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# RELAXATION KINETICS OF E. COLI RIBOSOMES

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Relaxation kinetics measurements on two types of ribosome preparations were performed by the pressure-jump and temperature-jump techniques, using light scattered at 90° as detector. For freshly prepared ribosomes isolated as 70S tight coupled from 26 000 RPM sucrose gradient sedimentation in 10 mM $Mg^{2+}$, surprisingly large reaction amplitudes were found in 10 mM $Mg^{2+}$ with both techniques, leading to an overall formation constant for 70S couples approximately three orders of magnitude smaller than that reported for tight couples. For pelleted two-times salt-washed ribosomes, amplitude titration versus $Mg^{2+}$ in the pressure-jump apparatus showed an amplitude maximum near 10 mM $Mg^{2+}$ with a relaxation time near 20 ms, and a second amplitude maximum near 2.5 mM $Mg^{2+}$ with a relaxation time near 25 s. Both types of preparation on reanalysis on sucrose gradients at 5 mM $Mg^{2+}$ showed approximately 15% of subunits, with a distinct zone in the 50S region. 70S tight couples recovered from a sucrose density gradient separation at 5 mM $Mg^{2+}$ on pelleted two-times salt-washed ribosomes behaved in the same way as the original sample in pressure-jump experiments at 10 mM $Mg^{2+}$. These findings have been interpreted as follows (1) the processes observed at 10 mM $Mg^{2+}$ are due entirely to the relatively small loose couple content of the samples, even in the case of material isolated as 70S tight couples, (2) the processes observed at 2.5 mM $Mg^{2+}$ are due almost entirely to the preponderant tight couple population of the material, and (3) samples isolated as 70S tight couples from sucrose gradients at 5 mM $Mg^{2+}$ spontaneously revert within hours into micro-heterogeneous material containing about 15% loose couples, for both types of ribosomes.

## 1. Introduction

E. coli MRE600 ribosomes have been subjected to kinetic analysis using temperature-jump [1,2] and pressure-jump [3,4] relaxation methods arranged with 90° scattered light detection [5–7]. A brief report of an early portion of this work has appeared elsewhere [8].

Since that report, we have studied two different types of preparations in some detail. Two series of concentrations were measured at 10 mM $Mg^{2+}$ in both temperature-jump and pressure-jump on Type I ribosomes, prepared by the slow-cooling method [9] and isolated as 70S couples from sucrose gradients at 10 mM $Mg^{2+}$. Two-times salt-washed pelleted ribosomes, Type II [10], were studied for their reaction amplitude dependence on $Mg^{2+}$ concentration at constant optical and electronics sensitivity and at constant ribosome concentration in the pressure-jump apparatus. Pressure-jump studies were also made on these ribosomes as a function of concentration at 10 mM $Mg^{2+}$ and 2.5 mM $Mg^{2+}$, and temperature-jump studies were made on these ribosomes as a function of concentration at 2.5 mM $Mg^{2+}$.

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## 2. Material and methods

### 2.1. Buffers

Buffer A: 10 mM Tris-HCl, pH 7.8, 10 mM magnesium acetate and 6 mM 2-mercaptoethanol. Buffer B: 10 mM Tris-HCl, pH 7.8, 60 mM KCl, magnesium acetate as indicated and 6 mM 2-mercaptoethanol (or 1 mM dithiothreitol). Buffer C: 20 mM Tris-HCl, pH 7.8, 50 mM $NH_4Cl$, 10 mM magnesium acetate and 1 mM dithiothreitol. Buffer D: 20 mM Tris-HCl, pH 7.8, 1.0 M $NH_4CL$, 10 mM magnesium acetate and 1 mM dithiothreitol. Buffer E: 40 mM Tris-HCl, pH 7.8, 1.0 M $NH_4Cl$, 20 mM magnesium acetate and 2 mM dithiothreitol.

### 2.2. Ribosomes

Ribosomes were prepared from E. coli MRE600 by one of two methods: (I) Type I ribosomes were prepared essentially as in Ron et al. [9], with some modifications. E. coli MRE600 was grown in L broth with aeration at 37°. After 6 h the cells were kept at 12° for 15 min, then harvested at 4° by centrifugation. Cells were washed once with sterile media, then suspended in Buffer A containing 1 mg/ml lysozyme. This solution was frozen in dry ice-acetone, then thawed. After a second freeze-thawing, 10% deoxycholate was added to make a final concentration of 0.3%. After 3 min on ice, cell debris was removed by a 10 min centrifugation at 30 000 X g. The supernatant was made up to 60 mM KCl by addition of appropriate volumes of 3 M KCl and aliquots were layered onto 15–30% sucrose gradients at 10 mM $Mg^{2+}$ in Buffer B. Following centrifugation at 26 000 rpm for 4 h in a Spinco SW27 rotor, the gradients were analyzed using a recording spectrophotometer, and the 70S ribosome peak was pooled and pelleted by centrifuging 2 h at 49 000 rpm in a Spinco 50 Ti rotor. The pellet was suspended at 10 mM $Mg^{2+}$ in Buffer B and diluted to desired concentrations. Kinetics experiments were generally performed on these ribosomes within 48–72 h after preparation, and the material was not frozen prior to examination. Samples were dialyzed against 10 mM $Mg^{2+}$ Buffer B for 12 h at 0° before kinetics experiments. (II) Salt washed Type II ribosomes were prepared from freshly grown E. coli MRE600 cells [10]. Fifteen liter cultures were grown with aeration at 37° to an absorbancy of 1.0 at 650 nm. Growth was stopped by the addition of crushed ice and the cultures were allowed to cool slowly to 5°. All subsequent operations were carried out at 0–4° unless otherwise stated. The cells were harvested in a Sharples continuous flow centrifuge and were washed once with Buffer C. Fifty grams of cells were ground with 100 g of alumina and extracted with 50–60 ml of Buffer C. Cell fragments and alumina were removed by centrifugation at 17 000 rpm for 25 min in a SS-34 rotor of the Sorvall centrifuge. The supernatant was incubated with 3.0 $\mu$g of DNase (Worthington, electrophoretically purified) per ml for 20 min at 37°, centrifuged for 30 min at 30 000 X g and the residue discarded. The S30 extract was then centrifuged for 2 h at 60 000 rpm in a Spinco 65 rotor. The ribosomal pellets were suspended in approximately 50 ml of Buffer D and stirred gently overnight. The suspension was centrifuged for 20 min at 30 000 X g and the sediment was discarded. The supernatant fraction was centrifuged for 2 h at 60 000 rpm and the resulting supernatant fluid was used for the preparation of chain initiation factors. For further purification of the ribosomes, a dark brown layer on top of the ribosomal pellets was carefully scraped off and discarded. The pellets were then suspended in approximately 50 ml of Buffer D and stirred gently for 4 h. The suspension was then centrifuged for 20 min at 30 000 X g. Ten ml aliquots of the supernatant were layered over 15 ml of 30% RNase-free sucrose solution in Buffer D and centrifuged for 12 h at 50 000 rpm in a Spinco 60 Ti rotor. The supernatant was discarded and the pellets were rinsed gently with Buffer D to remove any lipid material. The pellets were then suspended in Buffer E and diluted with an equal volume of 100% glycerol.

Prior to experiments, stock solutions of Type II ribosomes, stored at −80° in 50% glycerol, were diluted to a concentration of 14.5–445 $A_{260}$ units/ml and dialyzed against Buffer B at the appropriate $Mg^{2+}$ concentration for 12 h at 0°. Dialyzed solutions were incubated for 15 min at 37°, and cooled to room temperature before all kinetics measurements, except those reported earlier [8].

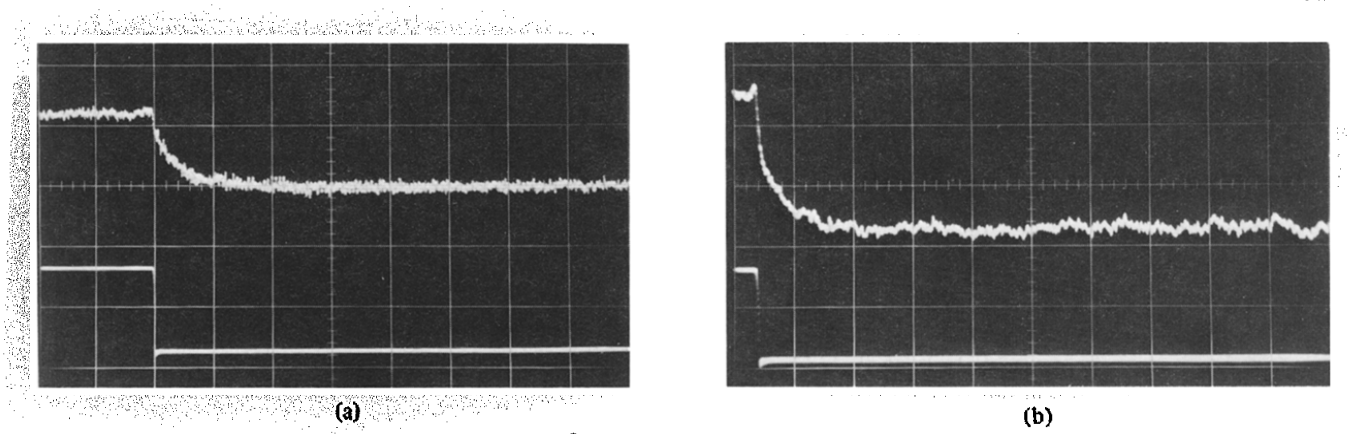


Fig. 1. Pressure-jump relaxation patterns at 10 mM $Mg^{2+}$ in Buffer B for Type I (a) and Type II (b) ribosomes. The system was pressurized from 1 atm to the final pressure of 750 lb/in$^2$ with helium in 2 ms. In (a), the concentration was 0.66% by weight, total light-to-dark deflection is 110 vertical divisions, and the horizontal oscilloscope scanning speed is 100 ms/div. In (b) the concentration was 0.25% by weight, total light-to-dark deflection is 100 vertical divisions, and the horizontal oscilloscope scanning speed is 50 ms/div. The lower traces in both patterns show the time course of the pressure, downward deflection representing increased pressure. The temperature was 25°C.

## 3. Results and discussion

Fig. 1 shows typical pressure-jump relaxation patterns at 10 mM $Mg^{2+}$, for Type I and Type II ribosomes. It is noted that for both types of ribosomes, a faster relaxation effect is often observed during the pressurization period, which for pressurization by helium has been reduced to 2 ms [7].

Since this effect cannot be resolved within the time range of this apparatus, our light-scattering temperature-jump relaxation apparatus [5] was used to extend the time resolution, as previously reported [8]. A similar fast process found in pressure-jump experiments has been attributed to refractive index changes [11]. For Type I ribosomes illustrated by the pressure jump record in fig. 1a, a temperature-jump relaxation in the time range of 1 ms, such as shown in fig. 2, has been found, at 10 mM $Mg^{2+}$. Concentration series run at 10 mM $Mg^{2+}$ on Type I ribosomes in both P-jump and T-jump yielded slow and fast relaxation times. Both T-jump and P-jump relaxation processes appear concentration-dependent. For the postulated two-step reaction mechanism

$$30\,S + 50\,S \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} 70\,S^* \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} 70\,S \qquad (1)$$

we have been able to arrive at the corresponding rate constants and equilibrium constants shown in table 1. Results shown in the top line of table 1 were obtained in one series of experiments using various Type I preparations, and assuming the first step in scheme (1) to be much faster than the second. The results in the second line of table 1 were all obtained on a single sample of Type I ribosomes. Identical concentrations [13] were measured in P-jump and T-jump, and evaluation according to reaction scheme (1) was performed by the general procedure described by Haysteen [12].

Attempts are now made to compare these results with those in the literature [11,13–16]. It is noted that rate constants were reported by Wishnia et al. [14] and Noll and Noll [15] who obtained data on "tight"

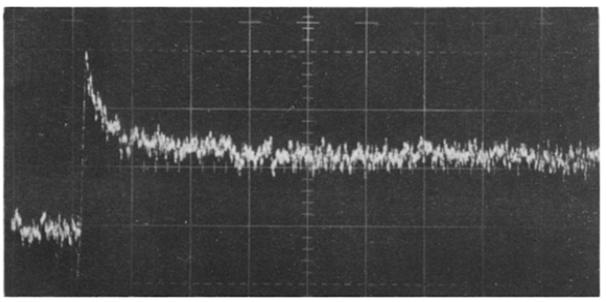

Fig. 2. Temperature-jump relaxation pattern at 10 mM $Mg^{2+}$ in Buffer B for type I ribosomes. The temperature rise was 4°C, final temperature 27.5°C. The electrical time constant for heating was $RC/2$ = 30 μs. The concentration was 1% by weight, total light-to-dark deflection is 160 vertical divisions, and the horizontal oscilloscope scanning speed is 2 ms/div. The first division of the trace at the left is the baseline before heating and the large sudden rise in scattering takes place during the heating period, after which the chemical relaxation takes place [5].

Table 1
Rate constants for Type I ribosome loose couples at 10 mM $Mg^{2+}$, assuming 15% "loose" couple, moles/l scale

| $k_{12}$ | $k_{21}$ | $K_{12}$ | $k_{23}$ | $k_{32}$ | $K_{23}$ | $K_{overall}$ |
|---|---|---|---|---|---|---|
| $7.86 \times 10^8$ | $5.6 \times 10^2$ | $1.4 \times 10^6$ | 7.7 | 6.3 | 1.2 | $3.08 \times 10^6$ |
| $3.47 \times 10^8$ | $6.15 \times 10^2$ | $5.6 \times 10^5$ | 10.3 | 13.5 | 0.76 | $9.9 \times 10^5$ |

couples [17,18] at lower $Mg^{2+}$ concentrations, whereas Wolfe et al. [13] and Schulz et al. [11] worked with ribosomes showing optimal [11] or considerable reactivity near 10 mM $Mg^{2+}$ and did not report rate constants. These authors have evidently been reporting results on heterogeneous mixtures [13] or on "loose" couples [11]. Görisch et al. [16] were able to interpret their light-scattering stopped-flow measurements as deriving contributions from both tight and loose couples. Our results in table 1 which give equilibrium constants three orders of magnitude smaller than reported for tight couples, would therefore seem to be for ribosomes of the same type as those studied by Wolfe et al. [13] and Schulz et al. [11], in spite of the fact that the method of isolation of Type I ribosomes should result in the isolation of "tight" couples. This quandary would also seem to be true for Type II ribosomes as judged from the pressure-jump behavior of fig. 1b. This matter was resolved by the pressure-jump reaction amplitude titration versus $Mg^{2+}$ concentration referred to above, in this case performed on Type II ribosomes. Whereas the process at 10 mM $Mg^{2+}$, illustrated in fig. 1b, is characterized by a relaxation time of about *20 milliseconds*, that at 2.5 mM $Mg^{2+}$, illustrated in fig. 3, has a relaxation time of about *25 seconds*. The reaction amplitude titration is shown in fig. 4. The finding of two distinct amplitude maxima near 10 mM $Mg^{2+}$ and 2.5 mM $Mg^{2+}$ is interpreted as being due to a microheterogeneous population of ribosomes. At 10 mM $Mg^{2+}$, "tight" couples are essentially undissociated [14,15]. Hence the reaction amplitude maximum at this $Mg^{2+}$ level, similar to that already reported by Schulz et al., represents entirely the interaction of "loose" couples, even though in our case these may represent a minor constituent of the total ribosome population. At 2.5 mM $Mg^{2+}$, the "loose" couples are nearly completely dissociated, as evidenced by the very small residual amplitude in the approximate range of 25 ms. Consistent with the static light-scattering $Mg^{2+}$ titration of Wishnia et al. [14] the reaction amplitude maximum at 2.5 mM $Mg^{2+}$ in the 25 s time range represents interaction of "tight" couples. Examination, on a sucrose density gradient in buffer containing 5 mM $Mg^{2+}$, of a typical sample of Type II ribosomes showing such a relaxation pattern as found in fig. 1b indicated approximately 15% of material sedimenting more slowly than the 70 S region. Even when the 70 S region was isolated from such a gradient and the concentrated recovered material was resubjected to pressure-jump relaxation at 10 mM $Mg^{2+}$, a relaxation pattern such as that in fig. 1b was again obtained. Apparently, once the generation of "loose" couples has started the process is not stopped by the isolation of "tight" couples. A further sucrose density gradient experiment was performed on Type I ribosomes. The isolated 70 S region was layered on a sucrose density gradient, as quickly as possible. Recentrifugation generated a pattern showing three resolved zones at 26 000 RPM, which according to the companion paper [17] means microheterogeneity. It should be pointed out that, because of the

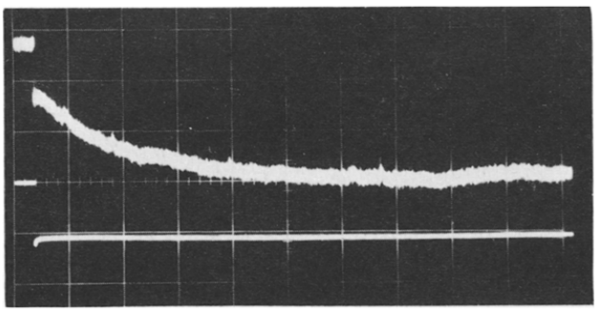

Fig. 3. Pressure-jump relaxation pattern at 2.5 mM $Mg^{2+}$ in Buffer B for Type II ribosomes. The system was pressurized from 1 atm to 510 lb/in$^2$ with nitrogen in 8 ms. The concentration was 0.59% by weight, total light-to-dark deflection is 50 vertical divisions, and the horizontal oscilloscope scanning speed is 14 s/div. The lower trace shows the time course of the pressure. The temperature was 24.8°C. The sudden drop in scattering at the beginning of the process is partly due to the process shown in fig. 2, and partly due to a slight residual loose couple interaction.

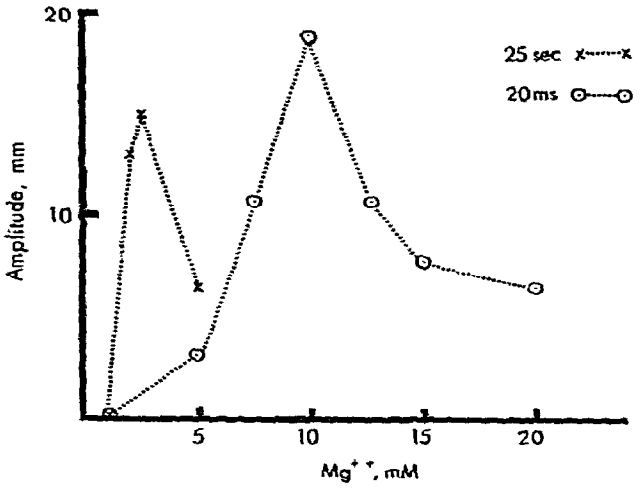


Fig. 4. Pressure-jump relaxation ampitude, in arbitrary units, versus $Mg^{2+}$ concentration for Type II ribosomes in Buffer B. All experiments performed at constant optical and electronic sensitivity settings on solutions at 0.26 weight percent concentration. For the rapid process (circles), only the amplitudes of the step having relaxation times near 20 ms are shown, and for the slow process (crosses), only the amplitudes of the step having relaxation times near 25 s are shown.

excellent time resolution and the excellent resolution on the scale of $Mg^{2+}$ concentration, a titration such as that shown in fig. 4 is more sensitive to the presence of a minor amount of loose couples than is the more classical analysis by means of density gradient sedimentation [18,19]. The demands on material and the amount of manipulation and equipment required hardly recommend this as a method of analysis, however. When reactions are driven by a large change of $Mg^{2+}$ in the stopped-flow apparatus [13,14,16] all of the reaction processes driven by the shift of $Mg^{2+}$ become superposed, and these have to be resolved by detailed statistical analysis [16], if a mixture of loose and tight couples is present. The advantage of working at a fixed $Mg^{2+}$ level, in the neighborhood of a reaction amplitude maximum in fig. 4, using a relaxation method, is that each type of ribosome reaction can be studied alone.

Attempts to study the more rapid relaxation process for salt-washed Type II ribosomes with the temperature-jump technique have met with considerable difficulty. It has been necessary to raise the concentration of ribosomes from 2 to 3 times, at 2.5 mM $Mg^{2+}$, to observe the rapid temperature-jump process. At 10 mM $Mg^{2+}$, this process has been generally undetected for Type II ribosomes at concentrations of 1%, possibly reflecting a small population of loose couples in most of these preparations. Table 2 shows the raw data for temperature-jump relaxation times at 2.5 mM $Mg^{2+}$ obtained from least-squaring log amplitude versus time. Despite the large scatter, a definite trend is shown by the averages of replicate experiments.

Table 2
Temperature-jump data for Type II ribosomes, 2.5 mM $Mg^{2+}$

| Concentration [wt. %] | $(s^{-1})\ \tau_1$ | Mean of $1/\tau_1$ |
|---|---|---|
| 2.97 | 248 | 280 |
| | 273 | |
| | 302 | |
| | 276 | |
| | 304 | |
| 2.38 | 278 | 258 |
| | 238 | |
| 1.78 | 198 | 235 |
| | 266 | |
| | 199 | |
| | 278 | |
| 1.19 | 176 | 225 |
| | 238 | |
| | 263 | |

One might well ask whether such a small trend in the presence of so much scatter in the data is really to be taken seriously. Do we really have any experimental evidence here of the existence of a rapid bimolecular process, or might we be observing just an isomerization? It is the purpose of the ensuing analysis to demonstrate that even under the extreme circumstance of an elementary bimolecular process taking place as fast as ribosomal subunits can meet, i.e. under diffusion control, the small trend shown in the data of table 2 is essentially all that one might ever expect to find in similar experiments. According to reaction scheme (1), the equilibrium concentrations of subunits A and B and of initial complex AB and ribosomes C are given by the equilibrium formation constants $K_{12} = k_{12}/k_{21}$ and $K_{23} = k_{23}/k_{32}$ according to

$$[\overline{AB}] = K_{12}[\overline{A}][\overline{B}], \qquad (2)$$

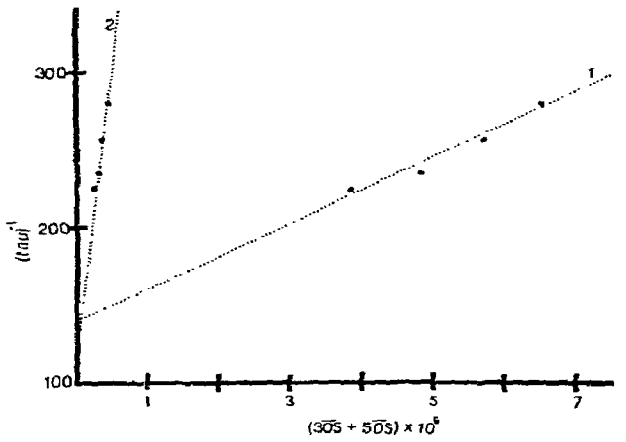


Fig. 5. Reciprocal relaxation times from temperature-jump experiments on Type II ribosomes at 2.5 mM $Mg^{2+}$ in Buffer B, shown in table 2, plotted against sum of subunit concentrations, assuming (curve 1) an overall formation constant of $8.2 \times 10^5$ liters/mole and (curve 2) an overall formation constant of $2.66 \times 10^8$ liters/mole. The points shown indicate mean values for each concentration, but the least square calculation utilized all of the data in table 2.

$$[\overline{C}] = K_{23}[\overline{AB}] = K_{12}K_{23}[\overline{A}][\overline{B}], \quad (3)$$

$$[\overline{AB}] + [\overline{C}] = K_{12}(1 + K_{23})[\overline{A}][\overline{B}]. \quad (4)$$

From

$$c = M_A[\overline{A}] + M_B[\overline{B}] + M_C K_{12}(1 + K_{23})[\overline{A}][\overline{B}], \quad (5)$$

where $c$ represents the total weight concentration in all forms, assuming equimolar total subunit population in A and B, we obtain

$$c/M_C = [\overline{A}] + K_{12}(1 + K_{23})[\overline{A}]^2 \quad (6)$$

from which

$$[\overline{A}] + [\overline{B}] = \frac{-1 + \sqrt{1 + 4K_{12}(1 + K_{23})c/M_C}}{K_{12}(1 + K_{23})}. \quad (7)$$

The overall equilibrium formation constant which can be observed by methods based on molecular weights is $K_{12}(1 + K_{23})$, which has been reported in the range $10^7$ liters/mole to $10^9$ liters/mole, depending on the level of $Mg^{2+}$ [14,15]. At total weight concentrations, of the order of 10 g/liter, we might then expect the second term under the radical in eq. (7) to be 10 times the first term, or larger. Eq. (7) can then be written in the approximate form

$$[\overline{A}] + [\overline{B}] = 2(1/\sqrt{K_{12}(1 + K_{23})})\sqrt{c/M_C} - 1/K_{12}(1 + K_{23}). \quad (8)$$

Thus the reciprocal relaxation time for the elementary bimolecular step in reaction scheme (1) can be written

$$1/\tau_1 = 2k_{12}(1/\sqrt{K_{12}(1 + K_{23})})\sqrt{c/M_C} + k_{21}k_{32}/(k_{23} + k_{32}). \quad (9)$$

In fig. 5 are shown the primary experimental data of table 2, plotted under two widely different assumptions. Line 1 is the result of least-squaring the data of table 2 according to the assumption that the overall formation constant of ribosomes $K_{12}(1 + K_{23})$ is $8.2 \times 10^5$ liters/mole. On the other hand, if $K_{12}(1 + K_{23})$ is taken as $2.6 \times 10^8$ liters/mole, which corresponds to setting $K_{12} = 6.6 \times 10^9/141$ liters/mole, and leaving $K_{23} = 0.078/0.017$ unchanged from its value for line 1, the points at the left side of the plot, showing a very large slope, are direct plots of the data of table 2.

Another way to say the same thing is that

$$k_{12} \equiv \frac{d(1/\tau_1)}{d([\overline{A}] + [\overline{B}])} = \frac{1}{2}\sqrt{K_{12}(1 + K_{23})}\,\frac{d(1/\tau_1)}{d\sqrt{c/M_C}}. \quad (10)$$

Since the primary data are values for $\tau_1$ and $c/M_C$, the slope $d(1/\tau_1)/d\sqrt{c/M_C}$ is completely experimental, and is unaffected by any assumptions as to mechanism or values of equilibrium constants. On the other hand, according to eq. (10), the value of the derived recombination rate constant $k_{12}$ for the assumed elementary bimolecular step in reaction scheme (1) depends directly on the value assumed for the overall formation constant $K_{12}(1 + K_{23})$. If this is relatively small, as in the case of line 1 in fig. 5, then the plot of $1/\tau_1$ versus $[\overline{A}] + [\overline{B}]$ will have a small slope (i.e. $k_{12}$ will be small), and one might be led to doubt that one is really seeing any bimolecular process at all. If $K_{12}(1 + K_{23})$ is chosen to be very large consistent with the literature values [14,15], then the same primary data of table 2 give rise to a plot of $1/\tau$ versus $[\overline{A}] + [\overline{B}]$ with a very large slope, line 2, from which one might feel conclusive evidence has been obtained for a bimolecular process at nearly the diffusion-controlled limit. Yet all of this obtains from the same experimental data of table 2, which shows only a small trend of relaxation time with total weight concentration amidst

a large amount of scatter. This analysis makes it clear that it is not possible to obtain much better evidence than this for the elementary bimolecular step. Although the signal to noise ratio may be improved by purely experimental refinements, no enhancement in the small concentration-dependence of the relaxation time can be expected, even for a diffusion-controlled bimolecular step.

A most disturbing feature of this analysis should be emphasized. If one expects to find a large recombination rate constant $K_{12}$ and assumes a large overall formation constant $K_{12}(1 + K_{23})$ one derives a large recombination rate constant from this assumption. If one expects a smaller recombination rate constant and assumes a smaller overall formation constant, one derives a smaller recombination rate constant from this assumption, a self-fulfilling prophecy with somewhat alarming implications.

Under the assumption of the steady-state mechanism of reaction scheme (1), we develop below a method for combining temperature-jump and pressure-jump data which overcomes this problem and allows first estimates of all the rate constants of reaction scheme (1) to be derived directly from the experimental kinetics data. The best justification we can offer for the steady state assumption itself is the very careful tests made by Wishnia et al. [14] proving that for tight couples under a wide range of conditions the kinetics in the slow time range fit a one-step bimolecular process. This should ensue under reaction scheme (1) only if the intermediate 70S* species is in a steady state during observation by stopped-flow light scattering or by pressure-jump methods.

The small amplitude process shown in fig. 5 is consistent with the amplitude function for the first step of reaction scheme (1)

$$A \propto \frac{1}{1/[30S] + 1/[50S] + 1/[70S^*]} \qquad (11)$$

in which either the subunits 30 S and 50 S or the intermediate ribosomal conformer 70 S* must be present at very small concentration. When [70S*] is very small, a rapid attainment of the 70 S* steady state level would then be followed in reaction scheme (1) by a slow relaxation (or in standard reaction kinetics, by a slow rate-determining process), which could satisfy the rate equation

Table 3
Overall pressure-jump constants assuming 15% loose couples

| $k_f$ | $k_r$ | $K_{12}K_{23} = k_f/k_r$ |
|---|---|---|
| $6.1 \times 10^6$ | 5.1 | $1.3 \times 10^6$ a) |
| $8.7 \times 10^6$ | 5.4 | $1.6 \times 10^6$ b) |
| $9.1 \times 10^3$ | 0.0147 | $6.2 \times 10^5$ c) |

a) Type I ribosome values reported in 1975 (for 20.5°C) [8], recalculated to moles/liter scale, assuming 15% loose couples.
b) Type II ribosome values at 10 mM $Mg^{2+}$, from plots of $1/\tau^2$ versus $c$.
c) Type II ribosome values at 2.5 mM $Mg^{2+}$, from $K_{12}k_{23}$ in table 4, assuming 85% tight couples.

$$\frac{d[70S]}{dt} = k_f[30S][50S] - k_r[70S] \qquad (12)$$

where $k_f = (k_{12}/k_{21})k_{23}$ and $k_r = k_{32}$. It is noted that in this pseudo-bimolecular process, the overall forward rate constant is diminished below that ($k_{12}$) for the elementary bimolecular step by the ratio $k_{23}/k_{21}$, which could be several orders of magnitude. Moreover, the overall reverse rate constant is essentially that for the transconformational change 70S → 70S*, *rather than for the dissociation of the 70S ribosome or its conformer 70S** into subunits. Assuming that the pressure-jump process of fig. 3 is such a pseudo-bimolecular steady-state process, we have derived for Type I and Type II ribosomes at 10 mM $Mg^{2+}$ and at 2.5 mM $Mg^{2+}$ the rate constants $k_f$ and $k_r$ shown in table 3, which are compared with the overall forward and reverse rate constants determined by Wishnia et al. [14], by Noll and Noll [15] and by Görisch et al. [16]. Our 10 mM data should be compared with loose couples [16] and our 2.5 mM data with tight couples [14–16].

Based on the assumption that the 70S* species in the reaction scheme (1) is in a steady state and that eq. (8) represents a good approximation to the sum of the subunit concentrations, it is possible to write an expression corresponding to eq. (9), for the slower relaxation time of the pressure-jump experiments:

$$1/\tau_2 = 2k_f(1/\sqrt{K_{12}(1 + K_{23})})\sqrt{c/M_C} + k_r - k_f/K_{12}(1 + K_{23}), \qquad (13)$$

where $k_f \approx (k_{12}/k_{21})k_{23}$ and $k_r \approx k_{32}$.

From eqs. (9) and (13) it follows that separate concentration series can be run on the same sample of ribo-

Table 4
Rate constants for Type II ribosome tight couples at 2.5 mM $Mg^{2+}$ assuming 85% "tight" couples, moles/l scale

| $k_{12}$ | $k_{21}$ | $K_{12}$ | $k_{23}$ | $k_{32}$ | $K_{23}$ | $K_{overall}$ |
|---|---|---|---|---|---|---|
| $2.24 \times 10^7$ | $1.42 \times 10^2$ | $1.58 \times 10^5$ | 0.058 | 0.0147 | 3.91 | $7.8 \times 10^5$ |

somes, but not necessarily at identical concentrations, in the two series. A least square of each set of data, independently, against $\sqrt{c/M_C}$ is then made, yielding two independent slopes and two independent intercepts, without any prior assumptions about the magnitude of equilibrium constants. If $S_1$ and $I_1$ represent slope and intercept for eq. (9), using temperature-jump data, and $S_2$ and $I_2$ represent slope and intercept for eq. (13), using pressure-jump data, then with some algebraic manipulation it can be shown that, in the case of the 2.5 mM $Mg^{2+}$, where the separation between slow and fast relaxation times is greater than three orders of magnitude,

$$K_{23} = \sqrt{I_1/I_2}/\sqrt{S_1/S_2}, \quad (14)$$
$$k_{23} = I_2 K_{23}(1 + K_{23}), \quad (15)$$
$$k_{32} = I_2(1 + K_{23}), \quad (16)$$
$$k_{12} = 0.25\, K_{23}(S_1^2/I_1), \quad (17)$$
$$k_{21} = I_2(1 + K_{23})K_{23}(S_1/S_2). \quad (18)$$

From eqs. (15)–(18), a first approximation is made to all of the four rate constants of reaction scheme (1), independent of external data. The resulting value of $K_{12}(1 + K_{23})$ is then inserted in eq. (7) to calculate the sum of subunit concentrations, $[\overline{A}] + [\overline{B}]$. This sum is then inserted into the general expression for the fast relaxation time

$$1/\tau_1 = k_{12}([\overline{A}] + [\overline{B}]) + k_{21} \quad (19)$$

and the values of $1/\tau_1$ are least-squared against $[\overline{A}] + [\overline{B}]$. The resulting new value of $K_{12} = k_{12}/k_{21}$ is used to calculate a new value of the overall equilibrium constant $K_{12}(1 + K_{23})$, which is again inserted into eq. (7) to calculate values for the sum of subunit concentrations at the weight concentrations used in the pressure-jump experiments. The steady-state equation is used for the slow relaxation time

$$1/\tau_2 = k_f([A] + [B]) + k_r, \quad (20)$$

where $k_f \approx K_{12}k_{23}$ and $k_r \approx k_{32}$. The pressure-jump relaxation times are least-squared against the sum of subunit concentrations according to eq. (20), and the slope and intercept are used to obtain a new value of $K_{23}$, which is required to converge to the previous value to within 1%. If this has not occurred, the new value of $K_{23}$ is used to calculate a new value of the overall equilibrium constant $K_{12}(1 + K_{23})$, which is inserted into eq. (7) to calculate a new set of values of the sum of subunit concentrations at the weight concentrations used in the temperature-jump experiments. Least-squaring of the temperature-jump data according to eq. (19) is repeated, and the entire calculation is recycled until convergence for $K_{23}$ is obtained to within 1%. Using the estimates generated directly from the kinetics data and eqs. (14)–(18) as the starting point, convergence was obtained to the values shown in table 4 in 9 cycles. Using the value for the overall equilibrium constant reported by Noll and Noll [15], convergence to values close to those shown in table 4 was obtained in 14 cycles. Thus, this method of successive approximations has effectively constrained the values of the rate constants in table 4 to fit the experimental data supplied, in fact to within 1% of the value reported for $K_{23}$. This does not at all mean that the results are accurate to this degree: it is clear from the scatter of the data for relaxation times in table 2 that this could not possibly be the case.

When comparison is made between our best estimates of the rate constants reported in tables 1 and 4 and values reported in the literature [14–16], it should be noted that the present report is the only one which has attempted to interpret the kinetics in terms of a two-step mechanism for a single population of ribosomes, making direct comparison somewhat complicated. Wishnia et al. [14] reported that their fastest recombination reaction corresponded to a rate constant only 1/350 of that predicted for diffusion control [20,21]. The rate constants $k_f$, table 3, at 10 mM $Mg^{2+}$ for Type II ribosomes agree almost to within a factor of two with their largest value, and the value $K_{12}k_{23}$ from table 1 for Type I ribosomes at 10 mM $Mg^{2+}$ is in even closer agreement if we can make the steady state assumption at 10 mM $Mg^{2+}$, which was not done

in evaluating the data. However, we have come to the conclusion that the data obtained in this study at 10 mM $Mg^{2+}$ are for loose couples, while the data of Wishnia et al. [14] are stated to be for pure tight couples. It is interesting to compare our results on loose couples with the only other report of rate constants for such species [16]. Görisch et al. report recombination rate constants for a final $Mg^{2+}$ concentration of either 21 mM or 5 mM. Their values at 21 mM $Mg^{2+}$ range from $6.4 \times 10^6$ to $9.8 \times 10^6$ liters/mole/s and their values at 5 mM $Mg^{2+}$ are given as $2.6–3.2 \times 10^5$, which might be directly compared with our value of $7.7 \times 10^6$ for $k_f$ in table 3 at 10 mM $Mg^{2+}$. This appears to represent a measurement of the same quantity in both types of measurement. However, it should be pointed out that if the two-step mechanism of reaction scheme (1) is accepted, the recombination rate constant for the elementary bimolecular step is really given by $k_{12}$ in tables 1 and 4. It is then noted that we report values of $3.5 \times 10^8$ and $7.9 \times 10^8$ liters/mole/s for loose couples in different preparations of Type I ribosomes at 10 mM $Mg^{2+}$, and a value of $2.3 \times 10^7$ liters/mole/s for tight couples of Type II ribosomes in 2.5 mM $Mg^{2+}$. Our largest value, for loose couples, is within a factor of eight of the limit for diffusion control, $6.6 \times 10^9$ liters/mole/s [20,21]. For tight couples at 2.5 mM $Mg^{2+}$, we can compare results in tables 2 and 4 with those reported by Wishnia et al. [14]. The value of $k_f = K_{12}k_{23}$ is $0.91 \times 10^4$, in fair agreement with the recombination rate constant of $1.7 \times 10^5$ reported by Wishnia et al., and with the value of $0.77 \times 10^5$ reported by Noll and Noll [15] at 2 mM $Mg^{2+}$ and 37°C, again suggesting that the same quantity is being measured by the three techniques. According to Görisch et al. [16], tight couples show a smaller recombination rate constant than loose couples within a single experiment. On the other hand if we were underestimating the loss of tight couples, as suggested by the dialysis experiments of Görisch et al. [16] at 2 mM $Mg^{2+}$, our values of $k_{12}$, $K_{12}$ and $K_{12}(1 + K_{23})$ would all be too small by a simple scale factor equal to the fraction of tight couples present. Again, if the two-step mechanism is accepted, the elementary recombination rate constant at 2.5 mM $Mg^{2+}$, $k_{12}$, table 4, is $2.2 \times 10^7$, considerably closer to that predicted for diffusion control. It is easier to compare dissociation to rate constants, because in the two-step mechanism of reaction scheme (1), the reverse rate constant for the pressure-jump observation is $k_r = k_{32}$, if the steady state applies. While the *interpretation* of $k_{32}$ is quite different from that of a dissociation rate constant, it is a measure of the overall rate of dissociation once the steady state is achieved and this is what will be measured in any steady-state observation. For loose couples, our measurements made at 10 mM $Mg^{2+}$, tables 1 and 3, can only be compared with those of Görisch et al. [16] made at a final concentration of 3 mM $Mg^{2+}$. Thus we report (tables 1 and 2) values of 5.1/s, 6.3/s and 13.5/s for different preparations of Type I ribosomes and a value of 5.4/s for one preparation of Type II ribosomes, all at 10 mM $Mg^{2+}$, compared to values of 0.66 to 0.87/s at 3 mM $Mg^{2+}$ [16]. For tight couples of Type II ribosomes at 2.5 mM $Mg^{2+}$, we report (tables 3 and 4) the value 0.0145/s, compared to 0.0025/s at 2.5 mM $Mg^{2+}$ and 3 mM $Mg^{2+}$ by Wishnia et al. [14], to 0.0022/s at 2 mM $Mg^{2+}$ and 37°C by Noll and Noll [15] and to 0.029/s to 0.032/s by Görisch et al. [16] at 3 mM $Mg^{2+}$. It is likely that the same quantity is being reported here, and that the differences reflect real differences in the ribosomes themselves, the tightest couples showing the smallest rate constants, especially since one need not know the concentration of reacting species to obtain this rate constant. Again, from reaction scheme (1), we would now *interpret* these rate constants in the case of tight couples as the rate constants for the rate-controlling step in the two-step process, which is the step in which 70S ribosomes rearrange to form a 70S* intermediate prior to dissociation into subunits. Comparison of tables 1 and 4 shows that the rate constants for the elementary bimolecular steps for loose couples at 10 mM $Mg^{2+}$ and for tight couples at 2.5 mM $Mg^{2+}$ are not tremendously different, which is what one might expect if the step is largely governed by diffusion control. The huge difference between loose and tight couples shows up in the rate constants for the slow step ($k_{23}$ and $k_{32}$), which are at least two orders of magnitude larger for the tansconformational changes in loose couples.

## 4. Summary

In summary, we have confirmed our earlier reported

finding [8] of two distinct relaxation processes which give rise to changes in the intensity of light scattered at 90°. These two processes have been found in the case of "tight" couples as well as in the case of "loose" couples. The faster process, found by temperature-jump experiments, is characterized by a recombination rate constant close to that predicted for diffusion control [20,21], $6.6 \times 10^9$ liters/mole/s. It has been extremely difficult to detect and measure this faster process for salt-washed Type II ribosomes [10] suggesting that in such preparations the primary reaction product between subunits is a 70S* conformer at a very small steady-state concentration. Pressure-jump reaction amplitude titrations versus $Mg^{2+}$ have demonstrated that there is an amplitude maximum near 10 mM $Mg^{2+}$, corresponding to "loose" couples, and another maximum near 2.5 mM $Mg^{2+}$, corresponding to "tight" couples. Such behavior has been found in samples in which the ratio of "tight" to "loose" couples is at least 5 : 1, and probably more nearly 10 : 1, suggesting that the pressure-jump reaction amplitude titration versus $Mg^{2+}$ is very sensitive to relatively small populations of "loose" couples. Evidence indicates that isolated tight couples of both Type I and Type II ribosomes revert rapidly into mixtures containing loose couples. The pressure-jump experiments at 2.5 mM $Mg^{2+}$ for "tight" couples, showing relaxation times in the neighborhood of 25 s, provide data for overall forward and reverse rate constants for a pseudo-bimolecular process which are in approximate agreement with the results of Wishnia et al. [14], Noll and Noll [15] and Görisch et al. [16]. The picture in which a slow conformational change of a small steady-state 70S* ribosome is the rate-determining step following the elementary bimolecular reaction step between subunits could account for observations of apparent bimolecular processes in a time range too slow for diffusion control. The observable overall reverse rate constant in that case becomes that for reversion of 70S to 70S*, instead of the rate constant for dissociation into subunits.

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