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Ribosomal Subunit Interaction as Studied by Light Scattering. Evidence of Different Classes of Ribosome Preparations†

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ABSTRACT: The Mg^{2+}-dependent equilibrium of ribosomal subunits was studied by light scattering in the absence of any other factor of protein synthesis. The difference of intensity in the light scattered by dissociated 30S–50S couples and by 70S particles was used as a method to measure the percentage of association as a function of Mg^{2+} under conditions of nonperturbation of the equilibrium. We found that pressure-resistant (type A) and non-pressure-resistant (type B) ribosomes may be characterized by the different behavior of their association equilibrium curves. The thermodynamic parameters of this equilibrium and their comparison for the two classes of ribosomes were calculated from experiments at different temperatures.

For many years a rather static view of ribosomes prevailed, and it was only recently that ribosomal structure was visualized as undergoing cyclic conformational changes during protein synthesis (Kurland, 1972; Springer et al., 1971; Grunberg-Manago et al., 1973). It is not surprising, therefore, that although many physical data are available on ribosome structure, very little is known about their dynamics: interactions between ribosomal proteins and RNA, between protein factors and individual 30-, 50-, and 70S subunits, as well as between the 30- and 50S subunits.

Interaction between the free subunits is the most sensitive and critical structural manifestation of active ribosomes and should provide important information on the association of the subunits during the cycle of protein synthesis. While it has been known since the work of Spirin (1971) and of Infante and Baierlein (1971) that the ribosomes are in dynamic equilibrium with their subunits, the quantitative data are still very scarce.

Two main difficulties have complicated most attempts to study this equilibrium. The first difficulty is technical; the sedimentation methods most commonly used do not permit kinetic measurements. Moreover, the association equilibrium of the subunits can be strongly perturbed by the hydrostatic pressure existing in the ultracentrifugation cells during high-speed sedimentation (Infante and Baierlein, 1971).

The other difficulty is due to artifacts arising from the ribosomes themselves which are heterogeneous. Two types of 30S + 50S couples have been described by the group of Bosch (Van Diggelen and Bosch, 1973; Van Diggelen et al., 1973) and by that of Noll et al. (1973); these couples were discriminated by sucrose gradient centrifugation at 60,000 rpm at 10 m*M* Mg^{2+}. Two peaks were identified, corresponding to apparent *s* values of 60 and 70 S; however, it was later found that at low speed they had the same *s* value but could be characterized by a different sensitivity to hydrostatic pressure.

It was found by both the above-mentioned groups that at 4–5 m*M* Mg^{2+} (under conditions of protein synthesis in vitro) the pressure-resistant (or "tight") couples are fully associated, while the others ("loose" couples) show a significant dissociation. Moreover, it was shown by Noll that the former couples are all active in the protein initiation complex and therefore represent the active ribosome preparation. We will simply call these categories A-type ("tight") and B-type ("loose") ribosomes.

Recently, the light scattering technique has been used to measure the molecular weight of *Escherichia coli* ribosomes (Igarashi et al., 1973; Reale-Scafati et al., 1971); this technique was also applied by Zitomer and Flaks (1972) to demonstrate a true chemical equilibrium between the ribosomes and their subunits over a wide range of conditions. However, their results appeared to be obtained with non-pressure-resistant ribosomes (where the 50% dissociation point is 9 m*M* Mg^{2+} at 37°), and it was important to

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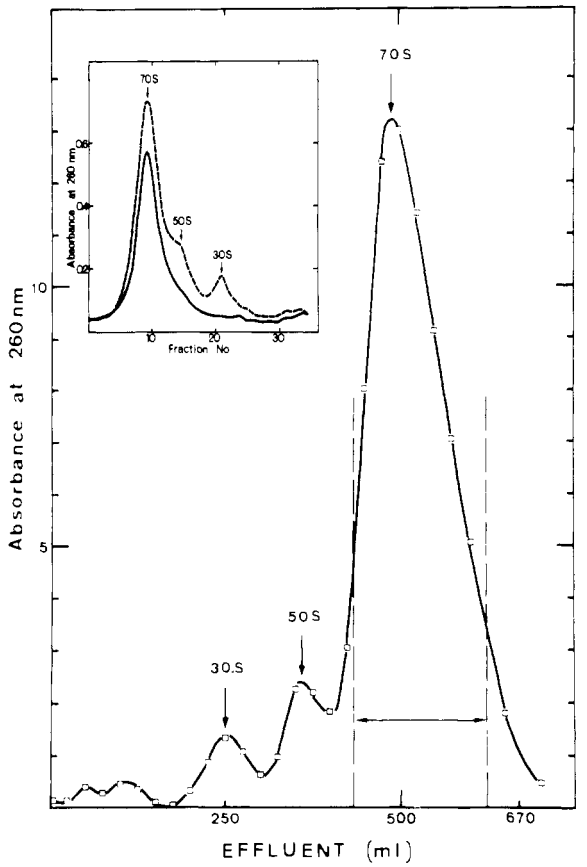

FIGURE 1: Ribosome fractionation by zonal centrifugation. Washed ribosomes (150 mg) were layered on a 10–30% linear sucrose gradient in the following buffer: Tris-HCl (pH 7.5), 10 m*M*; NH_4Cl, 60 m*M*; $Mg(OAc)_2$, 5 m*M*. Centrifugation was for 17 hr at 31,000 rpm in the Spinco Ti 14 rotor. Fractions 88–120 were pooled and concentrated. The final yield was 100 mg of "purified 70S ribosomes". Insert: Sucrose gradient centrifugation of two batches of high salt washed, unpurified ribosomes. Five A_{260} units of ribosomes was layered on top of a linear 10–30% sucrose gradient buffered as follows: Tris-HCl (pH 7.5), 10 m*M*; NH_4Cl, 50 m*M*; magnesium acetate, 5 m*M*. Centrifugation was for 120 min at 50,000 rpm in the Spinco SW 56 rotor: I (—), the ribosomes were prepared from fresh exponentially growing *E. coli* MRE 600 (generation time at 37°, 25 min) as described in Materials and Methods; II, (- - -) the ribosomes were prepared from commercial *E. coli* MRE 600 (Microbiological Research Establishment, Salisbury, U.K.) which had been kept frozen for 6 months at −20°. The original ribosomal pattern of these bacteria was almost identical with pattern I.

know whether these results also applied to true active ribosomes.

We are reporting here on our results with A-type and mixtures of A- and B-type ribosomes. We made a comparative study showing that the thermodynamic properties of their Mg-dependent association equilibrium are quite different.

Materials and Methods

Preparation of Ribosomes. "UNPURIFIED" WASHED RIBOSOMES. *E. coli* MRE 600 were ground by 30-g fractions in the presence of alumina, 60 g (Alcoa A 305 Bacterial grade), and suspended in 1.5 vol of the following buffer: Tris-HCl (pH 7.5), 10 m*M*; magnesium acetate, 10 m*M*; NH_4Cl, 10 m*M*; and β-mercaptoethanol, 7 m*M*. The extract was then centrifuged 20 min at 20,000*g* and the alumina precipitate was eliminated; the supernatant was then centrifuged 30 min at 30,000*g* in order to eliminate debris. The supernatant was again centrifuged 18 hr in rotor 42 (Spinco $L_2$65 B) and the supernatant discarded. The pellet was then washed twice: first in the above buffer and, after discarding the supernatant, in the following buffer: Tris-HCl (pH 7.5), 10 m*M*; NH_4Cl, 1500 m*M*; magnesium acetate, 10 m*M*; and β-mercaptoethanol, 7 m*M*. This yields a supernatant which constitutes the crude initiation factors and ribosomes which are resuspended in the first buffer and contain both A-type and B-type ribosomes.

"A-TYPE" 70S SUBUNITS. The "washed" ribosomes were further purified on a sucrose gradient 10–30% in the following buffer: Tris-HCl (pH 7.5), 10 m*M*; NH_4Cl, 60 m*M*; magnesium acetate, 5 m*M*. The gradient was centrifuged 17 hr at 31,000 rpm in a Beckman T 14 zonal rotor (Figure 1). The 70S fractions were collected and brought to 7 m*M* β-mercaptoethanol and 10 m*M* $Mg(OAc)_2$ before concentration by further centrifugation 24 hr at 25,000 rpm in rotor 30 (Spinco centrifuge). The 70S pellet was then rehomogenized in buffer A (Tris-HCl (pH 7.5), 10 m*M*; NH_4Cl, 100 m*M*; $Mg(OAc)_2$, 10 m*M*; β-mercaptoethanol, 7 m*M*) and stored frozen at −90°.

Before experiments each sample was reactivated by heating 15 min at 37° and centrifuged 30 min at 14,000*g*.

Solutions. The medium generally used (buffer B) had the following composition: sodium cacodylate (pH 7.0), 50 m*M*; NH_4Cl, 70 m*M*; $Mg(OAc)_2$, between 0 and 20 m*M*. The pH of cacodylate buffers varies only slightly with temperature and can be used at any temperature without corrections. We carefully checked that cacodylate did not interfere with the in vitro measurements of ribosomal activity. In some experiments, Tris-HCl (pH 7.5, 50 m*M*) was used instead of cacodylate, and KCl (50 m*M*) instead of NH_4Cl (buffer C).

Light Scattering and Turbidimetric Measurements. Light scattering measurements were carried out in an Aminco-Chance DW 2 spectrophotometer modified by the addition of a photomultiplier and of a 436-nm interference filter at 90° of the incident light. Turbidimetric measurements were also performed by measuring the absorbancy at 340 nm on the same apparatus. Both methods used the same cell which can be thermostated at any temperature between +40 and −80 ± 1°, as already described (Maurel et al., 1974).

In a typical experiment, 1 ml of buffer B containing the given Mg concentration was placed in the spectrophotometer cuvet and the scattered light or absorbancy was measured after temperature equilibration (5-min incubation). Unpurified washed or A-type 70S ribosomes (5 to 20 μl) in buffer A were then quickly added and the final stable scattering or absorbancy value was measured. After each experiment, the final concentration of the ribosomes was checked by absorption spectrophotometry at 260 nm and the experimental values were eventually corrected for small changes in the concentration.

The final ribosomal concentration, constant for each experimental curve, was limited to the range of 96–168 n*M* (4–7 A_{260} units/ml). The absence of aggregates of 70S ribosomes was tested for each experiment by the constancy of the ratio:

$$R = (I_{15} - I_0)/I_0$$

I_{15} and I_0 being the intensities of the signal of scattered or transmitted light at 15 m*M* Mg (100% association) and 0 m*M* Mg (0% association), respectively. According to the known molecular weights of the 30S subunits (0.85–0.9 × 10^6) and 50S subunits (1.7 × 10^6), the value of R must be 0.8–0.83.

Table I: Influence of Mg^{2+} Concentration on Initiation Complex Formation.[a]

Ribosomes	[^{3}H] fMet-tRNA Bound (pmol)		
	5 m*M* Mg^{2+}	10 m*M* Mg^{2+}	5 m*M*/10 m*M*
Washed ribosomes	5.36	0.609	8.80
Aged washed ribosomes	5.02	0.78	6.43
Purified 70S (0.56 A_{260})	5.46	0.37	14.75
30S (0.138 A_{260}) +50S (0.27 A_{260})	2.35	0.68	3.44

[a] The incubation mixture (100 μl) contained: Tris-HCl (pH 7.5), 50 m*M*; NH_4Cl, 50 m*M*; $Mg(OAc)_2$, as indicated; GTP, 1 m*M*; poly-(A,U,G), 0.13 A_{260} unit; [^{3}H] fMet-RNA, 14 pmol (sp act. 2553 cpm/pmol); IF_1, 12 pmol; IF_2, 10 pmol; IF_3, 18.6 pmol. Incubation 15 min at 37°. The binding effect was measured by the Millipore filtration technique. For further details on purification, materials, and methods see Dondon et al. (1974).

In some experiments, the percentage of 70 S was measured during reassociation at increasing Mg concentrations, following dissociation of the ribosomes at 1 m*M*; the exposure time of the ribosomes to 1 m*M* varied from 20 sec (which is sufficient to reach equilibrium at this concentration) to 1 min. The Mg concentration was increased up to 15 m*M* by stepwise additions of a 0.5 *M* solution of Mg.

Results

Functional Difference between Purified and Unpurified Ribosomes. In agreement with Noll et al. (1973) we found a functional difference between unpurified and A-type ribosomes. The latter were prepared by separating the 70S particles from the B-type 30S–50S couples on a sucrose gradient at 5 m*M* Mg^{2+}; the dissociated B-type 30S–50S couples were discarded and the pressure-resistant "A-type" 70S particles collected. As can be seen in Table I, with purified particles initiation is almost suppressed at high magnesium concentration, whereas "unpurified" washed ribosomes maintain nearly half of their original initiation efficiency.

Magnesium-Dependent Association Equilibrium of Ribosomal Subunits. Figure 2 shows the percentage of 70S particles as a function of Mg^{2+} concentration and indicates that $[Mg^{2+}]_{1/2}$, that is, the Mg^{2+} concentration yielding 50% association, is very comparable, under identical conditions, for both A-type (Figure 2A) and unpurified (Figure 2B) ribosomes, although the curves are much steeper for the former. On the other hand, a decrease in temperature results for both types of preparation in a decrease of $[Mg^{2+}]_{1/2}$. The values of $[Mg^{2+}]_{1/2}$ recorded at 25 and 10° with unpurified preparations appear to change somewhat with the experimental conditions of buffer and ionic strength, but are always significantly lower than the values reported by Zitomer and Flaks (1972) on presumably B-type ribosomes (Table II). Indeed our unpurified preparations usually contained less than 50% B-type ribosomes.

The simplest equilibrium scheme proposed by these authors for both Mg^{2+} and ribosome concentration dependence of association is:

$$30S + 50S + nMg^{2+} \rightleftharpoons 70S - Mg^{2+}{}_n \qquad (1)$$

where n is the difference between the number of Mg^{2+} bound to the 70S and that bound to the free subunits.

The equilibrium constant for such an equilibrium is given by:

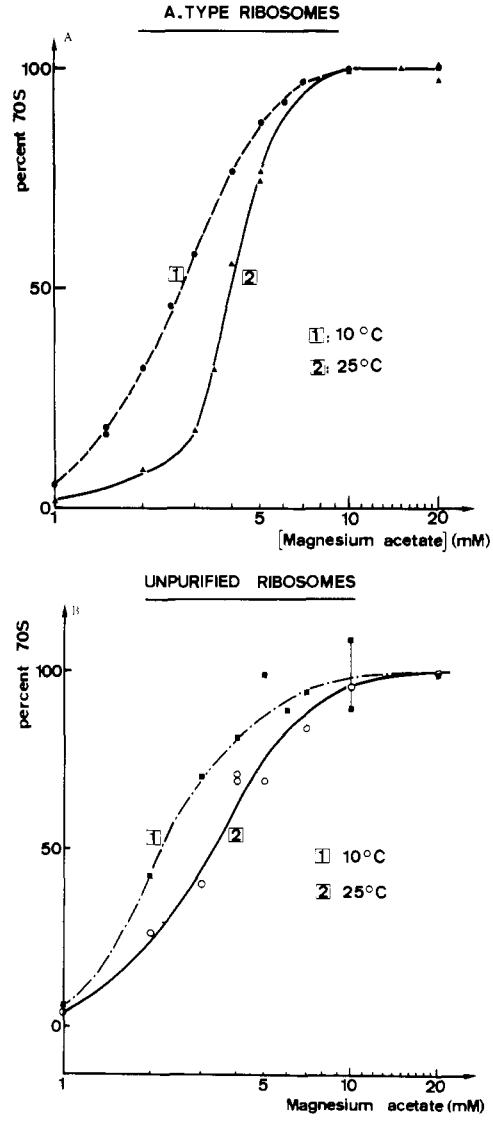

FIGURE 2: Percentage of 70S ribosomal subunits as a function of Mg^{2+} concentration, measured by light scattering or turbidimetry. The absolute light scattering at any Mg^{2+} concentration was measured as described in Materials and Methods after dilution of the stock suspension of ribosomes in buffer A into buffer B containing the specified Mg^{2+} concentration. The 0 and 100% associations were, respectively, obtained from the light scattering in buffer B containing 0 and 15 m*M* Mg^{2+}. In the case of 100% association we checked that the amount of Mg^{2+} was saturating and that further addition of Mg^{2+}, up to 20–25 m*M*, did not change the signal. Temperatures are as indicated on the figure: (A) A-type ribosomes (final concentration 5 A_{260} units/ml); (B) unpurified ribosomes (final concentration 4.3 A_{260} units/ml).

$$K_a = \frac{[70S - Mg^{2+}{}_n]}{[30S][50S][Mg^{2+}]^n} \qquad (2)$$

and by converting to log form:

$$\log \frac{[70S - Mg^{2+}{}_n]}{[30S][50S]} = \eta \log[Mg^{2+}] + \log K_a \qquad (3)$$

Under these conditions, a plot of the log of the ratio of the determined species vs. the log of Mg^{2+} concentration is linear with a slope of n and an ordinate intercept of log K_a. Examples of such log–log plots for the two different types of ribosomes are shown in Figure 3.

In the case of unpurified ribosomes (Figure 3B) the linearity obtained fits the above scheme; the values we obtained for $[Mg^{2+}]_{1/2}$, n, and K_a are reported in Table II. These

Table II: Thermodynamic Parameters of the Mg^{2+}-Dependent Association Equilibrium of Unpurified Ribosomes under Various Conditions.

Medium	$[Mg^{2+}]_{1/2}$ (m*M*)		*n*[c]		K_a[d]		ΔH (kcal/mol)
	10°	25°	10°	25°	10°	25°	
Cacodylate (50 m*M*) (pH 7)–NH_4Cl (50 m*M*) buffer B[a]	2.3	3.2	3.7	3.16	2.1×10^{17}	1.5×10^{15}	−45
Tris (50 m*M*) (pH 7.5)–KCl (50 m*M*) buffer C[a]	2	4	6.4	5.1	8×10^{24}	2.5×10^{19}	−85
Tris (50 m*M*) (pH 7.8)–KCl (50 m*M*)[b]	5.5	6.3	8.9	8.1	31.4×10^{26}	25×10^{24}	−70
Tris (50 m*M*) (pH 7.8)–KCl (150 m*M*)[b]	8.8	10.5	5.2	4.6	71×10^{16}	2.5×10^{16}	−58

[a] 4.3 A_{260} units/ml. [b] Values taken from Zitomer and Flaks (1972). [c] *n* values were measured as described in the text by slopes of log–log plots similar to Figure 3. [d] K_a was measured by ordinate intercepts of log–log plots; units, $M^{-(n+1)}$ as shown by equations in the text.

Table III: Comparison between Unpurified Ribosomes and A-Type Couples.

	Temp (°C)	$[Mg^{2+}]_{1/2}$ (m*M*)	*n*[a]	K_a
Unpurified ribosomes in buffer B	25	3.2	3.2	2.1×10^{17}
	10	2.3	3.7	1.5×10^{15}
A-type 70S,[b] I	35	4	8	2.3×10^{26}
	25	3.4	7.1	8.7×10^{24}
	14	2.65	5.4	104×10^{18}
II	35	3.7	6.2	10^{22}
	25	3.25	5.3	8×10^{19}
	10	2.4	4.7	1.62×10^{19}
III	35	3.6	10.9	1.8×10^{37}
	25	2.85	7.35	7.5×10^{25}

[a] *n* values measured as in Table II. [b] Three different preparations: I, 4.15 A_{260} units/ml in buffer B; II, 6.5 A_{260} units/ml in buffer B; III, 4.6 A_{260} units/ml in buffer C.

parameters again greatly depend on the experimental conditions but also on the preparation, although in all these cases linear log–log plots have been found.

The log–log plots for A-type ribosomes, under similar conditions, are linear only for a section around $[Mg^{2+}]_{1/2}$ and present a significant curvature at the lower and higher Mg^{2+} concentrations (see Figure 3A), indicating that the value of *n* is not constant over the whole saturation curve. In this case the above scheme is no longer appropriate; K_a and the ΔH value (determined from the Arrhenius plot of K_a) cannot be defined. However, empirical *n* values have been measured at half-saturation. and empirical values have been determined for K_a by the *y* intercept of the linear section of the curve. These are listed in Table III with $[Mg^{2+}]_{1/2}$ values and are compared to values obtained with unpurified ribosomes.

Effect of Temperature on the Association Equilibrium. The Mg^{2+} titrations at different temperatures (Figures 2 and 3) show that, depending on the type of ribosomes, the effect of temperature on the equilibrium parameters is quite different. Lower temperatures favor the association of both types of ribosomes, as can be seen by the decrease in $[Mg^{2+}]_{1/2}$ (Figure 2).

Furthermore, *n* and K_a increase with decreasing temperature for unpurified samples (Figure 3B and Table II); in this case association occurs at lower Mg^{2+} concentration, but with a higher "net excess" of ions bound to the 70S particles than to the free subunits. The value of ΔH obtained from these experiments is −85 kcal/mol, indicating that the overall association process is exothermic. Since any error in the titration curve results in great errors in the determination of K_a, the ΔH values are only approximate but always fall within the same order of magnitude.

On the contrary, *n*, measured at half-saturation, decreases with temperature in the case of A-type 70S particles (Figure 3A and Table III). As already mentioned for these ribosomes it is impossible to define an association constant, K_a. However, the *y* intercept of the linear portion of the Mg^{2+} titration curve yields an empirical value of K_a which, since both $[Mg^{2+}]_{1/2}$ and *n* decrease with temperature, also decreases at lower temperatures. Thus, in the case of A-type 70S particles, the variations of K_a with temperature are the opposite of what is observed with B-type and unpurified ribosomes.

Reversibility of Subunit Association. With both purified 70S unpurified ribosomes the titration curves obtained for association and for dissociation are independent of origin and direction of the titration, provided dissociation and reassociation are carried out quickly, the lower Mg^{2+} concentration being 2 m*M*. Under this value the results are different, depending on the ribosome preparation.

We showed (Figure 4B) that unpurified ribosomes, dissociated at 1 m*M* Mg^{2+} with an incubation time varying from 20 sec to 1 min, could be fully reassociated by increasing the Mg^{2+} concentration up to 10 m*M*. However, the equilibrium curve shows a "hysteresis", with respect to the normal dissociation curve, $[Mg^{2+}]_{1/2}$ being shifted from 2 to 3 m*M*. This is probably due to some reversible inactivation of the 30S particles which could be reactivated by exposure to high Mg^{2+} (Zamir et al., 1971). Both the association and dissociation curves of the unpurified ribosomes are noncooperative and apparently follow the simple equilibrium scheme.

On the contrary, A-type ribosomes, dissociated at 1 m*M* Mg^{2+} (and even at 0.5 m*M* Mg^{2+}) with an incubation time of 1 min, do not show the above phenomenon, suggesting that dissociation of purified particles is fully reversible (Figure 4A).

Discussion

The association equilibrium has been studied as a function of Mg^{2+} concentration and of temperature, with A-type ribosomes (corresponding to Noll's "tight couples"). The results obtained differ somewhat from those reported by Zitomer and Flaks (1972) on B-type ribosomes ("loose couples").

(1) The Mg^{2+} concentration at which ribosomes are half-associated at a given ribosomal concentration, $[Mg^{2+}]_{1/2}$, is much higher for B-type (5.5–9 m*M*) than for A-type (3 m*M*) (see Table II). This difference in stability is in fact what defines the two classes. However, for both types $[Mg^{2+}]_{1/2}$ increases as temperature increases.

(2) In the case of A-type ribosomes the double logarithmic plot of the equilibrium constant vs. Mg^{2+} is linear only

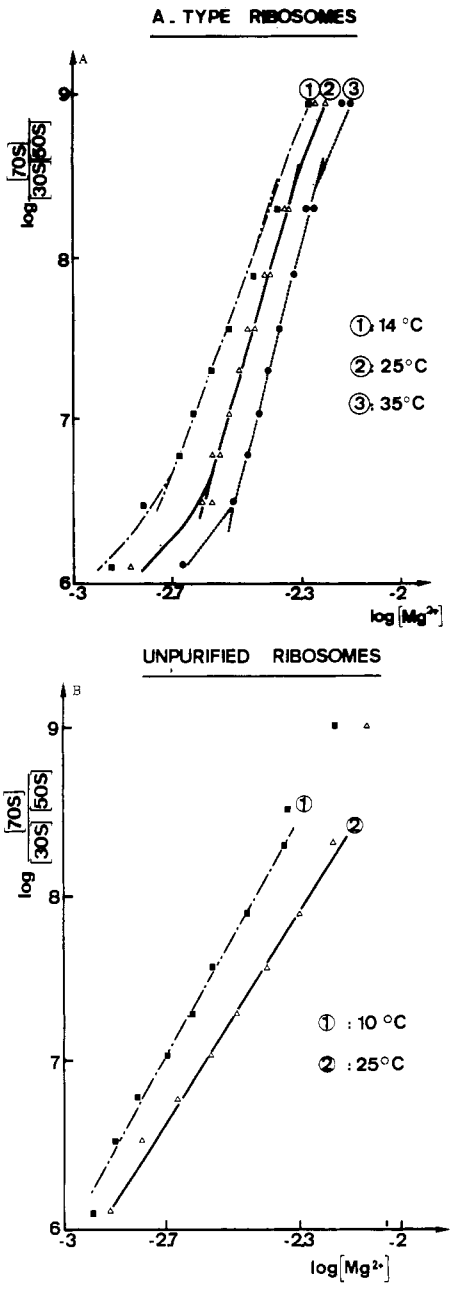

FIGURE 3: Log-log plots of the association equilibrium curves. Values for [70S]/[50S][30S] were calculated from curves similar to those of Figure 1. Temperatures are as indicated on the figure: (A) A-type ribosomes (final concentration 4.15 A_{260} units/ml in buffer B); (B) unpurified ribosomes (same final concentration as for 2B).

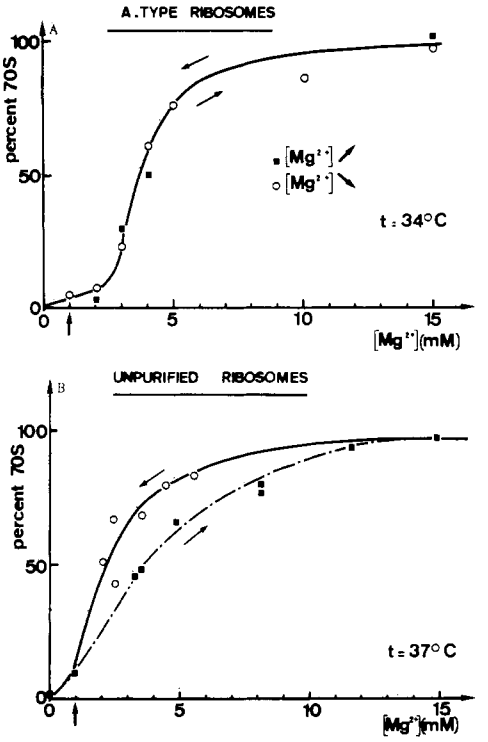

FIGURE 4: Reversibility of subunit association. Association equilibrium curves were obtained either by direct association of the ribosomes in buffer B containing the specified Mg^{2+} concentration (○) or reassociation after incubation in buffer B containing 1 mM Mg^{2+} (■): (A) A-type ribosomes (final concentration 5 A_{260} units/ml); incubation at 1 mM Mg^{2+} was 1 min at 37°; (B) unpurified ribosomes (final concentration 5 A_{260} units/ml); incubation time at 1 mM Mg^{2+} was between 20 sec and 1 min at 37°.

in the central part of the curve (between 20 and 90% association), whereas linearity extends to the whole range of measurements for the B-type ribosomes. Thus, the results for A-type ribosomes are not adequately explained by the equilibrium scheme 1 postulated by Zitomer and Flaks, nor by the resulting eq 2. We did, however, use eq 2 in the central part of the curves to define *descriptive* constants, n and K_a.

(3) n is not constant with temperature, which rules out a rigid model where a fixed number of Mg^{2+} molecules would bind during association. Furthermore, with unpurified, as well as with B-type, ribosomes (Zitomer and Flaks (1972), n decreases when temperature increases, whereas the reverse occurs with A-type ribosomes.

(4) Within a given class of ribosomes the equilibrium parameters vary somewhat with the preparation (see Table III). In fact, a small contamination of A-type by B-type ribosomes may be responsible for the observed variations. Likewise, heterogeneity of the unpurified preparations explains their n values being much smaller than the typical values for either B-type (Zitomer and Flaks, 1972) or A-type preparations.

Thus, instead of being an element of equilibrium 1, Mg^{2+}—as well as other mono- and divalent cations—should rather be considered as an independent variable acting, as do pressure and temperature, on the association equilibrium constant:

$$K = \frac{[70S]}{[30S][50S]} \qquad (4)$$

The problem is then to explain the law of variation of K with Mg^{2+}, $K = A[Mg^{2+}]^n$, where n is a function of $[Mg^{2+}]$ and depends on the preparation.

At this point it is not possible to make a precise model of ribosome association. We shall only try to discuss the possible roles of the different types of forces which may contribute to the interaction between the subunits. Electrostatic repulsion between the highly charged subunits certainly makes a large contribution, ΔG_{el}, to the free energy of binding, which has to be overcome by other forces such as hydrogen bonding, van der Waals interactions, and even chelation. Indeed a rough calculation of ΔG_{el} has been done by Wishnia et al. (1975) who consider the 30S, 50S, and 70S particles as spherical macroions, uniformly charged and accessible to the solvent. Binding of Mg^{2+} ions (experimentally determined) was taken into account for calculating the net charge of the ribosome, and was shown to vary suffi-

ciently with the Mg^{2+} concentration to account for the observed variations of K.

The effect of univalent salts, already discussed by a number of authors (Walters and Van Os, 1970, 1971; Oosawa, 1968; Choi and Carr, 1967; Manning, 1969, 1972; Wishnia et al., 1975) cannot be elucidated by the present data.

The electrostatic forces, however, are certainly not the only ones involved in ribosome stability. First, the stabilities of A- and B-type ribosomes are very different although they seem to bind Mg^{2+} to the same extent (A. Wishnia and A. Boussert, unpublished observations). Secondly, electrostatic interactions—repulsing as well as short-range attracting forces—should not greatly vary with temperature, since ϵT is approximately constant (ϵ is the dielectric constant). Therefore, the observed decrease of $[Mg^{2+}]_{1/2}$ when the temperature is decreased reflects the effect of temperature on terms other than electrostatic, and perhaps also its effect on the enthalpy of ionization of dissociable groups. Among the nonelectrostatic terms, attracting forces like hydrogen bonding and hydrophobic interactions may be very important at short distances (Walters and Van Os, 1970); the results of Spirin and Lishnevskaya (1971) seem to favor hydrogen bonding. Involvement of cations in salt bridges, that is, geometrically defined complexes, could also contribute to the association process and be one of the reasons for the specificity of Mg^{2+} and Mn^{2+} (Belitsina et al., 1971; Choi and Carr, 1967).

The structural differences between A- and B-type ribosomes are not clear as yet.

Cross experiments led to the conclusion that in most cases the 50S subunits are the ones determining the nature of the association (Van Diggelen and Bosch, 1973; Noll et al., 1973). The two-dimensional polyacrylamide electrophoretic patterns of B-type ribosomes and A-type 70S particles look remarkably similar (results not shown); therefore, the different behaviors of the two preparations do not seem to depend on a qualitative difference in protein composition. Quantitative differences, however, cannot be excluded (Van Diggelen et al., 1973), nor is it excluded that some specific polyamine is responsible for the tightness of A-type ribosomes (Van Knippenberg et al., 1974). In addition, cleavage of the ribosomal RNA (especially the 23S RNA which is more fragile) tends to reduce the stability of couples (Noll et al., 1973).

Thus, it is quite possible that heterogeneity would originate during the preparation or storage of the ribosomes; this is supported by the fact that extensively salt-washed, or aged ribosomes, behave as loose couples. However, heterogeneity also depends on the batch of *E. coli* used; some batches, even after the most gentle treatments, always yield a significant proportion of B-type ribosomes (Van Diggelen and Bosch, 1973) whereas ribosomes conventionally prepared from fresh fast-growing cultures turn out to be purely tight couples (Figure 1, insert). This is consistent with the finding that pressure-resistant ribosomes are the most active ones in protein synthesis, in the presence of phage RNA (Noll et al., 1973). Degradation of A-type ribosomes into B-type may therefore occur in the cell as well as in vitro, as a result of RNA and protein turnover in slowly growing cultures.

We would like to emphasize that at 5 mM Mg^{2+}, the association equilibrium of A-type 70S ribosomes is very much toward association. The effect of the initiation factors is to shift the overall equilibrium toward dissociation (IF 3) (Sabol et al., 1970; Subramanian and Davis, 1970; Dubnoff and Maitra, 1971; Kaempfer, 1972; Noll and Noll, 1972; Sabol et al., 1973; Godefroy-Colburn et al., 1975), and to increase the spontaneous dissociation rate (IF 1) (Noll and Noll, 1972; Godefroy-Colburn et al., 1975). However, the percentage of associated 70S ribosome at 5 mM Mg^{2+} largely depends on the ribosomal preparation, which would explain the conflicting reports concerning the requirements of initiation factors.

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Structure–Function Relationships in Glucagon: Properties of Highly Purified Des-His[1]-, Monoiodo-, and [Des-Asn[28],Thr[29]](homoserine lactone[27])-glucagon†

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ABSTRACT: We have compared the ability of glucagon and three highly purified derivatives of the hormone to activate hepatic adenylate cyclase (an expression of biological activity of the hormone) and to compete with [^{125}I]glucagon for binding to sites specific for glucagon in hepatic plasma membranes. Relative to that of glucagon, biological activity and affinity of [des-Asn[28],Thr[29]](homoserine lactone[27])-glucagon, prepared by CNBr treatment of glucagon, were reduced equally by 40- to 50-fold. By contrast, des-His[1]-glucagon, prepared by an insoluble Edman reagent and highly purified (less than 0.5% contamination with native glucagon), displayed a 15-fold decrease in affinity but a 50-fold decrease in biological activity relative to that of the native hormone. At maximal stimulating concentrations, des-His[1]-glucagon yielded 70% of the activity given by saturating concentrations of glucagon. Thus, des-His[1]-glucagon can be classified as a partial, weak agonist. Highly purified monoiodoglucagon and native glucagon displayed identical biological activity and affinity for the binding sites. Our findings suggest that the hydrophilic residues at the terminus of the carboxy region of glucagon are involved in the process of recognition at the glucagon receptor but do not participate in the sequence of events leading to activation of adenylate cyclase. The amino-terminal histidyl residue in glucagon plays an important but not obligatory role in the expression of hormone action and contributes to a significant extent in the recognition process.

The first event in the cascade of reactions leading to hormone response is the interaction of the hormone with its recognition site termed the "receptor". It is commonly assumed that binding of the hormone to the receptor induces certain transformations in the responding system. Studies of the structural requirements for hormonal recognition and action should provide further understanding of the mechanism of hormone action.

Although the structure of glucagon has been known since 1957 (Bromer et al., 1957), the structure–function relationships for this polypeptide hormone have not been elucidated clearly as yet. The discovery of a primary target for glucagon, namely the adenylate cyclase system (for review, see Rodbell, 1972; Sutherland, 1972), has provided a means of evaluating these relationships. Numerous studies have been reported with chemically modified derivatives of glucagon in attempting to establish the structure–functional role of each amino acid residue in the peptide (Spiegel and Bitensky, 1969; Rodbell et al., 1971a; Grande et al., 1972; Lande et al., 1972; Epand and Epand, 1972; Epand, 1972; Epand et al., 1973). One major obstacle in obtaining clear-cut results is the purity of the various glucagon derivatives. Chemical modification seldom produces complete conversion of substrate to product. Therefore, extensive purification is essential to remove any remaining native glucagon. Characterization of each derivative requires the study of its affinity for the receptor as well as its intrinsic activity in stimulating adenylate cyclase activity. Obviously, a significant level of contaminating glucagon would make interpretation difficult.

[Des-Asn[28],Thr[29]](homoserine lactone[27])-glucagon (CNBr-glucagon),[1] prepared by cyanogen bromide cleavage of glucagon, has been examined in two studies (Spiegel and Bitensky, 1969; Epand, 1972). In both cases, attempts to separate glucagon from CNBr-glucagon by either gel filtration or gel electrophoresis proved unsuccessful. Unreact-

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[1] Abbreviations used are: CNBr-glucagon, [des-Asn[28],Thr[29]](homoserine lactone[27])-glucagon; DH-glucagon, des-His[1]-glucagon; cyclic AMP, 3′,5′-adenosine monophosphate.