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Analysis of Cosolvent and Divalent Cation Effects on Association Equilibrium and Activity of Ribosomes†

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ABSTRACT: Interactions between *Escherichia coli* ribosomal subunits were studied as a function of divalent cations in water and various water–organic solvent solutions, and conditions favoring the association equilibrium of the two subunits were established. We show that the association constant, K_{assoc}, at fixed magnesium concentration, as determined by light scattering, was first stimulated and then inhibited when the concentration of organic solvent was raised, while the magnesium concentration $(Mg^{2+})_{1/2}$ yielding half-associated 70S species first decreased and then increased. Maximum K_{assoc} was obtained at higher concentrations (EGOH, 6.26 M; Me_2SO, 1.4 M) for hydrophilic solvents and at lower concentration (1-butanol, 0.33 M) for hydrophobic solvents. 1-Propanol (a linear alcohol) and *tert*-butyl alcohol (a branched alcohol) presented interesting cases since they exhibited no inhibitory effects till moderate concentrations (15% v/v or respectively 1.95 and 1.56 M); under these conditions, high stimulation of K_{assoc} ($\Delta K_{assoc} \gg 4$ units at 2 mM Mg^{2+}) and maximum decrease of $(Mg^{2+})_{1/2}$ ($\ll 0.5$ mM) were reached. These results are discussed in the light of experiments carried out on simpler systems taken as "models" (interactions of RNA, ATP, and GTP with Mg^{2+} and Ca^{2+}) and in the light of possible different physicochemical contributions of organic solvents. It is shown that changes in dielectric constant, pK values and solvation of divalent cations are not critical parameters in the perturbation of the equilibria. It is suggested that hydrophobic interactions between the cosolvent and nonpolar patches on the ribosomal protein surface might be initially responsible for the observed effect and possibly induce favorable conformation changes of ribosomal subunits. These structures are very stable, as shown by dialysis experiments against classical inactivating buffers, and very active, as shown by the corrected binding experiments of fMet-tRNA to 30 and 70 S.

Association of *Escherichia coli* ribosomal subunits 30 and 50 S to form the functional 70S particles is known to depend on the structure of the subunits and the ionic environment of the medium (Tissières & Watson, 1958; Tissières et al., 1958, 1959; Walters & Van Os, 1971; Debey et al., 1975; Noll & Noll, 1976). The degree of interaction between subunits as measured by the equilibrium association constant, K_{assoc}, at fixed (Mg^{2+}), should provide the basis for a most sensitive structural test of active ribosomes.

Because of the polyelectrolyte character of ribosomal RNA,[1] the behavior of ribosomal subunits is that of polyanions (Kliber et al., 1976). Analyzing experimental kinetics of divalent cation-binding data, Wishnia & Boussert (1977) showed that the main effects of divalent cations on K_{assoc} conform to the nonspecific charge-neutralization model. However, the need

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[1] Abbreviations used: EGOH, ethylene glycol; Me_2SO (DMSO in the figures), dimethyl sulfoxide; ATP, adenosine triphosphate; GTP, guanosine triphosphate; RNA, ribonucleic acid; poly(A,U,G), random copolymer of adenylic, uridylic, and guanylic acids.

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remains to establish the precise mechanism of subunit interactions and the nature of the different forces involved in these interactions (Debey et al., 1975; Hui Bon Hoa et al., 1977).

Study of association–dissociation equilibrium raises two problems: first, the nature of the interaction between the two subunits, and, second, the mechanism controlling this equilibrium at each subunit level and leading to other types of interactions. These two problems are often confused and most reports in the literature are only concerned with the nature of subunit's interactions (Peterman, 1964; Golberg, 1966; Walters & Van Os, 1971; Spirin & Lishnevskaya, 1971; Hui Bon Hoa et al., 1977). For instance, experiments of Spirin and Lishnevskaya relating the effect of a number of nonionic agents on the stability of association of ribosomal subparticles showed that, except for urea which destabilized association, hydrophobic agents promoted the association between subparticles by decreasing the magnesium concentration required for half-association. They conclude that the association mechanism involves hydrogen bonds. This inference appears logical but is not conclusive since cosolvents can also affect the mechanism controlling this association–dissociation equilibrium, thus leading to similar observations. Cosolvents may either decrease the electrostatic free energy (ΔG_{el}) of the system by increasing the affinity of Mg^{2+} toward the RNA phosphate or affect the nonelectrostatic free energy (ΔG_{nel}) of the system through cosolvent–subunit interactions, leading to subtle conformation changes favoring the two subunits associations.

If the necessary physicochemical studies had been made in water and water–cosolvent mixtures, then one could choose between these two presumed modes of cosolvent action. This constitutes the object of our article.

Before setting forth our results, let us recall that the use of cosolvents as perturbing tools of overall reactions as well as elementary equilibria and rate processes is becoming routinely applied to highly organized systems involving DNA, RNA, and proteins synthesis (Hamel, 1972; Crepin et al., 1975; Nakanishi et al., 1974; Brody & Leautey, 1973). In some cases, cosolvents mimic physiological effectors (Voigt et al., 1974; Ballesta & Vasquez, 1973) in producing similar conformations and activities of the macromolecule. It is clear that the knowledge of the mechanism of cosolvent activation could help in the study of that of the physiological effectors.

The experiments presented here which constitute the first of a series describe changes of the Mg^{2+}-dependent K_{assoc} of vacant ribosomes upon increasing the concentration (1) of polar cosolvents (primary alcohol, polyol, and Me_2SO) and (2) of cosolvents bearing increasing hydrophobic character (*tert*-butyl alcohol, 1-propanol, and 1-butanol). In addition, tests of fMet-$tRNA_f^{Met}$ binding to 70 and 30 S were performed at increasing concentrations of cosolvents in activating and inactivating mixed buffers. Results indicate that there is no correlation between changes of physicochemical parameters of mixed solvents and the observed increase in K_{assoc} of subunit interactions and that hydrophobic interaction between the solvent and nonpolar patches on the ribosomal protein surface might be initially responsible for the observed effect. The role of cosolvent and magnesium as effectors is therefore discussed.

Materials and Methods

Chemicals and Solutions. ATP (disodium salt), magnesium chloride (as a solution of 1 g/mL in pure water), and cacodylic acid were from Sigma Chemical Co., St. Louis; GTP (trilithium salt) was from Boehringer-Mannheim. Highly polymerized yeast RNA was from Calbiochem, San Diego. Poly(A,U,G), ratio 1:1:1, was synthesized with polynucleotide phosphorylase and was a gift of Dr. Thang (IBPC). Unfractionated tRNA from *E. coli* was obtained from Schwarz Bio-Research. [*methyl*-^{3}H]-L-Methionine, 7.3 Ci/mmol, was purchased from CEA (Saclay). Calcium chloride (suprapure) was from Merck, Darmstadt; ammonium chloride was from Prolabo; β-mercaptoethanol was from Merck, Schuchardt. Ethylene glycol and dimethyl sulfoxide were from Merck. 1-Butanol, 1-propanol, and *tert*-butyl alcohol were from Carlo Erba.

Preparation of A-Type Ribosomes. *E. coli* ribosomes were prepared as previously described (Debey et al., 1975; Hui Bon Hoa et al., 1977).

Before use, each ribosomal stock solution was reactivated in activating medium by heating for 15 min at 37 °C and then centrifuged for 45 min at 14000*g* at 5 °C to eliminate dust or precipitates and kept at 0 °C until final use. The concentrations of ribosomal stock solutions were estimated by measuring absorbancy at 260 nm (for 1-cm light path) by using the molar extinction coefficient $\epsilon_{70S} = 4.15 \times 10^7$ M^{-1} cm^{-1}. Stock ribosomal solution ($\simeq$350 A_{260} units/mL) was diluted just before use to a final concentration of 4 A_{260} units/mL. The buffer used was cacodylate buffer, 50 mM, pH 7.50, 50 mM NH_4Cl, 7 mM β-mercaptoethanol, and stepwise addition of magnesium or calcium chloride.

Total (unfractionated) *E. coli* tRNA was charged by tritiated methionine (300 Ci/pmol) in the presence of formyltetrahydrofolate. The tRNA was purified by phenol extraction in a sodium acetate buffer, 0.1 M, pH 5.5, and precipitated by alcohol. The discharge of unformylated Met-$tRNA_f^{Met}$ was enzymatically performed, and the fMet-$tRNA_f^{Met}$ thus obtained was purified by chromatography on a Sephadex G-50 column according to the procedure of Lelong et al. (1970).

Crude initiation factors were obtained according to the procedure of Dondon et al. (1974).

Light-scattering measurements of equilibrium association constants of ribosomal subunits were at 25 °C in the presence of divalent cations and cosolvents.

All the measurements were done at 25 °C in 2 mL of buffer by using a Jobin-Yvon "Bêarn" fluorometer as described previously (Hui Bon Hoa et al., 1977). The intensity of light scattered at 90° to the incident beam of intensity Iinc is proportional to (Iinc)$\Sigma(n_i M_i^2)$ where n_i and M_i are respectively molar concentrations and molecular weights of the scattering species (Wishnia et al., 1975; Wishnia & Boussert, 1977). The fluorometer was interfaced to a Tektronix 31/53 programmable computer in order to give automatically data concerning % 70 S, K_{assoc}, and ΔG_{assoc}. The total error of the measurement was about ±5% in % 70 S (Hui Bon Hoa et al., 1977) and ±0.02% in log K_{assoc}.

Binding Constants of Mg^{2+} and Ca^{2+} Complexed with Various Ligands. Binding constants between divalent cations (Mg^{2+} and Ca^{2+}) and nucleotides (ATP and GTP) were potentiometrically determined by the method of Taqui Khan & Martell (1967). In the pH range investigated (between 6.0 and 7.0) where the species $ATP^{3-}H$–Mg^{2+} or $GTP^{3-}H$–Mg^{2+} (or Ca^{2+}) was negligible, only the following equilibria were considered:

$$ATP^{3-}H \rightleftharpoons ATP^{4-} + H^+ \quad (1)$$

$$ATP^{4-} + M^{2+} \rightleftharpoons (ATP^{4-}\text{–}M^{2+})^{2-} \quad (2)$$

At fixed pH and no M^{2+}, the concentration of the protonated form of nucleotide was known; as the total M^{2+} concentration increased, the equilibrium in eq 1 shifted toward dissociation, and the proton released was then titrated by the pH-stat method. The binding constants in the pK region [$(H^+) = k$

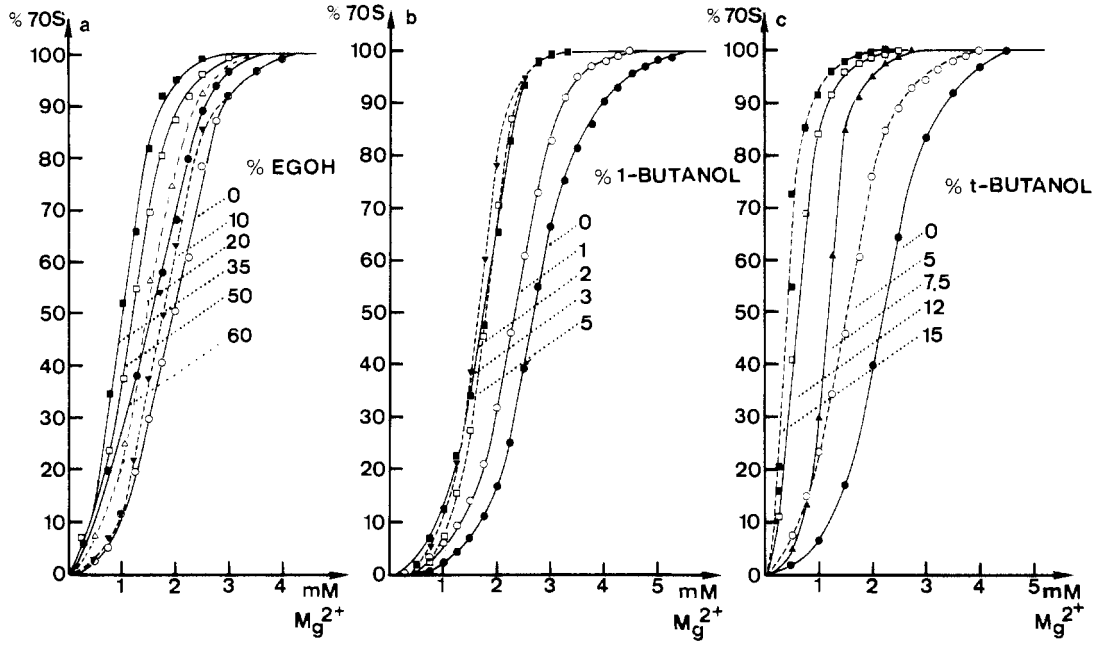

FIGURE 1: Reassociation equilibria of ribosomes as a function of magnesium concentrations at increasing cosolvent concentration (v/v): (a) EGOH; (b) 1-butanol; (c) *tert*-butyl alcohol. Ribosome concentration was 1.6×10^{-7} M. Buffer used was cacodylate, 50 mM, pH 7, 50 mM NH_4Cl, 7 mM β-mercaptoethanol, and the appropriate volume ratio of cosolvent. T = 25 °C.

and $A_t = M_t$] were then determined: $K = 2R/[A_t(1 - R)^2]$ where R is the fraction of deprotonated forms of nucleotide and k and A_t are the ionization constant and total concentration of nucleotides. M_t was the total M^{2+} concentration added.

All the measurements were carried out in the presence of 0.1 M NaCl as the supporting electrolyte, at 25 °C, with a Mettler titrator assembly (DK10, DK11, DK12, and DV11) (Larroque et al., 1976).

Concentrations of ATP and GTP (usually in the range of 4 mM) were determined spectrophotometrically on a Beckman Acta III by using the absorption coefficient ϵ_{259}(ATP) = 15.4 $\times 10^3$ M^{-1} cm^{-1} and ϵ_{252}(GTP) = 13.7 $\times 10^3$ M^{-1} cm^{-1}.

Binding constants of Mg^{2+}–RNA were determined according to the method described by Pörsche (1976) using a Beckman Acta III spectrophotometer at 20 °C and λ = 258 nm. The concentration of RNA was 50 mg/L and the supporting buffer was 10 mM Tris-HCl, pH 7.5, in the presence of 4 mM NaCl.

Binding of fMet-tRNA. The binding reaction mixture contained the following in 100 μL: 50 mM Tris-HCl, pH as indicated; 50 mM NH_4Cl; 1 mM GTP; 0.15 A_{260} unit of poly(A,U,G); 10 pmol of [^{3}H]fMet-tRNA (unfractionated) (sp act. 3100 cpm/mol); crude initiation factor extract as indicated; 70S ribosomes, 20 pmol, or 30S subunits, 15 pmol; magnesium acetate and ethylene glycol as indicated. Incubated 20 min at 37 °C, the reaction was then stopped by dilution with 3 mL of ice-cold 50 mM Tris-HCl (pH 7.4), 5 mM magnesium acetate, and 50 mM ammonium chloride, followed by filtration on nitrocellulose filters (Millipore). After being washed twice with cold buffer, the filters were dried at 90 °C, and radioactivity was measured in the standard toluene–PPO scintillation liquid.

Puromycin Reaction. The above assay mixture was first incubated at 37 °C for 15 min and then 140 μg of puromycin was added and the incubation was prolonged for 5 min. The reaction was stopped by addition of 1 mL of sodium acetate, 0.1 M, pH 5.25, and then 1 mL of pure ethyl acetate. After stirring and centrifuging, we counted the radioactivity from 1 mL of the organic layer. The amount of fMet-tRNA bound in the absence of initiation factors was subtracted.

Dialysis experiments of subunits were against low magnesium concentration (0.5 mM) and 50 mM Na^+ during 5 h at 4 °C in water or water–ethylene glycol, 65%:35% v/v; the buffer used was cacodylate, 50 mM, pH 7, and 7 mM β-mercaptoethanol. Control experiments using conductimetric measurements showed that under the above conditions, the cations were equilibrated inside and outside the dialysis tubing in water or mixed cosolvent.

Results

(1) K_{assoc} Changes as a Function of Increasing Mg^{2+} and Cosolvent Concentration. The effect of increasing organic solvent concentration on the association curve of ribosomes (percent of 70S particles as a function of magnesium concentration) was systematically studied and analyzed. The results obtained with three selected organic solvents (ethylene glycol, 0–60% v/v; 1-butanol, 0–5% v/v; *tert*-butyl alcohol, 0–15% v/v) are shown in parts a, b, and c of Figure 1. As the organic solvent concentration increases, the Mg^{2+} reassociation curves appear to shift toward low Mg^{2+} concentration, with a decrease of $(Mg^{2+})_{1/2}$; the range of cosolvent effect was quite different: 0–35% v/v for ethylene glycol, a hydrophilic solvent; 0–3% v/v for 1-butanol, a hydrophobic linear alcohol; and 0–15% v/v for *tert*-butyl alcohol, a hydrophobic branched alcohol. Further increase in organic solvent concentration results in an inversion of the effect. Moreover, for hydrophilic solvent (60% v/v EGOH), the association becomes less cooperative than in pure water, while for hydrophobic solvent, the association becomes more cooperative (5% v/v 1-butanol and 15% v/v *tert*-butyl alcohol).

The variations of both K_{assoc} and $(Mg^{2+})_{1/2}$ as a function of cosolvent concentration are symmetrical, an increase in K_{assoc} corresponding to a decrease in $(Mg^{2+})_{1/2}$ and vice versa (Figure 2). For the six cosolvents used, there is a stimulation of K_{assoc} at fixed Mg^{2+} (2 mM) and then an inhibition (except for *tert*-butyl alcohol and 1-propanol) when the concentration of cosolvent is raised. Control experiments showed that the decrease of K_{assoc} (which remained greater than that in water) is due to a reversible inhibition by high alcohol concentration

Table I: Change in Dielectric Constant D and Protonic Activity pa_H^* as a Function of Cosolvent Concentrations[a]

		at cosolvent concentration (% v/v)						
	cosolvent	0	10	20	30	35	50	60
D	EGOH	80.4	77.7	75.1	72	70	64.5	61.1
	Me_2SO	80.4	79.4	78.8	78.6			
$10^2/D$	EGOH	1.24	1.287	1.33	1.388	1.428	1.55	1.637
	Me_2SO	1.24	1.256	1.269	1.27			
pa_H^*								
cacodylate	EGOH	7.0	6.9	7.0	7.05	7.1	7.3	7.4
	Me_2SO	7.0	6.95	7.2	7.4			
tris	EGOH	8.0	7.8	7.8	7.8	7.8	7.8	7.9
	Me_2SO	8.0	7.74	7.75	7.76			

[a] pa_H^* values were measured with a glass electrode in mixed solvent as described by Larroque (1976).

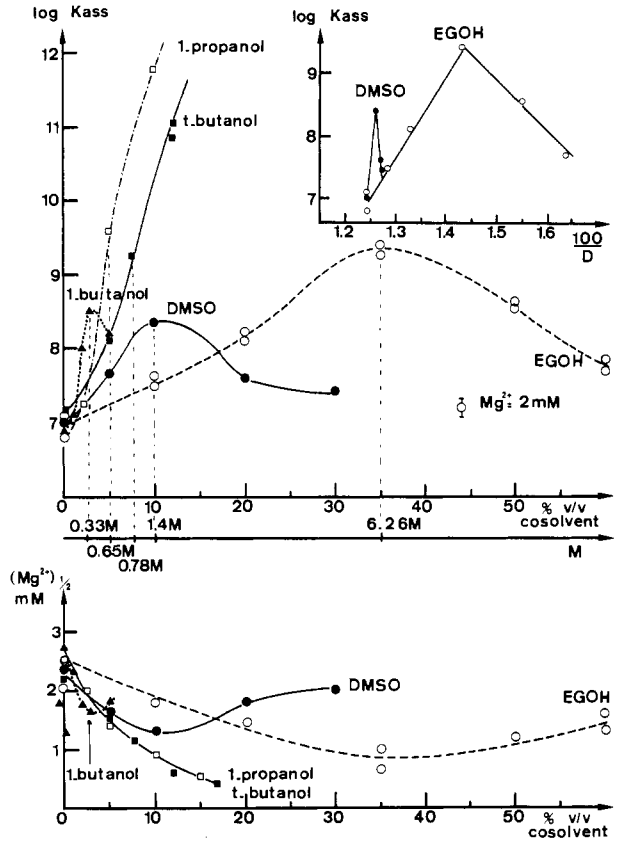

FIGURE 2: Plots of log K_{assoc} of ribosomal subunits at fixed magnesium concentration and of $(Mg^{2+})_{1/2}$ as a function of increasing volume ratio of some hydrophilic and hydrophobic cosolvents. Curves: (○) for EGOH; (●) for Me_2SO (DMSO in figure); (□) for 1-propanol; (■) for *tert*-butyl alcohol; (▲) for 1-butanol (Mg^{2+} = 2 mM; same ribosomal concentration and buffer as in Figure 1). Insert: plot of log K_{assoc} at 2 mM Mg^{2+} against the reciprocal of the dielectric constant for two cosolvents: (●) Me_2SO; (○) EGOH.

and not due to irreversible denaturation. The maximum stimulated effect on K_{assoc} was obtained at higher cosolvent concentration for hydrophilic than for hydrophobic solvent, and in all cases the value of log K_{assoc} was higher than that in water. Two hydrophobic cosolvents (1-propanol and *tert*-butyl alcohol) showed a most efficient effect: the K_{assoc} value increasing sharply (as compared to that of the other cosolvents) and reaching for 15% v/v a value about 4 times higher than that in water [log K_{assoc} = 11.8 at 1.3 M 1-propanol (10% v/v) and log K_{assoc} = 11 at 1.25 M *tert*-butyl alcohol (12% v/v)]. Parallel to the K_{assoc} increase, the $(Mg^{2+})_{1/2}$ decreased more sharply for hydrophobic than for hydrophilic solvents.

(2) *Change in Dielectric Constant and Protonic Activity in Mixed Solvent.* When cosolvents of low dielectric constant (D) are added to water, it is known that the dielectric constant of the mixed solution decreases as the volume ratio of cosolvent increases (Douzou et al., 1977; Travers & Douzou, 1970, 1974); simultaneously, the protonic activity pa_H^* of buffers is changed: first slowly increased (or decreased depending on the nature of buffers) up to 50% v/v of cosolvent and then rapidly increased for higher concentrations of cosolvent (Hui Bon Hoa & Douzou, 1973; Larroque et al., 1976).

Table II: Thermodynamic Data of Ribosomal Subunit Association at 25 °C

	in solvent (v/v)		
parameters	H_2O	35% EGOH	10% Me_2SO
D	80	70	79.4
M^{2+}	Mg^{2+}; Ca^{2+}	Mg^{2+}; Ca^{2+}	Mg^{2+}
log K_{assoc} (2 mM M^{2+})[a]	7	9.4; 8.8	8.4
ΔG_t (2 mM M^{2+}) (kcal/mol)	−10	−12.8; −12	−11.5
$\Delta G_{el_{calcd}}$ (2.5 mM M^{2+}) (kcal/mol)[b]	+9.5	+9.5	+9.5
$\Delta G_{nel_{calcd}}$	−19.5	−22.3; −21.5	−21

[a] Buffer used is cacodylate, 50 mM, pH 7, 50 mM NH_4Cl, 7 mM β-mercaptoethanol, and 2 mM $MgCl_2$ or $CaCl_2$. [b] According to Wishnia et al. (1975).

Data obtained in two cosolvents are summarized in Table I. Results show that ethylene glycol (D = 41.9) and dimethyl sulfoxide (D = 45) differently affect the macroscopic dielectric constant of their mixture with water: a very small change of D for the Me_2SO–water mixture ($\Delta D \simeq 1.6$) up to 30% v/v of cosolvent and a change of $\Delta D \simeq 19.3$ units for the EGOH–water mixture up to 60% v/v of EGOH.

A plot of log K_{assoc} of subunit interaction as a function of the reciprocal of D (inset, Figure 2) shows that for two different solvents (EGOH and Me_2SO) log K_{assoc} varies differently: the same value of D obtained from different cosolvents induces quite different values of log K_{assoc}.

pa_H^* of buffers was also affected by the presence of cosolvent. Table I shows that the effect remains small at low concentration of cosolvents: maximum increase in pa_H^* of cacodylate buffer of 0.4 unit from 0 to 60% v/v of EGOH and 0 to 30% v/v of Me_2SO. Tris buffers, on the contrary, exhibit a small decrease in pa_H^* (about 0.2 unit) and then a small increase (0.1 unit) when EGOH or Me_2SO is raised from 0 to 30% and then to 60% v/v. If one tries to plot log K_{assoc} as a function of the known pa_H^* of each mixed solution (Table I), no continuous curve is obtained, but a discrepancy of the values over 3 units of log K_{assoc} around pa_H^* 6.9 and 7.3 is obtained, which exceeds largely the precision of the experiments.

Thermodynamic data of associative processes of ribosomal subparticles as a function of electrostatic and cosolvent effects are summarized in Table II.

Table III: Binding Constants of Various Complexes at 25 °C (See Materials and Methods for Details)

		values for				
	parameter	ATP-Mg^{2+}	ATP-Ca^{2+}	GTP-Mg^{2+}	GTP-Ca^{2+}	RNA-Mg^{2+}
H_2O	K (M^{-1}) × 10^{-3}	13.9 ± 0.5	5.9 ± 0.3	7.6 ± 0.4	4.5 ± 0.5	26.4 ± 1.0
	log K	4.14	3.77	3.88	3.65	4.42
	ΔG (kcal/mol)	−5.72	−5.20	−5.35	−5.04	−6.11
EGOH, 30%	K (M^{-1}) × 10^{-3}	22.0 ± 1.0	8.2 ± 0.5	7.7 ± 0.4	3.1 ± 0.5	25.5 ± 1.0
	log K	4.34	3.91	3.89	3.49	4.41
	ΔG (kcal/mol)	−6.0	−5.4	−5.37	−4.82	−6.10
Me_2SO, 10%	K (M^{-1}) × 10^{-3}					22.5 ± 1.0
	log K					4.35
	ΔG (kcal/mol)					−6.01

(3) Hydration Changes of Divalent Cations and Interactions with Nucleotides and RNA. The organic solvents could affect the association equilibrium of ribosomes by altering the hydration of divalent metal cations and their interactions with the phosphate groups of ribosomal RNA. It was therefore decided to investigate the effect of ethylene glycol and dimethyl sulfoxide on the K_{assoc} of ribosomal subunits in the presence of Mg^{2+} and Ca^{2+} which have widely different hydration (Stuehr, 1978) and on the association constant of complexes formed between these cations and model compounds such as RNA and nucleotides ATP and GTP.

The effect of various concentrations of EGOH on K_{assoc} remained the same whether in the presence of magnesium or calcium (2 mM) (Figure 3).

Organic solvents have no effect on the binding constant of divalent metal cations (Mg^{2+} and Ca^{2+}) and RNA or nucleotides ATP and GTP (30% v/v ethylene glycol and 10% v/v dimethyl sulfoxide at 25 °C) except in the case of ATP–Mg^{2+} where the binding constant increased from 13.9 × 10^3 M^{-1} to 22 × 10^3 M^{-1}, but ΔG is not significantly affected (Table III).

Other experiments (results not shown), using bacterial virus R17-RNA taken as model, indicated that the interaction with Mg^{2+} was not affected by the presence of 35% v/v of ethylene glycol but the macromolecular structure of this RNA was slightly destabilized by the cosolvent (decrease of $T_m \simeq 12$ °C).

(4) Initiation Steps of Protein Synthesis in Water and Water–Ethylene Glycol Media. (a) Subunit Inactivation. Experiments of Miskin et al. (1970) and Zamir et al. (1971) showed that ribosomal subunits 30 and 50 S lose their associativity and activities when certain specific monovalent and divalent cations were either removed or decreased below a critical concentration.

These experiments were repeated here in water and in the presence of 35% v/v of ethylene glycol. Dialysis experiments of 30S and 50S subunits against low magnesium concentration (0.5 mM) and 50 mM Na^+ showed a loss of associativity for both ribosomal particles in water: 33% for 50 S and 72.4% for 30 S. To the contrary, in the presence of ethylene glycol (35% v/v) there was no loss of associativity for the 50S subunit and only a slight loss for the 30S subunit (~11.5%).

fMet-$tRNA_f^{Met}$ binding tests in aqueous activating buffer showed that when the 30S subunit was dialyzed in water, no activity could be detected, whereas when dialyzed in the presence of ethylene glycol (35%), binding occurred [0.66 pmol of fMet-tRNA bound to ethylene glycol dialyzed 30 S as compared to 0.4 pmol for active 30 S (2.57 × 10^{-8} M)].

These experiments clearly showed that the cosolvent (35% v/v EGOH) protected ribosomal subunits against inactivation by depletion of specific cations.

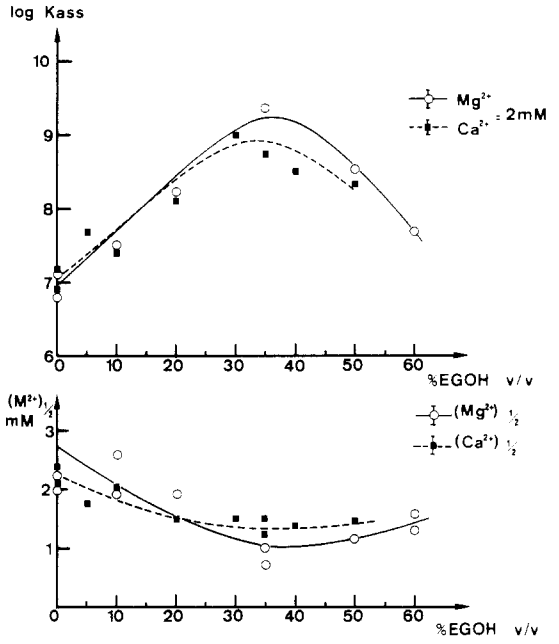

FIGURE 3: Plots of log K_{assoc} for various divalent cations at fixed magnesium concentration and of $(M^{2+})_{1/2}$ as a function of increasing volume ratio of ethylene glycol. Curves: (○) for Mg^{2+} and (■) for Ca^{2+} (M^{2+} = 2 mM). Same ribosomal concentration and buffer as in Figure 1.

(b) Activation of Binding to 70 S. Figure 4a shows the effect of ethylene glycol in the binding of fMet-$tRNA_f^{Met}$ to 70S ribosomes. At fixed magnesium concentration (2 mM Mg^{2+}), the binding was first proportional to the ethylene glycol concentration up to 30% (v/v) ethylene glycol, at which point the optimum binding was obtained; the binding then decreased for higher ethylene glycol amounts. This curve is quite similar to that obtained in Figure 2: there are some correlations between enhancement of activity and enhancement of associativity of the two subunits by cosolvent.

In addition, the binding of fMet-$tRNA_f^{Met}$ to 70S ribosomes was highly dependent on magnesium concentration in aqueous as well as in each cosolvent medium (Figure 4b); as cosolvent increased, the Mg^{2+} ion concentration for optimum binding was shifted toward a lower value and reached 2 mM Mg^{2+} at 20% v/v ethylene glycol. At higher ethylene glycol concentrations, the Mg^{2+} for optimum binding remains 2 mM Mg^{2+} in the range from 20 to 50% v/v EGOH. The optimum binding follows the same bell-shaped curve as does Figure 4a as a function of EGOH concentrations with stimulation and inhibition phases.

The cosolvent (here 30% v/v EGOH) did not suppress the requirement for initiation factors and messenger RNA (Table IV). The binding stimulation was only observed in the presence of both poly(A,U,G) and initiation factors, whatever

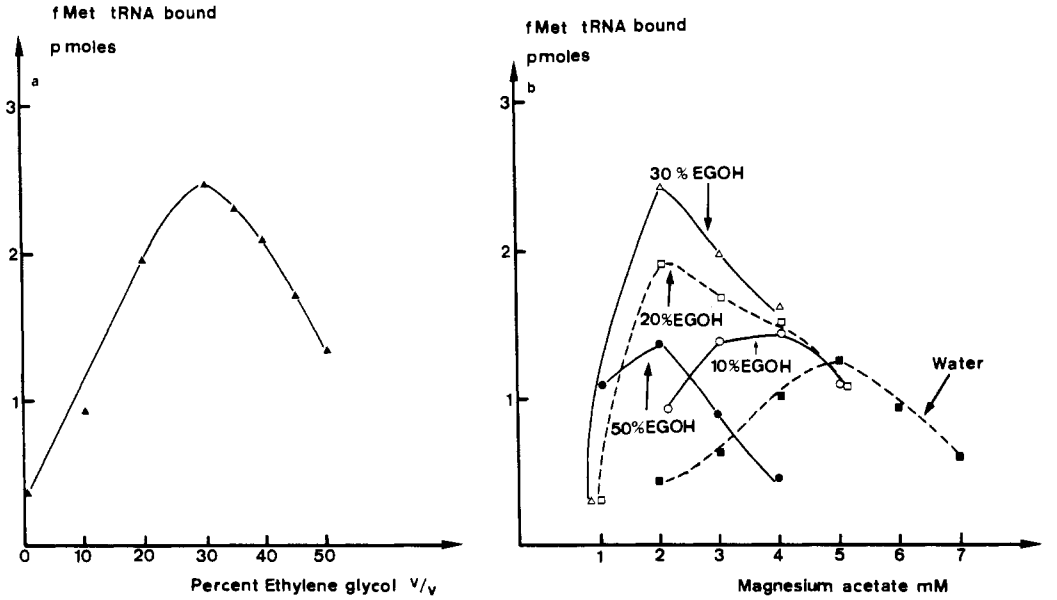

FIGURE 4: (a) Plot of the binding of fMet-tRNA$_f^{Met}$ to 70S ribosomes as a function of ethylene glycol concentration. The incubation mixture (100 μL) contained the following: 50 mM Tris-HCl buffer; 50 mM NH_4Cl; 0.11 A_{260} unit of poly(A,U,G); 1 mM GTP; 15 μg of crude initiation factors; 15 pmol of ribosomes, 70 S; 12 pmol of fMet-tRNA$_f^{Met}$ (sp act. 3130 cpm/pmol). Magnesium acetate = 2 mM. The ordinate indicates the amount of fMet-tRNA$_f^{Met}$ (in picomoles) bound to 70S ribosomes; the abcissa indicates the cosolvent medium expressed in percent ethylene glycol–water (v/v). Ribosomes were 30% active. (b) Plot of fMet-tRNA$_f^{Met}$ binding to 70S ribosomes as a function of magnesium concentration for various proportions of ethylene glycol. The cosolvent–water mixture is expressed as volume/volume. Same experimental conditions as for (a). Ethylene glycol: (■) 0; (○) 10; (□) 20; (△) 30; (●) 50%.

Table IV

Binding of fMet-tRNA$_f^{Met}$ to 70S Ribosomes in Cosolvent

medium	additions	fMet-tRNA$_f^{Met}$ bound (pmol)	fMet-Puro formed (pmol)
aqueous	+IF +poly(A,U,G)	1.05	1.10
ethylene glycol, 30%	+IF +poly(A,U,G)	2.20	2.39
	−IF +poly(A,U,G)	0.10	
	+IF −poly(A,U,G)	0.20	

Reversibility of Ethylene Glycol Action

preincubation	incubation	fMet-tRNA bound (pmol)
aqueous medium	aqueous medium	1.05
30% ethylene glycol	30% ethylene glycol	2.11
30% ethylene glycol	10% ethylene glycol	0.90
10% ethylene glycol	10% ethylene glycol	0.90

the proportion of cosolvent. In addition, fMet-tRNA$_f^{Met}$ was correctly positioned at the P site, as shown by puromycin reaction at 30% v/v EGOH. Moreover, the effect of ethylene glycol is reversible up to 30% v/v EGOH: stimulation of initiator tRNA binding disappeared after dilution of EGOH, thus eliminating the trivial effect of partial denaturation of the macromolecular system in the stimulative part of the process.

(*c*) *Activation of Binding to 30 S.* The binding of initiator tRNA to the 30S subunit exhibited similar features than with 70S ribosomes (Figure 5). The binding was highly dependent on the cosolvent proportion and on Mg^{2+} concentration until 8 mM Mg^{2+} was reached. In both aqueous and ethylene glycol media, the yield of binding was first proportional to Mg^{2+} concentration and then reached a plateau; as the amount of ethylene glycol increased, the plateau was obtained for lower

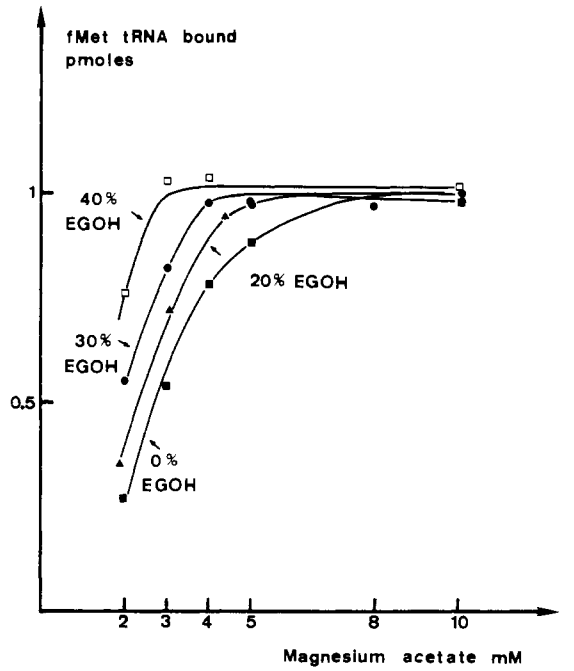

FIGURE 5: Plot of fMet-tRNA$_f^{Met}$ binding to 30S ribosomal subunits as a function of magnesium concentration for various proportions of ethylene glycol. Same incubation medium as in Figure 4b, except 10 pmol of 30S ribosomes and 8 pmol of [^{3}H]fMet-tRNA (sp act. 3150 cpm/pmol) were used. Ethylene glycol: (■) 0; (▲) 20; (●) 30; (□) 40%.

Mg^{2+} concentration but with the same yield of binding: 8 mM in aqueous medium and respectively 4, 3, and 2 mM in solution containing 20, 30, and 40% (v/v) of EGOH.

Discussion

The association equilibrium of ribosomal subparticles is characterized by the constant K_{assoc} and the free energy change $\Delta G_t = -RT \ln K_{assoc} = \Delta G_{el} + \Delta G_{nel}$ where ΔG_{el} (>0) and

ΔG_{nel} (<0) represent respectively the change in electrostatic and nonelectrostatic free energy during the association. This system is balanced by external factors such as divalent cations (electrostatic factors) and organic cosolvent (nonelectrostatic factors) which can easily tip it one way or the other. The association equilibrium of ribosomes might then be visualized as a two-step process. Step I consists in the neutralization (by divalent cations) of the electrostatic potential (predominantly promoted by ribosomal RNA) of both 30 and 50 S (Wishnia & Boussert, 1977). Step II consists in the association of the partly neutralized particles through nonelectrostatic interactions such as van der Waals forces, hydrogen bonds, and salt bridges (Spirin & Lishnevskaya, 1971; Walters & Van Os, 1971; Wishnia et al., 1975; Hui Bon et al., 1977).

Interaction of ribosomes is cosolvent dependent; cosolvent favored association by decreasing $(Mg^{2+})_{1/2}$; its effect must be considered as resulting from many possible contributions. Taking advantage of the large background of physicochemical data gathered previously for a number of water–organic solvent mixtures (Hui Bon Hoa & Douzou, 1973; Maurel & Douzou, 1975; Maurel et al., 1975; Travers & Douzou, 1970, 1974; Douzou et al., 1977; Maurel, 1978), we are able now to discuss some of these contributions in connection with the picture given above for the association equilibrium.

(a) Changes in dielectric constant have undoubtedly been the most widely suspected contribution of the organic solvent effect on biochemical reactions (Clement & Bender, 1963; Fink, 1974; Amis, 1953; Laidler & Ethier, 1953). But careful analysis of the results of Table I and the insert of Figure 2 does show that this parameter cannot account for either the associating or antiassociating effect of solvent. In addition, a decrease in dielectric constant upon addition of increasing amounts of organic solvent should increase electrostatic repulsions so that K_{assoc} would decrease; this is not what we observe.

(b) An induced change in pK of ionizable groups and pH of buffers by cosolvents is also probably not the main contribution of the observed phenomena. First, this is because the most stimulating effect was obtained at a small amount of cosolvent where the pa_H* or pK* changes were small (Δpa_H* < 0.4 unit in 50% EGOH; Table I). Second, a tentative titration plot of log K_{assoc} as a function of pa_H* indicates that no appreciable ionizing groups are implicated in the association binding site (Beaudry et al., 1978). Third, the pK values of the phosphate groups of nucleic acid are too acidic ($\leq$1) to be seriously perturbed by the organic solvents in the pH range used for this work.

(c) Binding constants between divalent cations (Mg^{2+} and Ca^{2+}) and model compounds (RNA and nucleotides) in water and mixed solvents are not significantly affected by the presence of cosolvent (Table II). This means that the solvent-induced partial dehydration of Mg^{2+} cations probably does not strengthen their interaction with phosphate groups (Pörsche, 1976). This is not surprising since $(Mg^{2+})_{1/2}$ and $(Ca^{2+})_{1/2}$ are affected to the same extent (Figure 3) although the hydration of these cations is known to be widely different (Stuehr, 1978). However, this does not exclude other solvation processes since all the solute species (ribosomal proteins) present in the medium are susceptible to changes in solvation.

(d) Since the cosolvents do not appear to affect directly the electrostatic interactions, they might affect the nonelectrostatic ones. In a recent work, Wesley et al. (1978) showed that when cosolvent (ethylene glycol) was used at low concentration (<50% v/v), no changes of the overall structural properties of the ribosome (both RNA and proteins) were observed by classical physical techniques; however, the perturbations could result from discrete and subtle conformation changes of subunits with drastic effects on K_{assoc} (Figure 2) and on functional activity (Figures 5 and 4).

It is not unreasonable to assume that cosolvent-induced conformational changes take place by exposing previously masked groups or by changing their orientation on the surface of ribosomal proteins. This might influence subtle spatial rearrangement of the structure of RNA allowing, for example, the formation of one supplementary hydrogen bond between 16S RNA and 23S RNA (ΔG_H = −2.5 kcal). If we assume that ΔG_{el} is not affected in order to simplify the analysis, the ΔG_{nel} becomes −22 kcal and ΔG_t = 12.5 kcal (Table II) so that K_{assoc} is increased by 2 orders of magnitude ($K_{assoc} = 10^9\ M^{-1}$). This is indeed what we observed in 35% (v/v) ethylene glycol.

This rough picture seems to be illustrated by the experiments using organic solvents having hydrophobic character. The higher the hydrophobic character of cosolvent, the lower is the concentration ensuring an optimum enhancement of K_{assoc} (Figure 2; except if the inhibition process is too important). This suggests that the activation and inhibition processes reflect exposure of nonpolar regions of the protein to the surrounding medium. The large number of hydrophobic residues in the proteins of the ribosomal subunits (Kaltschmidt & Wittmann, 1970) might cause these proteins to be solvated preferentially by hydrophobic cosolvents, leading to the formation of α-helical structure.

Finally, to account for the observed decrease in $(M^{2+})_{1/2}$ in the presence of cosolvent, three possible interpretations are proposed.

(1) The favorable change in free energy affecting ΔG_{nel} is larger than −2.5 kcal; this excess amount of free energy might therefore be spent at the cost of the unfavorable increase in ΔG_{el} resulting from the decrease in $(M^{2+})_{1/2}$. (2) Cosolvent by increasing α-helix content of ribosomal proteins could bring about a spatial and local redistribution of their positive charges so that a larger neutralization of the phosphate groups of RNA would follow. Under these conditions ΔG_{el} must be decreased so that a lesser amount of M^{2+} cations is necessary in order to reach the energetic balance controlling the association. (3) The conformational changes of ribosomal proteins induced by cosolvent might influence spatial arrangement of the structure of RNA in such a way as to change the binding affinity or the number of binding sites of Mg^{2+}. One could expect to determine cosolvent effect on Δn (the change of the average of Mg^{2+} bound by ribosome species) (Zitomer & Flasks, 1972). Unfortunately, the log–log plot of K_{assoc} as a function of Mg^{2+} is not linear (slope Δn is not constant) for A-type ribosomes (Debey et al., 1975; Noll & Noll, 1976); in addition, in the presence of cosolvents, the deviation from linearity increases with a shift in $(Mg^{2+})_{1/2}$ (plots not shown). These results showed that binding of Mg^{2+} is more cooperative.

(e) Cosolvents affect two tests of ribosomal activity: enhancement of both formation of 70 S and the binding of fMet-tRNA (Figures 2, 4a, and 5); it is likely that this reflects structural changes of ribosomal subunits. Cosolvent activation was highly Mg^{2+} dependent (Figures 4b and 5), indicating that Mg^{2+} and cosolvent are additive activators; on the other hand, cosolvent protects subunits against inactivation by depletion of (Mg^{2+}). Because of their difference in nature, they probably act in a different way, leading to the same results.

In conclusion, the above results suggest that cosolvents induce subtle conformational changes of ribosomal proteins by exposure of nonpolar patches on their surface to the surrounding medium. Changes in protein organization might then

favor the association of subunits into 70S particles possibly in a manner analogous to that of classical allosteric effectors (Dreyfus et al., 1978). The fact that cosolvent can produce identical activation as the divalent cations leads us to wonder if the principal electrostatic contribution of these cations could not mask the conformational one. However, at the present time, standard techniques have not been able to evidence these small conformational changes; high-resolution techniques like specific fluorescent probes should be more adequate methods.

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