The development of a robust industrial crystallography program for the bacterial 50S ribosomal subunit

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Although structure-based drug design (SBDD) is now a core technology in drug development, access to SBDD of the ribosome can still be limited by difficulties in obtaining high quality crystals. We have systematically assessed correlations between crystallization and the purity, integrity and activity of 50S ribosomal subunits from *Deinococcus radiodurans* as part of a program to develop a robust SBDD program. The work has informed changes to purification that have increased the success rate so that every preparation crystallizes without fail. Crystals diffract similarly to those from other laboratories, but with routine generation of crystals large enough to obtain a whole data set from a single crystal. The effects of altering purification parameters on subsequent crystallization are reviewed, including two novel ribosome chromatography methods that we have developed as alternatives to standard purification by centrifugation. The resulting system now satisfies our needs to inform synthesis of antibiotic candidates, with a one month turnaround between selecting compounds and delivering co-crystal structures.

Keywords antibiotics; ribosomes; structure-based drug design; 50S subunit; purification; bacteria; X-ray crystallography

1. Introduction

The use of X-ray crystallography for structure-based drug design (SBDD) has become an integral part of drug discovery. However, despite rapid progress over the last two decades, technical hurdles still remain for the most difficult targets, such as membrane proteins or large complexes [1]. One such challenge is the bacterial ribosome, a 2.4 MDa complex of three RNAs and over fifty proteins, upon which about 40% of antibiotics act. Despite the fact that the first structures of the bacterial ribosome were solved a decade ago, the unpredictability of this technology makes the routine generation of such structures for antibiotic design a daunting challenge for pharmaceutical companies.

The bacterial ribosome comprises one large subunit, called the 50S according to its sedimentation coefficient (in Svedbergs) and one small (or 30S) subunit, which together comprise a single (70S) ribosome. Pfizer markets successful drugs such as Zyvox and Zithromax that act on the 50S ribosomal subunit, so that X-ray crystallography of this subunit was a natural choice for a ribosome crystallography program to drive SBDD. Structures of the 50S subunit alone are available from only two organisms. These are the halophilic archaeon *Haloarcula marismortui* [2] and the mesophilic gram positive bacterium *Deinococcus radiodurans* [3]. A third, from a undisclosed gram positive bacterium [4], has not been published. High resolution structures of the 30S subunit from *Thermus thermophilus* are also available [5-6]. However, structures for the 70S ribosome, which are available for both *T. thermophilus* and *E. coli* were of insufficient resolution to be useful for drug design when the program at Pfizer began in 2003 [7-8].

The 50S subunits from *H. marismortui* readily crystallize to provide high resolution diffraction. However, being halophilic, they also require high salt concentrations (typically 2-3M) for structural integrity. These high salt concentrations can limit the solubility of antibiotic candidates that must be soaked into the crystals to yield drug: 50S co-crystal structures. The fact that *H. marismortui* is an archaeon rather than a bacterium further limits its usefulness, since archaeal ribosomes share many features with eukaryotic ribosomes [9], and small but significant differences in the way that antibiotics interact with archaeal and bacterial 50S subunits have been reported [10]. Subunits from the bacterium *D. radiodurans* thus represent a better model for the pathogens of interest to antibacterial research and so were chosen for the crystallography program at Pfizer.

However, like other bacterial ribosomes, obtaining purified subunits from *D. radiodurans* that crystallize was an unreliable process, where even a tried and tested protocol can often fail to produce subunits that crystallize, and even experienced laboratories can suffer periods where the ability to obtain crystals is inexplicably lost. To operate efficiently, the cycles of design, synthesis and testing of antibiotic candidates in industrial structure-based drug design require tight timelines that cannot tolerate costly delays caused by an unreliable SBDD system. The uncertainty in crystallization was thus a central challenge to be addressed before X-ray crystallography of the bacterial 50S subunit could be useful in an industrial setting. This limitation has been successfully addressed to produce a system that routinely and reliably generates high resolution drug:50S co-crystal structures. Using this robust system, a one month turnaround between the selection of compounds of interest to the delivery of the corresponding co-crystal structures allows crystallography to synchronize with the other activities. I describe below how this was achieved, after a brief introduction to traditional ribosome purification methodology.

2. Ribosome purification

Ribosomes are normally purified by ultracentrifugation. A cell lysate is prepared (typically by French press) and then clarified by a low speed centrifugation to remove unbroken cells. Subsequent spins remove contaminants that sediment much faster or slower than the ribosomes in order to arrive at a crude ribosome extract. The contents of a typical clarified bacterial lysate after centrifugation through a sucrose-density gradient are displayed in Figure 1. The profile of absorbance (at 254 nm) shows a peak of soluble debris (such as proteins and tRNA) that barely penetrates the gradient, followed by peaks of 30S and 50S subunits, then 70S ribosomes and finally polysomes (multiple 70S ribosomes on a messenger RNA). Larger debris have pelleted and so are not visible.

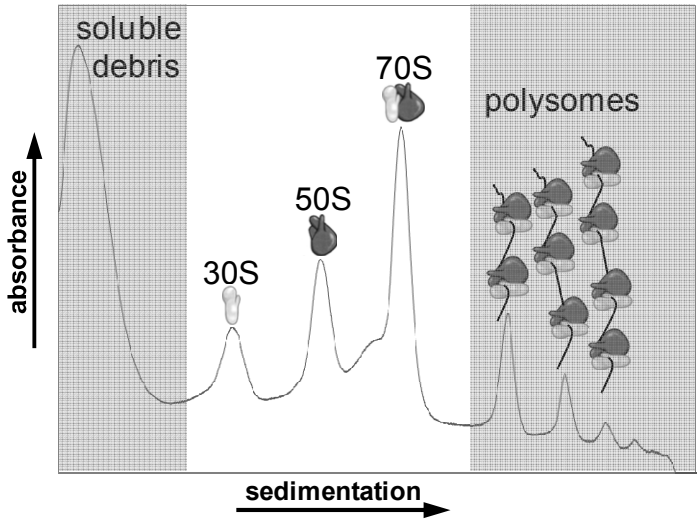

Fig 1. Contents of a *D. radiodurans* lysate separated by centrifugation on a sucrose-density gradient and detected by absorbance (at 254 nm). Absorbance peaks containing 30S ribosomal subunits, 50S subunits, 70S ribosomes and polysomes are labelled and indicated pictorially. Shaded areas indicate fractions that may be separated by differential centrifugation.

Often, this large debris and some of the larger polysomes are first pelleted from the clarified lysate by a high speed spin. The supernatant is then centrifuged again, but now long enough to pellet the subunits and 70S ribosomes so that the soluble debris can be discarded with the supernatant. Placing a layer of 1.1M sucrose at the bottom of the tube before centrifuging helps to separate any remaining membrane fragments from the ribosomes, which are more dense. The pellet of ribosomes is resuspended in buffer and sedimented on a sucrose-density gradient to separate 30S subunits, 50S subunits and 70S ribosomes. Fractions containing the desired ribosomal species are pooled and centrifuged to pellet them before final resuspension in a small volume of storage buffer. These standard techniques have changed little since the 1960's.

3. Magnesium ions and subunit association

The 30S and 50S subunit associate together largely via RNA to RNA contacts mediated by magnesium ions. Reducing the concentration of magnesium ions therefore dissociates the 70S into subunits. By lowering the magnesium ion concentration to 6 mM, about half of the 70S, known as "loose couples", dissociate into subunits, whereas the remaining "tight couple" 70S dissociate at the lower concentration of 1 mM magnesium ion. During translation, the small subunit rotates against the large subunit in the 70S couple. This rotation (or "ratcheting") is a fundamental mechanism of translation involved in the movement of transfer RNAs through the ribosome. Loose couple 70S seem to correspond to the ratcheted state, with tight couple 70S as the un-ratcheted ground state [11]. Tight and loose couple 70S are therefore conformers that differ in their mode of subunit association.

Thus, 50S subunits that are unassociated can be isolated from associated 50S provided that the concentration of magnesium ion is well above 6 mM. At 6 mM Mg^{2+}, 50S are released from loose couples, while at 1 mM Mg^{2+} all the 50S are in their unassociated form. The experiment of Fig 1 used 30 mM Mg^{2+} so that none of the 70S ribosomes or polysomes had dissociated into subunits, leaving only the "unassociated" 50S in the 50S peak. At 6 mM Mg^{2+} about half of the 70S and polysomes (the loose couples) would have dissociated into free subunits, while at 1 mM only peaks of 30S and 50S subunits would be seen sedimenting beyond the soluble debris. The magnesium ion concentration is therefore manipulated in traditional purification to obtain subunits or 70S ribosomes as desired.

Differences in association state were also exploited in our own experiments to provide different sub-populations of 50S subunits for crystallization trials. Although the difference between loose and tight 70S lies in how the subunits interact, there is some evidence that subunits from loose and tight couple 70S have differences that can persist even after the subunits separate [12-13]. Furthermore, the different conformations are likely to be acted on differently by the endogenous bacterial nucleases and proteases present in the early stages of purification . Separating the different classes

offered both a means to increase homogeneity, but also to provide subtly different samples (each of which might crystallize) from a single lysate.

4. Purification of *D. radiodurans* 50S for crystallization

4.1 Initial strategies

Our earliest attempts to obtain crystallizable 50S subunits from *D. radiodurans* followed a procedure obtained from a collaborator. This method pelleted the polysomes from the cell lysate [14]. These polysomes were then resuspended in buffer and centrifuged on a sucrose-density gradient made in buffer containing 1 mM Mg^{2+} to dissociate all of the polysomes into subunits. The 50S were recovered from the fractions by pelleting and resuspension in buffer. Since polysomes consist of actively translating ribosomes, it was suggested that the resulting subunits had superior activity that promoted crystallization [14]. Indeed, although little was known of the factors required to promote crystallization, early studies had suggested that high activity was critical [15]. However, despite numerous purifications and a close collaboration, none of the 50S subunit preparations we made by this method crystallized (under conditions where subunits obtained from the collaborator did crystallize). This was not entirely unexpected since the technology is known to be unreliable and hard to transfer between different laboratories. A second protocol that instead isolated the 50S from the rest of the ribosomes (after pelleting of the polysomes) also failed despite numerous attempts. We therefore set out to develop our own purification methods.

Our starting point was a standard method of preparing active ribosomes from *E. coli* [16]. These are the tight couple ribosomes referred to above. Since, in *E. coli* at least, these have been found to be more active than loose couple 70S for *in vitro* assays [17], and since good activity was known to be required for crystallization, it was logical to use these as the source of our 50S. The method requires that the tight couple 70S be isolated first on a sucrose–density gradient at a magnesium concentration (6 mM) at which the loose couple 70S have already dissociated into subunits. The 50S subunits obtained at 6 mM Mg^{2+} are a mixture of subunits that were unassociated and those that were loosely associated (this mixture is referred to hereafter as free/loose). The tight couple 70S isolated from this first sucrose gradient are then dissociated on a second gradient made in 1 mM Mg^{2+} buffer to derive 50S from the tight couples.

The first such preparation yielded subunits from tight couple ribosomes that had improved activity as expected (Fig 2), and which crystallized. The free/loose mix from this purification was less active, but in fact behaved the same in crystallization. About half of subsequent purifications were also successful, yielding tight and free/loose preparations that either both crystallized or both did not.

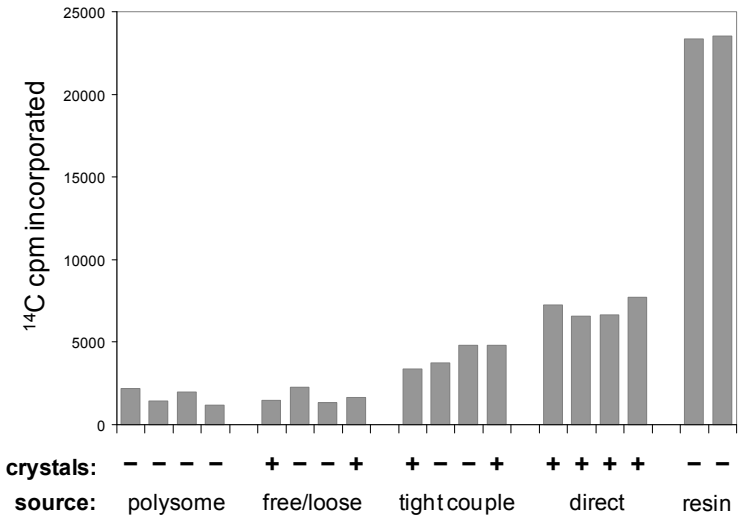

Fig 2. Activity of 50S subunits in polyU-directed synthesis of ^{14}C-polyphenylalanine *in vitro*. Typical examples are given for 50S purified from polysomes, mixed free/loose couple-derived 50S, tight couple-derived 50S, or 50S purified by the direct-isolation protocol (without or with a cysteine-Sulfolink resin pre-column). The success or failure of each preparation to crystallize is indicated below the plot by a '+' or a '–', respectively. Figure adapted from [18] (http://journals.iucr.org/) with permission.

Separation of 50S subunits according to their association status was thus an immediate success, but crystals were small and gave diffraction patterns that extended only to 10Å [18]. However, they provided a starting point for iterative improvement. Several strategies were adopted to improve the subunits. The purification protocol was varied and each subunit preparation was tested not only for performance in crystallization, but also for activity (Fig. 2), purity and integrity. This could guide improvement by helping us to understand how alterations to purification protocols affect the properties of the resulting subunits, while also perhaps revealing correlations of specific properties with crystallization. Contamination by the 30S subunit was monitored by centrifugation on analytical sucrose density gradients and kept below 3% by judicious selection of fractions from the preparative gradients. The content and integrity of ribosomal proteins was tracked by LC-MS [18]. Comparison of the LC chromatograms revealed that four proteins varied in

content. Two of these, proteins L19 and CTC are subject to C-terminal proteolytic degradation, while the other two, called L9 and L12 appear to simply dissociate, since no fragments were detected by MS. Surprisingly, this protein degradation and loss did not seem to influence crystallization [18].

In some experiments, homogeneity was further increased by separating unassociated 50S from those that were loosely associated by centrifuging first on a gradient made in buffer containing 30 mM Mg^{2+} to isolate the unassociated 50S. The 70S from this gradient was then run on a second gradient in 6 mM Mg^{2+} to isolate 50S only from loose couples [18]. A third gradient in 1 mM Mg^{2+} isolates 50S from the remaining (tight couple) 70S. 50S subunits from either the pure loose couple or the tight couple 70S crystallized, but not the subunits that had been unassociated. Thus, either form of 70S is a better source than unassociated 50S.

The ribosomal RNA ("5S" and "23S" rRNA) was extracted from 50S subunits and the integrity of the 23S compared by denaturing polyacrylamide gel electrophoresis (PAGE) as shown in Fig 3. The 23S rRNA in all preparations was somewhat degraded, but less so for 50S from tight couples or pure loose couples (Fig 3). Subunit association likely protects 23S rRNA at the subunit interface from nucleases; subunits may also be less flexible when associated and so be less prone to nuclease action. However, it seems that some degradation, at least, is tolerable and no obvious correlation with crystal formation was seen in these early comparisons. Studies on degradation of the 23S rRNA during purification showed that much of the degradation occurred during the early pelleting steps that precede the first sucrose-density gradient.

It was expected that the separate free/loose and tight couple derived 50S preparations were more homogeneous than a mixture of the two, and that this accounted for their ability to form crystals. However, recombining the crystallizable preparations of 50S from free/loose populations with those from tightly-associated 50S did not disrupt crystal formation, so that homogeneity could not explain the success of the separate preparations. We surmised that the use of a higher magnesium ion concentration in the first sucrose gradient (6 mM instead of 1 mM used in the earlier protocols) had somehow conferred an advantage.

4.2 ”Direct” isolation

The accumulating evidence suggested that isolating 50S that are associated with the 30S in a high magnesium buffer and without prior steps would improve the quality of the resulting subunits. This was confirmed by loading lysates directly onto sucrose density gradients (containing 30 mM Mg^{2+}) without any prior pelleting steps other than a short spin to remove unbroken cells. This represents a departure from traditional preparative methods, where ribosomes are normally isolated first from the lysate (as described in section 2) before separation on a sucrose-density gradient.

The 70S were isolated from the gradient fractions and recentrifuged on a second gradient made in 1 mM Mg^{2+} to separate the subunits. The 50S were recovered and found to have excellent crystallization properties. Diffraction patterns extended to 3.5 Å and every preparation made this way over a period of several years (twenty preparations in all) crystallized. The 70S-containing fractions from the first gradient had to be concentrated before loading onto the second gradient and it was found that concentrating by ultrafiltration, rather than by pelleting and resuspension, further improved the crystals so that they now gave diffraction patterns extending to 2.9 Å [18] and were large enough to yield a whole data set from a single crystal when used with a microfocussed synchrotron beam.

The reliability of this more direct isolation satisfied our needs for a robust system for SBDD, but also allowed efficient testing of further variants. Prior removal of the polysomes by pelleting reduced yields but did not affect crystal quality. However, prior pelleting and resuspension of the ribosomes that were to be loaded on the first sucrose gradient abolished crystallization. Thus the operative factor seems to be rapid and gentle isolation of the 70S ribosomes. This encouraged us in parallel efforts, discussed later, to replace centrifugation with more rapid and gentle chromatographic methods.

Varying the protocols and tracking the properties of the resulting subunits was thus a successful strategy to inform improvements to purification. However, it remained difficult to identify the key properties of crystallizable subunits. The final “direct” isolation method results in subunits with good activity (Fig. 2), yet crystals were also formed by preparations with much lower activity (e.g. free/loose in Fig. 2). As mentioned above, subunits from tight couple 70S had better activity than the free/loose preparations, but did not crystallize more readily, so no clear correlation of crystal formation with activity was found. It was concluded that activity had not been a limiting factor for the different 50S preparations made.

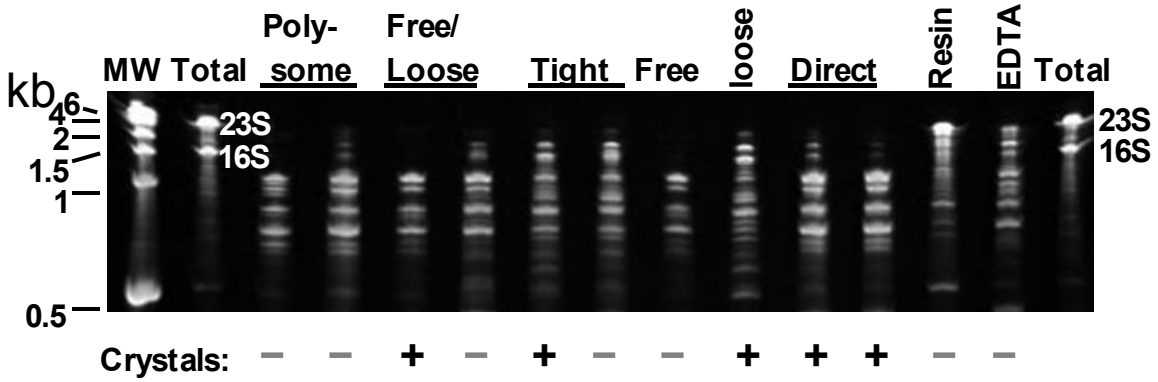

Fig 3. Ethidium bromide-stained rRNA isolated from different 50S preparations and resolved by denaturing PAGE.

MW= molecular weight standards, Total= intact 16S and 23S rRNA standards (16S rRNA is a component of the 30S ribosomal subunit and so not present in the other lanes). The source of the 50S subunits from which the rRNA was extracted is indicated above the plot. Free/Loose= unassociated 50S and 50S derived from loose couples. Loose= 50S derived from loose couples alone. Direct= direct isolation method. Resin= direct isolation preceded by cysteine-Sulfolink chromatography. EDTA= direct isolation in the presence of EDTA. The success or failure of each preparation to crystallize is indicated below the plot by a '+' or a '–', respectively. Figure adapted from [18] (http://journals.iucr.org/) with permission.

5. Purification by chromatography

5.1 Cysteine-Sulfolink chromatography

A second effort, pursued in parallel with purification of ribosomes by centrifugation was to find a suitable chromatography method to purify subunits for crystallization, since this could offer the advantage of scalability and automation, as well as parallel processing to compare purification in different buffers or from cells grown in different conditions. Chromatography methods for ribosome purification have been available for decades and have worked well for ribosomes from extremophiles such as *T. thermophilus* [19-22]. However, they have been much less used for ribosomes from mesophilic species such as *E. coli*. This is in part because high salt concentrations are required either for binding (hydrophobic interaction chromatography), elution (ion exchange), or for concentration prior to chromatography (size exclusion chromatography; [26]). In some cases this can damage *E. coli* ribosomes [23-25] and the same is likely true for *D. radiodurans* ribosomes.

This limitation was overcome by developing a novel chromatography that separates ribosomes from the rest of the lysate without using high salt concentrations. An agarose resin with an 18-carbon alkyl chain linker capped by cysteine is used to capture the ribosomes under mild conditions [27]. Elution is achieved by a moderate increase in salt concentration (from 150 mM to 300 mM NH_4Cl). The mechanism is not fully understood, but, like anion exchange, interaction is with the RNA of the ribosome and requires low salt concentration, while substituting a less hydrophobic linker prevented binding, so that the mechanism seems most analogous to mixed mode chromatography, with elements of both hydrophobic interaction and ion exchange [27].

Once purified away from contaminants, the subunits could then be separated as normal on sucrose density gradients. This chromatography proved particularly efficient for purifying the ribosomes away from nucleases present in the cell lysates, so that the resulting subunits had unusually intact ribosomal RNA and unusually high activity (see "resin' samples in Figs. 2 and 3). This was useful for the preparation of highly active ribosomes from clinical isolates of pathogens for *in vitro* assays [27], but could not be used to prepare 50S subunits from *D. radiodurans* for crystallization, as none of the preparations were found to crystallize.

Importantly, it was found that subunits prepared by centrifugation alone, and which had been shown to crystallize, could subsequently be bound to the resin, eluted and recovered without losing their ability to form crystals, so the chromatography itself was not the problem. To test whether the unusually intact RNA may prevent crystallization, the standard preparation by centrifuge was performed as normal, but with the inclusion of 1 mM ethylene diamine tetra acetic acid (EDTA) in the lysis buffer. EDTA inhibits nucleases that otherwise would degrade the rRNA, so that subunits with highly intact 23S rRNA result (see "EDTA" sample in Fig. 3). These also do not crystallize, yet incubation of purified subunits that do crystallize with EDTA did not impair their ability to crystallize, suggesting that as with the chromatography, it was not the EDTA itself that was the problem, but rather its prevention of nuclease action during the early steps of purification. This was quite unexpected, as 50S subunits from *H. marismortui* that readily crystallize have fully intact 23S rRNA (B.A. Maguire, unpublished). Similarly, *E. coli* 70S ribosomes that

crystallize are typically prepared from RNase I$^-$ strains, whose 23S rRNA is stable during purification. It was concluded that, unlike subunits from the other species, intact 23S rRNA in *D. radiodurans* 50S subunits actually prevented their crystallization. Since the 50S subunit is naturally poised between different conformations that it must adopt during the functional cycle of translation, it may be that selective nicking of the 23S rRNA relaxes the *D. radiodurans* subunit into a particular conformation and so promotes crystal formation by increasing its conformational homogeneity.

5.2 Purification by association with affinity-tagged 30S subunits

The advent of tag based affinity chromatography has also enabled the use of mild binding and elution conditions, but had previously been applied to analytical studies on ribosome components and co-factors rather than the bulk purification of ribosomes. An affinity tag was therefore placed on the *D. radiodurans* ribosome in order to purify the 50S subunit. However, we chose not to tag the 50S itself, but rather the 30S subunit, so that the association of the 50S subunits with the tagged 30S could be exploited to both capture and elute the 50S. A FLAG tag sequence was introduced onto the C-terminus of protein S16 of the 30S subunit so that both free 30S subunits and 70S ribosomes are retained when a cell lysate is passed through an anti-FLAG column [11]. Unassociated 50S and other debris pass straight through (Fig 4). These 50S can later be separated from the contaminating debris on a sucrose-density gradient.

Lowering the magnesium ion concentration to 1.75 mM releases the 50S subunits from loose couples (Fig. 4), with those from tight couples released later when the magnesium ion concentration is reduced further to 0.25 mM. The 70S dissociate at a lower magnesium concentration (1.75 and 0.25 mM) than during centrifugation on sucrose-density gradients (6 and 1 mM) because the high hydrodynamic pressures generated by centrifugation promote dissociation [28].

The method produced highly pure subunits with very similar characteristics to the crystallizable subunits produced by the direct ultracentrifugation protocol [18], yet they did not crystallize under standard conditions or even extensive alternative crystallization conditions tested. The reasons for this unexpected failure likely hold additional clues as to what potentiates crystallization.

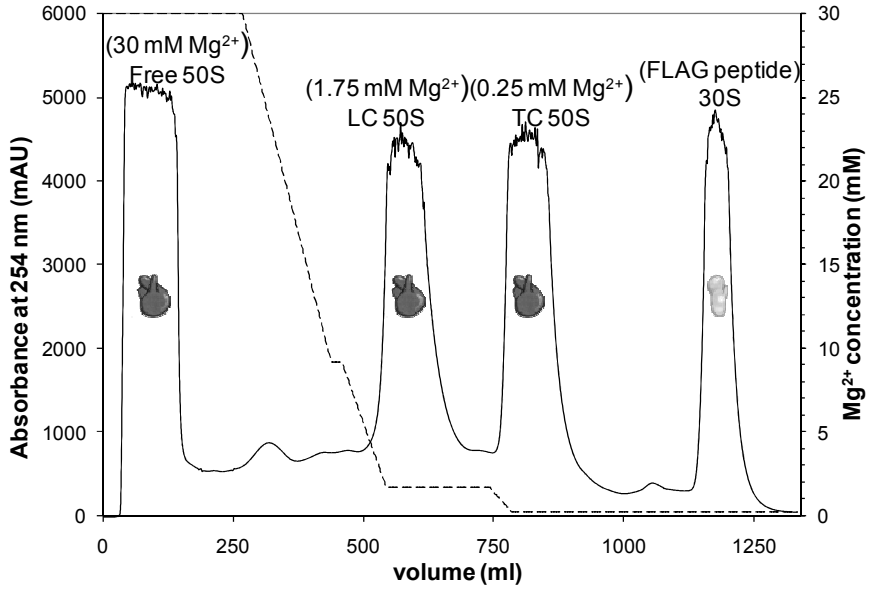

Fig. 4. Chromatogram of a standard purification of FLAG-tagged ribosomes. Absorbance (at 254 nm) is given by a solid line and Mg^{2+} concentration by a dotted line. Eluting peaks of 30S and 50S subunits are indicated with eluting buffer conditions in parentheses. LC= loose couple and TC= tight couple. Figure adapted from [11] with permission.

6. Future prospects

These studies on the *D. radiodurans* 50S subunit are the most systematic attempt so far to determine what governs the ability of ribosomal subunits to crystallize. Although the precise factors remain poorly understood, the work suggests further aspects to pursue. While the macromolecular components (ribosomal proteins and rRNA) have been analyzed here, smaller components such as metal ions [29] and polyamines [30] may also be worth studying since they are important for both the integrity and activity of subunits and so may influence their crystallization. Furthermore, although the conformational flexibility of the subunits used is surely of paramount importance, its contribution to crystallization has not yet been systematically evaluated.

The failure to leverage chromatography for more efficient production of crystallizable 50S subunits from *D. radiodurans* was disappointing given that chromatography has been successfully used to provide crystallizable ribosomes from other bacteria. However, such methods are likely to find application to the many new challenges facing the field. While bacterial ribosomes from *E. coli*, *T. thermophilus*, or *D. radiodurans* have served so far as a model for

ribosomes from pathogens, the ability to crystallize ribosomes from clinical isolates of new and emerging pathogens (with the latest resistance-conferring ribosome modifications) would replace these models with the real thing. High levels of degradative enzymes naturally present in clinical isolates made it difficult to isolate active ribosomes by centrifugation, but cysteine-Sulfolink chromatography has been successfully used to provide ribosomes from such strains [27] that may be sufficiently intact to be suitable for crystallization attempts. The cytoplasmic eukaryotic ribosome can also be purified by cysteine-Sulfolink chromatography, and this may prove useful in the ongoing race to provide a high resolution crystallographic structure.

The mitochondrial ribosome is of particular interest to antibacterial research because it is more closely related to bacterial ribosomes than the cytoplasmic one, so that unwanted side effects of antibiotics that target translation often arise from sensitivity of the human mitochondrial ribosome to the antibiotic. Prominent examples are the ototoxicity conferred by aminoglycosides [31] and myelosuppression by oxazolidinones [32]. The structure of the human mitochondrial ribosome would thus assist the design of new antibiotics with fewer side effects, but its purification is particularly challenging. However, tag-based affinity purification has already been used to isolate mitochondrial ribosomes for proteomic studies [33] and may also prove useful for preparative scale isolations for crystallization trials. Despite significant recent progress, the key factors controlling crystallization even of the (relatively) simple bacterial ribosome remain unclear, and the more challenging ribosomal targets will require further advances before they yield their first high resolution structures, let alone multiple structures on the routine basis required for industrial use.

Acknowledgements I give thanks to the numerous colleagues at Pfizer who contributed to the success of this work.

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