesis.

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*Institute de Biologie Physico-chimique, 13, rue Pierre et Marie Curie, 75005 Paris, France

** Institut de Biologie Physico-chimique, Service de Biochimie, 75005 Paris, France

[†] Centre de Biochimie et de Biologie Moléculaire, CNRS, 13009 Marseille, France

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