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Effects of ethidium bromide and berenil on protein synthesis

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Abstract. The effects of ethidium bromide, an intercalating dye and berenil, a nonintercalating dye on the biological activities of *Escherichia coli* ribosomes have been studied. Ethidium bromide treatment drastically reduced both enzymatic and nonenzymatic initiation complex formation, enzymatic as well as nonenzymatic binding of phenylalanyl tRNA, peptidyl transferase, GTPase as well as the overall protein synthesising activity as measured by the poly U-dependent polymerization of phenylalanine. On berenil treatment, however, only enzymatic formation of the initiation complex is marginally reduced. Other reactions are not markedly affected except the enzymatic phenylalanyl tRNA binding which is slightly decreased only at high Mg^{2+} concentration; the treated ribosome has lowered polymerizing activity at sub-optimal Mg^{2+} concentration (10 mM). Although it has already been shown in this laboratory that treatment with either dye leads to the unfolding of the structure of the ribosome, the present studies indicate that berenil treatment does not alter the structure of the ribosome drastically in contrast to ethidium bromide treatment.

Keywords. Ribosome; ethidium bromide; berenil; protein synthesis.

Introduction

Intercalating dyes like ethidium bromide, acridine orange, etc., are known to unwind the superhelical structure of DNA (Crawford and Waring, 1967; Waring, 1970). It has further been reported that berenil, a nonintercalating dye, can also unwind the superhelical structure of mitochondrial DNA (Rastogi and Koch, 1974). It has been shown in this laboratory (Suryanarayana and Burma, 1975) as well as by others (Stevens and Pascoe, 1972; Ballesta *et al.*, 1976) that ethidium bromide binds to the *Escherichia coli* ribosome most probably by intercalation into the

Abbreviations :

DNase, deoxyribonuclease; GTPase, guanosine triphosphatase; DTT, dithiothreitol; IF, initiation factor; EF, elongation factor; P_i, inorganic phosphate; PEP, phosphoenol pyruvate; poly U, polyuridylic acid; RNase, ribonuclease.

double-stranded regions of rRNAs in the ribosome. The binding of berenil to the *E. coli* ribosome has also been demonstrated in this laboratory (Sinharay *et al.*, 1977), although the mechanism of the binding is not known. Preliminary studies have indicated that the binding may not be through salt linkage (Burma *et al.*, 1978). It has also been demonstrated that the structure of ribosome is unfolded on treatment with ethidium bromide or acridine orange (Suryanarayana and Burma, 1975). Similar unfolding as measured by RNase I action was also observed on treatment of the ribosome with berenil (Sinharay *et al.*, 1977). In this respect, the mode of action of the intercalating and nonintercalating dyes appears to be the same. Ballesta *et al.* (1976) studied the effect of ethidium bromide treatment on the peptidyl transferase, GTPase (E.C. 3·1·5·1) and phenylalanine polymerizing activities of ribosomes. Therefore, it was of interest to compare the effects of treatment of ribosome with ethidium bromide and berenil on the various steps of protein synthesis. The objective of the studies was to find out whether the two dyes have similar effects on the function of the ribosome as they have on the overall structure of the ribosome. In the present studies, the phenylalanine polymerising activity as. well as the individual steps of protein synthesis as indicated below were measured: (i) enzymatic and non-enzymatic initiation complex formation, (ii) enzymatic and nonenzymatic binding of phenylalanyl tRNA, (iii) peptidyl transferase and (iv) GTPase activities.

Materials and methods

Materials

ATP, GTP, CTP and DNase (E.C. 3·1·4·5) were obtained from Worthington Biochemicals, Freehold, N.J., U.S.A. Poly U was the product of Miles Laboratories, Elkhart, Indiana, U.S.A. Ethidium bromide, GDP, and phosphoenol pyruvate (PEP) were procured from Sigma Chemical Company, St. Louis, MO, U.S.A. Berenil was a gift from H. Loewe of Farbweke Hoechst, A.G., Frankfurt, Germany. Sephadex G–25 and Dextran T-500 were supplied by Pharmacia Fine Chemicals, Uppsala, Sweden. Dowex-1 (Cl-) and Dowex-50 (H^+) were supplied by BioRad, Richmond, CA, U.S.A. Polyethylene glycol (Carbowax 6000) was purchased from Union Carbide Corporation, New York, U.S.A. National Biochemicals, Cleveland, Ohio, U.S.A., was the source of puromycin hydrochloride. PEP kinase (E.C. 2·7·1·40) was the product of Boehringer Mannheim, Germany. (^{14}C)-Phenylalanine and ^{32}Pi were purchased from New England Nuclear Corporation, Boston, Mass., and Bhabha Atomic Research Centre, Bombay, respectively. Millipore filter (0·45 μ) was of Millipore. Company, Bedford, Mass., U.S.A.

Methods

Ribosomes were prepared from *E. coli* MRE 600 (RNase I^-) by alumina grinding, DNase treatment and two washings with 1 M NH_4Cl (Datta and Burma, 1972). The supernatant after removal of ribosomes at 100,000 g was used for tRNA preparation according to the method of Zubay (1966). The crude initiation factors were prepared from 1 M NH_4C1 wash according to Dubnoff and Maitra (1971)

Elongation factor T (EF-T) and elongation factor G (EF–G) were partially purified and separated from each other by the method of Gordon *et al.* (1971). [^{14}C]-Phenylalanyl tRNA was prepared by charging tRNA with [^{14}C] phenylalanine in absence of other amino acids according to the method of Scott (1968). N-Acetyl-[^{14}C]-phenylalanyl tRNA was prepared by acetylating [^{14}C]-phenylalanyl tRNA according to the method of Haenni and Chapeville (1966). [γ-^{32}P] GTP was prepared by photo-phosphorylation as described by Nishizuka *et al.* (1968).

Treatment with ethidium bromide or berenil : Ribosomes (7 mg) in 1 ml of 0·01 M tris-HCl buffer, pH 7·4, 0·3 M KCl and 10 mM magnesium acetate were incubated with 0·8 mM ethidium bromide or berenil at 4° C for 16 h. Treated and untreated ribosomes were isolated by passing through Sephadex G–25 column. Under this condition, 140 moles of berenil and 200 moles of ethidium bromide were bound per mole of ribosome.

Enzymatic and nonenzymatic initiation complex formation : The initiation complex formation was measured according to the method of Ravel and Shorey (1971). The incubation was carried out at 25° C for 20 min in a mixture (0·1 ml) containing 0·05 M tris-HCl buffer, pH 7·6, 0·08 M KCl, 0·08 M NH_4C1, 5 mM dithiothreitol (DTT), 0·28 mg ribosome, 40 μg poly U, 24 pmol of N-acetyl [^{14}C]-phenylalanyl tRNA, varying concentrations of Mg^{2+} (as indicated) and with or without 100 μg crude initiation factors and 0·25 mM GTP. The complex formed was measured by millipore filter binding technique (Ravel and Shorey, 1971).

Enzymatic and nonenzymatic binding of phenylalanyl tRNA : The assay was carried out according to Ravel and Shorey (1971) with slight modifications. For the measurement of binding, 0·43 mg ribosome, 50 μg poly U, 21 pmol [14C]-phenylalanyl tRNA were incubated with (enzymatic) or without (nonenzymatic) 7 mg EF–T and 0·1 mM GTP in 0·25 ml of the buffer used in the assay of initiation complex. The incubation was carried out at 25° C for 20 min. The subsequent procedure was the same as for the assay of initiation complex formation.

Peptidyl transferase activity : The assay of peptidyl transferase activity was carried out by the puromycin reaction according to Igarashi *et al.* (1971), however, N-acetyl [^{14}C]-phenylalanyl tRNA was used as donor in place of phenylalanyl tRNA. Immediately before use, the initiation complex was formed as described above. The requisite volume of the reaction mixture was added to a solution (final volume 0·5 ml) containing 0·05 M Tris-HCl buffer, pH 7·6, 0·08 M KCl, 0·08 M NH_4C1, 5 mM DTT and 0·9 μmol of puromycin. After incubation at 25° C for 45 min, the puromycin derivative formed was extracted into ethylacetate and counted in the liquid scintillation spectrometer (Mark II of Nuclear, Chicago, U.S.A.

EF–G-dependent GTPase activity : The GTPase activity was measured as described by Nishizuka *et al.* (1968). The reaction mixture contained in a total volume of 0·25 ml, 0·05 M tris-HCl buffer, pH 7·4, 0·16·M NH_4C1, 12 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 5 nmol of [γ-^{32}P] GTP, 25 μg EF–G and 0·1 mg ribosome, After incubation for 10 min at 30° C, the released $^{32}P_i$ was complexed

with ammonium molybdate and extracted into isobutanol-benzene and the radioactivity measured.

Phenylalanine polymerising activity : The method of Wahba and Miller (1974) was followed with slight modifications. The reaction mixture contained in a total volume of 0·2 ml, 0·05 M Tris-HCl buffer, pH 7·6, 0·08 M NH_4CI, 14 mM 2-mercaptoethanol, different amounts of magnesium acetate (as indicated), 1 mM ATP, 0·2 mM GTP, 3 mM PEP, 10 μg PEP kinase, 0·3 mg dialysed S–100 protein (the supernatant fraction obtained during ribosome preparation by centrifugation of the extract at 100,000 g), 10 μg poly U, 40 μg ribosome, 550 μg tRNA and 12 nmol [^{14}C]-phenylalanine. The incubation was carried out at 37° C for 30 min. The radioactivity in the trichloroacetic acid precipitate was measured after collecting the precipitate on the millipore filter.

In all the assays described above, the values were corrected for the blank values which did not exceed 10% of the experimental values.

Results

Effects of ethidium bromide and berenil treatment on E. coli ribosomes

(*a*) *Enzymatic and nonenzymatic initiation complex formation* : The enzymatic and nonenzymatic (without IF1, IF2, IF3 and GTP) initiation complex formation with the treated and untreated ribosomes was measured at various Mg^{2+} concentrations (figure 1). As expected, the initiation complex formation is dependent on the concentration of Mg^{2+} and there is a small but significant amount of initiation complex formed in absence of initiation factors and GTP. Ethidium bromide treatment drastically reduces both enzymatic and nonenzymatic complex formation whereas berenil treatment has comparatively less effect on the initiation complex formation, specially he enzymatic one.

(*b*) *Nonenzymatic and enzymatic phenylalanyl tRNA binding* : The EF–T dependent as well as independent binding of phenylalanyl tRNA to both treated and untreated ribosomes was measured at various Mg^{2+} concentrations (figure 2). The enzymatic binding was found to be optimum at about 8 mM Mg^{2+} and the enzymatic and nonenzymatic binding became comparable at high Mg^{2+} concentration (15 mM). As in case of initiation complex formation, phenylalanyl tRNA binding capacities (enzymatic as well as nonenzymatic) of the ethidium bromide-treated ribosomes are very much reduced at the different Mg^{2+} concentrations studied. This is found not to be true in the case of berenil-treated ribosomes. The enzymatic binding is slightly affected only at high Mg^{2+} concentrations whereas the nonenzymatic binding appears to be somewhat stimulated under certain conditions.

(*c*) *Peptidyl transferase activity*: Varying amounts of initiation complex of ribosome with N-acetyl-[^{14}C]-phenylalanyl tRNA were used to measure peptidyl transferase by puromycin reaction (figure 3). Unfortunately, however, due to small amount of initiation complex formed by ethidium bromide-treated ribosome (figure 1) only limited amount of initiation complex could be added in this case,

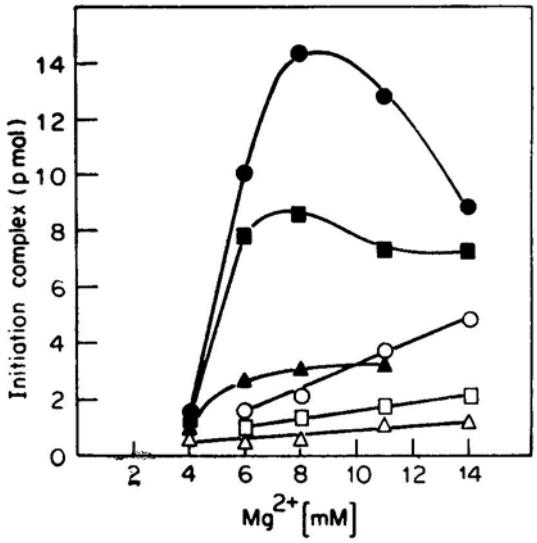

Figure 1. Effects of ethidium bromide and berenil treatments of the E. coli ribosomes on the initiation complex formation.

The methods of treatment of ribosomes with ethidium bromide and berenil have been described in the text. The initiation complex formation was followed according to Ravel and Shorey (1971), which has been described in the text.

Nonenzymatic : O untreated Δ ethidium bromide-treated ❑ berenil-treated

Enzymatic: ● untreated ▲ ethidium bromide-treated ■ berenil-treated

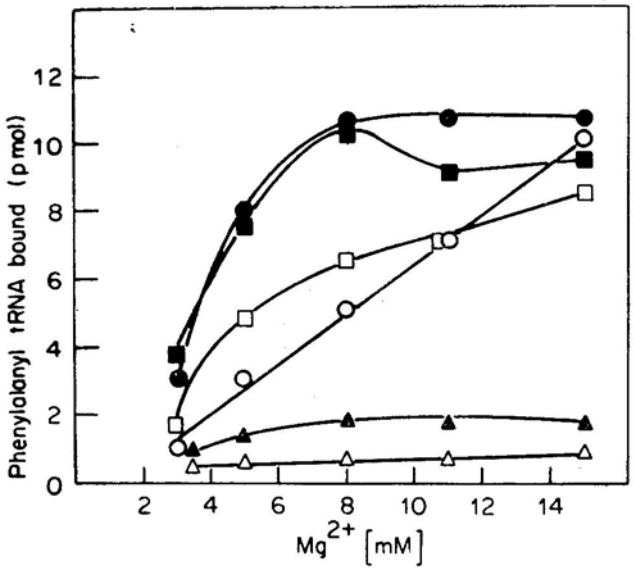

Figure 2. Effects of ethidium bromide and berenil treatments of E. coli ribosomes on the binding of phenylalanyl tRNA to ribosomes.

Symbols as in figure 1.

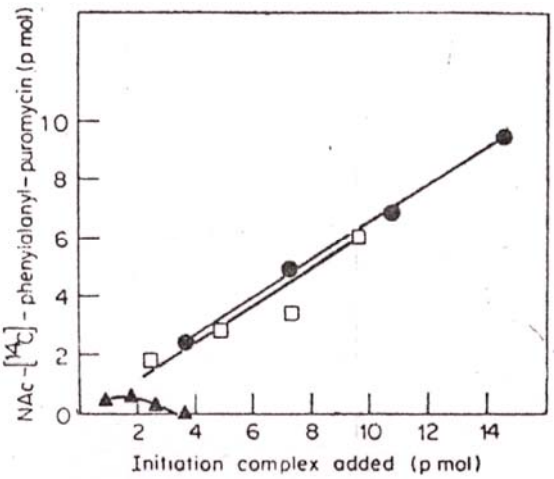

Figure 3. Effects of ethidium bromide and berenil treatments of *E. coli* ribosomes on the peptidyl transferase activity of ribosomes.

● untreated ▲ ethidium bromide-treated ■ berenil-treated

Very little peptidyl transferase activity could be measured under this condition. This is in agreement with the data presented by Ballesta *et al.* (1976). Berenil treatment, however, has practically no effect on the peptidyl transferase activity.

(*d*) *EF–G dependent GTPase activity*: EF–G dependent GTPase activity of the treated and the untreated ribosomes was measured at various Mg^{2+} concentrations (figure 4). The optimum concentration of Mg^{2+} was found to be around 12 mM. Ethidium bromide-treated ribosomes display one-half to one-third of GTPase activity. Ballesta *et al.* (1976) observed a gradual loss of GTPase activity upon treatment of ribosome with increasing concentrations of ethidium bromide. As in the earlier case, berenil treatment, however, has very little effect on the GTPase activity.

(*e*) *Polymerisation of phenylalanine* : The polymerising activity of the ribosome is very much dependent on the concentration of Mg^{2+} (figure 5). With the untreated ribosome, maximum activity is obtained at about 15 mM Mg^{2+}. Ethidium bromide-treated ribosome was found to have very low activity under optimum condition, as expected from earlier results. Berenil treatment has very little effect on the polymerising activity at optimum Mg^{2+} concentration (15 mM). At 10 mM Mg^{2+} concentration, however, the treated ribosome has half as much activity as the untreated ribosome.

Discussion

It is well established that the double helical structure of DNA is distorted due to intercalation of a dyelike ethidium bromide, between the base pairs (Crawford

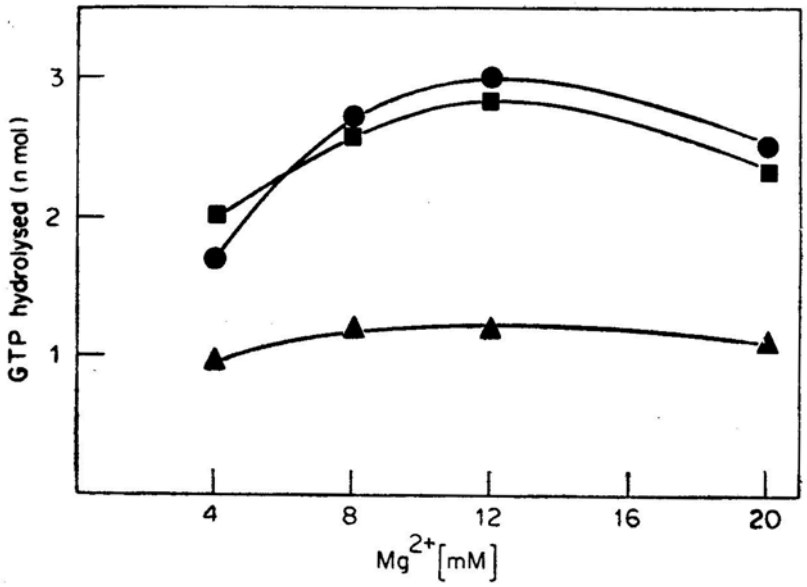

Figure 4. Effects of ethidium bromide and berenil treatments on GTPase activity of ribosomes.

Symbols as in figure 3.

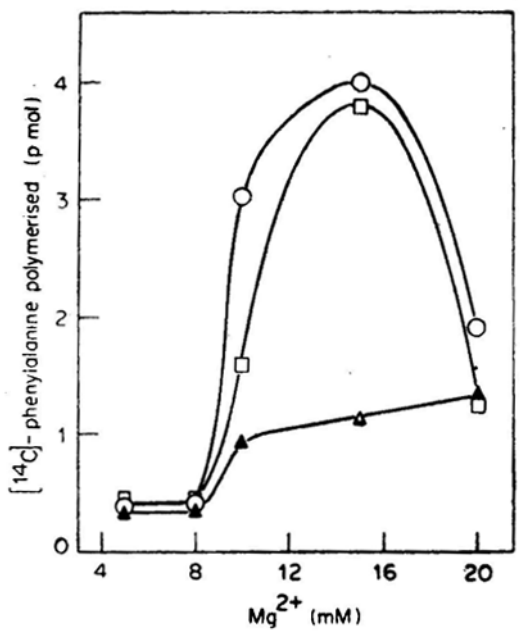

Figure 5. Effects of ethidium bromide and berenil treatments on the polymerization of phenylalanine by ribosome. (Results of this figure were presented at the Int· Symp. on "Biomolecular structure, conformation, function and evolution", Madras, Burma *et al*., 1978).

Symbols as in figure 3.

and Waring, 1967). This is also likely to happen if the dye intercalates into the double stranded regions of rRNA. The conformational change induced by the drug may lead to the weakening of the interaction between rRNAs and proteins and concomitant unfolding of the structure of the ribosome (Suryanarayana and Burma, 1975). This might explain the release of some proteins from the 70S ribosome on treatment with ethidium bromide and subsequent decrease in peptidyl transferase, GTPase and phenylalanine polymerising activities as observed by Ballesta *et al.* (1976) and also confirmed in this paper, although the present experiments have been done under somewhat different conditions. Ballesta *et al.* (1976) performed the experiments in presence of the dye while in our experiments the presence of free dye was avoided as far as possible. Further, in the present investigations, the initiation complex formation as well as the binding of phenylalanyl tRNA to the ribosome was also found to be considerably affected. Berenil treatment did not bring about such an effect. The initiation complex formation and the binding of phenylalanyl tRNA are only partially affected whereas peptidyl transferase and GTPase activities practically remain unaffected on treatment with berenil. The overall protein synthesising activity of the ribosome was also affected on treatment with ethidium bromide as observed by Ballesta *et al.* (1976) whereas the effect of berenil treatment depends on the concentrations of Mg^{2+}. At optimum Mg^{2+} concentration (15 mM) there is very little effect.

The mechanism of binding of berenil to the ribosome is not known. Although the release of one or two proteins cannot be completely ruled out under such a condition, the less drastic effect on the steps of protein synthesis indicates that the conformational change induced in the ribosome due to binding of the dye might be responsible for the reduced initiation complex formation and slightly decreased binding of phenylalanyl tRNA at high Mg^{2+} concentration. The steric hindrance caused by the bound dye to the functional proteins (or rRNA chains) may also be responsible for these effects. Further studies are in progress to understand the mechanism of binding of berenil to the ribosome.

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