Plastid-specific ribosomal proteins (PSRPs) have been proposed to play roles in the light-dependent regulation of chloroplast translation. Here we demonstrate that PSRP1 is not a *bona fide* ribosomal protein, but rather a functional homologue of the *Escherichia coli* cold-shock protein pY. Three-dimensional Cryo-electron microscopic (Cryo-EM) reconstructions reveal that, like pY, PSRP1 binds within the intersubunit space of the 70S ribosome, at a site overlapping the positions of mRNA and A- and P-site tRNAs. PSRP1 induces conformational changes within ribosomal components that comprise several intersubunit bridges, including bridge B2a, thereby stabilizes the ribosome against dissociation. We find that the presence of PSRP1/pY lowers the binding of tRNA to the ribosome. Furthermore, similarly to tRNAs, PSRP1/pY is recycled from the ribosome by the concerted action of the ribosome-recycling factor (RRF) and elongation factor G (EF-G). These results suggest a novel function for EF-G and RRF in the post-stress return of PSRP1/pY-inactivated ribosomes to the actively translating pool.

Chloroplasts are intracellular organelles present in higher plants and algae; they contain the entire machinery with which the process of photosynthesis is conducted. According to the endosymbiotic theory of chloroplast evolution (1–3), this organelle originated through engulfment of a photosynthetic unicellular prokaryote by a eukaryotic host cell, and the subsequent integration of the two genomes (that of the engulfed prokaryote, and the eukaryotic nucleus) through a process of gene transfer from the chloroplast to the nuclear genome. Thus, although the chloroplast carries its own transcriptional and translational machineries, the development and maintenance of the chloroplast are dependent on the coordinated expression of chloroplast- and nuclear-encoded gene products.

The light-dependent process of photosynthesis is the primary function of the chloroplast. Because the components that are crucial for the biogenesis of the photosynthetic apparatus are encoded by both the chloroplast and nuclear genomes, the plant cell has evolved several mechanisms to achieve concerted regulation of gene expression in the two cellular compartments, in response to changes in illumination (4–6). Regulation of gene expression is primarily post-transcriptional, and is achieved through altered mRNA processing and stability, and the control of the translational apparatus itself in response to environmental signals like light (7–9). It has been demonstrated that the redox state of the chloroplast achieved in response to photosynthetic electron transport can regulate protein synthesis within the chloroplast at the stages of initiation and elongation (10–12).

A detailed analysis of the chloroplast translational machinery, the chloroplast ribosome together with its trans-acting translational factors, will provide important clues as to how such gene regulation is achieved. Proteomic characterization of the chloroplast ribosomes (chlororibosomes) from spinach has revealed the presence of six plastid-specific ribosomal proteins (PSRPs), four of which are associated with the 30S subunit and two with the 50S subunit, in addition to the plastid orthologs of bacterial r-proteins (13–16). All six PSRPs are encoded by genes located in the nucleus and are synthesized in the cytoplasm as precursor polypeptides. However, the functional roles of these PSRPs have not yet been elucidated.

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Here, we demonstrate that PSRP1 can interact with *Escherichia coli* 70S ribosomes, both *in vivo* and *in vitro*. Cryo-EM and three-dimensional image reconstruction reveal that PSRP1 binds within the intersubunit space of the ribosome, overlapping the positions of the anticodon stem-loops of A- and P-site tRNAs. Binding of PSRP1 induces conformational changes within several ribosome intersubunit bridges and stabilizes the ribosome against dissociation. Additionally, we show that binding of PSRP1 to the 70S ribosome is stabilized by the presence of the ribosome-recycling factor (RRF), but unaffected by the presence of initiation factor 3 (IF3). However, in the presence of elongation factor G (EF-G-GTP) and RRF, PSRP1, and pY appear to be recycled from the ribosome analogously to tRNA. We propose a model describing how PSRP1/pY-inactivated ribosomes are returned to the translation cycle, once stress conditions have abated.

**EXPERIMENTAL PROCEDURES**

*Expression of PSRPs in E. coli and Preparation of the E. coli 70S-PSRP1 Complexes—*The gene encoding the mature PSRP1 was amplified from cDNA template and cloned into pET32b, thus introducing N-terminal thioredoxin (Trx) and 6× histidine (6×His) tags onto the mature PSRP1. *E. coli* pY and IF3 genes were amplified from *E. coli* gDNA and cloned into pET-14b. All plasmids were transformed into BL21(DE3), protein expression was induced at mid-log phase with 1 mM isopropyl-1-thio-β-D-galactopyranoside, and the cells were harvested at late log-phase. Cells were lysed by freeze thawing and were resuspended on ice in Buffer A (20 mM Hepes-KOH, pH 7.8, 10 mM MgCl₂, 60 mM NH₄Cl, 4 mM β-mercaptoethan), in the presence of lysozyme (1 μg/ml) and phenylmethylsulfonyl fluoride (0.2 mM). Lysates were clarified by centrifugation at 30,000 × g for 30 min, and the supernatant (S30) was then applied to 10–30% sucrose gradients in Buffer B.

**Binding Assays**—Binding assays were performed as described previously (17). Dissociation assays were performed using tight-couple *E. coli* 70S ribosomes that were incubated in the presence or absence of protein factors in Buffer B (20 mM Hepes-KOH pH 7.8, 2.5 mM MgCl₂, or 5 mM MgCl₂, 200 mM NH₄Cl, 4 mM β-mercaptoethanol), before being applied to a 10–30% sucrose gradient in Buffer B.

**Figure 1.** Binding of spinach PSRP1 to *E. coli* ribosomes. *a,* Coomassie Blue-stained SDS-PAGE with lysate (L) and purified 70S ribosomes (R) from *E. coli* cells overexpressing PSRP1 (lanes 4 and 5) or Trx control (lanes 2 and 3). *b,* 30S subunits (lanes 4–6) or 70S ribosomes (lanes 7–9) were incubated in the absence (−) or presence of a 2.5× (+) or 5× (+++) excess of PSRP1 protein, before being centrifuged through a 10% sucrose cushion, with the pellet then subjected to SDS-PAGE and Coomassie Blue staining. Lysate (lane 2) and 70S ribosomes (lane 3) from *a* are included for reference. Arrows in *a* and *b* indicate ribosomal protein S1 (RPS1), PSRP1 positions in 70S ribosome (lanes 3 and 5 in panel *a*), and relevant protein marker sizes (lane 1).

**Figure 2.** Binding position of PSRP1 on the *E. coli* 30S ribosomal subunit. Both the 30S subunit (yellow) and PSRP1 (green) masses were derived from the cryo-EM maps of 70S-PSRP1 complexes (see supplemental Fig. S2) that were obtained under *a* (in vivo) and *b* (in vitro) conditions; *c,* the 30S subunit portion of the chloroplast 70S map (26), with density corresponding to PSRP1 shown in green. The 30S ribosomal subunit has been computationally removed from all three maps, to reveal the PSRP1-binding position. The 30S subunit maps are shown from their subunit-subunit interface side. Landmarks of the 30S subunit: *b,* body; *h,* head; *pt,* platform; *sp,* spur; and h44, 16S rRNA helix 44.
incubated with varying amounts of protein factors (5× PSRP1 and 10× molar excess of RRF over 70S ribosomes; 5× molar excess of PSRP1 and IF3 over 30S ribosomes; 10× molar excess of PSRP1, RRF and EF-G over 70S ribosomes in the presence of 0.5 mM GTP and GDPNP) for 20 min at 37 °C in binding buffer (20 mM Hepes-KOH, pH 7.6, 10 mM MgCl₂, 30 mM NH₄Cl) before being loaded on a 10% sucrose cushion in binding buffer and centrifuged for 30 min at 75,000 rpm in a TLN 100 rotor at 4 °C. For each condition, aliquots of the initial reaction (R), supernatant (S) and pellet (P) after centrifugation were analyzed by filtration on Millipore filters (0.45 μm), before being loaded on a 10% sucrose cushion in binding buffer and centrifuged in a SW40 rotor at 19,000 rpm for 16.5 h at 4 °C. Gradients were monitored at 254 nm, from top to bottom.

Cryo-electron Microscopy and Three-dimensional Image Reconstruction—All EM data were collected on a Tecnai F20 field emission gun electron microscope (FEI, Eindhoven, The Netherlands), equipped with low-dose kit, and an Oxford cryo-transfer holder, at a magnification of ×50,760. For the in vitro and in vivo assembled E. coli 70S-PSRP1 complexes, 53 and 37 micrographs, respectively, were scanned on a Zeiss flatbed scanner (Z/I Imaging Corporation, Huntsville, AL), with a step size of 14 μm, corresponding to 2.76 Å on the object scale. The projection-matching procedure within the SPIDER software (21) was employed to obtain three-dimensional maps. We used a 11.5 Å resolution E. coli 70S ribosome map (22) as the initial reference. For the in vitro complex, 32,611 images sorted into 18 groups (according to defocus values, ranging from 1.2 to 4.2 μm), were selected, after visual and cross-correlation-based screening and removal of images from over-represented groups among the initial set of 83 representative views of the ribosome, and were used in the final reconstruction. For the in vivo complex, 18,317 images were sorted into 15 defocus groups ranging from 1.6 μm to 4.3 μm. The resolution of the final CTF-corrected three-dimensional map, estimated using the Fourier

### tRNA Binding Assays—tRNA binding assays were performed as described previously (19). Briefly, in a total volume of 25 μl, E. coli ribosomes (0.4 μM) were incubated separately with 12 μM of PSRP1, pY, and/or RRF in the presence of 0.4 μM [³²P]tRNAfMet (and the presence or absence of 5 μM MF-mRNA (19)). The binding of [³²P]tRNAfMet to ribosomes was monitored at 20 °C, and centrifuged for 30 min at 75,000 rpm in a TLN 100 rotor at 4 °C. For each condition, aliquots of the initial reaction (R), supernatant (S) and pellet (P) after centrifugation were analyzed by filtration on Millipore filters (0.45 μm), before being loaded on a 10% sucrose cushion in binding buffer and centrifuged in a SW40 rotor at 19,000 rpm for 16.5 h at 4 °C. Gradients were monitored at 254 nm, from top to bottom.

Ribosome-recycling Assays—Recycling assays were performed as described previously (20) with the following modifications. Briefly, E. coli 70S ribosomes (0.4 μM) were incubated with 10× molar excess of protein factors indicated for each reaction in binding buffer (20 mM Hepes-KOH, pH 7.6, 8.2 mM MgCl₂, 80 mM NH₄Cl, 4 mM β-mercaptoethanol) for 20 min at 37 °C in a total volume of 150 μl, before being loaded on a 30–50% sucrose gradient in the same buffer and centrifuged in a SW40 rotor at 19,000 rpm for 16.5 h at 4 °C. Gradients were monitored at 254 nm, from top to bottom.

Cryoelectron microscopy and Three-dimensional Image Reconstruction—All EM data were collected on a Tecnai F20 field emission gun electron microscope (FEI, Eindhoven, The Netherlands), equipped with low-dose kit, and an Oxford cryo-transfer holder, at a magnification of ×50,760. For the in vitro and in vivo assembled E. coli 70S-PSRP1 complexes, 53 and 37 micrographs, respectively, were scanned on a Zeiss flatbed scanner (Z/I Imaging Corporation, Huntsville, AL), with a step size of 14 μm, corresponding to 2.76 Å on the object scale. The projection-matching procedure within the SPIDER software (21) was employed to obtain three-dimensional maps. We used a 11.5 Å resolution E. coli 70S ribosome map (22) as the initial reference. For the in vitro complex, 32,611 images sorted into 18 groups (according to defocus values, ranging from 1.2 to 4.2 μm), were selected, after visual and cross-correlation-based screening and removal of images from over-represented groups among the initial set of 83 representative views of the ribosome, and were used in the final reconstruction. For the in vivo complex, 18,317 images were sorted into 15 defocus groups ranging from 1.6 μm to 4.3 μm. The resolution of the final CTF-corrected three-dimensional map, estimated using the Fourier
shell correlation with a cut-off value of 0.5 (22), was 9.8 Å (or ~7.6 Å by the 3σ criterion) and 14.1 Å (or ~10.1 Å by the 3σ criterion), respectively, for the in vitro and in vivo complexes.

The fall-off of the Fourier amplitudes toward higher spatial frequencies was corrected for the map of in vitro complex as described previously (22). Mass corresponding to PSRP1 was isolated through comparison with the cryo-EM map of the ligand-free E. coli 70S ribosome. The homology model of PSRP1 was generated using Swiss-Model (23). Visualization and interpretation of the map, and docking of crystallographic structures, were performed using SPIDER, IRIS Explorer (Numerical Algorithms Group, Inc., Downers Grove, IL), O (24), and Ribbons (25).

RESULTS

Binding of Spinach PSRP1 to the E. coli Ribosome—The mature form of spinach PSRP1 was cloned into pET32b; the construct introduces an N-terminal Trx fusion linked to the protein via a six-histidine (6×His) affinity tag. Trx-PSRP1 was overexpressed in E. coli, and the ribosomes isolated from log-phase cells were subjected to sucrose-density gradient centrifugation. SDS-PAGE and Western blotting against the 6×His tag indicated that Trx-PSRP1 migrated with 70S ribosomes. PSRP1 remained bound at near-stoichiometric levels to 70S ribosomes purified from the sucrose gradients, consistent with a tight association (Fig. 1a, lane 5), whereas no equivalent band was observed for the Trx control (Fig. 1a, lane 3).

To complement the in vivo results, we performed in vitro ribosome binding assays using purified Trx-PSRP1 protein. The use of Trx fusion enables the binding of PSRP1 to be monitored directly by Coomassie Blue-stained SDS-PAGE gels, because the Trx (17 kDa) fusion increases the molecular mass of PSRP1 (27 kDa) to 44 kDa, such that it migrates above the majority of the E. coli ribosomal proteins (<30 kDa). Consistent with the in vivo results, Trx-PSRP1 binds to E. coli 70S ribosomes (Fig. 1b, lanes 8 and 9), as well as to 30S subunits (Fig. 1b, lanes 5 and 6), and binding increases with increasing excess of PSRP1 protein, saturating at 1:1 factor/ribosome ratio (supplemental Fig. S1a). Furthermore, the binding is specific; Trx-PSRP1 does not bind to E. coli 50S subunits (supplemental Fig. S1b). The in vivo purified 70S-Trx-PSRP1 complex, and the in vitro 70S-Trx-PSRP1 complex prepared with 20-fold excess of PSRP1 protein, were then analyzed by cryo-EM and single particle image reconstruction, to determine the binding site of PSRP1.

Site of Binding of PSRP1 on the E. coli 70S Ribosome—Comparison of the cryo-EM maps of the in vivo and in vitro assembled E. coli 70S-PSRP1 complexes with the map of empty E. coli 70S ribosome (control) shows extra mass-density in the inter-subunit space on the 30S subunit; this mass-density could be

**Figure 3.** Conformational change in bridge B2a upon PSRP1 binding. a, 30S (semitransparent orange) and 50S (semitransparent indigo) subunits are shown side-by-side, with bridge B2a region boxed. The right panel shows the enlarged boxed area, with control 50S subunit map (solid blue) superimposed to show the proximity of PSRP1 (green) to density corresponding to 23S RNA helix 69 (H69C) and shift in H69 position in the PSRP1-bound map (H69P, semitransparent indigo). In the inset, the ribosome masses from the far and near planes have been computationally removed for visual clarity. b, stereo-representation of h44 (orange), H69 (indigo), and PSRP1 (green) shown in ribbons, as derived by fitting of coordinates of those components into the in vivo PSRP1-bound 70S ribosome map. The control h44 and H69 are shown in gray. The arrow indicates the direction of movement of H69 upon PSRP1 binding. Landmarks of the 30S subunit: sh, shoulder; the rest of the landmarks are the same as in the legend to Fig. 2. Landmarks of the 50S subunit: CP, central protuberance; St, L7/L12 stalk.

**Figure 4.** Conformational change in bridge B2a upon PSRP1 binding. a, 30S (semitransparent orange) and 50S (semitransparent indigo) subunits are shown side-by-side, with bridge B2a region boxed. The right panel shows the enlarged boxed area, with control 50S subunit map (solid blue) superimposed to show the proximity of PSRP1 (green) to density corresponding to 23S RNA helix 69 (H69C) and shift in H69 position in the PSRP1-bound map (H69P, semitransparent indigo). In the inset, the ribosome masses from the far and near planes have been computationally removed for visual clarity. b, stereo-representation of h44 (orange), H69 (indigo), and PSRP1 (green) shown in ribbons, as derived by fitting of coordinates of those components into the in vivo PSRP1-bound 70S ribosome map. The control h44 and H69 are shown in gray. The arrow indicates the direction of movement of H69 upon PSRP1 binding. Landmarks of the 30S subunit: sh, shoulder; the rest of the landmarks are the same as in the legend to Fig. 2. Landmarks of the 50S subunit: CP, central protuberance; St, L7/L12 stalk.
Function of Plastid-specific Ribosomal Protein 1

**FIGURE 6. Binding of PSRP1 to the ribosome in the presence of different factors.**

*a* Binding of PSRP1 to 70S ribosomes in the absence or presence of RRF (−RRF or +RRF, respectively). The binding of PSRP1 increases 1.6 ± 0.2-fold in the presence of RRF. 
*b* SDS-PAGE result showing the binding of PSRP1, in the absence or presence of IF3 (−IF3 and +IF3, respectively), to the small ribosomal subunit (30S). The presence of IF3 does not influence the binding of PSRP1 (IF3/PSRP1 binding stoichiometry is 1.1 ± 0.2). 
*c* Binding of PSRP1 and RRF to 70S ribosomes, and the effect of binding upon addition of different nucleotides together with EF-G. In the reaction with EF-G-GDPNP in comparison to EF-G-GTP, the binding of EF-G increases 3.0 ± 0.3-fold, the binding of PSRP1 remains unaffected (PSRP1(GDPNP)/PSRP1(GTP) binding stoichiometry is 0.9 ± 0.1), and the binding of RRF decreases about 0.6 ± 0.2-fold, respectively. In all cases, at least three independent experiments were performed, and the errors represent the S.D. from the mean. For each condition, aliquots of the initial reaction (R), supernatant (S), and pellet (P) were loaded on 15% gel and stained with Coomassie Blue. M denotes marker lanes. Proteins bands are marked on the right.

readily assigned to PSRP1 (Fig. 2, a and b, supplemental Fig. S2). The shape and location of the density feature representing PSRP1 is very similar, between the in vitro and in vivo complexes. However, the in vitro map shows finer details because of its higher resolution (9.8 Å), relative to the in vivo map (14 Å). The density, when viewed from the subunit-interface side, spans across the neck region of the 30S subunit, making contacts with the tip of helix 44 (h44) as well as components of the 30S head. The region of the 50S subunit closest to the PSRP1 density is helix 69 (H69) of the 23S rRNA. The binding position of PSRP1 seen in the present eubacterial 70S ribosome-PSRP1 complex maps closely match with that observed in the spinach chloroplast 70S ribosome (26, Fig. 2c). This comparison strongly supports our assignment of PSRP1 within the eubacterial 70S ribosome-PSRP1 complexes, given that the N-terminal Trx domain was not present in the chloroplast map. However, density corresponding to the Trx domain is visible only in the difference maps, suggesting that this domain is highly flexible on the ribosome.

**PSRP1 Is a Ribosome-binding Factor Rather Than a Ribosomal Protein.—** Genes homologous to spinach PSRP1 can be found in all completely sequenced plant (nuclear) genomes, where they also encode signal sequences targeting the PSRP1 protein into the plastid. PSRP1 homologues are also found in cyanobacteria, green algae, and plasmodium species, as well as in several eubacterial lineages. However, the chloroplast PSRP1 proteins are generally larger than the eubacterial homologues, especially the γ-proteobacteria. For example, the mature spinach PSRP1 protein is 236 amino acids in length (27), whereas the E. coli homologue is less than half that length, with a sequence of only 113 amino acids homologous to the N-terminal region of PSRP1 (supplemental Fig. S3). The solution structures of the E. coli (28, 29) and Haemophilus influenzae (30) PSRP1 homologues reveal a single globular domain comprising two α-helices and a four-stranded β-sheet. We used these structures as templates to generate a homology model for the N-terminal domain (NTD) of spinach PSRP1, which we subsequently docked into the PSRP1 density from...
tRNAs (Fig. 3b) (31, 32). Consistently, we show that PSRP1 and pY inhibit the binding of tRNA to 70S ribosomes (light green bars in Fig. 3c), as has been reported previously for pY (17, 33). pY was much more effective than PSRP1, and the presence of mRNA appeared to increase the stability of the tRNA on the ribosome (Fig. 3c). Indeed, the binding position of PSRP1 reported here on an E. coli ribosome, as well as in the homologous chloroplast ribosome (26), is incompatible with translation. Furthermore, the similarity in binding sites of PSRP1 and pY (17) on the ribosome, together with their high sequence homology suggests a functional similarity between the two proteins, leading us to suggest that PSRP1 is a pY-like stress response factor, rather than a ribosomal protein.

The weaker density for the CTD of PSRP1 region suggests that this segment is flexible and does not contribute significantly to ribosome binding. This observation is consistent with the fact that overexpression of the CTD alone does not lead to significant binding of PSRP1 to 70S ribosomes. Additionally, a PSRP1 mutant, in which a 56-amino acid “acidic region” (supplemental Fig. S3) was deleted from the CTD, still binds to 70S ribosomes (34). PSRP1 Contacts Intersubunit Bridge Elements and Stabilizes the 70S Ribosome—The binding of PSRP1 induces conformational changes in various regions of both the 30S and 50S subunits. Several of these changes involve ribosomal components that form intersubunit bridges, including the 16S rRNA helix 44 (h44) and 23S rRNA helix 69 (H69) that interact with one another to form bridge B2a (Fig. 4a), one of the largest and most conserved intersubunit bridges in the ribosome (32). Apparent reorganization of regions involved in the formation of this and other bridges (supplemental Fig. S4) suggested to us that PSRP1 binding significantly alters the strength of association of the two ribosomal subunits. Binding of the PSRP1 shifts H69 slightly toward the 50S subunit, such that α-helix 1 of the PSRP1 is situated at the junction of H69 and h44 (Fig. 4b). The observed shift in H69 might have been facilitated by the electronegative surface potential of the PSRP1 α-helix 1 facing the H69 (supplemental Fig. S5). Whereas the direction of H69 shift would imply a weakened bridge B2a, direct interactions of PSRP1 with both h44 and H69 appear somehow to further strengthen the bridge (see below).

To further investigate the effect of PSRP1 on the association of two ribosomal subunits, two different types of dissociation assays were performed in the presence and absence of PSRP1/pY and/or initiation factor 3 (IF3) (Fig. 5). IF3 is known as an anti-association factor, which can split weakly associated 70S ribosomes (17, 35). First, E. coli 70S ribosomes were incubated in a buffer containing low magnesium, in the absence (Fig. 5a) or presence of PSRP1 (Fig. 5, b and c) or E. coli pY (Fig. 5d). In the absence of PSRP1 (or pY), the low magnesium leads to dissociation of the 70S ribosomes into 30S and 50S subunits. However, the presence of increasing concentrations of PSRP1 leads to stabilization of the 70S ribosomes (Fig. 5, b and c), analogous to what is observed in the presence of E. coli pY (Fig. 5d) (17). Second, 70S ribosomes were incubated in a buffer that induces minimal dissociation of 70S ribosomes into subunits, in the absence of E. coli IF3 (Fig. 5e), but clear dissociation in the presence of IF3 (Fig. 5f). In contrast, the addition of PSRP1 (Fig. 5g) or E. coli pY (Fig. 5h) counteracts the effects of IF3 to stabilize the 70S ribosomes to a greater extent than that observed in the presence of IF3 (Fig. 5e). Collectively, these observations suggest that PSRP1 is functionally analogous to E. coli pY (17), at least in terms of stabilizing the 70S ribosome against dissociation. This observation further supports our assignment of PSRP1 as a stress response factor rather than an innate ribosomal protein.

PSRP1 Is Recycled from the Ribosome, Analogous to tRNA, by the Tandem Actions of RRF and EF-G—The RRF works in conjunction with a GTPase, EF-G, and initiation factor 3 (IF3), to split post-termination complexes (PoTC) in a GTP-dependent manner, and to recycle the component ribosomal subunits for the next round of translation (36). In the chloroplast ribosome, RRF has been found to directly contact PSRP1 (26). This led us to hypothesize that perhaps RRF, EF-G, and IF3 play a role in recycling PSRP1 from the ribosome after the stress conditions are relieved. To investigate the interplay between these factors, we first performed a series of binding assays. We found that the presence of RRF significantly promotes the binding of PSRP1 to 70S ribosomes (Fig. 6a). Consistently, in our tRNA binding assays, we observe that the competitive effects of RRF and
PSRP1/pY with tRNA binding to 70S ribosomes are additive (dark green bars in Fig. 3c). In contrast, binding of PSRP1 to the 30S subunit is not influenced by the presence of IF3 (Fig. 6b). In the next experiment, we pre-bound PSRP1 and RRF to the ribosome (as in Fig. 6a) and then monitored the binding of EF-G in the presence of either GTP or a non-hydrolyzable analog, GDPNP (Fig. 6c). In the presence of GDPNP, very strong binding of EF-G was observed; binding of PSRP1 remained unaffected, whereas binding of RRF was decreased (Fig. 6c), suggesting that PSRP1, RRF, and EF-G-GDPNP cannot co-occupy a given ribosome. In contrast, in the presence of GTP, low-level binding of EF-G is observed, whereas PSRP1 and RRF binding remains unaffected. One possible explanation for the low stoichiometry of EF-G on the ribosome is that EF-G is cycling on and off the ribosome; however, it is unclear whether subunit dissociation is taking place under these conditions. To test this, we monitored the splitting of 70S ribosomes into subunits, on sucrose gradients (Fig. 7). As previously reported (20, 37, 38), this reaction is dependent on the presence of RRF and EF-G-GTP (Fig. 7, a–c). In these experiments, the concentration of IF3 used exerts no dissociative effect alone (Fig. 7a), but is instead needed to prevent the dissociated subunits from reassociating during centrifugation through the sucrose gradients (Fig. 7c) (20). When 70S ribosomes were prebound with pY (or PSRP1), the dissociative effects of RRF, EF-G, and IF3 were significantly reduced (Fig. 7d). The pY-mediated reassociation and the EF-G/RRF-mediated dissociation appeared to be competitive events, because further increasing the concentrations of RRF and EF-G shifted the equilibrium toward dissociation (Fig. 7e).

**DISCUSSION**

The PSRPs from higher plants show extensive sequence similarity with cyanobacterial PSRP1 homologues (supplemental Fig. S3). In the photosynthetic cyanobacterium *Synechococcus* PCC 7002, the PSRP1 homologue is termed LrtA (Light-repressed transcript A), because the transcript encoding the protein has been shown to be light-regulated (39). The *lrtA* transcript was not detectable upon continuous illumination, but after a transfer to dark the transcript level became very high. Upon re-illumination (20 min), the transcript level was reduced to 20% and after 40 min *lrtA* was not detectable any longer (39). Therefore, we suggest that expression of PSRP1 may also be light-regulated, and that PSRP1 has a function analogous to that of *E. coli* pY, in stabilizing 70S ribosomes under conditions unfavorable for translation. Whereas *E. coli* pY performs this function under cold-shock conditions, PSRP1 may function in dark conditions; however, further work will be required to verify this.

Based on our results, we propose a model for the role of pY and PSRP1 during conditions of stress, in particular, how the stored ribosomes are returned to the actively translating pool once growth conditions are restored (Fig. 8). Under optimal growth conditions, post-termination complexes are split into
the component subunits through the action of RRF and EF-G (Fig. 8, a–d) (36). IF3 prevents reassociation of the subunits by direct binding to the 30S subunit (Fig. 8, e and f) (20, 37, 38). Under stress conditions, such as cold shock for E. coli or darkness for plants, the stress response factors, pY and PSRP1, are up-regulated (39, 40), leading to binding of stress factor to empty 70S ribosomes or 30S subunits (Fig. 8, g and h). Binding of the stress protein to 30S subunits can occur independently of the presence of IF3 (Figs. 5b and 7g); stress factor promotes binding of the 50S subunits to form 70S ribosomes (Fig. 7d), and thus leads to a loss of IF3 binding (Fig. 8h) (41). Protein Y-stabilized ribosomes have been shown to be translationally inactive (33), consistent with the mapping of pY and PSRP1 to the site overlapping the mRNA and A- and P-tRNA binding sites (Fig. 3b). The pY/PSRP1-bound ribosomes thus evidently represent a storage state, such that a pool of mature ribosomes is present and ready to participate in translation once the stress conditions are alleviated. The shift to optimal growth conditions switches off the stringent response leading to an up-regulation of components involved in translation, including translation factors such as RRF and EF-G (reviewed in Ref. 42). Because RRF, EF-G, and PSRP1/pY cannot occupy a given ribosome simultaneously (Fig. 6c), and because the presence of RRF and EF-G-GTP leads to splitting of PSRP1-70S complexes (Fig. 7d), we propose a novel shared role for RRF and EF-G during the post-stress response. This role is the recycling of the stress factor from the ribosome (Fig. 8, i and j), so that the 30S and 50S subunits can be returned to the translation cycle (Fig. 8k). Mechanistically, we envisage the process as operating in a fashion similar to the recycling of the PoTC by RRF and EF-G, in that the RRF progresses through the ribosome from the A-site toward the E-site, and in doing so it disrupts the interaction between h44 and H69. Intersubunit bridge B2a is thereby broken, leading to dissociation of the 70S ribosome into subunits (43, 44). Along this pathway, RRF would also be expected to dislodge the stress protein; however, we have not thus far been able to distinguish between the possibility that the factor remains bound to the 30S subunit and the possibility that it re-binds following dissociation. Whereas our study allows us to propose a functional role of PSRP1 in plastid translation, the possibility of involvement of this protein in other physiological events of the plastids cannot be ruled out.

In addition to cold-shock conditions, pY is found bound to 70S ribosomes in stationary-phase E. coli cells, suggesting that the pY family of proteins play a more general role in the stress response. In the stationary-phase, 70S ribosomes also dimerize to form 100S particles, a process that is mediated by the ribosome modulation factor (RMF) and hibernation promotion factor (HPF) (45). Interestingly, HPF shows 40% sequence homology with pY (46), perhaps hinting that HPF also binds within the intersubunit space, and be similarly subject to RRF- and EF-G-mediated post-stress recycling.

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