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Formation of 70S ribosomes: large activation energy is required for the adaptation of exclusively the small ribosomal subunit☆

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Abstract

Association of ribosomal subunits is an essential reaction during the initiation phase of protein synthesis. Optimal conditions for 70S formation in vitro were determined to 20 mM Mg^{2+} and 30 mM K^{+}. Under these conditions, the association reaction proceeds with first order kinetics, suggesting a conformational change to be the rate-limiting step. 70S formation separates into two sub-reactions, the adaptation of the ribosomal subunits to the association conditions and the association step itself. The activation energy of the process was determined to 78 kJ/mol and revealed to be required exclusively for the adaptation of the small subunit, rather than the large subunit or the association step. The presence of mRNA [poly(U)] together with cognate AcPhe-tRNA, accelerates the association rate significantly, forming a well-defined 70S peak in sucrose gradient profiles. mRNA alone provokes an equivalent acceleration, however, the resulting 70S couple impresses as an ill-defined, broad peak, probably indicating the readiness of the ribosome for tRNA binding, upon which the ribosome flips into a defined state.

Keywords: Protein synthesis; Ribosome; Activation energy; Association of ribosomal subunits

1. Introduction

After translation of mRNA, a ribosome falls off and dissociates into a large and small subunit. The small subunit commences protein synthesis again by recognizing the initiation signals on the mRNA, with the help of initiation factors and initiation tRNA, fMet-$tRNA^{fMet}$ in eubacteria. Both association of the large subunit and release of the initiation factors result in the formation of the 70S initiation complex and mark the transit to the elongation phase of protein synthesis.

70S formation can be considered as the last major step in the assembly of ribosomes, a remarkably complicated process that integrates 57 different components, namely ribosomal RNAs (rRNAs) and ribosomal proteins (r-proteins), into a super-molecular machine [1]. This process can now be studied at the atomic level, since atomic

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models of both ribosomal subunits were published recently [2–4], enabling a new level in defining and solving problems of ribosomal assembly and function. An example of this new quality, is the identification of a small rRNA region of 56 nt that is the primary binding site of both L24, one of the two assembly initiator proteins, and L4, one of the five proteins responsible for an essential early assembly step during the formation of the 50S subunit [5,6]. Another example is the identification of binding sites of antibiotics, leading to atomic models of the action of some drugs [7].

Assembly of multi-component complexes can be considered as the quaternary structure phase of folding, whereas in a strict sense, folding is generally understood as the folding of proteins and thus represents the tertiary structure phase. However, RNAs also have a defined tertiary structure; a prominent example is the L-shaped tRNA. Ribosomal assembly combines the tertiary structures of both rRNAs and r-proteins. The association of the ribosomal subunits as the last assembly step involves mainly salt bridges and ion pairs, rather than hydrophobic interactions; the former forces stabilize the 70S particle as indicated by early high-pressure equilibrium studies of Jaenicke et al. [8,9]. This conclusion was confirmed by recent crystal structure analysis of the ribosome: Mainly rRNAs rather than ribosomal proteins play a dominant role in the interactions between the subunits [10].

The analysis of the association process of ribosomal subunits reveals a surprisingly large activation energy of approximately 80 kJ/mol. Unexpectedly, the 30S adaptation process optimized for maximal yields of 70S ribosomes takes the lion's share of the activation energy rather than that of the 50S or the association step itself.

2. Experimental

Ribosomes and ribosomal subunits were isolated from *E. coli* strain K12-D10 (RelA$^-$, Met$^-$, RNase I$^-$; [11]) as described previously [12]. Poly(U), with a length of 250 ± 150 nt was kindly provided by Ayse Özlem Tastan, MPI für Molekulare Genetik, Berlin.

Sucrose-gradient analysis of the associated ribosomal 30S and 50S subunits (the amounts of subunits and the presence of poly(U) or Ac[^{14}C]Phe-tRNA^{Phe} are indicated) was performed in a SW40-rotor (Beckman) with a 10–30% sucrose gradient in the presence of 20 mM HEPES-KOH, pH 7.6 at 0 °C, 4 mM β-mercaptoethanol, 30–100 mM KCl and 6–20 mM Mg^{2+}, as indicated. The centrifugation was performed at 4 °C with 22 000 rpm, for 16 h. The gradient was fractionated monitoring the A_{254} nm absorption. The relative particle fractions were determined by integrating the areas of the gradient A_{254} profile. The sedimentation coefficients (*S*-values) were estimated with the help of parallel centrifuged 30S and 50S standard particles.

The relative association yield of the 70S particles is given in percent (100% 70S corresponds to 36 pmol and to the total signal area of a gradient profile).

For non-enzymatic binding of Ac[^{14}C]Phe-tRNA^{Phe}, 10 pmol of ribosomal component were incubated with 13 pmol of Ac[^{14}C]Phe-tRNA^{Phe} in the presence or absence of poly(U) (as indicated) under the ionic conditions of 20 mM HEPES-KOH, pH 7.6 at 0 °C, 4 mM β-mercaptoethanol, 30 mM KCl and 20 mM $MgCl_2$, for 30 min on ice. The binding of Ac[^{14}C]Phe-tRNA^{Phe} was determined via nitrocellulose filtration followed by scintillation counting.

Data processing: with the assumption that the rate-limiting step is a conformational change and thus a first-order law, the equation holds:

$$\ln[(70S_{max} - 70S_t)/70S_{max}] = -kt, \quad (1)$$

where the $70S_{max}$ is the maximal 70S formation observed (50% 70S) and $70S_t$ is the association yield at the time *t*. The linear dependence of $\ln[(70S_{max} - 70S_t)/70S_{max}]$ shown in Fig. 2c justifies the assumption of a first-order law. On the basis of the temperature dependence of the derived rate constants, the activation energy was determined according to the Arrhenius equation:

$$\ln k = E_a/RT + \ln A, \quad (2)$$

where *A* is the frequency factor, E_a the activation energy, *R* the universal gas constant with 8.314 J/(mol·K) and *T* the absolute temperature.

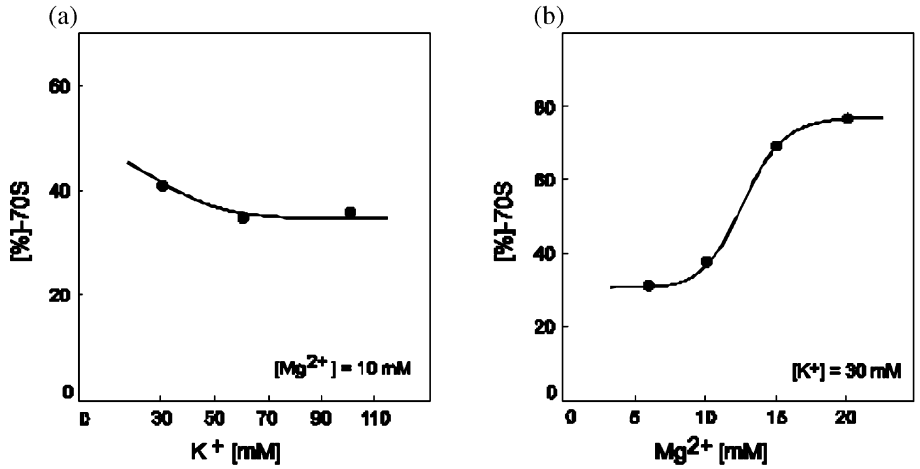

Fig. 1. Association of ribosomal subunits at various K^+ and Mg^{2+} concentrations. 1 A_{260} units of 30S and 50S subunits corresponds to 72 and 36 pmol, respectively. (a) Yields of 70S formation at 30, 60 and 100 mM K^+. (b) Yields of 70S formation at 6–20 mM Mg^{2+}. 100% 70S formation corresponded to 75% (=36 pmol) of the signal area of the total A_{254} profile. For further details see Section 2.

3. Results and discussion

3.1. Optimization of the association conditions

Analytical amounts of ribosomal subunits were incubated under various conditions, the association products were analyzed and quantified by a density gradient centrifugation. The range of the analyzed parameters was set giving attention to previous association studies [13,14]. A follow-up check was necessary, since the preparation conditions of the ribosomal subunits were significantly different and, more importantly, the ribosomes were washed with high salts, thus generating heterogeneous populations of ribosomes [15].

In the first experiment, the K^+ concentration was tested, keeping Mg^{2+} at 10 mM. One A_{260} unit of each of the small and large ribosomal subunits (72 and 36 pmol, respectively) were incubated in 100 μl at 40 °C for 30 min. The yield of 70S ribosomes was determined via sucrose gradient centrifugation (see Section 2), followed by the integration of the peak signals representing 30S and 50S ribosomal subunits and 70S ribosomes. The results are shown in Fig. 1a, indicating an almost constant yield of 70S ribosomes, approximately 40% with a weak tendency of increasing products towards lower concentration. The sedimentation coefficients of the association products were determined according to McEwen [16], as 55S (100 mM K^+), 62S (60 mM) and 67S (30 mM, not shown). It follows that not the yield, but rather the property of the association products, depend on the K^+ concentration. The optimal value was therefore 30 mM.

Fixing the K^+ concentration at 30 mM, the Mg^{2+} concentration was tested in the range of 5–20 mM in the next experiment (Fig. 1b). Now a dramatic effect was observed, with a maximal association at approximately 20 mM Mg^{2+}; the association products increased from 31% at 6 mM to 77% at 20 mM. The sedimentation coefficients were 70S at 20 and 15 mM, 67S at 10 mM and 60S at 6 mM Mg^{2+} (not shown). It follows that good yields of 70S ribosomes were obtained at 30 mM K^+ and 20 mM Mg^{2+}.

3.2. Kinetics of association

Stoichiometric amounts of 30S and 50S subunits (0.5 A_{260} and 1 A_{260} units, respectively) were incubated at various temperatures up to 60 min. At various times, an aliquot of the incubation mix was withdrawn and the association process inter-

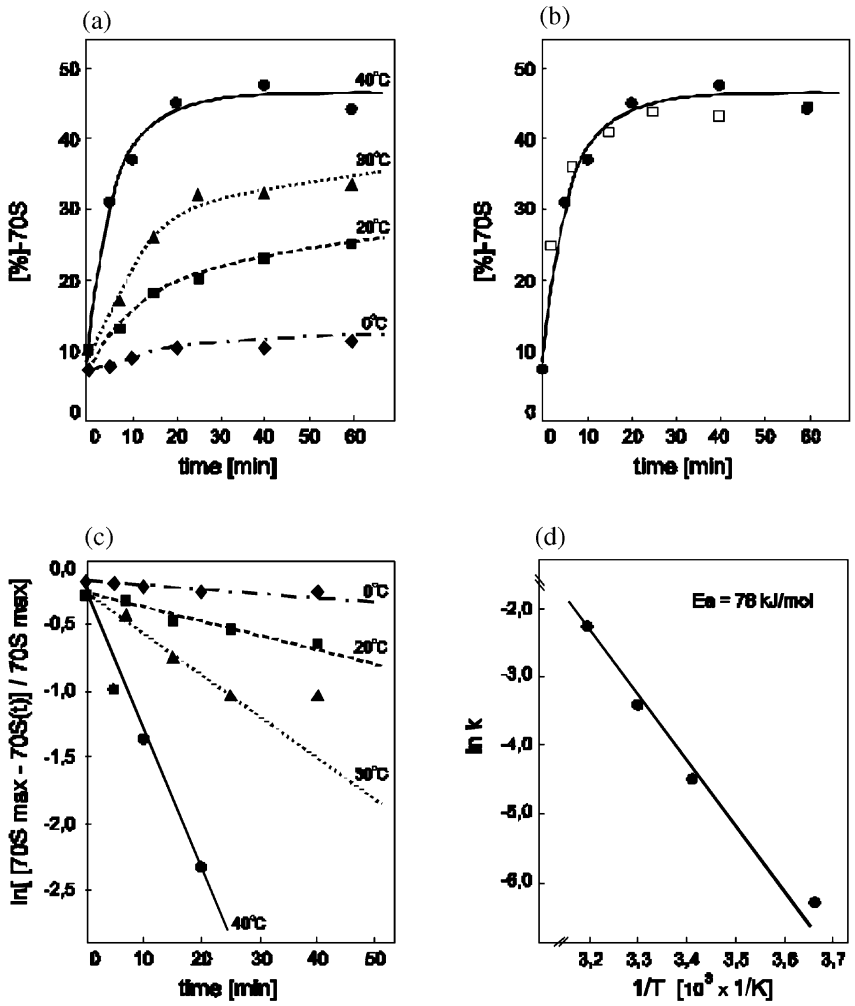

Fig. 2. Kinetics of 70S formation. (a) 0.5 A_{260} units 30S subunits and 1 A_{260} units 50S subunits (36 pmol in both cases) were mixed and incubated at the indicated temperatures, under the ionic conditions of 20 mM HEPES·KOH, pH 7.6 at 0 °C, 20 mM Mg^{2+}, 30 mM K^{+} and 4 mM β-mercaptoethanol. 100% 70S formation (equivalent to 36 pmol) corresponds to the total signal area of the gradient profile. (b) 30S and 50S subunits were separately incubated under the association conditions at 40 °C for the times indicated, mixed and incubated for an additional minute at 40 °C (open squares). For comparison, the data obtained in (a) for 40 °C are also given (closed circles). (c) Graph of the term $\ln((70S_{max}-70S_t)/70S_{max})$ vs. the incubation time. The slopes of the straight lines give the rate constants: $k_0=0.0018$ min^{-1} (0 °C), $k_{20}=0.0112$ min^{-1} (20 °C), $k_{30}=0.0327$ min^{-1} and $k_{40}=0.1044$ min^{-1}. (d) The rate constants are plotted according to the Arrhenius equation. For details see text and Section 2.

rupted by shock-freezing in liquid nitrogen. The analysis shown in Fig. 2a verified that the degree of association was conserved via the centrifugation step. Finally, all samples were thawed at 4 °C and subjected to a sucrose-gradient centrifugation (see Fig. 2a). The data were processed assuming a first order law, i.e. the rate-limiting step would be a conformational change in this case. The assumption is justified and verified, if straight lines are obtained. Fig. 2c demonstrates that this is indeed the case and that the rate-limiting step of the reaction forming 70S ribosomes is a conformational change. The corresponding rate constants were determined to $k_0=0.0018$ min^{-1} (0 °C), $k_{20}=$

0.0112 min^{-1} (20 °C), $k_{30}=0.0327$ min^{-1} (30 °C) and $k_{40}=0.1044$ min^{-1} (40 °C). The temperature dependence of the rate constants was determined according to the Arrhenius equation (Fig. 2d, see Section 2). The slope of the resulting straight line is the negative quotient $(-Ea/R)$, where Ea is the activation energy and R the universal gas constant. The activation energy amounts to 78 kJ/mol.

70S formation as performed here separates into two steps. The first is the adaptation of the separated ribosomal subunits to the association condition, the second is the association step itself, where the subunits couple to form the 70S ribosomes. To clarify which step is a rate-limiting step and thus determines the activation energy, we performed incubation kinetics of the separated subunits in the association milieu for up to 45 min at 40 °C before mixing them and incubating for 1 min at 40 °C. The association reaction was interrupted by shock freezing the aliquots. The analysis of the amount of formed 70S ribosomes was done as described above in Section 2. The latter incubation for 1 min does not compromise the analysis, since the formation of 70S ribosomes requires more than 10 min, when adaptation and association is performed at the same time (Fig. 2a). The data clearly demonstrate that the formation of 70S ribosomes is practically the same if the kinetics were performed at the adaptation conditions with the separate subunits (open squares in Fig. 2b) or if both adaptation and association is allowed to proceed during the same incubation (closed circles).

In order to test the energetic requirements of the individual subunits, incubation kinetics of both subunits were performed. In the first set of experiments the 50S subunits were incubated at 40 °C for 45 min prior to mixing with the 30S subunits. The latter had been incubated at 40 °C for the times indicated. After mixing, the solution was kept at 40 °C for 1 min before the 70S formation was analyzed via sucrose gradient centrifugation. In the second set of experiments, the 30S subunits were incubated correspondingly at 40 °C for 45 min prior to mixing with the 50S subunits, which had been incubated at 40 °C for the times indicated. The mixed solution was kept at 40 °C for 1 min before subjecting to a sucrose gradient centrifugation.

The results reveal that only the small subunit requires an adaptation step in contrast to the large subunit (Fig. 3a). It follows that the activation energy is essentially used for the adaptation of the small ribosomal subunits.

In the presence of poly(U) and AcPhe-tRNA, the association rate was faster than in the absence of these ligands (Fig. 3b), while poly(U) alone (open circles) had an intermediate effect. We used short poly(U) with a relatively defined length (250 ± 150 nt), in order to avoid problems with the resolution in sucrose gradients. Corresponding sucrose gradient profiles revealed an additional detail induced by the ligands (Fig. 3c,d). Presence of both poly(U) and AcPhe-tRNA resulted in a highly defined and sharp peak (dashed lines in Fig. 3c,d), whereas poly(U) alone gave a peak of the same area that was broad and ill-defined (dotted lines). mRNA without a Shine–Dalgarno (SD) sequence only weakly interacts with the ribosome in the absence of cognate tRNAs, as demonstrated via nitrocellulose filtration [17]. Here, we demonstrate that the SD-less poly(U) interacts with 70S almost to the same extent, but the broad peak indicates an 'open' structure of the 70S couple, probably ready for tRNA binding to the P site. Only tRNA binding to programmed ribosomes induces a defined conformational state of the ribosome. This effect was convincingly demonstrated by cryo-electron microscopy, where a programmed ribosome containing a tRNA at the P site showed an improved resolution, as compared to empty ribosomes [18]. This complex played an important role for the elucidation of the tRNA positions during the elongation cycle (see for details [19]).

In the next experiment, we tried to correlate the energetic requirements of the small subunit with its tRNA binding capacity. To this end, the binding of Ac[^{14}C]Phe-$tRNA^{Phe}$ was tested in the absence or presence of cognate mRNA poly(U). Heat activated 30S subunits (45 min at 40 °C) in the absence of mRNA do not bind any tRNA (experiment 1 in Table 1) confirming a previous observation [20] and non-heat-activated 30S subunits in the presence of mRNA also did not bind any

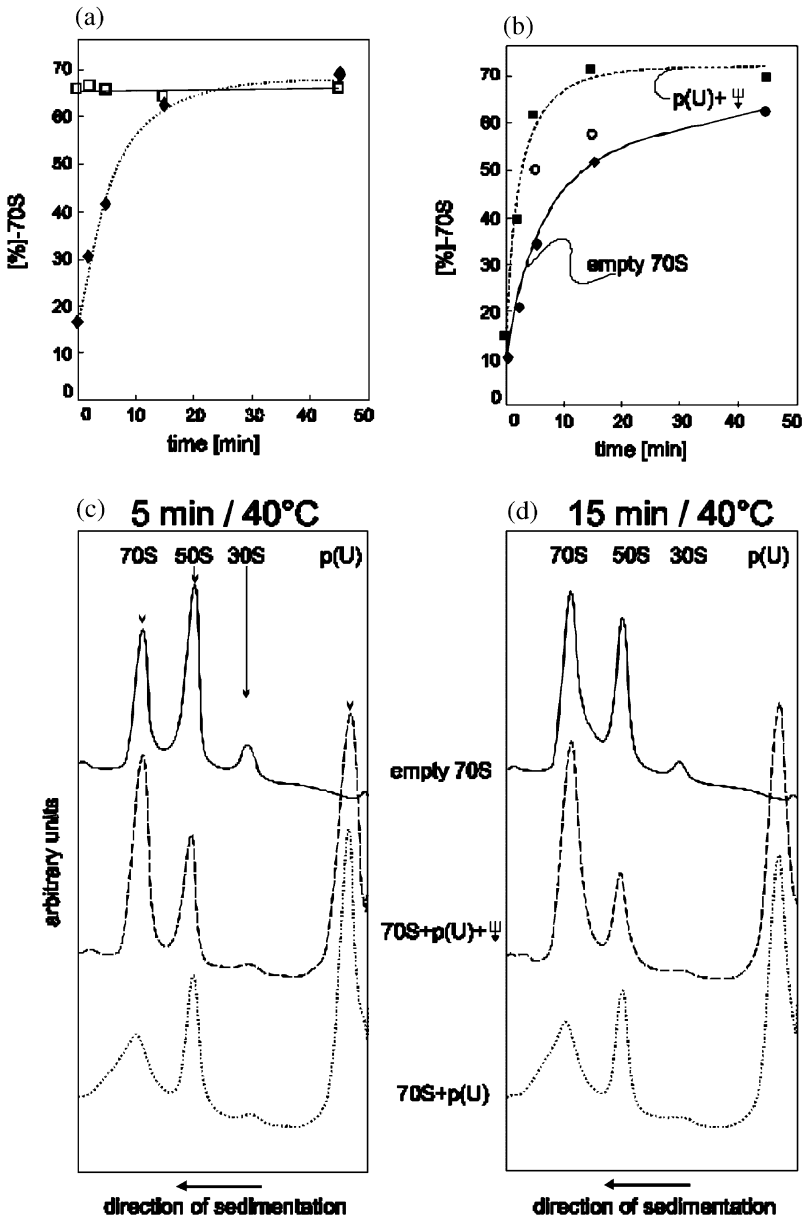

Fig. 3. 70S formation upon pre-incubation of the ribosomal subunits. (a) Kinetics of incubation at 40 °C of 36 pmol 30S subunits (dotted line and diamonds); after mixing with 50S subunits pre-incubated at 40 °C for 45 min, the mixture was incubated for 1 min at 40 °C before analysis via a sucrose-gradient centrifugation. Likewise, kinetics of incubation at 40 °C of 36 pmol 50S subunits (solid line and open squares) were performed and mixed with 30S subunits pre-incubated at 40 °C for 45 min; the mixture was also incubated for 1 min at 40 °C and subjected to a sucrose-gradient centrifugation. (b) Activation of 30S subunits as in (a), but in the presence of poly(U) and Ac-Phe-tRNA^{Phe}. Solid squares and dashed line, 30S activation in the presence of poly(U) and Ac-Phe-tRNA^{Phe}; open circles, activation in the presence of only poly(U); filled diamonds and solid line, control without poly(U) and Ac-Phe-tRNA^{Phe}. (c) and (d) gradient profiles showing 70S formation with activated 30S subunit. Solid line, 30S activation without ligands; dashed line, 30S activation in the presence of poly(U) and Ac-Phe-tRNA^{Phe}; dotted line, in the presence of poly(U) only. 30S activation for 5 and 15 min, respectively, at 40 °C: p(U), poly(U); Ψ, Ac-Phe-tRNA^{Phe}.

Table 1
Binding of Ac-[^{14}C]Phe-tRNA^{Phe} to 30S subunits and re-associated 70S ribosomes

Exp.	30S	Ψ	Poly(U)	50S	Binding of Ψ per ribosome
1	+	+	–	–	0.03
	+ (0′/40 °C)	+	+	–	0.05
	+	+	+	–	0.61
2	+	+	–	+ (0′/40 °C)	0.17
	+	+	+	+ (0′/40 °C)	0.92
3	+	+	–	+ (1′/40 °C)	0.44
	+	+	+	+ (1′/40 °C)	0.99

0′/40 °C and 1′/40 °C, no or 1 min incubation at 40 °C of the subunits, respectively, before mixing with the complementary subunits. The mixture was incubated for 30 min at 0 °C, in the presence of the indicated ligands before nitrocellulose filtration. 30S were pre-incubated for 45 min at 40 °C if not otherwise indicated: exp., experiment; Ψ, Ac-Phe-tRNA^{Phe}.

tRNA. Non-heat activated 30S were hardly able to associate with activated 50S subunits (17%, Fig. 3a), and thus, as expected, the presence of 50S subunits did not show significant binding values (not shown).

Very low binding values were obtained upon addition of 50S subunits to the heat-activated 30S subunits (0.17 per 70S ribosome, first line in experiment (2). The addition of mRNA provoked a drastic increase for 30S as well as for (30S + 50S) subunits to approximately one AcPhe-tRNA^{Phe} per 70S ribosome, although a 1.3-fold excess of AcPhe-tRNA^{Phe} over 70S was used (last line of experiment 1 and 2 in Table 1). This finding confirms the 'exclusion principle' for peptidyl-tRNA binding [21,22], which probably reflects the insertion of the polypeptide residue into the corresponding tunnel of the large subunit: If a peptidyl residue has been inserted into the tunnel, a second peptidyl-tRNA cannot bind any more to the ribosome, regardless of whether the tRNA moiety of the first peptidyl-tRNA is present at the P or A site.

Interestingly, the binding of AcPhe-tRNA^{Phe} to non-programmed 70S depends on a short incubation of the 50*S* subunits (1 min at 40 °C). Without this incubation, a low binding of 0.17 AcPhe-tRNA^{Phe} is observed, with incubation an almost three-fold higher value of 0.44 is found. The tRNA binding to isolated 30S subunits in the presence of mRNA is fully dependent on an incubation of the 30S subunits (experiment 1 in Table 1).

4. Conclusions

For almost three decades it has been known that both ribosomal subunits require an activation energy of approximately 100 kJ/mol in order to be ready for protein synthesis [23]. After activation of both subunits, association to 70S ribosomes occurs with relative ease, indicated by a low activation energy of approximately 23 ± 7 kJ/mol [24,25].

Here, we analyzed the global activation energy for 70S formation, starting with subunits that have not been activated before. Surprisingly, the exclusive adaptation of the small subunit rather than that of both subunits, or the association step itself, requires most of the large activation energy of approximately 80 kJ/mol. This is unexpected, since isolated 50S subunits are inactive in, e.g. peptide-bond formation and need the presence of approximately 30% of alcohol to be activated [26]. 70S ribosome are active in peptide-bond formation, also indicating an activation step of the large subunit during the association reaction. We note that the required Mg^{2+} concentration is very high (20 mM) as compared to the in vivo conditions, where the Mg^{2+} concentration is between 2–4 mM, flanked and supported by polyamines. Spermidine and spermine seem to play the major role of the polyamines [27]. The high Mg^{2+} concentration alone is not activating the 50S subunit, since 70S ribosomes are hardly active in peptide synthesis under this condition: The optimal Mg^{2+} concentration for protein synthesis in the absence of polyamines is 8–11 mM [28,29], and in the presence of polyamines, between 3 and 5 mM Mg^{2+} (for discussion see [27]). The association and the programmed tRNA binding must induce a rearrangement of the peptidyl-transferase center responsible for the activation (compare lines 4 and 5, experiment 2 in Table 1), since in the absence

of mRNA, little binding is found (0.17 per 70S ribosome). Interestingly, the non-programmed binding is improved to 0.44, if the 50S subunit is incubated for only 1 min at 40 °C, indicating a subtle activation also of the large subunit. Furthermore, association rearranges the 30S subunit, opening the A site for the aminoacyl-tRNA; this site is not accessible in isolated 30S subunits [30,31].

It follows that both subunits change their conformation upon association and that the rate-limiting step of 70S formation is a conformational change of only the small subunit, requiring a high activation energy of approximately 80 kJ/mol. The question remains whether the strong activation effects observed with the 30S subunits also occurs in the cell or whether the inactivation of the small subunit is a consequence of standard procedures applied for the isolation of the ribosomal subunits. To answer this question in vivo, near conditions have to be maintained during the isolation procedure in the presence of initiation and termination factors, at least one of which, namely the initiation factor IF3, prevents association of the ribosomal subunits.

Acknowledgments

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