ACTION OF METHANOL ON THE ASSOCIATION OF RIBOSOMAL SUBUNITS AND ITS EFFECT ON THE GTPase ACTIVITY OF ELONGATION FACTOR G

J. Voigt and A. Parmeggiani

Gesellschaft für Molekularbiologische Forschung
3301 Stöckheim, Germany

Received April 16,1973

Summary: Methanol causes association of 30S and 50S ribosomal subunits from E. coli at $MgCl_2$ concentrations in which they are normally completely dissociated. The 70S ribosome formed under these conditions shows a lower sedimentation velocity and is functionally active in the EF-G GTPase. Association of ribosomal subunits in the presence as well as absence of methanol is affected by washing the ribosomes with 0.5 M NH_4Cl. Methanol reduces the Mg^{2+} concentration required for subunit association as well as for EF-G GTPase activity. The basic requirement for EF-G GTPase activity both with and without alcohol is shown to be the association of 30S and 50S subunits.

Since the work of Monro et al. (see 1) first pointed out the peculiar properties of organic solvents on the peptidyl transferase activity of bacterial ribosomes, the effect of alcohol on the other components involved in polypeptide chain elongation has been extensively investigated. Ribosomes extracted with ethanol and NH_4Cl have been found inactive in the reactions associated with the elongation factors G (EF-G) and T (EF-T); activity can be restored by addition of the 50S proteins L7 and L12 (2-5). Ballesta et al. have reported that methanol stimulates the ribosome-dependent GTP hydrolysis of EF-T (6) and the EF-G-dependent GTPase activity of their CsCl α- and β-cores from 50S subunits (7). Hamel and Nakamoto observed stimulation of EF-T GTPase activity and EF-G-dependent GTP binding to ribosomes by methanol while the "turnover cleavage" of GTP was mildly inhibited (8,9). A lack of information still exists, however, about the action of alcohol on association of the ribosomal subunits and its consequent effect on the functions of ribosomes. Starting from the observation that methanol induces EF-G-dependent GTPase activity at $MgCl_2$ concentrations normally too low for this activity, we have examined the behaviour of ribosomes in the presence of methanol in order to explain some aspects of the ribosome-dependent GTP hydrolysis of EF-G.

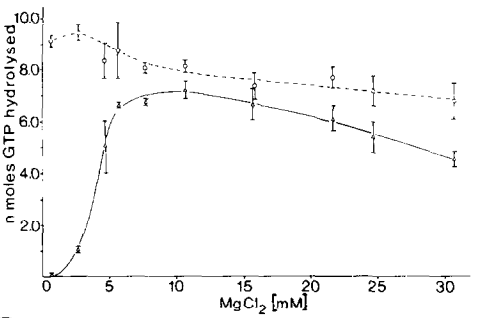
MATERIALS AND METHODS

Pure EF-G and ribosomes from E. coli B $T2^r$ or A 19 were prepared essentially as described (10). Ribosomes were either 2- or 5-times washed (see legends) with 0.5 M NH_4Cl - 20 mM Tris-HCl (pH 7.8) - 10 mM $MgCl_2$ - 60 mM KCl - 7 mM 2-mercaptoethanol. Ribosomal subunits were isolated by sucrose density gradient centrifugation at 0.5 mM $MgCl_2$ using a Spinco 15 Ti zonal rotor. The 30S were 98% pure and the 50S 96-97%, as measured by sedimentation in analytical sucrose density gradients. Poly(U)-dependent polyphenylalanine synthesis and poly(U)-dependent phe-tRNA binding indicated that the 50S were contamined with 4-6% 30S active in these reactions. EF-G-dependent GTPase activity of the 50S subunits was, however, not corrected for this contamination because the functional heterogeneity of the 30S subunits (11, 12), caused mainly by the purification procedure, indicates that the contamination as measured by these methods does not represent the total contamination with 30S subunits active in the EF-G GTPase reaction (13). γ-^{32}P-GTP was prepared by the method of Glynn and Chappell (14,15) and purified on DEAE-cellulose in bicarbonate form with a gradient of triethylammonium bicarbonate buffer. The 75 µl GTPase reaction mixture contained: 7.5 µmoles Tris-HCl, pH 7.8; 6 µmoles NH_4Cl; 1 µmole 2-mercaptoethanol; 25-32 nmoles γ-^{32}P-GTP (5-30 Ci/mole); 1-2% glycerol; $MgCl_2$, ribosomes, EF-G and methanol as indicated in the single experiments. After incubation for 10 min at 30°C, the GTPase activity was measured as the amount of $^{32}P_i$ liberated (16). Analytical density centrifugations were done either in 7-30% or 7-25% linear sucrose gradients containing 20 mM Tris-HCl, pH 7.8; 50 mM KCl; methanol and $MgCl_2$ as indicated in the legends. Protein determinations were done according to Lowry et al. (17) using crystalline bovine serum albumin as standard.

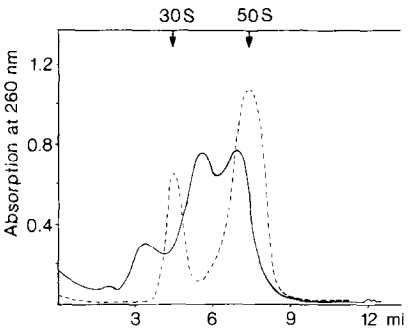
RESULTS AND DISCUSSION

Effect of methanol on the $MgCl_2$ optimum of the ribosome-dependent GTPase activity of EF-G

As Fig. 1 illustrates, methanol caused the ribosome-dependent EF-G GTPase to be active at unusually low $MgCl_2$ concentrations. In the presence of 20% methanol approx. maximum GTPase activity occurred already at 0.5 mM Mg^{2+}. Methanol strongly stimulated this activity and abolished its sigmoidal characteristic at $[Mg^{2+}] < 5$ mM. No striking effect was observed between 5 mM and 20 mM $MgCl_2$ which is the range for optimum EF-G GTPase activity in the absence of alcohol. Because GTPase activity induced by methanol is completely abolished by an excess of EDTA over Mg^{2+}, some Mg^{2+} must still be required even in the presence of alcohol. As shown in a later section of this paper, maximum activity in the presence of methanol becomes lower than the control if more EF-G than ribosomes is present in the assay.



1



2

Fig. 1. Effect of methanol on the $MgCl_2$ optimum of the EF-G-dependent ribosomal GTPase. The 75 µl reaction mixture contained: 2.5 A_{260} units of twice NH_4Cl-washed 70S ribosomes (62.5 pmoles); 1.0 µg EF-G (12 pmoles); 25 nmoles γ - ^{32}P-GTP; plus (o---o) and minus (Δ——Δ) 20% (v/v) methanol. Other conditions were as described in Methods.

Fig. 2. Effect of methanol on the association and sedimentation behaviour of ribosomal particles. 7.5 A_{260} units of twice NH_4Cl-washed 70S ribosomes were diluted into 100 µl of 20 mM Tris-HCl (pH 7.8) - 0.5 mM $MgCl_2$ - 50 mM KCl without or with 20% (v/v) methanol. After incubation at 30°C for 10 min the solutions were layered on 12.5 ml gradients of 7 to 25% sucrose in the same buffer with (———) and without (- - - -) 20% (v/v) methanol, and spun at 38,000 rpm for 210 min. The gradients were then analysed on an ISCO density gradient fractionator model 680.

Effect of methanol on the association of ribosomal subunits

The ability of methanol to induce ribosome-EF-G GTPase activity at $MgCl_2$ concentrations which cause dissociation of the 70S ribosome prompted us to test whether methanol affects the state of association of the ribosomal subunits. As illustrated in Fig. 2, three different ribosomal particles with the apparent sedimentation coefficients of 47S, 38S and 23S were observed in sucrose density gradients containing 20% methanol and 0.5 mM $MgCl_2$ while the control experiments without methanol showed only 30S and 50S subunits. To identify the particles observed in the presence of methanol, the different ribosomal fractions were sedimented at 105,000 g, dissolved in 20 mM Tris-HCl (pH 7.8) - 10 mM $MgCl_2$ - 30 mM KCl - 30 mM NH_4Cl and centrifuged in a sucrose-gradient in 15 mM $MgCl_2$ without methanol. The 47S fraction sedimented as 70S

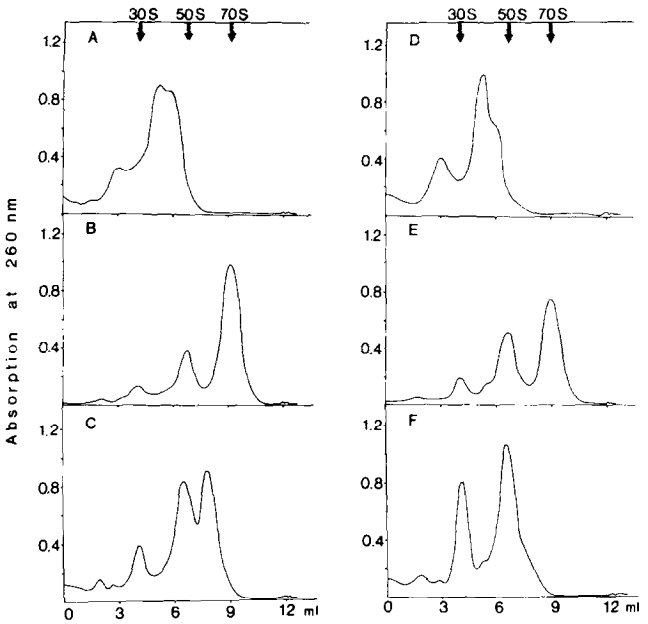


Fig. 3. Effect of NH_4Cl-washing on the association behaviour of the ribosomal subunits at different [Mg^{2+}] in the presence and absence of methanol. A,B,C: ribosomes twice washed; D,E,F: 5-times washed ribosomes. A and D: 0.5 mM $MgCl_2$ and 20% methanol; B and E: 15 mM $MgCl_2$ without methanol; C and F: 5 mM $MgCl_2$ without methanol. In each experiment, 7.5 A_{260} units of 70S ribosomes were diluted into 100 µl of a solution containing 20 mM Tris-HCl (pH 7.8); 50 mM KCl; $MgCl_2$ and methanol as already described. Other experimental conditions as in Fig. 2 except for the sucrose gradient (7 to 30%).

ribosomes while the 23S and 38S fractions corresponded to 30S and 50S subunits, respectively. Moreover, on sucrose gradients in 0.5 mM $MgCl_2$ the 47S fraction dissociated into 30S and 50S subunits. These control experiments are not illustrated.

The ability of ribosomal subunits to associate either in the presence of 15 mM $MgCl_2$ or in the presence of 0.5 mM $MgCl_2$ and 20% methanol was affected in a similar way by repeated NH_4Cl-washes. Sedimentation in sucrose gradients showed that three additional washes with 0.5 M NH_4Cl increased the amount of subunits present in both conditions (Fig. 3A, B, D and E) and practically eliminated the 70S ribosomes at 5 mM $MgCl_2$ (Fig. 3C and F). NH_4Cl-washes seem, therefore, to affect ribosomal components which play an essential role in the formation of the 70S ribosome in the absence as well as presence of methanol. As Fig. 4 illustrates, the $MgCl_2$ optimum for EF-G-dependent GTPase

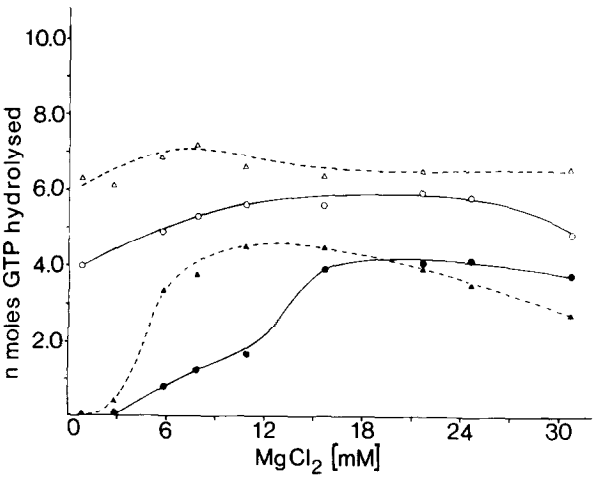


Fig. 4. Effect of washing with 0.5 M NH_4Cl on the $MgCl_2$ optimum of the EF-G-ribosome GTPase activity tested in the presence or absence of 20% (v/v) methanol. The 75 µl reaction mixture contained: 1.25 A_{260} units of 70S ribosomes (31 pmoles); 2.0 µg EF-G (24 pmoles); other conditions as described in Methods. Twice washed ribosomes tested in the absence (▲---▲) or presence (△---△) of 20% methanol; 5-times washed ribosomes tested in the absence (●——●) or presence (○——○) of 20% (v/v) methanol.

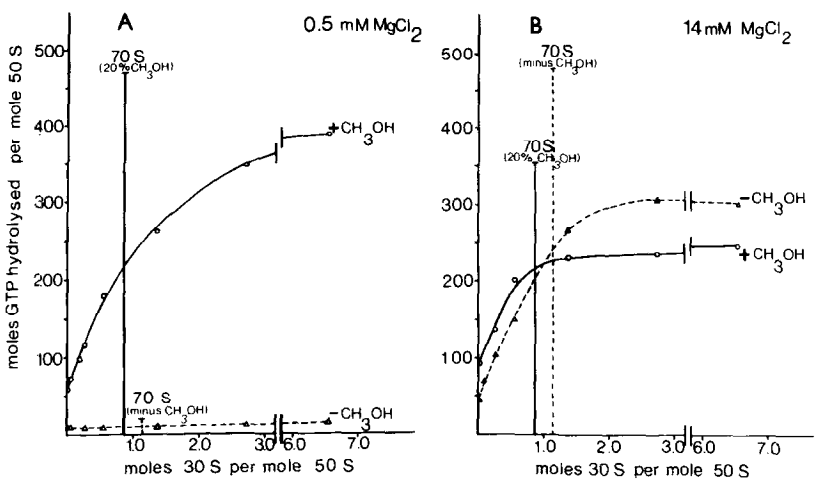


Fig. 5. Dependence of the EF-G GTPase on the concentration of 30S subunits in the presence and absence of 20% methanol. The 75 µl reaction mixture contained: 0.5 A_{260} units of 50S ribosomal subunits (20 pmoles); 8.0 µg of EF-G (96 pmoles); 32 nmoles ^{32}P-GTP; 30S subunits and $MgCl_2$ as indicated. Other conditions as described in Methods. Results are not corrected for the approx. 5% contamination of the 50S subunits by 30S which are active in the poly(U)-dependent binding of aminoacyl-tRNA. A: 1.4 mM $MgCl_2$; B: 14 mM $MgCl_2$; without (△---△) and with 20% (v/v) methanol (○——○).

activity both with and without alcohol is shifted to higher concentrations by repeated NH_4Cl-washes.

Role of the ribosomal subunits in the EF-G-dependent GTPase

Although the described experiments suggest that formation of 70S ribosomes is fundamental for EF-G-dependent GTPase activity, the extent of participation of 50S subunits alone in this reaction, particularly in the presence of methanol, remained to be investigated. To avoid effects due to functional heterogeneity of the 30S subunits, the EF-G-dependent GTPase activity was tested in the presence of increasing amounts of 30S subunits at different $MgCl_2$ levels with and without 20% methanol. An excess of EF-G over 50S subunits was used to improve the reaction velocity and, therefore, the accuracy of the activity measurements at low concentrations of 30S subunits. As shown in Fig. 5, methanol was found at 14 mM $MgCl_2$ to reduce both maximum activity and the molar ratio of 30S to 50S subunits required for maximum activity. The reduction in maximum activity which took place also in the 70S control was observed only when EF-G was in excess over ribosomes (compare with Fig. 1. where [EF-G] / [ribosomes] = 0.2). It is presently under investigation whether this difference caused by changing the ratio of EF-G to ribosomes depends on a reduction in the rate of exchange of EF-G between ribosomes which under normal conditions takes place at each round of GTP hydrolysis (18). The amount of 30S required for maximum activity depends on the number of NH_4Cl-washes and density gradient centrifugations carried out for isolation of 70S ribosomes and their subunits, and can be much higher than indicated. At 14 and 32 mM $MgCl_2$ (the results of the latter concentration are not shown) the 50S activity in the absence of alcohol represented only 10 to 15% of the maximum activity and was stimulated about two-fold by methanol (Fig. 5a). At 0.5 mM $MgCl_2$ in the presence of methanol, about 15% of the maximum activity was obtained without addition of 30S. The activity of the 50S subunit alone is certainly much smaller than these values

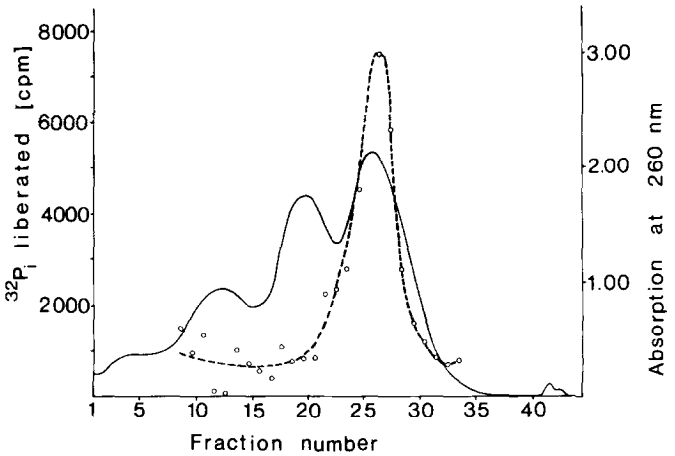


Fig. 6. EF-G-dependent GTPase activity of the ribosomal particles at 0.2 mM $MgCl_2$ in the presence of methanol. 15 A_{260} units of twice NH_4Cl-washed ribosomes (375 pmoles) were diluted into a 300 µl solution containing 20 mM Tris-HCl (pH 7.8); 50 mM KCl; 0.5 mM $MgCl_2$ and 20% (v/v) methanol. After incubation at 30°C for 10 min the solution was layered on a 12.5 ml gradient of 7 to 25% sucrose in the same buffer and spun at 38,000 rpm for 270 min in a SW 40 Ti rotor. Fractions were collected with an ISCO density gradient fractionator model 680 and 25 µl aliquots assayed for EF-G-GTPase activity. The 75 µl reaction mixture contained: 16 µg of EF-G (192 pmoles); 27.5 nmole ^{32}P-GTP (spec.act., 29 Ci/mole); 20% (v/v) methanol and 0.2 mM $MgCl_2$. Other components as described in Methods. In this case the reaction was allowed to proceed for 30 min. EF-G GTPase activity (o---o); absorption at 260 nm (———).

which neglect any correction for contaminating 30S subunits (see Methods). The importance of the association of the 30S and 50S subunits for EF-G-dependent GTPase is illustrated by the sucrose gradient of Fig. 6, which shows that the EF-G-dependent GTPase activity is essentially catalyzed by the 70S ribosome also in the presence of methanol at low $[Mg^{2+}]$.

The results presented in this paper demonstrate that methanol influences the state of association of the ribosomal subunits mainly at low $[Mg^{2+}]$ where ribosomes are otherwise dissociated. Methanol acts by reducing the $MgCl_2$ concentration required for association of the 30S and 50S subunits as well as for EF-G GTPase activity. The 70S ribosome induced by methanol at low $MgCl_2$ supports the GTPase activity of EF-G. At the same time, methanol stimulates the activity of the 50S alone. However, if the contaminating 30S are taken into account, this activity represents only a minor fraction of

the maximum activity reached in the presence of saturating amount of 30S subunits. Moreover, this stimulation is overemphasized in the case of an excess of EF-G over ribosomes by the reduction of the maximum activity of the GTPase reaction. In conclusion, this communication points out that in the presence of methanol as already demonstrated in its absence (19,20), the association of 30S and 50S subunits is the basic requirement for the catalytic turnover of the EF-G GTPase reaction.

Acknowledgement: We thank Drs. G. Chinali, R. C. Marsh and G. Sander for critically reviewing this manuscript. This work was supported by SFB 75 and by grant Pa 106 of the Deutsche Forschungsgemeinschaft.

REFERENCES

1. Monro, R.E., Staehelin, T., Celmo, M.L., and Vazquez, D., Cold Spring Harbor Symp.Quant.Biol. 34, 357 (1969)
2. Kisha, K., Möller, W., and Stöffler, G., Nature New Biol. 233, 62 (1971)
3. Sander, G., Marsh, R., and Parmeggiani, A., Biochem.Biophys.Res.Commun. 47, 872 (1972)
4. Brot, N., Yamasaki, E., Redfield, B., and Weissbach, H., Arch.Biochem. Biophys. 148, 148 (1972)
5. Hamel, E., Koka, M., and Nakamoto, T., J.Biol.Chem. 247, 805 (1972)
6. Ballesta, J.P.G., and Vazquez, D., Proc.Nat.Acad.Sci. U.S.A., 69, 3058 (1972)
7. Ballesta, J.P.G., Montejo, V., and Vazquez, D., FEBS Lett. 19, 79 (1971)
8. Hamel, E., and Nakamoto, T., J.Biol.Chem. 247, 6810 (1972)
9. Hamel, E., and Nakamoto, T., Biochemistry 11, 3933 (1972)
10. Parmeggiani, A., Singer, C., and Gottschalk, E.M., in S.P. Colowick and N.O. Kaplan (Editors), Methods in Enzymology, Vol.20, Academic Press, New York, 1971, p. 291
11. Kurland, C.G., Voynow, P., Hardy, S.J.S., Randal, L., and Lutter, L., Cold Spring Harbor Symp.Quant.Biol. 34, 17 (1969)
12. Zamir, A., Miskin, R., and Elson, D., J.Mol.Biol. 60, 347 (1971)
13. Parmeggiani, A., Voigt, J., and Chinali, G., unpublished results
14. Glynn, I.M., and Chappell, J.B., Biochem.J. 90, 147 (1964)
15. Maitra, U., Nakata, Y., and Hurwitz, J., J.Biol.Chem. 242, 4908 (1967)
16. Wahler, B.E., and Wollenberger, A., Biochem.Z. 329, 508 (1958)
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J.Biol.Chem. 193, 365 (1951)
18. Chinali, G., and Parmeggiani, A., Europ.J.Biochem. 32, 463 (1973)
19. Marsh, R., and Parmeggiani, A., Proc.Nat.Acad.Sci. U.S.A. 70, 151 (1973)
20. Sander, G., Voigt, J., Marsh, R., and Parmeggiani, A., unpublished results