The complete structure of the chloroplast 70S ribosome in complex with translation factor pY

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Abstract

Chloroplasts are cellular organelles of plants and algae that are responsible for energy conversion and carbon fixation by the photosynthetic reaction. As a consequence of their endosymbiotic origin, they still contain their own genome and the machinery for protein biosynthesis. Here, we present the atomic structure of the chloroplast 70S ribosome prepared from spinach leaves and resolved by cryo-EM at 3.4 Å resolution. The complete structure reveals the features of the 4.5S rRNA, which probably evolved by the fragmentation of the 23S rRNA, and all five plastid-specific ribosomal proteins. These proteins, required for proper assembly and function of the chloroplast translation machinery, bind and stabilize rRNA including regions that only exist in the chloroplast ribosome. Furthermore, the structure reveals plastid-specific extensions of ribosomal proteins that extensively remodel the mRNA entry and exit site on the small subunit as well as the polypeptide tunnel exit and the putative binding site of the signal recognition particle on the large subunit. The translation factor pY, involved in light- and temperature-dependent control of protein synthesis, is bound to the mRNA channel of the small subunit and interacts with 16S rRNA nucleotides at the A-site and P-site, where it protects the decoding centre and inhibits translation by preventing tRNA binding. The small subunit is locked by pY in a non-rotated state, in which the intersubunit bridges to the large subunit are stabilized.

Keywords chloroplast; cryo-EM; pY; ribosome; translation

Introduction

Chloroplasts are cellular organelles in algae and higher plants responsible for carbon fixation by the photosynthetic reaction (Eberhard et al., 2008). Consequently, these autotrophic organisms are the primary source of fixed carbon and chemical energy in most ecosystems on earth. The endosymbiotic theory states that plastids, including the chloroplast, evolved through the engulfment of a cyanobacterium by the eukaryotic progenitor cell (Sagan, 1967; Margulis, 1970). This primary endosymbiotic event occurred about 1 billion years ago and led subsequently to three evolutionary lines of plastid-containing organisms: the glaucophytes, the rhodophytes (red algae) and the chlorophytes (green algae), from which the higher plants diverged approximately 400–475 million years ago (Gould et al., 2008; Jensen & Leister, 2014). Although transfer of genes to the nuclear genome happened over time (Timmis et al., 2004; Bock & Timmis, 2008), plastids still contain their own genome (plastome). Plastids of higher plants, these approximately 100 genes encode proteins and RNA molecules of the transcription and translation machinery and components of the photosynthetic apparatus (Sugiura, 1989). To control the proper function of the chloroplasts under changing environmental conditions, algae and plant cells evolved to coordinate the expression of the plastid- and nuclear-encoded genes by regulating the levels of transcription, mRNA stability and translation (Jarvis & Lopez-Juez, 2013).

The protein biosynthesis in chloroplasts is catalysed by a bacterial-type 70S ribosome (Tiller & Bock, 2014), called chloroplast ribosome, composed of a 50S large subunit and a 30S small subunit. Although the chloroplast and the bacterial 70S ribosomes share a common ancestor, they have diverged considerably from each other as evident from proteomic analysis (Yamaguchi & Subramanian, 2000, 2003; Yamaguchi et al., 2000) and structural characterization at low resolution (Manuell et al., 2007; Sharma et al., 2007). Very recently, a cryo-EM reconstruction of the chloroplast large ribosomal subunit at 3.5 Å resolution was published (Ahmed et al., 2016) and their findings concerning the 50S subunit are in agreement with our study of the complete chloroplast 70S ribosome. The chloroplast ribosomal RNA (rRNA) (total 4,524 nucleotides in spinach) is about the same length as in the bacterial ribosome (total 4,566 nucleotides in Escherichia coli). However, it is fragmented to include a 4.5S rRNA molecule with sequence homology to the 3′ tail of the bacterial 23S rRNA. Although bL25 and uL30 are completely missing in the chloroplast 70S ribosome, the total protein mass is increased by more than 170,000 Da due to extension of ribosomal proteins sharing homology with bacteria and the acquisition of three plastid-specific ribosomal proteins (PSRPs) to the small and two PSRPs to the large subunit (Yamaguchi & Subramanian, 2000, 2003; Yamaguchi et al., 2000). The new components, together with plastid-specific translation factors, play an important role in the...

To better understand the evolution and the function of ribosomes in plastids, we determined the atomic structure of the chloroplast 70S ribosome in complex with the plastid translation factor pY using cryo-electron microscopy (cryo-EM).

Results and Discussion

Structure of the chloroplast 70S ribosome

Chloroplast 70S ribosomes were purified from spinach (Spinacia oleracea) leaves and investigated by cryo-EM single-particle analysis (Appendix Fig S1). The three-dimensional (3D) reconstruction of the complete chloroplast 70S ribosome was resolved to 3.4 Å (Figs 1A and EV1, and Appendix Fig S2). To improve the structural interpretation, the particle images were further classified using maximum-likelihood-based algorithms, masking, and signal subtraction approaches to yield cryo-EM maps of the 50S large subunit at 3.2 Å resolution (Fig EV1 and Appendix Fig S2) and of the 30S small subunit at 3.6 Å resolution (Fig EV1 and Appendix Fig S3). The obtained maps were of sufficient quality to allow building and refinement of an almost complete model of the chloroplast 70S ribosome (Fig 1A, and Appendix Figs S4 and S5; Appendix Tables S1–S3) revealing the chloroplast-specific ribosomal features at molecular detail.

We could identify and position all plastid-specific ribosomal proteins, which we also renamed to conform to the new convention of ribosomal protein nomenclature (Ban et al, 2014) (Fig 2). Several of these, bTHXc (PSRP4), cL37 (PSRP5) and cL38 (PSRP6), were de novo built into the electron density map (Figs 2A–C and EV2), whereas for the remaining two proteins, cS22 (PSRP2) and cS23 (PSRP3), which are bound to the more flexible foot of the small subunit, homology models were fitted as rigid bodies (Fig 2D and Appendix Fig S6). Compared to the bacterial 70S ribosome, the chloroplast 70S ribosome has different architectural features due to presence of additional proteins and N- and C-terminal chloroplast-specific extensions of ribosomal proteins with bacterial homologs (Fig 1B). The changes are particularly pronounced between the platform and shoulder of the 30S subunit and around the polypeptide exit site of the 50S subunit (Fig 1B). As a striking example, such protein extensions mediate the interactions between the ribosome steps.

Figure 1. Architecture of the chloroplast 70S ribosome.
A Structure of the chloroplast 70S ribosome. 50S subunit proteins are in blue, 23S rRNA in cyan, 5S rRNA in green, 4.5S rRNA in red, 30S subunit proteins in gold, 16S rRNA in pale yellow, E-site tRNA in pink and translation factor pY in green. Plastid-specific ribosomal proteins cS22, cS23, bTHXc, cL37 and cL38 are shown in red. Protein and rRNA elements conserved between chloroplast and bacterial 70S ribosome are in blue and grey, respectively. Chloroplast-specific rRNA elements are shown in purple. Plastid-specific ribosomal proteins and additional protein extensions are in red and yellow, respectively. Translation factor pY is shown in green. Structural landmarks of the 70S ribosome are indicated.
and the plastid-specific 4.5S rRNA (Whitfeld et al., 1978) (Fig EV3).

In addition, we identified an intersubunit bridge unique for chloroplast ribosomes, called B7c, formed by a plastid-specific extension of bS6c at the 30S platform and uL2c below the L1 stalk (Appendix Fig S7; Appendix Table S4).

In contrast to the solvent side, the rRNA core and the interface of the two subunits are structurally much more conserved relative to the bacterial ribosome (Fig 1B), implying also a conserved mechanism of mRNA decoding by the small subunit and the peptide bond synthesis by the large subunit.

**Structural insights into the role of the plastid-specific ribosomal proteins**

Plastid translation activity is absolutely required for the regular development and function of plants, and mutations of plastid ribosomal proteins and defective assembly of the translation apparatus influence the plant anatomy and morphology (Ahlert et al., 2003; Rogalski et al., 2006; Tiller et al., 2012). Although biochemical studies have not yet been conducted for all plastid ribosomal proteins, it appears that more ribosomal proteins are essential for proper translation activity in the chloroplast than in the bacterial (E. coli) 70S ribosome (Tiller & Bock, 2014). Interestingly, of the five chloroplast-specific ribosomal proteins, cS23 (PSRP3), bTHXc (PSRP4) and cL37 (PSRP5) are essential for plastid translation activity (Tiller et al., 2012), and furthermore, cS22 (PSRP2) plays a role in plant development under stress conditions and has RNA chaperone activity (Xu et al., 2013). Under the tested conditions, the knockdown of cL38 (PSRP6) had no measurable effect on plastid translation (Tiller et al., 2012). Our atomic structure reveals the role of these proteins in the context of the chloroplast 70S ribosome.
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The two proteins associated with the large subunit, cl37 and cl38, are lacking a homologue in bacteria, indicating that they were probably acquired later in evolution. cl37 forms a long helical structure and is positively charged (10.72 pI) due to many lysine and arginine side chains that interact with the RNA backbone (Figs 2A and EV2). Contrary to previous studies (Sharma et al., 2007) in which cl37 was incorrectly positioned close to the L1 stalk, we identified cl37 bound to a deep groove formed by several rRNA elements of 23S rRNA domain III (Fig 1A). Binding of cl37 at this position is accompanied by changes in helix H58 and the expansion of the loop connecting H54 and H55 when compared to bacteria (Fig EV2). Furthermore, the complete helix H63, which interacts with helices H58, H59 and H60 of domain III in bacteria, is absent in chloroplasts (Fig EV2), and it appears that cl37 is required to stabilize this rRNA fold through extensive interactions with the RNA backbone. This is in agreement with the biochemical data by Tiller et al. (2012), showing that knockdown of cl37 leads to reduced level of 50S subunits, probably due to incomplete folding and subsequent degradation of the 23S rRNA.

For plants with a knockdown of cl38, no obvious changes in the plant phenotype were observed under the experimental conditions, and only a slightly lower content of thylakoid complexes could be measured (Tiller et al., 2012). Our map revealed an elongated fold of cl38, which is bound to the L7/L12 stalk base via N-terminal interactions to the 23S and the 5S RNA and C-terminal protein–protein contacts to bL20c and bL21c (Figs 1A, 2B, and EV2). The hairpin D loop of 5S RNA domain γ interacting with cl38 is rearranged due to the insertion of an additional nucleotide in the plastid 5S rRNA. In bacteria, domain γ is further contacting ribosomal proteins bL25 and uL30, which are missing in chloroplast ribosomes. Although cl38 probably evolved to locally stabilize the stalk base and the attachment of the 5S rRNA, bL25 and uL30 are not structurally replaced by cl38 or by other plastid ribosomal proteins (Fig EV2).

Plastid ribosomal protein bTHXc is homologous to bacterial protein bTHX (Yamaguchi & Subramanian, 2003) found so far only in the Thermus bacterial genus (Leontiadou et al., 2001), indicating convergent evolution or gene capture at a later step in evolution. Bacterial bTHX is a small (26 amino acids) basic (12.1 pI) protein, and its structure has been visualized by X-ray crystallography as part of the 30S subunit of Thermus thermophilus bound to a cavity formed by 16S rRNA elements of the head (Wimberly et al., 2000). In our cryo-EM map, we identified bTHXc located at the same place (Figs 1A, 2C, and EV2). The overlay of bTHXc (46 amino acids) with bTHX (Wimberly et al., 2000) indicates a similar core fold interacting with the 16S rRNA helices h41 and h42 and a plastid-specific C-terminal extension forming hydrophobic interactions with uS13c (Fig EV2). Therefore, bTHXc stabilizes the 16S rRNA of the 30S head and the intersubunit bridge B1b between uS13c and the central protuberance (CP) of the 50S subunit. In plants with a bTHXc knockdown, reduced levels of plastid 30S subunits lead to a reduced plastid translation activity and severe growth defects (Tiller et al., 2012). Considering that bTHX is only found in thermophilic bacteria, plastid ribosomes possibly acquired this protein to stabilize the ribosomes as an adaptation to fluctuating temperatures that the plant cells are exposed to.

The foot of the chloroplast 30S subunit is highly reorganized due to the truncation of 16S rRNA helices h6, h10, h17 and the acquisition of new proteins and protein extensions (Fig 1). Despite the structural flexibility of the foot, the use of local 3D classification allowed us to obtain a map into which homology models of cS22 and cS23 could be fitted as rigid bodies (Fig 2D and Appendix Fig S6), at a position consistent with previous reports (Sharma et al., 2007). cS22 contains two RNA-binding motifs (RBM) connected by a flexible linker of 17 amino acids and shows RNA chaperone activity (Xu et al., 2013). Because only one RBM domain could be fitted into the density close to loop h10 and no other unassigned density in close proximity is visible, we suggest that the second RBM domain is flexibly attached to the first and may be used to bind mRNA during translation initiation or for localizing the ribosome within the chloroplast through interactions with RNA or ssDNA. The positioning of cS23 in the context of the changed structure of the rRNA suggests that this protein may function as a replacement for the truncated helix h6 (Fig 2D and Appendix Fig S6). The knockdown of cS23 leads to defective chloroplast translation with severe alterations of leaf anatomy (Tiller et al., 2012), indicating that cS23 is an important ribosomal protein with possible additional roles in ribosome assembly or in translation.

New features of the plastid ribosomal RNA

In the chloroplast 70S ribosome, the loss of rRNA mass through truncations is almost balanced by the acquisition of plastid-specific rRNA expansion segments, which mainly protrude from the solvent side of the 50S subunit (Fig 1B). The 16S rRNA of the 30S subunit (Appendix Fig S8) responsible for mRNA binding and stabilization of the codon–anticodon interactions shows relatively small structural adaptations compared to the bacterial 16S rRNA except for truncations of helices h6, h10 and h17, which are involved together with cS22 and cS23 in forming the remodelled foot of the small subunit (Fig 2D and Appendix Fig S6). In the 23S rRNA of the 50S subunit (Appendix Fig S9), rRNA helices H7, H9, H45, H63 and H98 are truncated or completely lost. New rRNA expansions segments, unique for chloroplast ribosomes, have evolved on the solvent side and are partially stabilized by ribosomal protein extensions (Fig 1B). The 5S rRNA forms large parts of the CP and is structurally rearranged in the area where it interacts with cl38 (Fig 2B). The 50S subunit also contains a third rRNA molecule, the 4.5S rRNA (Whitfield et al., 1978). In the plastome, the sequence of the 4.5S rRNA shows homology to the 3′ tail of bacterial 23S rRNA (55% sequence identity); however, it is separated from the 23S rRNA by a 115 nt spacer sequence (Fig EV3). Our map revealed the complete fold and interactions of the 4.5S rRNA (Fig 3A), which occupies a similar position on the plastid ribosome as the 3′ tail of the bacterial 23S rRNA and forms interactions with uL3c, uL13c, bL17c, bL19c and bL32c. A loop formed by nine nucleotides (comprising residues 30–38) unique to the 4.5S rRNA interacts with the N-terminal extension of protein bL19c and several loops of uL3c (Fig EV3). The interaction between the 5′ end of the 4.5S rRNA and 3′ end of the 23S rRNA is stabilized by a plastid-specific α-helical extension of uL13c that compensates the loss of helix H98 of the 23S rRNA and forms specific electrostatic interactions with the rRNA ends via two conserved basic residues, Arg48 and Lys49 (Fig 3B). This α-helix of uL13c is positioned through interactions with two plastid-specific domains of bL21c and uL22c, underscoring its importance for integration of the 4.5S rRNA into the subunit.
RNA elements located in the 5′-UTRs of mRNAs are proposed to be the major determinants of correct translation initiation in plastids. Nuclear-encoded trans-acting factors, which are partially regulated by abiotic factors as light or temperature, specifically bind to these cis-elements and enable efficient translation initiation either by rearranging the structure of the mRNA 5′-UTR or by mediating the interaction between the mRNA and the anti-SD sequence of the plastid 16S rRNA and the anti-SD sequence of the plastid 16S rRNA (Qu et al., 2012; Duval et al., 2013) to recruit it to the mRNA channel of the small subunit. 

In the chloroplast ribosome, the mRNA entry site is encircled by the ribosomal proteins uS3c, uS4c and uS5c. Ribosomal protein 

In chloroplasts, the polypeptide exit tunnel region is considerably different compared to the bacterial ribosome because of the

| A | Position of the 4.5S rRNA (red) at the surface of the 50S large subunit. The 3′ and 5′ ends of the 4.5S rRNA and the 23S rRNA (blue) are labelled. Ribosomal proteins interacting with or in close proximity to the 4.5S rRNA are shown in different colours. |
| B | Stabilization of the 5′ end of the 4.5S rRNA and the 3′ end of the 23S rRNA by the plastid-specific N-terminal tail of uL13c (yellow). Specific residues of uL13c, uL22c and 4.5S rRNA are labelled. |

**Adaptions of the mRNA entry and exit sites to chloroplast-specific translation initiation**

Our cryo-EM map of the chloroplast 30S subunit reveals new protein features around uS2c, which modify the mRNA entry and exit sites and represent parts of bS1c and uS5c (Fig 4A). In bacteria, ribosomal protein bS1 is essential for translation initiation as it binds (Boni et al., 1991) and unfolds the 5′-UTR of mRNA (Qu et al., 2012; Duval et al., 2013) to recruit it to the mRNA channel of the small subunit. Chloroplast bS1c contains three OB-folds and long C- and N-terminal extensions. We observe that in the chloroplast ribosome interactions between bS1c and uS2c are much more extensive compared to the bacterial system and involve not only one of the OB-folds of bS1c, but also chloroplast-specific C- and N-terminal extensions that wrap around uS2c (Fig 4B). The remaining two OB-folds of bS1c are not visible in our cryo-EM map (Fig EV4) as a gap in the rRNA backbone density at the putative cleavage site. Furthermore, our structure shows that the RNA-binding sequence of RH39 is accessible to the solvent and well-positioned for processing of the hidden break.

**Modifications of the SRP-binding site at the polypeptide tunnel exit**

In chloroplasts, the polypeptide exit tunnel region is considerably different compared to the bacterial ribosome because of the
truncation of 23S rRNA helix H7 and extensions of uL24c and uL29c (Fig 5A and Appendix Fig S11). Furthermore, in the course of evolution, the bacterial-ancestral uL23 was substituted by a variant of the eukaryotic-cytoplasmic uL23 (Bubunenko et al, 1994), which has a truncated hairpin loop pointing towards the polypeptide tunnel and an elongated α-helical C-terminus (Fig 5B). These architectural modifications at the tunnel exit region likely coevolved with the protein targeting mechanism that is specific for chloroplasts. In contrast to the bacterial system, the chloroplast lacks the RNA component of the signal recognition particle (SRP) (Richter et al, 2010) and consists only of protein cpSRP54. Consequently, two known docking sites of the bacterial SRP RNA (Halic et al, 2006; Jomaa et al, 2016), the C-terminal domain of bL32 and stem-loop H100 of 23S rRNA, have changed in chloroplasts (Fig 5A and Appendix Fig S11). In particular, bL32c has an elongated α-helix interacting with the surrounding rRNA backbone via many positively charged residues, and bacterial stem-loop H100 of 23S rRNA is structurally replaced by the extra loop of the plastid-specific 4.5S rRNA.

The NG-domain of bacterial SRP Ffh, which is homologous to cpSRP54, binds to conserved residues of uL29 and a binding pocket formed by uL23 and uL29 (Kramer et al, 2002; Jomaa et al, 2016). In chloroplasts, the residues on uL29c are partially conserved, but the putative binding pocket of uL23c is adapted by a plastid-specific extension of uL29c (Fig 5C). The C-terminal helix of uL29c interacts with the C-terminus of uL23c, thereby shielding the residues that mediate the interactions with the NG-domain.

### Plastid translation factor pY bound to the mRNA channel

Protein synthesis in chloroplasts responds to changes in light and temperature and is mainly regulated at the translational level, while the mRNA content in the organelle is maintained constant (Fromm et al, 1985; Kim & Mayfield, 1997; Marin-Navarro et al, 2007). We purified chloroplast ribosomes from plant tissue incubated in the cold and in darkness, conditions under which the protein synthesis is reduced (Fromm et al, 1985). As observed previously (Sharma et al, 2007), under these conditions ribosomes are associated with
plastid translation factor pY (previously called PSRP1) (Fig 6A and Appendix Fig S12), which is a homologue of bacterial cold shock protein Y (pY or YifA) that stabilizes 70S ribosomes under stress conditions by binding to the subunit interface (Agafonov et al., 2001; Vila-Sanjurjo et al., 2004; Sharma et al., 2010; Polikanov et al., 2012).

The high-resolution maps revealed the binding site of factor pY bound to the mRNA channel of the small subunit and allowed us to build an almost complete structure (Fig 6B). The identity of plastid pY was established by direct inspection of the density and confirmed by mass spectrometry analysis of the ribosome sample (Appendix Fig S12). The structure reveals the molecular interactions of pY with the 16S tRNA residues that form the decoding centre. Helix α1 binds to the rRNA backbone of helix h44 via positively charged residues, helix α2 extends above the mRNA channel, thereby preventing mRNA binding, and the four stranded β-sheet (β1-4) points towards the head (Fig 6A and B). In the A-site of the ribosome, negatively charged residues of helix α2 and the loop between helix α2 and sheet β4 (Fig 6C) stabilize the universally conserved bases A1441 (A1492 in E. coli) and A1442 (A1493), which are involved in decoding in a partially flipped out conformation that is between the empty and mRNA-tRNA bound states observed for bacterial ribosomes (Wimberly et al., 2000; Selmer et al., 2006). Furthermore, bases G478 (G530) and C1003 (C1054), which are also involved in decoding, are stabilized through respective contacts with Pro127 and Arg119 of pY. The loop between β2 and β3 located at the A-site is larger than in the eubacterial pY homologues and forms contacts to helices h18 and h34 (Fig 6C) at the mRNA entry site. Reaching towards the mRNA exit site, the C-terminal extension of helix α2 contains two histidine residues, His178 and His181, that mimic the mRNA bases at the E-site. All these specific interactions enable plastid pY to fulfil two of its functions: first, functionally most important areas of the 70S, including the key nucleotides of the decoding centre, are protected by plastid pY from being degraded during the translational arrest, in a similar way as suggested for bacterial pY (Vila-Sanjurjo et al., 2004), and second, the binding of pY to the mRNA channel prevents binding of the A-site and P-site tRNAs (Fig 6D) and inhibits translation. The C-terminal domain of plastid pY is disordered in our structure, in agreement with its possible role in pY activation that likely involves interactions with other factors (Bubunenko & Subramanian, 1994; Sharma et al., 2010).

Comparing the maps of the chloroplast ribosome in the pY-bound state with a reconstruction of an empty state plastid ribosome at lower resolution reveals that the small subunit is in a rotated state relative to the large subunit in the absence of pY (Fig EV5A–D). In this conformation, the body is rotated by 5.8° (ratcheting) and the head by 7.0° (swivelling) in comparison with the non-rotated state with bound pY, which reduces the number of intersubunit contacts (Fig EV5E–H and Appendix Table S4). Especially, the bridges B1a and B1b between the small subunit head and the large subunit A-site finger and the CP, respectively, as well as bridges B7a, B7b and B7c are weakened by the small subunit rotation. Coupled with body rotation and head swivelling, the tRNA moves from an E/E-state in the non-rotated to a P/E-state in the rotated conformation (Fig EV5I and J). Because we do not see empty 70S ribosome in a non-rotated state, it is likely that plastid pY stabilizes the chloroplast ribosomes from dissociation in the non-rotated state with increased intersubunit contacts under environmental conditions that do not require active protein synthesis.

**Relationship to apicoplast ribosomes**

The structure of the chloroplast ribosome also allows for better understanding of ribosomes found in a relict plastid, called...
apicoplast, in protozoan parasites (Wilson, 1993; McFadden et al., 1996) responsible for severe diseases like malaria (Plasmodium falciparum) and toxoplasmosis (Toxoplasma gondii). The “plant-like” apicoplast critical for proliferation of these organisms probably originated from red algae by secondary endosymbiosis (Waller et al., 2003) and contains its own active transcription and translation machinery. The chloroplast ribosome described here now provides the best starting point for understanding the structure of apicoplast ribosomes that have an rRNA reduced in length and are lacking several proteins (12 ribosomal proteins with bacterial homolog for P. falciparum and 14 for T. gondii) that are present in chloroplast ribosomes (Habib et al., 2016). Considering that several compounds targeting the bacterial ribosome also show activity against the malaria parasite (Goodman et al., 2007; Kalanon & McFadden, 2010; Wilson et al., 2015), the structure presented here can also be used as a starting model for designing better drugs capable of targeting the translation apparatus of plastids.

Conclusions

The structure presented here reveals the architecture of the chloroplast 70S ribosome with important implications for understanding
its relationship to ancestral bacterial ribosomes. We also observe important differences in the structure related to plastid-specific mechanism of translation initiation and membrane protein targeting. Furthermore, we reveal the molecular mechanism of how translation factor pY inhibits translation by binding to the mRNA channel region of the small subunit to protect the decoding site and stabilize the ribosome in an inactive form during the dark phase of the chloroplast day cycle. These results contribute to the mechanistic understanding of translation in chloroplast and its regulation and pave the way for future structure-based biochemical and genetic studies.

Materials and Methods

Preparation of chloroplast 70S ribosomes

Fresh leaves of spinach (S. oleracea) were obtained from the local supermarket and stored for a few hours in the dark at 4°C. The purification of chloroplasts from leaf tissue was done according to a previously described protocol (Bartsch et al., 1982). The enriched chloroplasts were lysed by gentle stirring (180 rpm, 4°C, 90 min) in lysis buffer (10 mM Tris–HCl pH 7.6, 25 mM KCl, 25 mM MgCl2, 2 mM DTT, 0.1 mM PMSF, 2 mM spermidine, 0.05 mM spermine, 2% (w/v) Triton X-100). The suspension was cleared by centrifugation (25,350 g, 30 min, 4°C) using a Beckman Type 45Ti rotor (Beckman-Coulter), and the supernatant was loaded onto 50% (w/v) sucrose cushions and centrifuged (101,390 g, 15 h, 4°C) using a Beckman Type 45Ti rotor (Beckman-Coulter). The ribosome pellets were dissolved in monosome buffer (25 mM Tris–HCl pH 7.6, 25 mM KCl, 25 mM MgOAc2, 2 mM DTT, 2 mM spermidine, 0.05 mM spermine). The sample was layered onto 10–40% (w/v) sucrose gradients and centrifuged (51,610 g, 15 h, 4°C) using a Beckman Type SW-32Ti rotor (Beckman-Coulter). The fractions containing most chloroplast 70S ribosomes were pooled (Appendix Fig S1), and the buffer was exchanged to sucrose-free monosome buffer using Amicon Ultra-4 spinning centrifugal filter units with 100,000 molecular weight cutoff (Merck Millipore).

Cryo-EM data acquisition and processing

The sample was diluted with monosome buffer to a final 70S ribosome concentration of 50 nM, and 5 μl was applied to Quantifoil R2/2 holey carbon grids (Quantifoil Micro Tools), which had been previously coated with a thin home-made carbon film and glow-discharged (negative, 25 mA, 30 s) using an Emitter K100X (Quorum Technologies). The grids were automatically blotted and flash-frozen by plunging into a 2:1 mixture of liquid ethane and propane using a Vitrobot (FEI Company). Cryo-EM data were collected on a Titan Krios cryo-tem transmission electron microscope (FEI Company) equipped with a Falcon II direct electron detector and operated at 300 kV with a magnification of 100,720× and a defocus range between −0.8 and −3.5 μm. The EPU software (FEI Company) was used for automated data acquisition by collecting seven movie frames with a combined dose of 20 electrons per Å2 per exposure (770 ms combined exposure time) after discarding the first frame (55 ms).

We used the software DOSEFGPU DRIFTCORR (Li et al., 2013) to correct for beam-induced specimen motion and CTFFIND (Mindell & Grigorieff, 2003) to estimate the CTF parameters from the drift-corrected micrographs. Micrographs were selected by evaluating the quality of the power spectra. From finally 2,796 selected micrographs, 326,094 particles were automatically selected using Batchboxer from the EMAN software package (Ludtke et al., 1999). Further steps of image processing were performed in RELION 1.4 (Scheres, 2012). In an initial 2D classification, binned particle images (5.56 Å/px on the object scale, 80 px frame size) were classified into 200 classes. Particles assigned to classes showing separated 30S and 50S subunits, 80S ribosomes and non-ribosomal particles were removed from the dataset. Multiple 3D classification steps in combination with masking and signal subtraction approaches (Appendix Figs S2 and S3) were applied to obtain homogenous particle image subsets for the 3D reconstructions of the complete 70S ribosome, the 50S subunit and 30S subunit. The final high-resolution refinements of these particle subsets at full-pixel size (1.39 Å/px on the object scale, 320 px frame size) resulted in 3D reconstructions of the chloroplast 70S ribosome at 3.4 Å resolution from 140,583 particle images, of the 50S subunit at 3.2 Å resolution from 154,332 particle images and of the 30S subunit at 3.6 Å resolution from 127,031 particle images according to the Fourier Shell Correlation (FSC) = 0.143 criterion (Fig EV1). Subsequently, the maps were sharpened and used for manual atomic model building, refinement and validation.

Structure building and refinement

To obtain a full atomic model of the 70S ribosome, the structures of the 30S and 50S subunits were initially built into the EM maps of the individual subunits using O (Jones et al., 1991; Jones, 2004) and COOT (Emsley et al., 2010) and the coordinates of an E. coli ribosome as a guide (PDB 4YBB; Noeske et al., 2015). The maps were of excellent quality and allowed building of almost all RNA and protein residues. In peripheral regions of the subunits with lower local resolution, a few protein extensions were built as unassigned UNK residues, and in the case of proteins cS22, cS23 and bS1c, PHYRE models were docked as rigid bodies (Kelley et al., 2015) (Appendix Tables S2 and S3). The unambiguous density for factor pY (PSRP1) allowed docking of a PHYRE model followed by rebuilding at atomic level (Appendix Fig S12). The atomic models were subsequently refined and validated using PHENIX (Adams et al., 2010) as described previously (Greber et al., 2014). In brief, the coordinates were refined in reciprocal space against structure factors back-calculated from the EM maps using the mlhl target to restrain the phases. The phases were weighted according to the FSC dropoff as described (Greber et al., 2014). During coordinate refinement of the subunits, an optimal geometry weighting value of wxc = 1.25 was established, which resulted in model geometry and R-factor values typical for the chosen resolution ranges (Urzhumtseva et al., 2009) (Appendix Fig S4; Appendix Table S1). Using high geometry weighting values results in low R-factors but worse model geometry and possible overrefinement, while at low geometry weighting values the R-factors are increased and the model geometry is overfitted. The refinement of the complete 70S was performed using the coordinates of the individually refined 50S and 30S subunits, which were rigid body fitted into the 3.4 Å cryo-EM reconstruction of the chloroplast 70S ribosome (Fig EV1). At the E-site of the 70S intersubunit
space, a density representing a mixture of tRNAs was observed. To account for this density, an optimized canonical E. coli tRNA-Phe derived from PDB 2300 was docked. Protein contacts between both subunits were adjusted, and the linker of bL31c, which bridges both subunits, was added. The complete 70S model was then fully refined against the 3.4 Å cryo-EM map using PHENIX (Appendix Fig S4; Appendix Table S1) in a similar procedure as described above for the subunits, using an optimal geometry weighting value of \( w_xc = 1.4 \).

Creation of figures

Figures showing cryo-EM reconstructions and molecular models were created using UCSF Chimera (Pettersen et al., 2004) and PyMOL (The PyMOL Molecular Graphics System, Version 1.7 Schrödinger, LLC). Local resolution plots were generated in ResMap (Kucukelbir et al., 2014).

Mass spectrometry analysis

Purified chloroplast 70S ribosomes (~50 µg) were mixed with SDS gel-loading buffer (final concentration: 50 mM Tris–HCl pH 6.8, 2% (w/v) sodium dodecyl sulphate, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, 100 mM β-mercaptoethanol) and heated for 10 min at 70°C before loading the sample on a 12% polyacrylamide gel (GenScript). The gel was stained with Coomassie brilliant blue G-250 (Sigma-Aldrich) and protein bands in the molecular weight range between 20 and 40 kDa and one band at 50 kDa have been cut out. The sliced protein bands were sent for protein identification by mass spectrometry (liquid chromatography MS/MS) performed at the Functional Genomics Center Zurich (FGCZ). The Mascot software packages (Perkins et al., 1999) was used for the database searches in SwissProt and Trembl, and the results were analysed applying stringent settings [1% protein false discovery rate (FDR), a cut-off of 0.05 for the Mascot score]. A minimum of two peptides per protein, 0.1% peptide FDR.

Accession numbers

The 3.4 Å cryo-EM map of the chloroplast 70S ribosome, the 3.2 Å cryo-EM map of the 50S subunit and the 3.6 Å cryo-EM map of the 30S subunit have been deposited in the Electron Microscopy DataBank with accession codes EMD-3533, EMD-3531 and EMD-3532, respectively. The refined coordinates of the atomic structure of the 50S subunit and the 30S subunit have been deposited in the Protein Databank with accession codes 5MMM, 5MMI and 5MMJ, respectively. The coordinates of the atomic model of the complete chloroplast 70S ribosome have been deposited as PDB 5MMM. A PyMOL script for display of the chloroplast 70S ribosome is available from the Ban Lab website (www.bangroup.ethz.ch).

Expanded View for this article is available online.

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Author contributions

PB and ML established the purification procedures. PB, ML and MS performed the preparation and the biochemical analysis of the chloroplast ribosome. PB and MS prepared cryo-EM samples. PB and DB acquired the cryo-EM data. PB and DB calculated the cryo-EM reconstructions. ML, PB, MS and NB interpreted the structures. PB wrote the manuscript. All authors contributed to the final version of the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

References


