A peer-reviewed version of this preprint was published in PeerJ on 17 September 2018.

View the peer-reviewed version (peerj.com/articles/5620), which is the preferred citable publication unless you specifically need to cite this preprint.

Levitskii S, Derbikova K, Baleva MV, Kuzmenko A, Golovin AV, Chicherin I, Krasheninnikov IA, Kamenski P. 2018. 60S dynamic state of bacterial ribosome is fixed by yeast mitochondrial initiation factor 3. PeerJ 6:e5620 https://doi.org/10.7717/peerj.5620

An intermediate state of bacterial ribosome dissociation is fixed by yeast mitochondrial initiation factor 3

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The processes of association and dissociation of ribosomal subunits are of great importance for the protein biosynthesis. The mechanistic details of these processes, however, are not well known. In bacteria, upon translation termination, ribosome dissociates into subunits which is necessary for its further involvement into new initiation step. The dissociated state of ribosome is maintained by initiation factor 3 (IF3) which binds to free small subunits and prevents their premature association with the large subunits. In this work, we have exchanged IF3 in *E.coli* cells by its ortholog from *Saccharomyces cerevisiae* mitochondria (Aim23p) and showed that yeast protein cannot functionally substitute the bacterial one and is even slightly toxic for bacterial cells. Our *in vitro* experiments have demonstrated that Aim23p does not split *E.coli* ribosomes into subunits. Instead, it fixes an intermediate state of ribosomes dissociation characterized by sedimentation coefficient about 60S. Using molecular modeling, we show that such fixation is due to mitochondria-specific terminal extensions of Aim23p that stabilize the position of the protein on the bacterial ribosome.

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ABSTRACT

The processes of association and dissociation of ribosomal subunits are of great importance for the protein biosynthesis. The mechanistic details of these processes, however, are not well known. In bacteria, upon translation termination, ribosome dissociates into subunits which is necessary for its further involvement into new initiation step. The dissociated state of ribosome is maintained by initiation factor 3 (IF3) which binds to free small subunits and prevents their premature association with the large subunits. In this work, we have exchanged IF3 in *E.coli* cells by its ortholog from *Saccharomyces cerevisiae* mitochondria (Aim23p) and showed that yeast protein cannot functionally substitute the bacterial one and is even slightly toxic for bacterial cells. Our *in vitro* experiments have demonstrated that Aim23p does not split *E.coli* ribosomes into subunits. Instead, it fixes an intermediate state of ribosomes dissociation characterized by sedimentation coefficient about 60S. Using molecular modeling, we show that such fixation is due to mitochondria-specific terminal extensions of Aim23p that stabilize the position of the protein on the bacterial ribosome.

INTRODUCTION

Upon termination of protein biosynthesis in bacteria, 70S ribosome dissociates into small (30S) and large (50S) subunits. Free small subunit then takes part in *de novo* formation of the initiation complex with mRNA, initiator tRNA, and several initiation factors. Binding of the large subunit promotes release of the initiation factors, and associated 70S ribosomes begins a new round of translation (for review, see (Laursen et al. 2005)).

The ribosome dissociation / association is, thus, of great importance for the whole process of translation. It is known that bacterial ribosomes dissociate into subunits when the translation termination stage is over; two proteins, namely RRF and EF-G, are responsible for this (Zavialov et al. 2005) (Peske et al. 2005). Once free 30S and 50S subunits appear, initiation factor 3 (IF3) binds to the small subunit in order to keep it apart from the large one (Zavialov et al. 2005). This stage is, in fact, the very first stage of the translation initiation process; 30S•IF3 complex becomes the basis for the full-size initiatory complex formation which includes Shine-Dalgarno sequence of mRNA, initiator tRNA, and initiation factors 1 and 2. It is worth mentioning that anti-association activity of IF3 is definitely of passive mode: it does not promote dissociation of

the ribosome into subunits but instead binds to free small subunit and prevents its re-association with the large one (Gualerzi et al. 1977) (Gottleib et al. 1975).

The exact mechanism of ribosome dissociation into subunits remains not clear. This is due to methodological complications of studying this fast and dynamic process. In kinetic study, a model was proposed that assumed the existence of several consecutive conformations of 70S ribosome in course of its dissociation; IF3 was hypothesized to be a potential effector of corresponding conformational changes which could shift the equilibria between different states of dissociating ribosome (Goss et al. 1980). It can be assumed that these conformations might be characterized by different sedimentation coefficients, less than 70S but probably more than 50S. Indeed, a ribosomal ~60S state was described in *in vitro* experiments; this state was formed under specific experimental conditions (Morimoto 1969). The authors used a term "60S component" and postulated that this was a new stable intermediate of the subunits dissociation / association reaction and that this intermediate was just "swollen 70S" (Morimoto 1969). Further investigations, however, have demonstrated that the exact sedimentation coefficient of this "swollen 70S" depends on the sedimentation speed and on the initial 70S concentration (Spirin 1971). These results fit very well with the above-discussed hypothesis about consecutive conformational changes of 70S ribosome during dissociation. However, none of these intermediate states has been seen as stable structure.

In this work, we investigated the effects of yeast mitochondrial IF3, Aim23p, on the *E.coli* translation. The idea comes from the recent work of Ayyub and colleagues where it has been demonstrated that mammalian mitochondrial IF3 (mtIF3), although being unable to fully substitute for IF3 in *E.coli*, exhibits some functional activity in bacterial cells (Ayyub et al. 2018). We exchanged *infC* gene (coding for IF3) in bacteria by AIM23 gene and found that Aim23p cannot substitute the cognate factor. Moreover, Aim23p was slightly toxic for the bacterial cell which was mediated by mitochondria-specific parts of the protein, namely its N- and C-terminal extensions. Our *in vitro* investigations have revealed that Aim23p does bind to *E.coli* ribosome and fixes its unusual state with sedimentation coefficient about 60S. This state can be further transformed into fully dissociated state if Am23p concentration is increased. Terminal extensions of Aim23p have been shown to be responsible for 60S state fixation. Thus, an intermediate state of bacterial ribosome dissociation has been for the first time detected as a stable structure.

MATERIALS AND METHODS

Plasmids, ***E.coli* strains** and **oligonucleotides** used in the work may be found in Tables 1, 2, and 3, respectively.

Cloning and standard procedures

Different versions of AIM23 (*S.cerevisiae*) and *infC* (*E.coli*) genes were cloned into above-mentioned vectors by standard PCR-restriction-ligation approach.

Western-blot was performed by standard protocol using the rabbit antibodies against 6-His-tagged recombinant Aim23p (produced on our order by Almabion).

Construction of mutant *E.coli* strains (Thomason et al. 2007)

Genomic disruption of *infC* gene coding for IF3 was carried out in the *E.coli* strain MG1655. Cassette for *infC* genomic disruption containing the chloramphenicol resistance gene was prepared by PCR from pKD3 plasmid. Primers (see Table S3) contained 5'-parts designed for the homologous recombination into the target genome site. The cassette was then delivered into *E.coli* cells by electroporation. These cells initially contained pKD46 plasmid encoding for recombinase, as well as pACDH plasmid containing *infC* gene. Clones where recombination took place were selected on chloramphenicol-containing medium and screened by PCR.

For transferring the bacterial genetic material to phage P1, 5ml of *E.coli* culture in logarhythmic growth phase was infected by 100 ul of phage suspension. The mixture was incubated at 37 ºC for 3 hours with shaking and centrifuged at 9200 g for 10 minutes. The phage-containing upper fraction was taken and filtered through 0.45 um filter.

For generation of the experimental *E.coli* strains, the MG1655 cells containing pBAD plasmid with cloned *infC* gene were transformed by the plasmids coding for IF3 or different variants of Aim23p. 2 ml of ON cultures were pelleted and resuspended in 1 ml of 10 mM $CaCl_2$, 5mM $MgSO_4$. Suspensions were 100 ul aliquoted, then half a volume of P1 lysate was added, and the mixtures were incubated at 37 ºC for 30 minutes without shaking. Then 1 ml of LB medium and 200 ul of sodium citrate were added followed by the incubation at 37 ºC for 1 hour

with shaking. The cells were plated on the agar dishes with antibiotics, 0,02% arabinose and 5mM sodium citrate. Screening of the clones was performed by PCR.

The growth curves of *E.coli* strains were registered in automatic mode using microplate reader Infinite M200 PRO (Tecan Instruments).

Ribosome purification and analysis

Ribosomes were isolated from *E.coli* strain MG1655 according to (Rivera et al. 2015) with minor changes. Briefly, bacterial cells from 1L culture with OD_{600} ~0.6 were collected, lysed, ribosomes from clarified lysate were sedimentated through 10% sucrose cushion, and dissolved in minimal volume of 10 mM Tris-HCl pH 7,0; 60 mM KCl, 60 mM NH_4Cl, 7 mM magnesium acetate, 7 mM β-mercaptoethanol, 0.25 mM EDTA. Isolated ribosomes were stored at -80°C. For dissociation reaction, approximately 24 pmoles (one unit of OD_{260}) of ribosomes were mixed with different amounts of recombinant Aim23p, IF3, or Aim23ΔNΔC in the above-indicated buffer. Mixtures were incubated at 37°C for 30 min, and then applied on 15% - 40% continuous sucrose gradients prepared on the same buffer. Samples were centrifuged for 18 hours at 100,000 g, and then fractionated from top to bottom (45 fractions each of 250 ul were taken). Absorbencies of all fractions at 260 nm were measured.

Molecular modeling

Homology model of Aim23p complex with *E.coli* 30S subunit was done with Modeller 9.17 (Sali & Blundell 1993) (script may be found in Supplementary Information). For building of this model, we have used known structure of bacterial 30S subunit complex with the cognate IF3 (Pioletti et al. 2001), as well as sequence alignment of Aim23p with *E.coli* IF3 and other orthologs (Atkinson et al. 2012).

Folding of Aim23p N-terminal extension was done with AbinitioFold protocol (Bonneau et al. 2002) (Bonneau et al. 2001) on base of fragments obtained from Robetta web server (Kim et al. 2004). Simulation was stopped after 180000 decoys were collected. Homology modeling with new conformation of N-terminal extension was done with Modeller 9.17 (Sali & Blundell 1993). Conformation of the C-terminal extension was equilibrated with FloppyTail protocol from Rosetta (Kleiger et al. 2009). Spatial structure visualization was done with the PyMOL Molecular Graphics System, Version 2.0 (Schrödinger, LLC).

RESULTS

Full-length Aim23p is undesirable for *E.coli* cells due to its terminal extensions.

As it has been already mentioned in Introduction, mammalian mtIF3 possesses some functional activity in *E.coli* cells (Ayyub et al. 2018). On the other hand, we have previously demonstrated that *E.coli* IF3 may partially rescue the growth defects of the yeast strain lacking Aim23p (Kuzmenko et al. 2014). Moreover, Aim23p was shown to bind to small subunit of bacterial ribosome *in vitro* (Atkinson et al. 2012). Taken together, these findings have allowed us to hypothesize that Aim23p might be at least partially functional in *E.coli* cells as initiation factor 3.

To verify this hypothesis, we have constructed three plasmids for further delivery into *E.coli* cells, coding for either cognate IF3 (positive control), Aim23p, or Aim23p without its mitochondria-specific N- and C-terminal extensions (Aim23ΔNΔC). This last construct was designed in order to specifically check possible effects of Aim23p terminal extensions on bacterial translation: theoretically, these protein parts, being mitochondria-specific, might not be needed for protein biosynthesis in *E.coli*. Cloned genes of Aim23p and Aim23ΔNΔC did not contain sequences coding for mitochondrial targeting sequence. Thereafter, we have disrupted *E.coli infC* gene coding for IF3. This gene contains the promoter for the expression of the downstream gene (Wertheimer et al. 1988), so we have removed only first 153 nucleotides of *infC* gene from the bacterial genome. Since IF3 is indispensable for bacteria, before disruption we have transformed *E.coli* with the plasmid bearing *infC* gene under control of glucose-repressible promoter. Finally, we have delivered the above-described plasmids into bacterial cells and disrupted the genomic copy of *infC* gene. The scheme of bacterial strains engineering is presented on Fig. 1A.

Then, we down-regulated the expression of *infC* gene in these strains with glucose and measured their growth rates. The resulting curves may be found in Fig. 1B. The strain bearing wild-type *infC* gene on the plasmid grows normally, with entering the logarithmic phase fast and reaching the plateau. The strain carrying the empty vector shows no growth at first 10 hours of incubation which is easily explained by the absence of *infC* gene. However, slow growth has been detected afterwards, probably as a result of the leakage of glucose-repressed promoter

which, in turn, allows minimal amount of IF3 to be synthesized. If *E.coli* cells contain Aim23ΔNΔC, the corresponding strain's growth curve is almost identical to that of the strain containing an empty vector. This clearly indicated the impossibility of Aim23ΔNΔC to functionally substitute IF3 in bacterial cells. The most interesting case is definitely the bacterial strain bearing the full-size Aim23p. This strain, although reaching finally the level of the strain containing an empty vector, grows measurably slower than strain containing an empty vector. This means that the full-size Aim23p, but not Aim23ΔNΔC, negatively affects the viability of *E.coli* cells. It is rather possible that the terminal extensions of Aim23p may somehow interrupt the bacterial translation.

Terminal extensions provide the ability of Aim23p to fix an intermediate form of *E.coli* ribosomes during dissociation.

The above-described unusual effect of Aim23p in *E.coli* cells has led us to study the interaction of Aim23p with *E.coli* ribosomes *in vitro*. It is well known that adding cognate IF3 to purified bacterial ribosomes shifts the equilibrium of the ribosome dissociation reaction making the dissociated state preferable (Gottleib et al. 1975). Based on this, we have purified the ribosomes from *E.coli* cells, incubated them with the recombinant IF3 (positive control), or Aim23p, or Aim23ΔNΔC, fractionated the reactions by sucrose gradient centrifugation and analyzed the corresponding sedimentation profiles by measuring the optical densities of the fractions at 260 nm. The results of our experiment are presented in Fig. 2A. First of all, the sedimentation of the ribosome sample with no proteins added was characterized by clear UV peaks of 30S and 50S subunits, as well as the whole 70S ribosomes, with the latter being most pronounced. Adding IF3, as expected, led to the complete dissociation of ribosomes into the subunits (disappearance of the 70S peak and significant increase of the 30S and 50S peaks) while adding of Aim23ΔNΔC gave no effect on the sedimentation profile. This was also expected: in our *in vivo* experiments, this protein could not substitute for the cognate IF3 in *E.coli* cells (see Fig.1). This seems to be mediated by the impossibility of Aim23ΔNΔC to bind the bacterial ribosomes. The profile of sedimentation has been curiously changed with adding the full-size Aim23p. This protein caused a fusion of 50S and 70S peaks with the formation of a single wide and poorly-resolved peak with maximum UV absorbance corresponded to approximately 60S sedimentation coefficient, exactly between 70S and 50S. At the same time, the 30S peak was

increased, but to the less extent than in case of the full dissociation promoted by IF3. Since adding of any protein might not increase the sedimentation coefficient of 50S subunit, we have to hypothesize that Aim23p somehow affects the whole ribosome leading to a slight decrease of its buoyant density. The most logical explanation of this phenomenon would be that Aim23p cannot promote the normal ribosome dissociation at concentrations used in the experiment (20:1 molar ratio in relation to the ribosomes concentration) but instead binds to it and fixes an intermediate dissociation state. This state might be less stable than usual ribosome, which should lead to its spontaneous partial dissociation. This is exactly what results from the above-mentioned observation that 30S peak is slightly increased with adding Aim23p. Speaking about the peak of dissociated 50S subunits, it seems to partially overlap with the peak of the "60S intermediate" and thus may not be clearly observed. The appearance of this "60S state" might be the reason of the Aim23p slight toxicity for *E.coli* cells observed by us (see Fig. 1B). It should be noted, finally, that such action of Aim23p on the bacterial ribosomes is definitely mediated by its mitochondria-specific terminal extensions.

After obtaining these intriguing results, we have decided to analyze the dose-dependency of the Aim23p effect on *E.coli* ribosomes. The resulting profiles of ribosomes sedimentation after adding Aim23p at different concentrations are presented at Fig. 2B. If Aim23p concentration is 2.5 times more than in previously described experiment (50:1 molar ratio in relation to the ribosomes concentration), then the peak of "60S state" is almost not observed. Instead, one can see a normal 50S peak which is slightly moved towards the increase of the sedimentation coefficient. Probably, Aim23p at this concentration causes almost complete dissociation of the ribosome with just trace amount of "60S state", which results in minimal shift of the corresponding peak. At the same time, the 30S peak is meaningfully increased relative to the situation when the "60S state" is clearly observed. When Aim23p concentration is increased twice more (up to 100:1 molar ratio in relation to the ribosomes concentration), the resulting profile is identical to that in case of IF3 adding to ribosomes. Summarizing these results, one can conclude that Aim23p in large concentrations does not fix the "60S state" of isolated *E.coli* ribosomes but instead promotes their dissociation into the subunits, like cognate IF3.

Aim23p and *E.coli* IF3 act jointly to dissociate bacterial ribosomes *in vitro*.

As it has been mentioned above, the discovered "60S state" of bacterial ribosomes might be the result of the decreased ribosome stability. In other words, the equilibrium of the dissociation reaction in this case may be slightly shifted towards free subunits without full dissociation. This, in turn, means that such state of the ribosome should be subjected to dissociation easier than the normal 70S state. In order to check this hypothesis, we performed *in vitro* dissociation experiments with Aim23p and IF3 being simultaneously added to ribosomes. While Aim23p was added in concentration sufficient for "60S state" fixation (10:1 molar ratio in relation to the ribosomes concentration; see Fig. 2C), the amount of IF3 used was not enough for full ribosome dissociation (10:1 molar ratio in relation to the ribosomes concentration). If both proteins were presented in the reaction together (each at the same concentration as alone), the complete dissociation was detected. The only possible explanation of this phenomenon is that "60S state" is indeed subjected to dissociation easier that the normal 70S state. If such intermediate state appears as a result of Aim23p action, the minimal amount of the free 30S subunits is immediately formed (this was also seen in our previous experiments, see Fig.2A and B). Adding a little amount of the cognate IF3 leads to the fixation of the 30S subunits in their free state and further shifts the reaction equilibrium towards the dissociated state of the ribosome. Thus, Aim23p and IF3 may act jointly to promote the dissociation of the bacterial ribosomes. To verify this, we performed a Western-blot analysis of the ribosomes fractions corresponding to the free subunits and to the whole ribosomes in presence of Aim23p, or IF3, or both proteins together. The results are presented at Fig. 2D. We used the home-made antibodies against 6-His-tagged recombinant Aim23p, and, luckily, they had a significant cross-reactivity with the 6-His-tag (data not shown). Thus, we were able to detect both Aim23p and IF3 in a single sample since IF3 used in our experiments was also 6-His-tagged. As a control, we have used Aim23ΔNΔC which, as we have shown before, does not promote any change in *E.coli* ribosomes sedimentation profile. Indeed, we have not detected this truncated protein neither in 30S, nor in 70 fractions which means that Aim23ΔNΔC is not able to bind the *E.coli* ribosomes. The possibility that our antibodies do not recognize this truncated Aim23p version can be excluded since this protein has been detected by the same antibodies in the yeast cells (data not shown). IF3 in this experiment has been found to bind exclusively free 30S subunits but not 70S ribosomes, exactly as expected. Aim23p, however, is detected both in free 30S subunits and in the 70S ribosomes fractions, and this does not qualitatively depend on presence or absence of IF3

in the reaction. This explains well the joint action of these two proteins resulting in the ribosomes dissociation which cannot be achieved when using Aim23p and IF3 at the same concentrations separately.

Terminal extensions of Aim23p ensure protein interaction with *E.coli* ribosomes by fixing Aim23p core part on the small subunit

A very interesting question rises from the above-described results: in which manner does Aim23p interact with bacterial ribosome and what is the role of its terminal extensions in such interaction? To answer this question, we have performed molecular modeling.

Previously (Atkinson et al. 2012) sequence alignment of Aim23p with *E.coli* IF3 and other orthologs has been done. On the base of this data, as well as the known structure of 30S complex with the cognate IF3 (Pioletti et al. 2001), we have built the homology model of Aim23p complex with *E.coli* 30S ribosomal subunit with the help of Modeller 9.18 (script may be found in Supplementary Information). Resulting structure eventually has a long and extended N-terminal tail (Suppl. Fig.2). Size of this tail was comparable with size of 30S subunit and model could not provide valuable information about N-terminal extension function. We have suggested that N-terminal extension is somehow structured and have built the corresponding model with Rosetta AbInitio protocol. From 18398 decoys of N-terminal extension, top ten had alpha helical structure with RMSD less than 10 Å. This observation reflects the fact that N-terminal extension does not possess certain spatial structure but probably has mobile helical packaging. N-terminal extension model with best Rosetta score was used to rebuild new homology model of 16S RNA and Aim23p complex. Resulting model has surprisingly revealed strong interaction of N-terminal extension with C-terminal domain of Aim23p core part and with long 3' terminal helix of 16S RNA. Additional distance restraints between centers of mass from 15 to 40 Å were applied to sample distance between Aim23p's N-terminal extension and C-terminal domain. As a result, top models have confirmed interaction of N-terminal extension with C-terminal domain, while interaction with 16S RNA does not look favorable. Best models of packed C-terminal extension showed interaction with N-terminal domain. The summary of molecular modeling is presented in Figure 3.

DISCUSSION

We have demonstrated previously that *E.coli* IF3 fused with the mitochondrial targeting sequence of Aim23p may complement to minimal extent the absence of AIM23 gene in yeast (Kuzmenko et al. 2014) which is a strong evidence of Aim23p being *bona fide* initiation factor 3 in mitochondria. This finding is not surprising taking into account that bacterial enzymes can often functionally substitute for their mitochondrial orthologues. This, for example, has been demonstrated for several aminoacyl-tRNA synthetases (Edwards & Schimmel 1987) (Chiu et al. 2009) and for the proteins involved in Fe-S clusters formation (Kispal et al. 1999). In this work, we have performed "reverse" experiment and investigated if Aim23p is able to substitute for cognate IF3 in *E.coli* cells. The cases of successful complementation of bacterial proteins by their mitochondrial orthologues have been described remarkably rarer than the opposite situations. However, mammalian mitochondrial initiation factor 2 has been shown to function in *E.coli* cells instead or two cognate factors at once, namely IF1 and IF2 (Gaur et al. 2008). Most probably, this is due to the short insertion domain of mammalian mtIF2 that is believed to execute the function of IF1 in mitochondria. Moreover, in a recent work it has been demonstrated that mammalian mtIF3, although not being able to fully substitute for IF3 in *E.coli*, exhibits some functional activity in bacterial cells (Ayyub et al. 2018). Speaking about Aim23p, this protein, as we have discovered in the present study, does not work as an initiation factor in *E.coli*, independently of presence or absence of the terminal extensions. We used an experimental system where cognate IF3 gene was disrupted in the bacterial genome but was presented on the plasmid under the control of glucose-repressible promoter. Such promoters are well known to leak if the amount of glucose is low. In our case, this allows the synthesis of minimal amount of IF3 and weak growth of the bacterial culture after a dozen of hours of incubation, when the main portion of glucose becomes utilized by bacterial cells (Fig. 1B). Surprisingly, this weak growth is even slower in presence of full-size Aim23p when comparing to Aim23p without terminal extensions. This means that these regions of Aim23p even make this protein slightly toxic for bacterial cells. Interestingly, mammalian mtIF3 behaves quite differently in *E.coli*. Full-size factor does not markedly affect the *E.coli* growth rate while deletion of the N-terminal extension leads to the severe growth impairment (Ayyub et al. 2018). However, to our opinion, these results should not be directly compared with the data presented in this work. The main reason for this is the difference in the experimental systems. Ayyub and colleagues used the mutant strain in which IF3 was devoid of first 55 amino acids and was

synthesized in normal quantities. Earlier, the same authors have shown that this truncated version of IF3 is enough for *E.coli* survival and can perform all main functions of the factor (Ayyub et al. 2017). This means that the action of any mtIF3 version in such cells is somewhat additional to the action of the cognate factor. On the contrary, our *E.coli* cells contained minimal amount of wild-type IF3 synthesized from repressed but leaking promoter, and the quantity of Aim23p encoded in the plasmid was much higher. In this case, the heterologous factor influence on the bacterial cells might be stronger than that discovered by Ayyub and colleagues.

The negative influence of Aim23p on *E.coli* cells, most probably, might realize *via* its interaction with bacterial ribosomes. This is exactly what we have demonstrated in the present work. In certain concentration range, Aim23p promotes the formation of a very unusual state of *E.coli* ribosomes *in vitro*. Our results presented in Fig.2 indicate that this state is characterized by the partial fusion of 70S and 50S peaks. The maximum of absorbance at 260 nm in this case approximately corresponds to the 60S sedimentation coefficient. We propose to call it "60S intermediate dissociation state". To our current knowledge, such ribosome state has never been detected *in vivo*. However, it was described in *in vitro* experiments (Morimoto 1969), notably at approximately the same magnesium concentrations as we used in our work (10 mM vs 7 mM, respectively). Morimoto used a term "60S component" and postulated that this was a new stable intermediate of the subunits association reaction and that this intermediate was just "swollen 70S" (Morimoto 1969). Further investigations, however, have demonstrated that the sedimentation coefficient of this "swollen 70S" depends on the centrifugation speed and on the initial 70S concentration (Spirin 1971). This clearly indicates that discussed ~60S zone on the sedimentation pattern is the consequence of the dynamic equilibrium of dissociation-association reaction rather that the stationary ribosomal structure.

In this work, the 60S intermediate dissociation state has been for the very first time fixed by adding a protein to the ribosomes. Aim23p possesses this activity due to its terminal extensions since we have not seen any changes in the ribosome sedimentation profile when adding Aim23ΔNΔC (Fig. 2A). This has been confirmed by the molecular modeling of Aim23p complex with 30S: terminal extensions of Aim23p (especially N-terminal one) have been shown to interact directly with the core protein part which probably makes Aim23p "fixed" on the small subunit (see Fig.3). Interestingly, the similar effect of mammalian mtIF3 has been described with regard to human mitochondrial ribosomes dissociation *in vitro* (Haque et al. 2008). Full-length

mtIF3 promotes normal dissociation while using its truncated version without terminal extensions causes the partial fusion of the 39S (large subunit) and 55S (whole mitoribosome) peaks. This clearly indicates some abnormal dissociation. Probably, terminal extensions of mitochondrial IF3s prevent the formation of intermediate dissociation states in case of mitoribosomes but promote their formation when acting on the bacterial ribosomes.

In the present work, the "60S state" has been demonstrated to dissociate by increased amount of Aim23p (Fig. 2B), or by a small amount of *E.coli* IF3 (Fig. 2C). The slight toxicity of Aim23p for *E.coli* cells (see above, Fig. 1B) may be also explained by fixing the "60S state" which dissociates poorly in presence of marginal IF3 quantities synthesized from leaking promoter. At the same time, the presence of even large amount of Aim23p in *E.coli* cells together with the physiological amount of the cognate IF3 has no effect on bacterial viability, as we could see when purifying recombinant Aim23p from wild-type *E.coli* cells (data not shown). Even if "60S state" is fixed in such conditions, it might rapidly dissociate to the subunits with the help of IF3. This may be also the case in the work of Ayyub and colleagues: having sufficient amounts of cognate *E.coli* IF3 allows bacterial ribosomes to keep the dissociated state *in vivo* properly regardless on the mammalian mtIF3 presence, and this could explain almost normal growth of the corresponding bacterial strains (Ayyub et al. 2018).

The question if "60S intermediate state" exists in wild-type bacterial cells is of high interest since it is worth proposing that *in vitro* ribosomes could not pose a conformation which is not pertained to them *in vivo*. The answer "no", however, seems to be obvious as bacterial IF3 is well-known to bind only free 30S subunits. This was also seen in the present work (Fig. 2D). At the same time, IF3 must bind 70S ribosomes, or at least keep bound to 30S when 70S is already formed, to fix any dissociation intermediate. The impossibility of this binding is not dogmatic. In the structural study, IF3 was found as a part of the fully assembled bacterial initiator complex, together with 70S ribosomes (Allen et al. 2005). The authors propose that IF3 does bind the free 30S subunit initially and then remains bound to 70S ribosomes for a short time after subunits association. Moreover, in a recent study binding of IF3 with 70S ribosome was confirmed by FRET experiments, and an alternative binding site of IF3 was identified on 50S subunit (Goyal et al. 2017). The subunits association in presence of IF3 might be realized *via* some intermediate states relative to the "60S state" detected in the present work.

The possible mechanisms of the *E.coli* ribosomes 60S intermediate state formation and dissociation are summarized in Fig.4.

CONCLUSIONS

The main result of this work is the detection of an intermediate state of *E.coli* ribosomes dissociation ("60S state"). This state was for the very first time fixed by protein (*S.cerevisiae* mitochondrial translation initiation factor 3, Aim23p). We also demonstrate that Aim23p and cognate *E.coli* IF3 actions on bacterial ribosome are of different modes and that these two proteins may bind it jointly. Using computer modeling, we show that the key players in the game of Aim23p binding to *E.coli* ribosomes are protein's mitochondria-specific terminal extensions that nestle the core part of Aim23p to ribosomal small subunit. Thus, the binding efficiency increases. Our results provide a basis for future structural studies of "60S state" which, in turn, will elucidate the fine mechanisms of bacterial ribosome dissociation / association.

ACKNOWLEDGEMENTS

We are grateful to Gemma Atkinson (Umea University, Sweden, and Tartu University, Estonia) for the *in silico* prediction of Aim23p terminal extensions. We also thank Konstantin Khodosevich (Copenhagen University, Denmark), Vasili Hauryliuk (Umea University, Sweden, and Tartu University, Estonia), Ivan Tarassov (Strasbourg University, France), Stanislav Kozlovsky and Alexey Kharitonov (Moscow University, Russia) for providing strains and chemicals. We appreciate the improvement of the figures quality by Alexey Fedyakov (Moscow University, Russia). Our special thanks are to Sergey Dmitriev (Moscow University, Russia) and to Vyacheslav Kolb (Institute of Protein Research, Russia) and his lab members for helpful discussions. The technical help of our students Maria Klimontova, Valeria Zinina, Anna Mirnaya, Anastasia Kapusta, and Margarita Chudenkova is greatly appreciated.

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FIGURE LEGENDS

Fig.1. Aim23p without terminal extensions is non-functional in *E.coli* cells while full-size Aim23p is even slightly toxic. **(A)** Scheme of the mutant *E.coli* strains production. At the first stage, the *infC* gene coded for *E.coli* IF3 was cloned into pACDH vector. The resulting plasmid was delivered into *E.coli* cells following by the genomic disruption of *infC* by the chloramphenicol resistance gene (Cat). The *infC* gene on the plasmid made the resulting strain viable. Then, the cells were inoculating by P1 phage in order to capture the genomic DNA region containing the disrupted *infC* gene. The result of the first stage was the phage with the above-mentioned genomic DNA region. On the second stage, the *infC* gene was cloned into pBAD vector (under control of glucose-repressible promoter), and genes of Aim23p and Aim23ΔNΔC

were cloned into pACDH vector. pBAD-*infC* and pACDH with one of the above-mentioned genes were then delivered into wild-type *E.coli* cells following by the inoculation by the phage from the first stage. This was resulted in the substitution of the wild-type *infC* genomic copy by the disrupted gene. As a result, a series of *E.coli* strains were generated with the following features: (1) genomic disruption of *infC*, (2) presence of *infC* on the pBAD vector, (3) presence of *infC* (positive control), Aim23p, or Aim23ΔNΔC on the pACDH vector. **(B)** Growth curves of the *E.coli* strains (indicated on the right) obtained as described in Fig. 3A. Bacteria were initially incubated without glucose, then the medium was changed to the glucose-containing one, and the optical density registration began. Each strain contained *infC* gene on the pBAD vector under control of glucose-repressible promoter. IF3: *infC* gene on the pACDH vector. Vector: empty pACDH. Aim23 and Aim23ΔNΔC: full-size and truncated AIM23 genes, respectively, on the pACDH vector.

Fig. 2. The unusual effects of Aim23p on *E.coli* ribisomes *in vitro*. **(A, B, C)** Ribosomes sedimentation profiles: optical densities at 260 nm (Y-axes of the each graph) of different fractions of *E.coli* ribosomes which were pre-incubated with the indicated proteins and sedimentated in the sucrose gradient. On the X-axes: 20-25 sequential fractions, from bottom to top of the gradient. Molar ratios protein:ribosomes are indicated near each sedimentation profile. The peaks corresponded to the ribosomes and their free subunits are marked with the vertical dotted lines and are designated below the graphs. **(D)** Western-blot hybridization of different fractions of *E.coli* ribosomes which were pre-incubated with the indicated proteins and sedimented in the sucrose gradient. In each case, the mixture of 2 fractions composing the peaks of 30S or 70S was analyzed (indicated on the top). Aim23ΔNΔC: 2 fractions composing the corresponding peaks on Fig. 2A. All other samples: 2 fractions composing the corresponding peaks on Fig. 2C. We used the antibodies against recombinant Aim23p with the significant cross-reactivity to the 6-His-tag which allowed us to detect both Aim23p and IF3 (indicated by arrows on the left) in the single analysis.

Fig. 3. Model of Aim23p interactions with *E.coli* 16S RNA. N-terminal extension is in light-pink, N-terminal domain is in hot-pink, C-terminal domain is in magenta and C-terminal extension is in deep-purple. 16S RNA is in black and white. **(A)** Overview of Aim23p location

on 16S RNA. **(B)** Close-up view in same orientation. **(C)** Close-up view with counterclockwise rotation around vertical axis displaying proximity of N-terminal extension, C-terminal domain and 16S RNA.

Fig.4. The hypothetic scheme of the formation and dissociation of *E.coli* ribosomes intermediate state *in vitro*. 1. Initially, the small (SSU) and large (LSU) subunits of the ribosome are associated one to another (70S). Adding of Aim23p (the terminal extensions are represented by black boxes) changes the ribosome conformation making the subunits more flexible relative to one another and allowing their reciprocal movements without full dissociation (60S). 2. This intermediate dissociation state cannot spontaneously dissociate to the subunits in presence of Aim23p. 3. Adding more Aim23p, however, shifts the dissociation reaction equilibrium which results in appearance of the free SSU and LSU (30S + 50S). 4. Full dissociation of the intermediate can also be reached by adding *E.coli* IF3 in amount insufficient for dissociation of 70S ribosomes.

Table 1(on next page)

Table 1. Plasmids used in this work.

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Plasmid	Description
pACDH	Low-copy vector for expression in *E.coli*
pACDHinfC*	pACDH with cloned *infC* gene from *E.coli*
pACDHAim23*	pACDH with cloned AIM23 gene lacking mitochondrial targeting sequence
pACDHAim23ΔNΔC*	pACDH with cloned AIM23 gene lacking mitochondrial targeting sequence and both terminal extensions
pBAD	Vector for *E.coli* expression containing glucose-repressible promoter
pBADinfC*	pBAD with cloned *infC* gene from *E.coli*
pKD3	Plasmid containing FRT-cat-FRT for preparation of *E.coli* disruption cassettes
pKD46	Plasmid with Lambda Red recombinase from phage λ for efficient gene disruption in *E.coli*
pET32a	Vector for the heterologous proteins expression in *E.coli*
pETIF3*	pET32a with cloned *infC* gene from *E.coli*
pETAim23*	pET32a with cloned AIM23 gene lacking mitochondrial targeting sequence
pETAim23ΔNΔC*	pET32a with cloned AIM23 gene lacking mitochondrial targeting sequence and both terminal extensions

* Generated in this work.

Table 2(on next page)

Table 2. *E.coli* strains used in this work.

Table 2. *E.coli* strains used in the work

Strain	Genotype / Description / Purpose
MG 1655	K-12 F^- λ^-ilvG$^-$ rfb-50 rph-1 For genetic manipulations, for ribosome isolation
MG_infC_ACDH*	MG 1655 + pACDHinfC + pKD46
MG_ΔIF3*	MG_infC_ACDH with first 153 nucleotides of *inf3* gene exchanged by chloramphenicol resistance cassette
MG_infC_BAD* *("vector" on Fig.3)*	MG 1655 + pBADinfC + pACDH
MG_IF3* *("IF3" on Fig.3)*	MG 1655 + pBADinfC + pACDHinfC
MG_Aim23* *("Aim23" on Fig.3)*	MG 1655 + pBADinfC + pACDHAim23
MG_Aim23ΔNΔC* *("Aim23ΔNΔC" on Fig.3)*	MG 1655 + pBADinfC + pACDHAim23ΔNΔC
Rosetta (DE3) pLysS	F^- ompT $hsdS_B(R_B^- m_B^-)$ gal dcm λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) pLysSRARE (Cam^R) For heterologous proteins synthesis and purification
Top10	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^- For molecular cloning

* Generated in this work.

Table 3(on next page)

Table 3. Oligonucleotides used in this work.

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(all synthesized by Evrogen)

Restriction sites are in capital letters.

1	Cloning of IF3 into pACDH and pBAD	tcagccatggctaaaggcggaaaacgagttc
2		tcaggaattcctactgtttcttcttaggagcga
3	Cloning of AIM23ΔNΔC into pACDH	tcagccatggcttggagcaccgggaca
4		tcaggaattcctatggtttaacgtcctttggta
5	Cloning of AIM23 into pACDH	tcagccatggctaatgcatcatctaccacag
6		tcaggaattcctacatttcattcatttttttctctg
7	Production of chloramphenicol resistance disruption cassette	tgcaacaagagattcgcagccgcagtcttaaacaattggaggaataaggtatgg agaaaaaaatcactgg
8		ccattatacgacaaaccggcggctcggcgttagggctgatctcgactaagtcatc gcagtactgttgta
9	Screening of IF3 disruption and transduction (PCR-product is synthesized in case of IF3 gene conservation only)	caggaagttcgcttaacagg
10		ggttagcgtgcttgtgc
11	Screening of IF3 disruption and transduction (PCR-products from IF3 gene and from disruption cassette are different in size)	gacgtaaatgaagtgatcgagaag
12		ggttagcgtgcttgtgc
13	AIM23 cloning into pET32a	gactCATATGaatgcatcatctaccacaga
14		ctagCTCGAGcatttcattcatttttttctct
15	AIM23ΔNΔC cloning into pET32a	gactCATATGtggagcaccgggacaga
16		ctagCTCGAGtggtttaacgtcctttggta
17	IF3 cloning into pET32a	atgcCATATGaaaggcggaaaacgagttc
18		actgCTCGAGctgtttcttcttaggagcg
19	Screening of pET32a-based constructs	gctagttattgctcagcgg
20		atgcgtccggcgtaga

Figure 1(on next page)

Aim23p without terminal extensions is non-functional in *E.coli* cells while full-size Aim23p is even slightly toxic.

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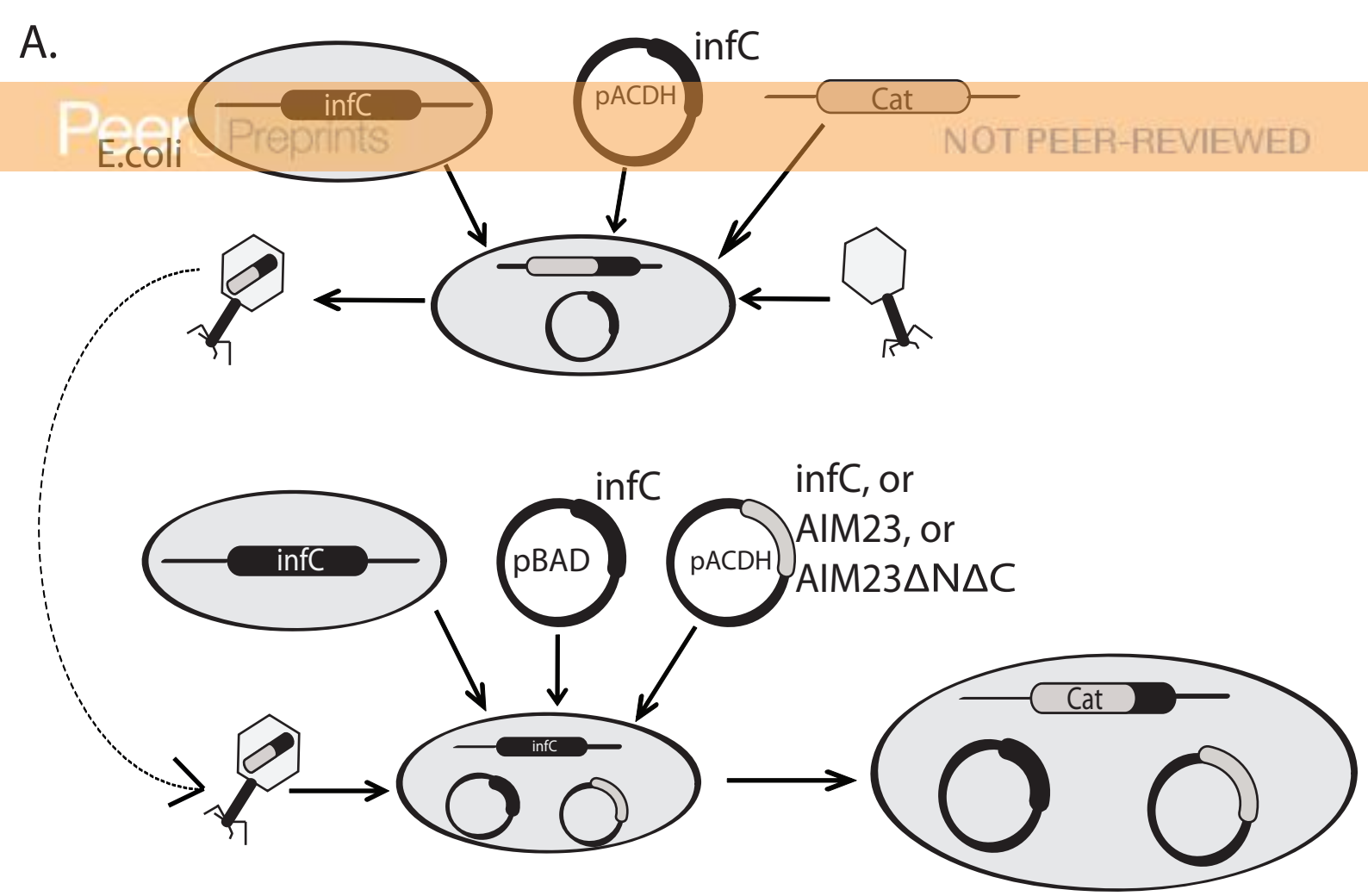

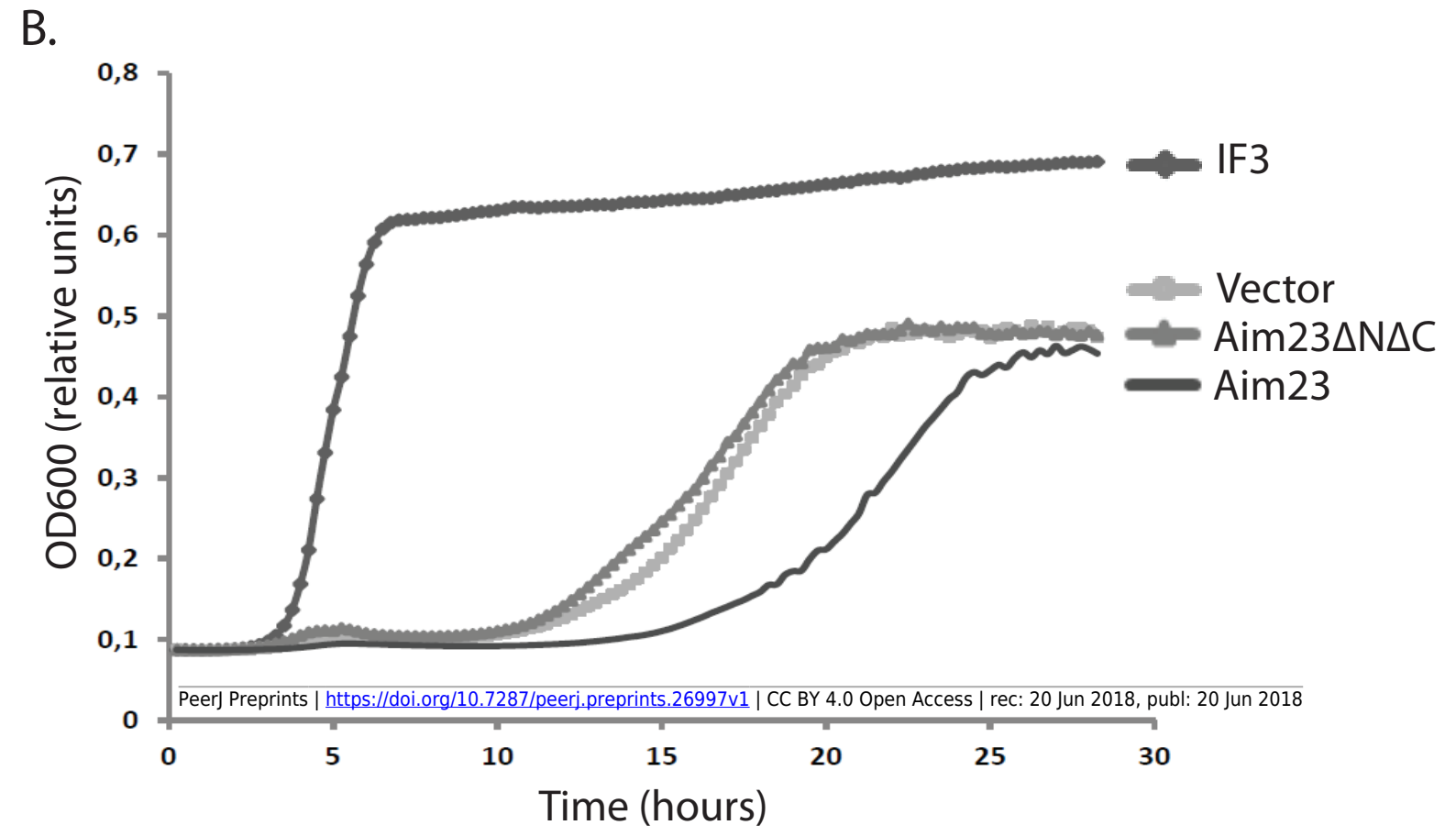

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Figure 2(on next page)

The unusual effects of Aim23p on *E.coli* ribisomes *in vitro*.

(A, B, C) Ribosomes sedimentation profiles: optical densities at 260 nm (Y-axes of the each graph) of different fractions of *E.coli* ribosomes which were pre-incubated with the indicated proteins and sedimentated in the sucrose gradient. On the X-axes: 20-25 sequential fractions, from bottom to top of the gradient. Molar ratios protein:ribosomes are indicated near each sedimentation profile. The peaks corresponded to the ribosomes and their free subunits are marked with the vertical dotted lines and are designated below the graphs. **(D)** Western-blot hybridization of different fractions of *E.coli* ribosomes which were pre-incubated with the indicated proteins and sedimented in the sucrose gradient. In each case, the mixture of 2 fractions composing the peaks of 30S or 70S was analyzed (indicated on the top). Aim23ΔNΔC: 2 fractions composing the corresponding peaks on Fig. 2A. All other samples: 2 fractions composing the corresponding peaks on Fig. 2C. We used the antibodies against recombinant Aim23p with the significant cross-reactivity to the 6-His-tag which allowed us to detect both Aim23p and IF3 (indicated by arrows on the left) in the single analysis.

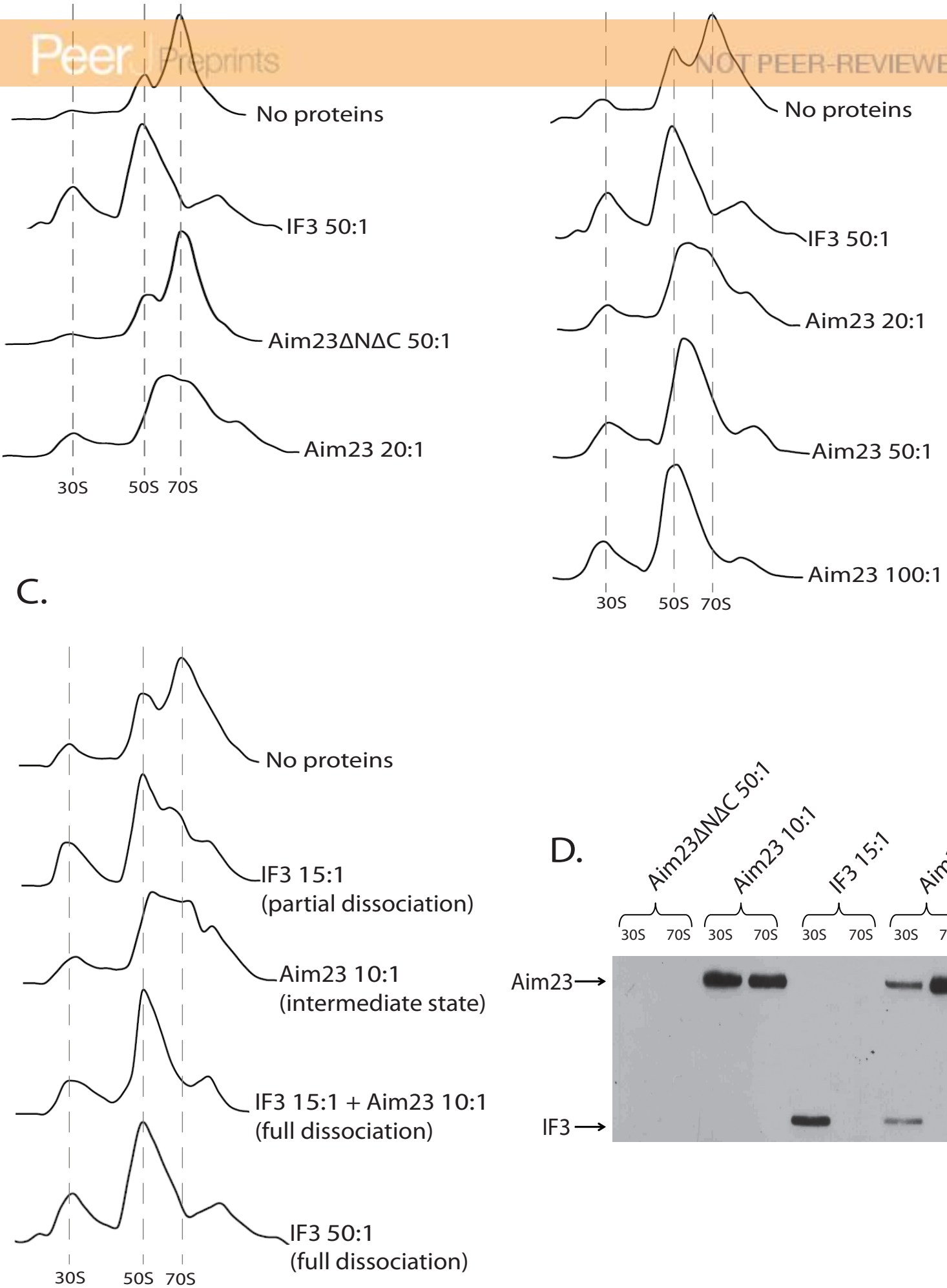

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Figure 3

Model of Aim23p interactions with *E.coli* 16S RNA.

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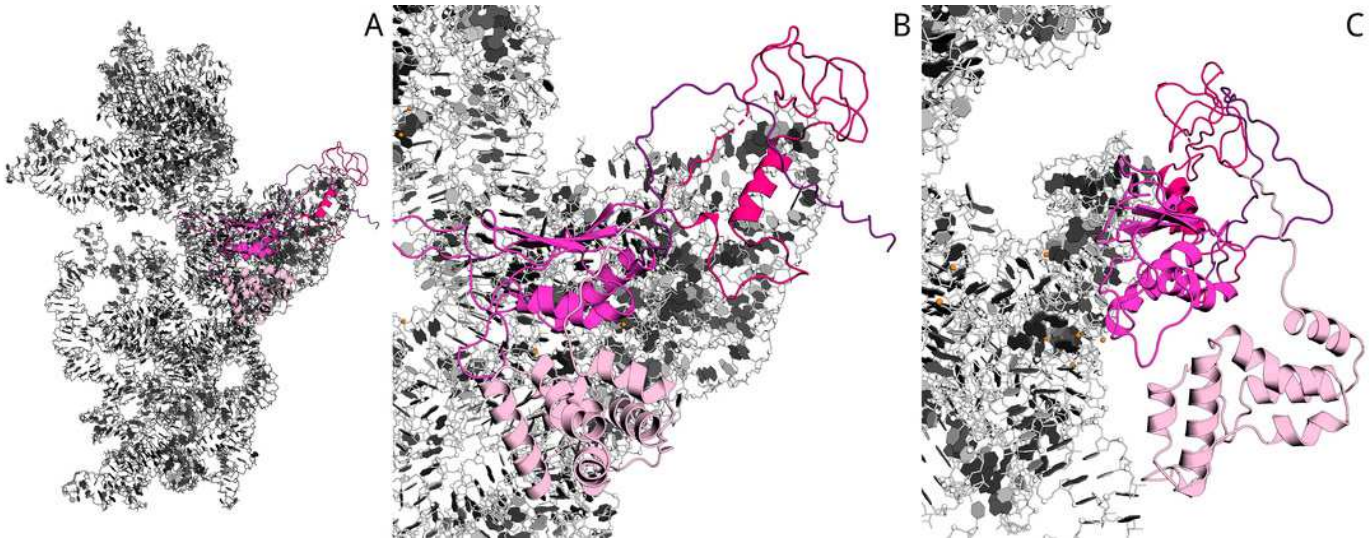

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Figure 4(on next page)

The hypothetic scheme of the formation and dissociation of *E.coli* ribosomes intermediate state *in vitro*.

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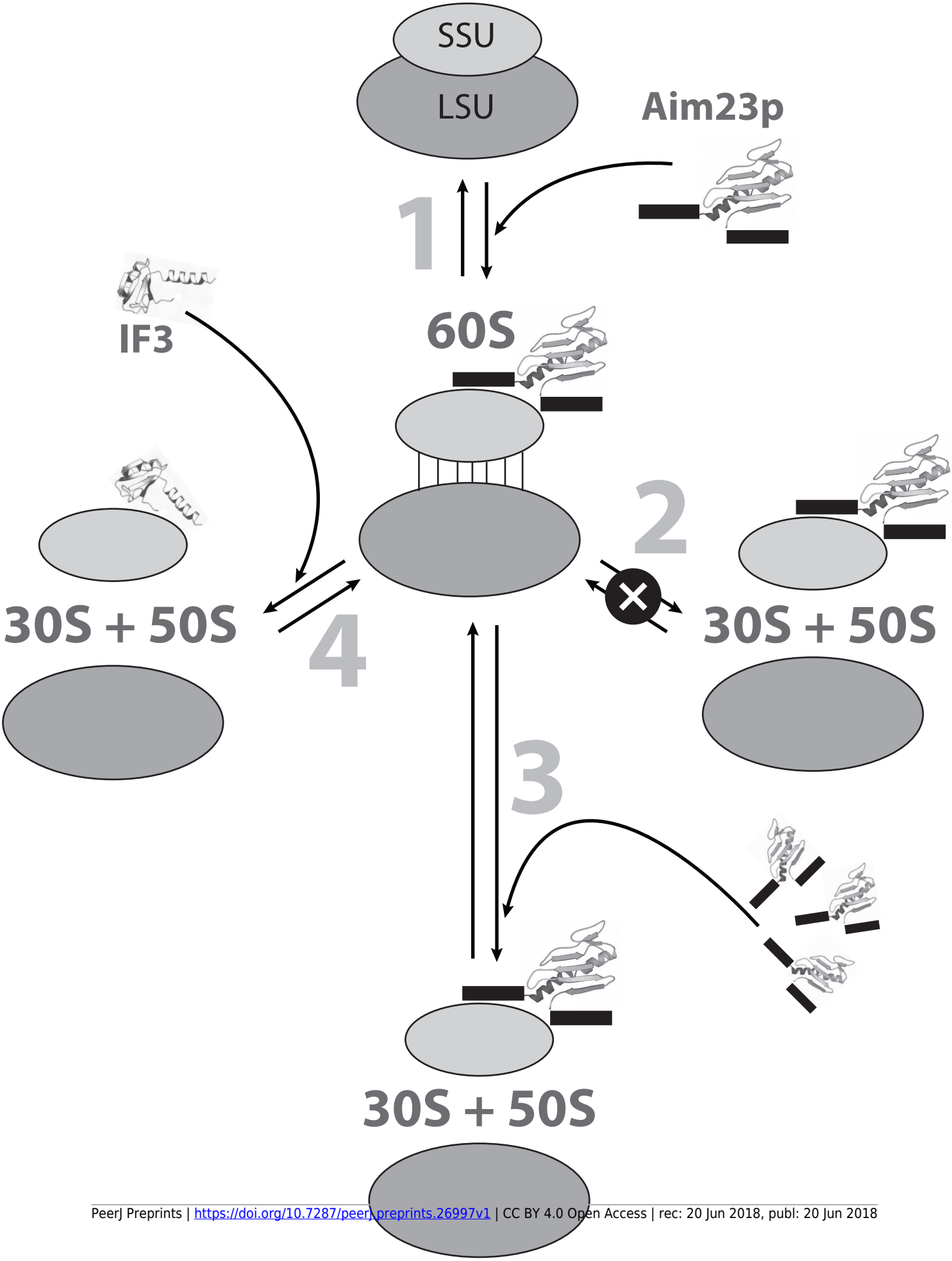
SSU
LSU
Aim23p
1
IF3
60S
30S + 50S
2
30S + 50S
4
3
30S + 50S