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70S Ribosomes Bind to Shine–Dalgarno Sequences without Required Dissociations

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In all living systems, proteins are synthesized on ribosomes by translating sequences of mRNA. Translation processes are complex and highly regulated by various translation factors together with ribosomes. It is known that translation initiation is the most dynamic step in building an initiation complex composed of a ribosome, mRNA, and an initiator tRNA.[1,2] In bacteria, it is thought that the 70S ribosome must dissociate into a 30S and 50S subunit triggered by initiation factors to interact with mRNA. It is generally accepted that the free 30S ribosomal subunit binds to a Shine–Dalgarno (SD) sequence, a 3–10 nucleotide purine-rich sequence (for example, AGGAGG), on a mRNA in the first step of the initiation translation, and then the 50S subunit is recruited to the mRNA–30S complex with an initiator tRNA on an AUG start codon downstream of the SD sequence.[3] On the other hand, the preassembled 70S ribosome can translate only a leaderless mRNA lacking the SD sequence with an AUG start codon at the 5′ terminus of the mRNA.[4,5] Although most free ribosomes exist in the 70S form under physiological conditions,[6] very few studies have investigated the binding of 70S to a mRNA with a SD sequence.[4,5] The interaction of the intact 70S ribosome with the SD sequence has either been overlooked or not established in the literature.

In this communication, we report that the intact 70S ribosome can bind directly to mRNA with the SD and AUG sequences without a required dissociation. Binding kinetics were studied using a mRNA-immobilized 27 MHz quartz-crystal microbalance (QCM; Figure 1), which is known to be a very sensitive mass measuring device in aqueous solutions. QCM resonance frequencies decrease linearly upon mass increases on the QCM electrode to the nanogram level.[7–9] Calibrations of the 27 MHz QCM in aqueous solutions are described in the Supporting Information (S1), and 1 Hz of frequency decrease was calibrated as an increase in 0.18 ng cm^{-2} of ribosomes in aqueous solution.

E. coli 70S ribosomes and 30S and 50S ribosomal subunits were prepared and purified as described previously.[4] Initiator tRNAs (tRNA^{fMet}) were prepared from an overexpressed strain and purified.[4] fMet-tRNA^{fMet} was enzymatically methionylated by methionyl-tRNA synthetase and then formylated by methionyl-tRNA formyltransferase.[4] mRNAs were prepared by in vitro transcription using a T7 RNA polymerase. For biotinylation of the 3′ terminus of the mRNA, the transcript was oxidized with $NaIO_4$ and then modified with biotin hydrazide. We prepared several kinds of biotinylated mRNAs with SD + AUG or SD + UUG sequences, the mRNA without the SD sequence (non-SD), the mRNA in which the SD sequence is hybridized with the antisense DNA (anti-SD), and the mRNA in which a 5′-untranslated region (5′-UTR) is hybridized with the antisense DNA (anti-UTR). The sequence of the transcripts are shown in Table 1, and preparation methods of mRNA and the sequence of antisense DNAs are described in the Supporting Information (S2 and S3). AFFINIX Q4 with four 500 μL cells equipped with a 27 MHz QCM plate at the bottom of each cell was used as a QCM instrument.[7–9] Biotinylated mRNA was linked by avidin–biotin interactions on a QCM plate covered with NeutrAvidin™ as previously reported.[7–9] After immobilization of mRNA, ribosomes were injected into aqueous buffer (10 mM HEPES-KOH, pH 7.3, 100 mM NH_4Cl, 5 mM $Mg(OAc)_2$, 0.5 mM $CaCl_2$, 25 °C) in a QCM cell. Experimental procedures are described in the Supporting Information (S4).

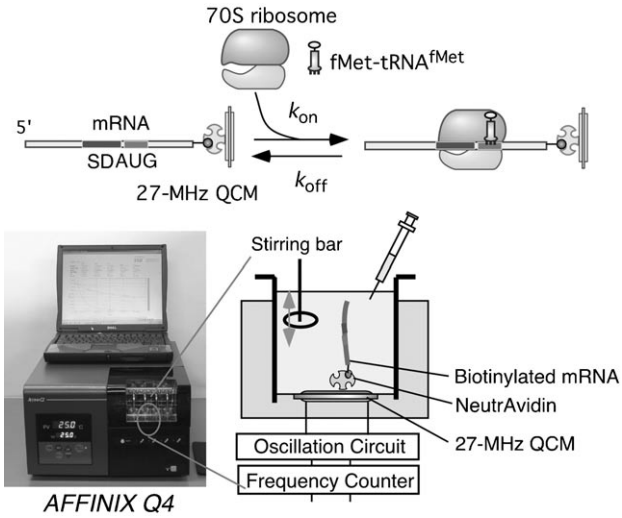

Figure 1. Experimental setup for measuring the binding kinetics of ribosomes (70S, 50S, 30S, and crosslinked 70S) with/without fMet-tRNA^{fMet} to a biotinylated mRNA-NeutrAvidin-immobilized 27-MHz quartz-crystal microbalance (QCM, AFFINIX Q4) in aqueous solutions.

Figure 2 A shows typical time courses of frequency decreases (mass increases) corresponding to additions of 30S, 50S, and 70S ribosomes in aqueous solutions. The 30S subunit showed relative binding to mRNA with SD and AUG sequences (SD + AUG), but the 50S subunit showed little specific binding. The

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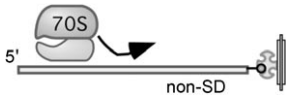
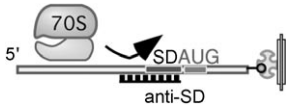
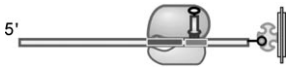
Table 1. Kinetic parameters for binding of ribosomes to mRNA-immobilized on a 27 MHz QCM.[a]

Run	Combination[b]		Δm_{max} [ng cm^{-2}]	Molar ratio of 70S versus 30S	K_a [10^7 M^{-1}]	k_{on} [10^4 M^{-1} s^{-1}]	k_{off} [10^{-4} s^{-1}]
1	(SD+AUG), 30S		48±8	1	5.6±1.5 (6.2)[c]	16±5	27±2.8
2	(SD+AUG), 50S		–	–	<0.1	–	–
3	(SD+AUG), 70S		110±15	0.8	4.3±1.0 (6.8)[c]	18±7	26±2.9
4	(non-SD), 70S		–	-	<0.1	–	–
5	30S-(SD+AUG), 50S		91±16	1.2	0.4±0.1	–	–
6	antiSD-(SD+AUG), 70S		–	–	<0.1	–	–
7	antiUTR-(SD+AUG), 70S		48±3	0.4	12±2.0	–	–
8	(SD+AUG), 70S+tRNA		120±9	0.9	10±1.6	–	–
9	(SD+UUG), 70S+tRNA		144±54	1.1	2.6±1.2	–	–
10	(SD+AUG), cross-70S		–	–	<0.1	–	–
11	(AUG), cross-70S		130±161	1.0	2.7±4.5 (9.2)[c]	5.0±4.0	5.4±1.7

[a] 10 mM HEPES-KOH, pH 7.3, 100 mM NH_4Cl, 5 mM $Mg(OAc)_2$, 0.5 mM $CaCl_2$, 25 °C. [b] Sequences of mRNA. SD+AUG: 5′-GGGAGAUUCCCCAUGAUAACAUGGAUUCAC**AGGAGG**CACAAUAC**AUG**AAAAACUAGUCCGGGUACCGAGCUCGAAUU-3′, SD+UUG: 5′-GGGAGAUUCCCCAUGAUAACAUGGAUUCAC**AGGAGG**CACAAUAC**UUG**AAAAACUAGUCCGGGUACCGAGCUCGAAUU-3′, non-SD: 5′-GGGAGAUUCCCCAUGAUAACAUGGAUUCAC**UCACGC**CACAAUAC**AUG**AAAAACUAGUCCGGGUACCGAGCUCGAAUU-3′, AUG: 5′-G**AUG**AGCACAAAAAAGAAACCAUUAACACAAGAGCAGCUUGAGGACGCACGUCGCCUUAAAGCAAUUUAUGGGAAUU-3′. [c] Association constants determined from k_{on}/k_{off}.

70S ribosome unexpectedly bound to the internal SD sequence, because it has been accepted that only the free 30S subunit can bind to the internal SD sequence.[4,5] 70S and 30S showed little specific binding to the mRNA without the SD sequence (non-SD; see Run 4 in Table 1). When the concentration of ribosomes was increased, saturation binding behaviors were observed (Figure 2B) and the maximum amount bound (Δm_{max}) and the association constant (K_a) were obtained from curve fitting analyses of Figure 2B. The results are summarized in Table 1, together with the binding rate constant (k_{on}) and the dissociation rate constant (k_{off}), which were obtained from curve fitting binding time courses as in Figure 2A.[7–9] Although the Δm_{max} value of the 70S was larger than that of the 30S, the molar ratio of the maximum bound 70S and 30S was calculated to be 1.0±0.2 considering their molecular weights (2520 and 930 kDa, respectively (Runs 1 and 3 in Table 1).

With these results, we considered cautiously two models of 70S binding onto the mRNA: 1) the 70S ribosome could bind directly to mRNA without dissociating into each subunit or 2) the 30S subunit, which might exist as an equilibrium mixture of the 70S ribosome, binds first to the mRNA and then the 50S subunit binds to the 30S-bound mRNA. To examine the possibility of the second binding route, we added several experiments. Dissociations of 70S to each subunit of 50S and 30S have been known to decrease with the increase of Mg^{2+} ions and the binding constant has been estimated to be approximately 10^9 M^{-1} in the presence of 5 mM $Mg(OAc)_2$.[10] In our experiments, 2–25 nM of the 70S ribosome was used in the presence of 5 mM Mg^{2+} ions, which indicates that the population of the free 30S subunit is negligibly small. In addition, binding amounts of 70S to the mRNA did not increase when the Mg^{2+} concentrations increased from 5 to 50 mM. This indicates that

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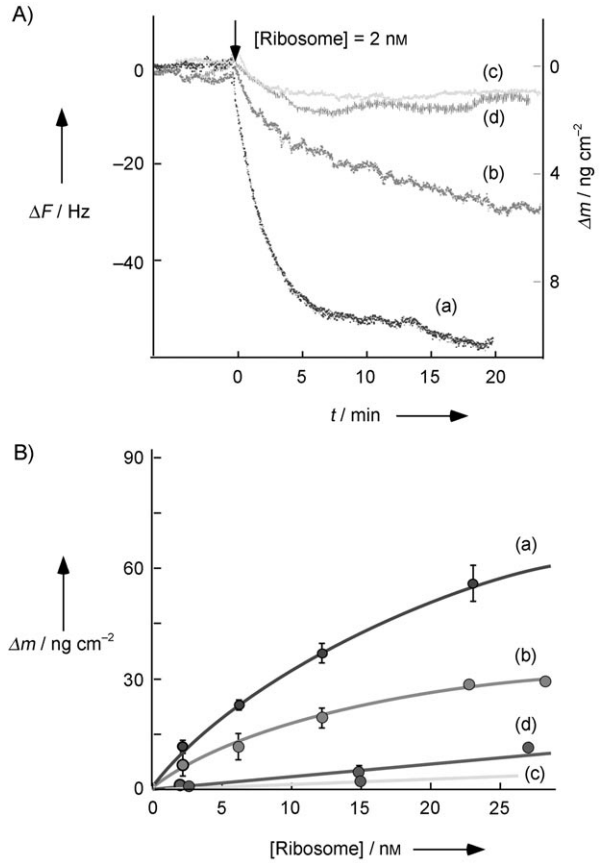

Figure 2. A) Typical time courses of frequency decreases (mass increases) of the mRNA (SD + AUG)–immobilized QCM, responding to the addition of 2 nM of ribosomes, and B) their saturation binding curves. Bindings of a) 70S ribosome to mRNA, b) 30S subunit to mRNA, c) 50S subunit to mRNA, and d) 50S subunit to the 30S-bound mRNA (10 mM HEPES-KOH, pH 7.3, 100 mM NH_4Cl, 5 mM $Mg(OAc)_2$, 0.5 mM $CaCl_2$, 25 °C).

5 mM of Mg^{2+} ions is enough to diminish the free 30S (see the Supporting Information S5 and Figure S1). The maximum amount of 70S bound to the mRNA was not affected in the presence of tenfold excess of 50S, which decreases the population of free 30S in an equilibrium mixture of 70S (see Supporting Information S6 and Figure S2). These results indicate that the direct binding of 30S is negligible under these conditions. As shown in Figure 2, curve d, 50S hardly bound to the 30S-bound mRNA. The K_a value of 50S to the 30S–mRNA complex was found to be $4\times10^6\,M^{-1}$, which was tenfold lower than $K_a = 4.3\times10^7\,M^{-1}$ of 70S to the mRNA (Run 5, in Table 1). Therefore, the second binding route (the recombination of 50S onto the 30S-bound mRNA) is excludable.

To confirm whether the 70S ribosome formed the ribosome–mRNA complex properly, we observed the 70S binding in the presence of the initiator tRNAs (see the Supporting Information S7 and Figure S3). The K_a value of the 70S ribosome (Run 3: $4.3\times10^7\,M^{-1}$) increased more than twice in the presence of fMet-tRNA^{fMet} that recognizes the AUG start codon (Run 8: $10\times10^7\,M^{-1}$). The K_a value of the 70S ribosome showed a large decrease (Run 9: $2.6\times10^7\,M^{-1}$) in the case of mRNA with UUG sequences instead of AUGs, which cannot be recognized by fMet-tRNA^{fMet}. These results also support the hypothesis that the intact 70S ribosome can include fMet-tRNA^{fMet} and recognize both the SD sequence and the AUG start codon without the ribosome dissociating into its subunits.

We further dissected the binding mode of 70S to the SD sequence on mRNA. It is generally considered that the 30S subunit laterally binds to the internal SD sequence on mRNA, and that the 70S ribosome can only bind to leaderless mRNA with an AUG start codon at the 5′ terminus and translates through sliding along the mRNA from its 5′ terminus.[2] In our experiments, the 70S ribosome could not bind to the SD sequence blocked with antisense DNA (anti-SD, Run 6 in Table 1), but showed a specific binding capability to mRNA whose 5′-UTR was blocked with anti-UTR DNA (Run 7). The aforementioned results indicate that the intact 70S ribosome and the 30S subunit cannot bind to the 5′ terminus and then slide to the internal SD sequence, but can hold laterally to the SD sequence. X-ray structural studies showed that 70S ribosomes have a cleft at the interface of each subunit.[11] Thus, the anti-SD in 70S ribosome could bind to the internal SD sequence that would pass this cleft laterally without ribosomal dissociation.

To verify this hypothesis, we prepared a crosslinked 70S ribosome, which was reported to not translate mRNA with an internal SD sequence, but to synthesize proteins from mRNA from an AUG start codon at the 5′ terminus.[5,12] The crosslinked 70S hardly bound to the internal SD sequence (Run 10 in Table 1), but could bind to the 5′ terminus of the AUG sequence with fMet-tRNA^{fMet} (Run 11). These results imply that the crosslinked 70S ribosome can only access the 5′ terminus of the 5′ AUG sequence via short sliding, and cannot bind to the internal SD sequence due to blocking from the crosslinker that prevents the lateral binding of mRNA to the ribosomal cleft. Both the k_{on} and k_{off} values for the binding of the crosslinked 70S ribosome to the 5′ terminus of the AUG sequence were three times lower than those for the binding of the normal 70S ribosome to the internal SD sequence (Runs 11 and 3, respectively). These results support the suppositions that the lateral-binding mode of the 70S ribosome is faster than the terminal-binding mode of the crosslinked ribosome, and that the intact 70S ribosome can bind to the internal SD sequence laterally with a binding rate similar to that of the free 30S subunit (Runs 3 and 1, respectively).

In conclusion, we first propose that the intact 70S ribosome can bind laterally to internal mRNA SD sequences without dissociating into each subunit as an additional pathway to form an initiation complex, in addition to the general pathway where the free 30S ribosome binds to the SD sequence. The present study shows that 70S ribosomes fundamentally have a binding ability to mRNA without initiation factors such as IF3, which would be required in the absence of initiation factors in evolution history. In fact, the 70S ribosome–mRNA complex can start translation in the absence of initiation factors.[4] This indicates that 70S can potentially translate the programmed mRNA without initiation factors. We speculate that IF3 can adjust a position of mRNA and tRNA of a 70S–tRNA–mRNA complex and helps to translate efficiently without subunit dissociation. We are now studying biochemically the translation

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process from the 70S–mRNA complex. The QCM technique is useful to determine these complicated molecular interactions by measuring binding kinetics and absolute bound amounts (ratio) to the nanogram level.

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Keywords: 30S subunit · 70S ribosome · quartz crystal microbalance · SD sequence · translation process

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