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Molecular localization of a ribosome-dependent ATPase on *Escherichia coli* ribosomes

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ABSTRACT

We have previously isolated and described an *Escherichia coli* ribosome-bound ATPase, RbbA, that is required for protein synthesis in the presence of ATP, GTP and the elongation factors, EF-Tu and EF-G. The gene encoding RbbA, *yhih*, has been cloned and the deduced protein sequence harbors two ATP-motifs and one RNA-binding motif and is homologous to the fungal EF-3. Here, we describe the isolation and assay of a truncated form of the RbbA protein that is stable to overproduction and purification. Chemical protection results show that the truncated RbbA specifically protects nucleotide A937 on the 30S subunit of ribosomes, and the protected site occurs at the E-site where the tRNA is ejected upon A-site occupation. Other weakly protected bases in the region occur at or near the mRNA binding site. Using radiolabeled tRNAs, we study the stimulating effect of this truncated RbbA on the binding and release of different tRNAs bound to the (aminoacyl) A-, (peptidyl) P- and (exit) E-sites of 70S ribosomes. The combined data suggest plausible mechanisms for the function of RbbA in translation.

INTRODUCTION

The functional domains of the ribosome include a peptidyl transferase center, a GTPase center and three tRNA binding sites. Of these, the A-site hosts the aminoacylated-tRNA, the P-site harbors the peptidyl tRNA, and the E-site is involved in the exit of free tRNA. The elongation facet of translation consists of three reactions: decoding of the mRNA by aminoacyl-tRNA, peptide bond synthesis by the peptidyl transferase and EF-G mediated translocation (1–3).

During decoding, the aminoacyl-tRNA is attached to the ribosome as a GTP•EF-Tu•aminoacyl-tRNA complex (4). The ribosomal proteins L7/L12 promote GTP hydrolysis and EF-Tu•GDP unbinds from the ribosome before a peptide bond can be formed. EF-Ts catalyzes the exchange of GDP in EF-Tu•GDP with GTP (1–3). Following peptide bond formation, the peptidyl-tRNA must be translocated from the A-site to the P-site and the deacyl-tRNA must also be translocated from the P- to the E-site. Translocation results in movement of the mRNA so as to expose the next codon of the mRNA in the A-site. This process is accelerated by EF-G in the presence of GTP (5,6). The hydrolysis of GTP also presumably induces the release of the EF-G from the ribosome (3,6).

Fungi harbor a factor, called EF-3, which is a ribosome-dependent ATPase (7,8). The fungal EF-3 allows the tRNA to be released from the E-site upon A-site occupation in a reaction analogous to that of a protein first discovered in *Escherichia coli* and called W, which ejects tRNAs from a post-translocation intermediate on 70S ribosomes (9). The W protein appears to be a truncated version of RbbA. The name of the W protein was changed to RbbA to indicate the fact that the protein is a ribosome-bound ATPase (10,11). Recently, it has been reported that the fungal EF-3 also promotes release of tRNA from a post-translocation intermediate on yeast ribosomes (12).

We have previously reported that the transfer of aminoacyl-tRNAs into peptides directed by MS2 RNA in a reconstituted system requires hydrolysis of both ATP and GTP (13). RbbA, an intrinsic ATPase that binds to 70S ribosomes and to 30S subunits, is responsible for the ATP requirement (10,11).

The gene encoding RbbA, *yhih*, has been identified. The amino acid sequence of RbbA reveals the presence of two ATP-binding domains in the N-terminal region of the protein. The C-terminal region of the protein bears extensive sequence similarity to the yeast EF-3, a ribosome-dependent ATPase (10).

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Here, we report the isolation of a truncated form of the RbbA protein that can be easily overproduced and purified. This protein was found to be stable and fully active in translation assays. We study the localization of the truncated RbbA on ribosomes using chemical protection against base-specific reagents. The ribosomal binding sites affected by the truncated RbbA and its sensitivity to antibiotic inhibition suggest possible mechanisms for its action in translation.

MATERIALS AND METHODS

Materials

[^{14}C]Phe (30 Ci/mM), [^{3}H]Phe (58 Ci/mM) and [γ-^{33}P]ATP (250 Ci/mM) were from the Amersham Corp. DNA, RNA, PCR purification and gel extraction kits were from Qiagen and AMV-reverse transcriptase was from Pharmacia. Dimethyl sulphate (DMS), diethyl pyrocarbonate (DEP), kethoxal (Ke) and carbodiimide (CMCT) were from Aldrich-Gold. [^{3}H]fMet-tRNA^{fMet} (1.1 Ci/mg) was a gift from Dr D.L. Shinabarger and S. Swaney (Pharmacia, Corp. Kalamazoo, MI). tRNA^{fMet} and tRNA^{Phe} were from Boehringer Mannheim. The sequencing reactions were performed with the USB sequencing kit.

Preparation of 70S ribosomes and tRNA labeling

70S ribosomes from mid-log *E.coli* MRE 600 cells were prepared as described previously (10,11). The tRNAs were labeled with [γ-^{33}P]ATP using T4 polynucleotide kinase and [γ-^{33}P]ATP. The labeled tRNAs were purified as described (14).

Overproduction and ATPase assay of His-tagged RbbA

XL1-blue *E.coli* cells harboring the plasmid, pQE9-*yhih* (10), were grown in Luria–Bertani (LB) media at 30°C until an OD_{600} ~0.65. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 2 mM and the cells were grown for an additional 4 h at 30°C. Cells were harvested and lysed by sonication in lysis buffer [50 mM KH_2PO_4/K_2HPO_4 (pH 8.0), 300 mM KCl]. The lysed cell suspension was centrifuged for 20 min at 15 000 × g and the supernatant was incubated with 0.5 ml Ni-NTA agarose beads (Qiagen) for 45 min at 4°C. The bead suspension was then packed in a 3 ml column and washed with 20 vol lysis buffer followed by 20 vol lysis buffer at pH 6.1. The N-terminally His-tagged RbbA was eluted with lysis buffer at pH 7.0 containing 250 mM imidazole and passed through a NAP-10 gel filtration column (Pharmacia) equilibrated with 50 mM HEPES-OH (pH 7.2), 50 mM KCl, 1 mM $Mg(OAc)_2$, 1 mM DTT. All purification buffers contained complete EDTA-free protease inhibitors (Boehringer Mannheim). For the ATPase assay, the hydrolysis of [γ-^{33}P]ATP was measured using ammonium molybdate as described previously (10) using 8 pmol ribosomes and 16 pmol of RbbA.

Poly rU-directed polyPhe synthesis

In vitro synthesis was as described (10) with a few modifications. Ribosomes (8 pmol) were programmed with 25 µg poly rU in 50 µl reaction buffer [20 mM Tris–HCl (pH 7.4), 40 mM NH_4Cl, 6 mM $Mg(OAc)_2$, 1 mM DTT, 0.6 mM ATP and 0.1 mM GTP] for 15 min at 37°C. Reactions also contained 20 pmol EF-Tu, 12 pmol C-terminally His-tagged EF-G, Phe-tRNA^{Phe} synthetase (1 µg) and 0.5 µCi [14C]Phe (30 Ci/mM). For RbbA stimulation, 16 pmol N-terminally His-tagged RbbA was used.

RNA modification and primer extension analysis

The 30S and 70S ribosomes with or without the RbbA protein were modified using DEP, DMS, kethoxal or CMCT (15). Adenines and cytosines were also determined (15). The primers were first selected to span the 1492 region of the 16S rRNA that confers resistance to hygromycin B which is a specific inhibitor of RbbA. Half pmol rRNA (modified or native; with or without the RbbA) and 0.5 pmol of primer (that specifically anneals to a region of 16S in 70S ribosomes) were added to 6 µl of 10 mM Tris–HCl (pH 6.9), 40 mM KCl and 0.5 mM EDTA. The solution was heated at 95°C for 30 s and transferred to 50°C for 20 min.

Three µl of the annealed primer-template mixture was transferred to a fresh tube where 2 µl of an extension mixture (25X reverse transcriptase buffer, 0.2 µl [α-^{35}S]dATP, 1.6 µl 0.75 mM dCTP, dGTP and dTTP and 1 U of AMV reverse transcriptase) was added. The reactions were incubated at 37–42°C for 15 min, and then chased with 2 µl of dATP for 15 min at 37–42°C. The extension reaction was terminated by adding 5 µl of stop solution (loading buffer containing 80% deionized formamide) provided by the Sequenase™ Version 2.0 DNA Sequencing Kit (USB, Cleveland, OH). The tubes were then heated for 3 min at 95°C, chilled on ice and 1 µl was loaded on a DNA sequencing gel. The DNA sequence was determined (16) according to the instructions from the manufacturer (USB, Cleveland, OH).

Purification of [^{3}H]Phe-tRNA^{Phe}

Preparation of [^{3}H]Phe-tRNA^{Phe} was according to our previous paper (17). Briefly, Phe-tRNA^{Phe} synthetase from *E.coli* was isolated from an 80% ammonium sulphate precipitate of the ribosomal wash fraction and purified. Aminoacylation of pure tRNA^{Phe} from *E.coli* was carried out in a 5 ml incubation mixture at 37°C for 20 min. The reactions contained: 7.5 mM Tris–HCl (pH 7.2), 15 mM KCl, 15 mM $Mg(OAc)_2$, 1.5 mM DTT, 5 mM ATP, 0.75 mg tRNA^{Phe}, 0.15 Ci[^{3}H] Phe (500 nM/mCi) and 25 µg Phe-tRNA^{Phe} synthetase from *E.coli*. After aminoacylation, the [^{3}H]Phe-tRNA^{Phe} was extracted with phenol/chloroform, and purified by chromatography on Mono-Q columns. Over 80% of the [^{3}H]Phe-tRNA^{Phe} was recovered.

Binding of labeled tRNAs to 70S ribosomes

Reactions (45 µl total volume) contained 16.6 mM $Mg(OAc)_2$, 35 mM Tris–HCl (pH 7.4), 93 mM NH_4Cl, 3.5 mM DTT, 0.35 mM GTP, 9 pmol $AUGU_3$, 8 pmol 70S ribosomes and either 20 pmol EF-Tu, 1.2 pmol [^{33}P]tRNA^{fMet} or 8 pmol tRNA^{Phe} and/or 16 pmol RbbA as described below. For RbbA, 0.2 mM ATP was supplemented. All reactions were incubated at 30°C for 10–12 min.

For A-site binding, the reactions contained the indicated concentrations of $AUGU_3$, EF-Tu, tRNA^{fMet} and [^{3}H]Phe-tRNA^{Phe} (10 000 d.p.m.). Where indicated, reactions were supplemented with 16 pmol RbbA and 0.2 mM ATP. Tetracycline

(0.1 to 0.5 mM) sensitivity was used as a further indication of A-site binding (18).

For P-site binding, 70S ribosomes, $AUGU_3$ and [^{3}H]fMet-tRNA^{fMet} (50 000 d.p.m.) were added to the reactions and P-site occupation was assessed by estimating the amount of puromycin reactivity.

E-site binding was detected by first binding $AUGU_3$, [^{33}P]tRNA^{fMet} and [^{3}H]Phe-tRNA^{Phe} or N-acetyl-[^{3}H]Phe-tRNA^{Phe} to 70S ribosomes and estimating the amount reactivity of each tRNA to either puromycin or tetracycline. In order to determine the effect of RbbA on A-, P- and E-sites as well as the stability of the complex, two of the differentially labeled tRNAs were used in the reactions. [^{33}P]tRNA^{fMet}, binds to the P-site as well as to the E-site (18) whereas fMet-tRNA^{fMet} preferentially binds to the P-site. Thus, to block the binding of the [^{33}P]tRNA^{fMet} to the P-site, fMet-tRNA^{fMet} was used to displace the [^{33}P]tRNA^{fMet} on the P-site. Further, [^{33}P]tRNA^{fMet} bound on the E-site is released from the ribosomes in the presence of ATP and the RbbA protein, and is insensitive to tetracycline inhibition. The [^{33}P]tRNA^{fMet} bound on the P-site is insensitive to RbbA.

Since the initial rate of binding of [^{33}P]tRNA^{fMet} to ribosomes was found to be insensitive to RbbA and ATP addition, a second assay was used to determine the release of [^{33}P]tRNA^{fMet} from ribosomes. Thus, the [^{33}P]tRNA^{fMet} was first bound to 70S ribosomes with $AUGU_3$ for 10 to 12 min at 30°C, ATP and RbbA were then added and the incubation was continued for 12 min. The reactions were terminated with 4 ml binding buffer at 4°C, the fractions were washed with the binding buffer and the filtrates were collected on Millipore filters (0.45 μ) prior to determining the radioactivity.

RESULTS

Purification of an RbbA fragment

The purification scheme for the RbbA protein described in Materials and Methods consistently yielded a protein of 61 kDa when analyzed by SDS–PAGE (Figure 1). The protein is considerably smaller than the 97 kDa full-length RbbA encoded by the *yhih* gene. The fragment corresponds to the N-terminal region of the complete RbbA protein and contains ATP-binding motifs. The sequence of the gene encoding the complete protein contains a transmembrane domain at the C-terminal end. This domain does not occur in this truncated form of RbbA. Mass spectrometric analysis confirmed that the polypeptide indeed corresponds to 61 000 Da and is about 98% homogeneous (data not shown). No other fragments of the protein could be observed by mass spectrometry or SDS–PAGE (Figure 1).

To examine whether the truncated form of RbbA resulted from a nonsense mutation, its entire open reading frame was sequenced and compared with that from the GenBank. No nonsense codons were found in the sequence. Our previous observations demonstrated that the full-length protein is difficult to over-express, extremely unstable and rapidly proteolyzed making it very difficult to isolate and to store. Although the reason for this instability is not known, the mass spectrum analysis suggests that the complete protein (894 amino acid residues) is probably processed by proteolysis at the proline residue #541. We asked whether this truncated protein (541 amino acid residues) harbors the ATPase and translation-promoting activity ascribed to the full-lengh RbbA protein.

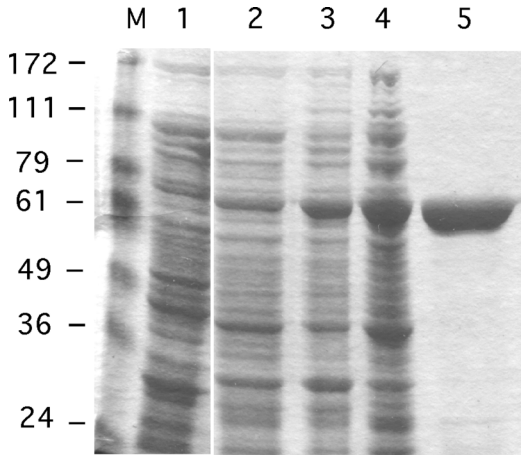

Figure 1. SDS–PAGE of the processed (truncated) recombinant RbbA protein. Lanes: M, Molecular weight markers; 1, Total *E.coli* lysate prior to IPTG induction; 2, *E.coli* lysates after IPTG induction; 3, An aliquot of supernatant after incubation with Ni-NTA resins; 4, An aliquot from the flow through of the NTA column used to purify the RbbA; 5, Eluted with 250 mM imidazole from the NTA column and finally dialyzed and concentrated RbbA protein.

RbbA stimulates synthesis and ribosome-dependent ATP hydrolysis

The full-length RbbA was originally isolated based partly on its ATPase activity and on its ability to stimulate *in vitro* polyPhe synthesis (10,11). This RbbA harbors both ribosome-independent and ribosome-dependent ATPase activities (10). Poly rU-directed polyPhe synthesis was examined in the presence of GTP, ATP and Phe-tRNA synthetase, EF-Tu and EF-G (10). EF-Tu or EF-G alone had no detectable activity. The combination of EF-Tu and EF-G promoted a small amount of synthesis due to the limiting concentration of GTP. The RbbA protein could not replace EF-Tu or EF-G in the assay (data not shown). With ATP, RbbA markedly increased the amount of polyPhe synthesis (Figure 2A). It is therefore concluded that the truncated RbbA protein can be scored by its ability to stimulate polyPhe synthesis.

We asked whether the truncated protein could also promote the ribosome-dependent hydrolysis of ATP. The truncated RbbA or ribosomes alone catalyzed a low level of ATP hydrolysis. Addition of this RbbA to ribosomes markedly enhanced the ribosome-dependent ATPase activity of 70S ribosomes (Figure 2B). Thus, the truncated RbbA is fully active in promoting synthesis and the ribosome-dependent hydrolysis of ATP.

Effect of RbbA on tRNA binding to the ribosome A-site

We examined whether the truncated RbbA affects the A-site bound tRNA. As shown in Figure 2C, the EF-Tu-dependent [^{3}H]Phe-tRNA^{Phe} binding is significantly stimulated by the presence of RbbA and ATP. Formation of the complex required that the [^{33}P]tRNA^{fMet} be bound to the ribosome

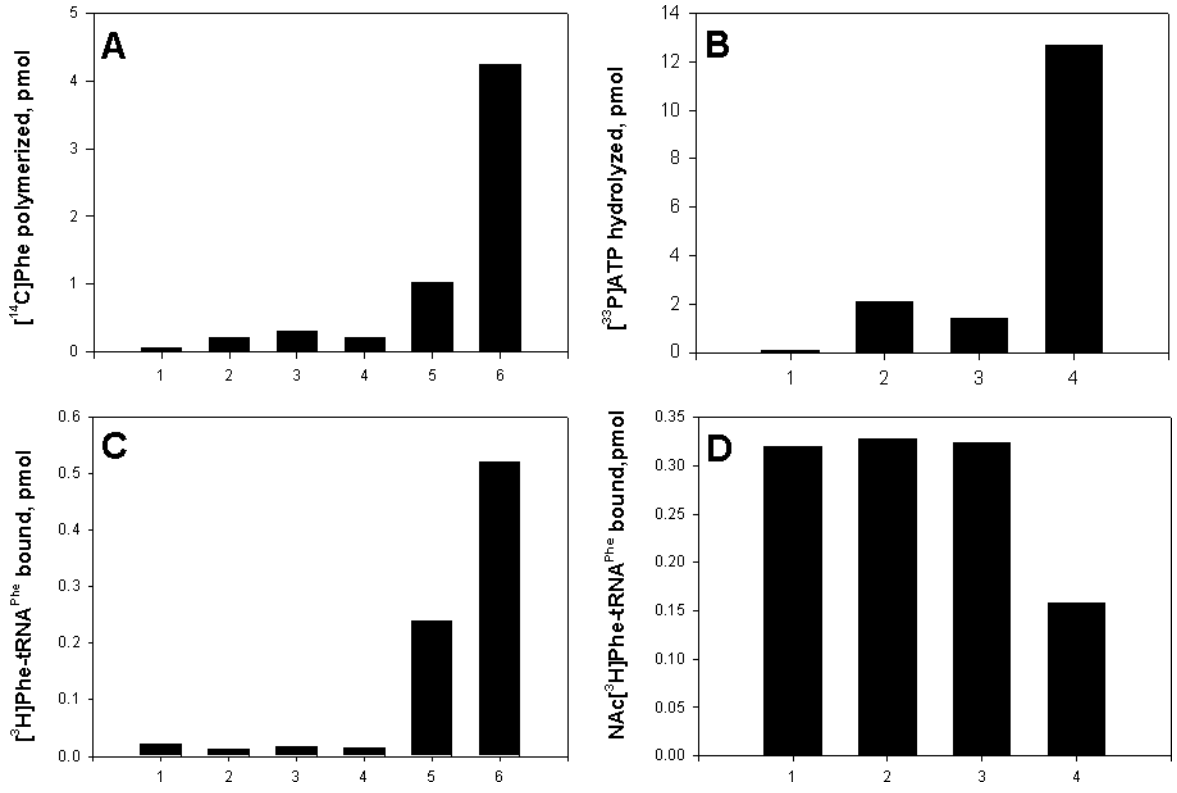

Figure 2. (**A**) Stimulation of polyPhe synthesis by RbbA. PolyPhe synthesis directed by poly rU was conducted as described in Materials and Methods using ATP, GTP and Phe-tRNA^{Phe} synthetase in the reactions. Lanes: 1, Reaction with 0.6 mM ATP, 0.1 mM GTP and 1 μg Phe-tRNA^{Phe} synthetase; 2, Lane 1 + RbbA; 3, Lane 1 + EF-Tu; 4, Lane 1 + EF-G; 5, Lane 1 + EF-Tu + EF-G; 6, Same as Lane 5 except with addition of RbbA. (**B**) ATP hydrolysis by 70S ribosomes in the presence of RbbA. Hydrolysis of [γ-^{33}P]ATP was measured as described in Materials and Methods. Lanes: 1, [γ-^{33}P]ATP only; 2, [γ-^{33}P]ATP + 70S ribosomes; 3, [γ-^{33}P]ATP + RbbA; 4, [γ-^{33}P]ATP+ ribosomes +RbbA. (**C**) Binding of [^{3}H]Phe-tRNA^{Phe} to the A-site in the presence of deacyl [^{33}P]tRNA^{fMet}. The binding of [^{3}H]Phe-tRNA^{Phe} (20 000 d.p.m.) to the A-site was described in Materials and Methods with deacyl-tRNA^{fMet} present in the reactions and ATP added where indicated. Lanes: 1, Reaction contained $AUGU_3$-programmed ribosomes, [^{33}P]tRNA^{fMet}; 2, Lane1+ [^{3}H]Phe-tRNA^{Phe} (16 000 d.p.m.); 3, Lane 1+ [^{3}H]Phe-tRNA^{Phe} + RbbA; 4, Lane 1+ EF-Tu; 5, Lane 1+ [^{3}H]Phe-tRNA^{Phe} + EF-Tu +RbbA; 6, Lane 1 + [^{3}H]Phe-tRNA^{Phe} + EF-Tu + RbbA + 0.2 mM ATP. (**D**) Binding of [^{33}P]tRNA^{fMet} and N-acetyl- [^{3}H]Phe-tRNA^{Phe} to 70S ribosomes. Binding was performed as described in Materials and Methods, and in the legend to Figure 2C except that N-acetyl-[^{3}H]Phe-tRNA^{Phe} was used instead of [^{3}H]Phe-tRNA^{Phe}. Lanes: 1, Reaction contained GTP, $AUGU_3$-programmed ribosomes, deacyl-[^{33}P]tRNA^{fMet} and N-acetyl-[^{3}H]Phe-tRNA^{Phe} (6000 dpm); 2, Lane1+ ATP; 3, Lane1+ RbbA; 4, Lane1+ ATP +RbbA.

P-site. The binding of [^{3}H]Phe-tRNA^{Phe} had no significant effect on the binding of the [^{33}P]tRNA^{fMet} (data not shown).

The specificity of these interactions was examined with N-acetyl-[^{3}H]Phe-tRNA^{Phe}. Again, at the early stages (10–12 min), ATP and RbbA had no effect on the binding of the [^{33}P]tRNA^{fMet} to ribosomes (data not shown). In contrast, at later stages (20 min), the binding of N-acetyl-[^{3}H]Phe-tRNA^{Phe} to the $AUGU_3$•tRNA^{fMet} •ribosome complex was markedly reduced by the combination of RbbA and ATP (Figure 2D). The [^{33}P]tRNA^{fMet} also remained bound to the ribosome after the 20 min incubation. Thus, RbbA in the presence of ATP had no effect on the [^{33}P]tRNA^{fMet} bound to the P-site when the A-site was occupied with an aminoacyl-tRNA or by the peptidyl-tRNA analogue.

We proceeded to study the effect of RbbA on the binding and unbinding of tRNAs to the ribosome P- or E-sites. To this end, [^{33}P]tRNA^{fMet} or [^{3}H]fMet-tRNA^{fMet} were bound to ribosomes with the $AUGU_3$ hexamer as the mRNA. We found no significant difference in the initial or subsequent reaction of the P-site bound [^{3}H]fMet-tRNA^{fMet} with the addition of RbbA and ATP (data not shown). In marked contrast, the [^{33}P]tRNA^{fMet} that is first bound to ribosomes with the $AUGU_3$ message is rapidly released from the particles by a subsequent incubation with RbbA in the presence of ATP (Figure 3A). Addition of a twenty-fold molar excess of deacyl-tRNA^{Phe} (8 pmol) relative to 0.4 pmol [^{33}P]tRNA^{fMet} to the reactions consistently increased the observed release of the tRNA. Tetracycline is an A-site specific inhibitor and has little or no effect on P- or E-site bound-tRNA (18). The presence of tetracycline has a small effect on the release of the [^{33}P]tRNA^{fMet} (Figure 3A). Therefore, the [^{33}P]tRNA^{fMet} must have bound to the E-site and RbbA must directly or indirectly act at this site to promote its release from the ribosome.

A summary of the experiments on the release [^{33}P]tRNA^{fMet} promoted by RbbA and ATP is shown in Figure 3B. The figure also summarizes the effect of the A-site inhibitor, tetracycline, and the non-cognate tRNAPhe on the RbbA-promoted release of [^{33}P]tRNA^{fMet}. Figure 3B shows that a 5–6% inhibition by tetracycline is consistently observed.

A consistent but relatively small competition of [^{33}P]tRNA^{fMet} binding occurred upon addition of excess

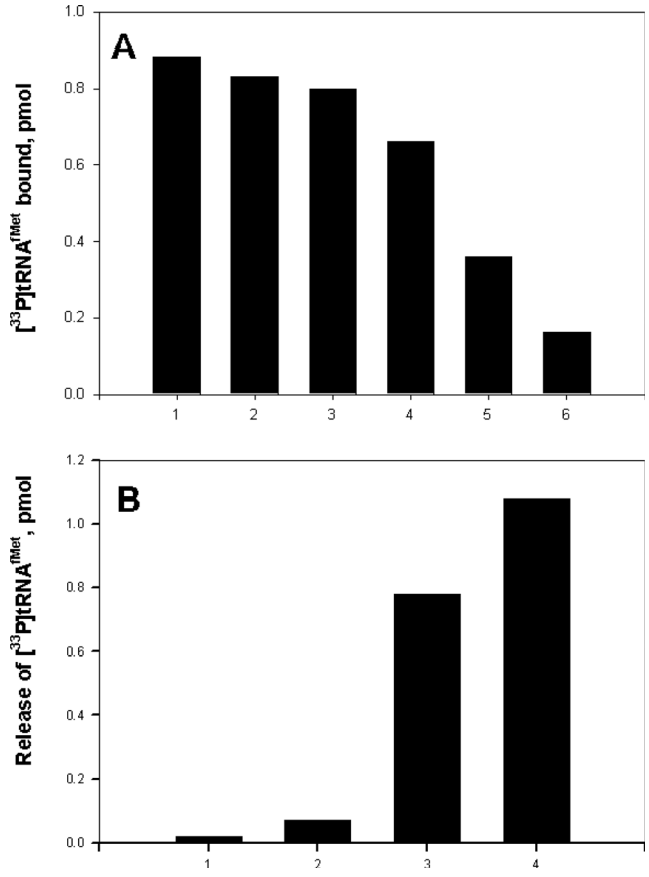

Figure 3. (**A**) Binding of [^{33}P]tRNA^{fMet} to 70S ribosomes. The reactions were as described in Materials and Methods. Lanes: 1, Reaction contained $AUGU_3$-programmed ribosomes, 1.2 pmol deacyl-[^{33}P]tRNA^{fMet}; 2, Lane 1 + RbbA; 3, Lane 1 + ATP; 4, Lane 1 + RbbA +0.1 mM tetracycline; 5, Lane 1 + ATP + RbbA; 6, Lane 1+ ATP + RbbA +tRNA^{Phe}. (**B**) Release of [^{33}P]tRNA^{fMet} from ribosomes. Release of deacyl [^{33}P]tRNA^{fMet} from 70S ribosomes programmed with $AUGU_3$was as described in Materials and Methods. Binding was limited to 10 min at 30°C followed by incubation with RbbA, ATP, deacyl tRNA^{fMet} or buffer as indicated. Lanes: 1, Reactions contained the AUGU3-programmed 70S ribosomes and deacyl [^{33}P]tRNA^{fMet}; 2, Lane 1 + 0.1 mM tetracycline; 3, Lane 1 + RbbA + ATP; 4, Lane 1 + RbbA + 0.2 mM ATP + 8 pmol tRNA^{Phe}.

non-cognate tRNA^{Phe} and not with smaller concentrations. The lack of extensive competition for the entrance of the [^{33}P]tRNA^{fMet} by the tRNA^{Phe} confirms that the predicted specificity of the sites was conserved in the given experimental conditions.

Binding site of RbbA on ribosomes

The RbbA protein binds to 70S ribosomes and to the 30S subunit. RbbA also binds specifically to the 16S rRNA nucleotides that make part of its binding site on the ribosome (10,11,19).

Study of the bases protected by binding of RbbA to the ribosome against the action of the base-specific reagents, DMS, kethoxal, CMCT or DEP indicated that A937 is strongly protected against DEP treatment by interactions of the protein with the 70S ribosome (Figure 4). RbbA did not confer protection against action of CMCT nor kethoxal (data not shown). Weak protections were observed with DMS and this may be due to other, possibly distal, interactions

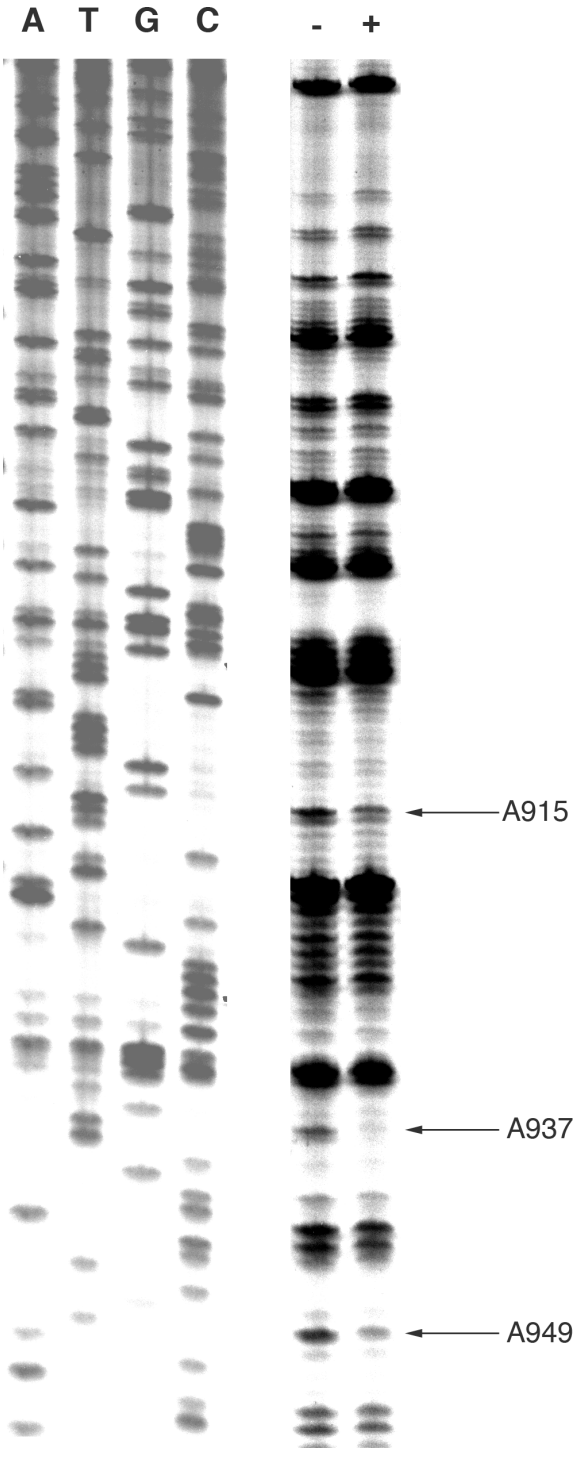

Figure 4. Chemical protection footprinting analysis of 70S ribosomes complexed to the RbbA protein. The 70S ribosome was treated with DMS with or without the RbbA protein as described in Materials and Methods. Reactions with (+) or without (−) RbbA were treated with DEP prior to annealing the primer and extension by reverse transcriptase.

of the protein with the ribosome. Such effects are commonly observed (3). Two other bases, A915 and A949 were also consistently observed to be weakly protected by the protein. No protections were observed around G889, G890 or G925 which are protected by RbbA on the protein-free 16S rRNA (19).

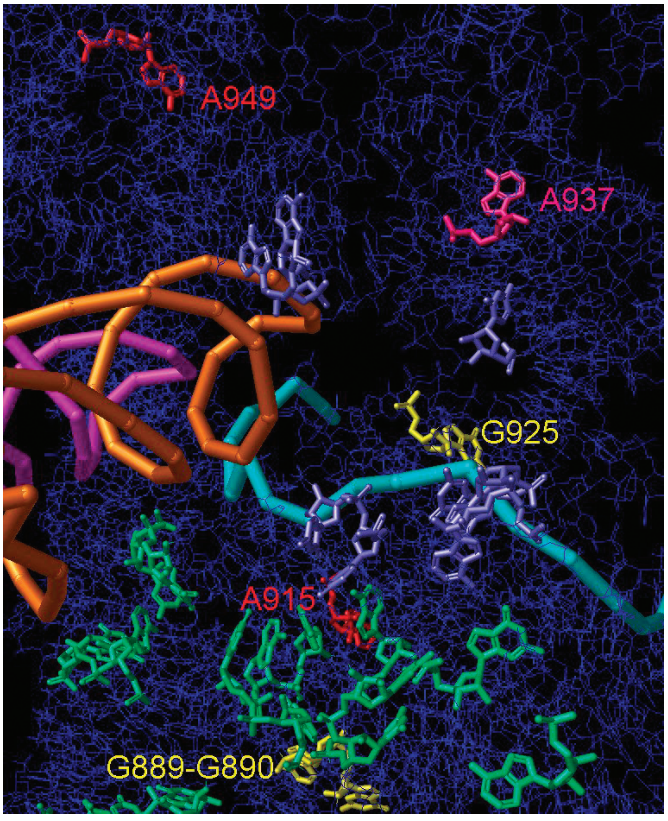

Figure 5. Mapping of protected bases. RbbA chemical protected footprinted bases were mapped on the public data base 1YL4 structure, which is made up of *Thermus thermophilus* bases but with *E.coli* numbering (20). The 16S rRNA backbone is shown in dark blue. The 16S rRNA bases that contact the 50S subunit are enlarged and in light green. Nucleotide bases that contact the E-site RNA are shown as enlarged purple bases. Enhanced truncated RbbA protected bases A949 and A915 are in red and A937 is in pink. Base A937 was also a base that has direct contact with the E-site tRNA (20). The pink and orange spirals represent A-site and P-site tRNAs, respectively. The cyan atomic structure is the mRNA that runs though the 30S subunit. Previous RbbA protected bases G889, G890 and G925 are shown in yellow. If a structure were available for the E-site tRNA, it would be right of the orange spiral tRNA and contacting the enlarged purple bases as well as A937.

Interestingly, the protected base (A937) resides in the E-site of the ribosome (20). This data suggests that RbbA protects the ribosomal E-site, but does not exclude other possible contacts of the protein with the mRNA binding region that occurs around the more weakly protected A915 and A949 (Figure 4).

The relative positions of the nucleotides that are protected by RbbA are shown in the 3D model of the 30S subunit (Figure 5). The A-site and P-site tRNAs (in pink and orange, respectively) are shown for reference. The most strongly protected base, A937, resides on the E-site of the 30S subunit (20). The other two bases that are weakly protected from modification of the 16S rRNA, A949 and A915, occur near the region that binds the mRNA on the 30S subunit. For comparison, G889, G890 and G925 that are protected by RbbA on the protein-free 16S rRNA are shown in the diagram. Thus, the position of the protected bases is consistent with the proposed function for the RbbA protein to release the tRNA from the E-site.

DISCUSSION

A ribosome-dependent ATPase, RbbA, has been identified and isolated from *Escherichia coli* cells (10,11). The gene encoding the full-length RbbA has also been identified as open reading frame *yhih* that potentially encodes 894 amino acids. The N-terminal sequence harbors two ATP-binding domains characteristic of many ABC transporters (21) and an aminoacyl-tRNA synthetase motif suggesting possible interactions with RNA (10,11).

As shown here, the truncated RbbA stimulates synthesis of polyPhe *in vitro* as well as the ATPase activity. The existence of conserved ATP-binding domains within this short form of RbbA suggests that the truncated RbbA, but not some intrinsic property of 70S ribosomes, is responsible for the ATPase activity.

At least two (22) or perhaps three molecules of GTP are hydrolyzed by ribosomes (one by each contact with aminoacyl-tRNA•GTP•EF-Tu and EF-G•GTP) in each round of translation (22). The need for additional energy from the hydrolysis of ATP is not understood, but ATP hydrolysis is required for the stimulation of synthesis in both *E.coli* and *Sacharomyces cerevisiae* when physiological concentrations of GTP and ATP are used with RbbA or EF-3 (9–12). Neither EF-3 nor RbbA possess nucleotidase activity.

Study of the 16S rRNA bases protected by RbbA in the intact 70S ribosome revealed that the E-site base, A937, is specifically protected by interactions of RbbA with the ribosome. This base is part of the E-site that is involved in the release of [^{33}P]tRNA^{fMet} that is postulated to occur upon occupation of the A-site during synthesis (18). The E-site may also have a function in translational fidelity (18,23). In addition, A915 and A949 were weakly protected against the action of DEP on the 70S ribosome. The latter bases reside in the neck of the 30S subunit which is involved in mRNA binding (24). Immune-EM data suggest that the mRNA binds around the neck of the 30S subunit such that its 5′ end occupies the platform side and the 3′ end is near the shoulder of the subunit (25). Recently, the position of the mRNA has been determined by X-ray diffraction analysis of the 70S ribosome (26). The mRNA was found to occur threaded through a channel that wraps around the neck of the 30S subunit (26). These data confirm previous studies of the position of the mRNA on the ribosome (24,25). Further, the channel through which mRNA threads during synthesis is closed and additional energy may be required to open it in order to enable mRNA movement. It is possible that the ribosome-dependent ATPase interacts with these protected sites in order to enhance mRNA movement. However, the strongest protection of A937 by RbbA occurs on the E-site of the ribosome.

Thus, the most obvious possible function of RbbA may be linked to the binding of the protein to the E-site and suggests that this interaction promotes the release of the deacyl-tRNA that must occur during synthesis. It has been amply documented that the A and the E-sites of the ribosome are allosterically linked in a negative fashion such that binding of the deacyl-tRNA to the E-site prevents aminoacyl-tRNA binding to the A-site and binding of aminoacyl-tRNA to the A-site prevents deacyl-tRNA binding to the E-site (18). We find that RbbA indeed stimulates the binding of Phe-tRNA^{Phe} to

the A-site in the presence of EF-Tu as well as release of the [^{33}P]tRNA^{fMet}, presumably from the E-site of the ribosome. By binding to the E-site, RbbA could stimulate the entrance of aminoacyl tRNA to the A-site. However, the sequence of the reactions stimulated by RbbA is unknown.

The peptidyl-tRNA analogue, N-acetyl-Phe-tRNA^{Phe}, was found to be released from the A-site by the RbbA protein in the presence of ATP. This suggests that the aminoacyl moiety of the tRNA may be essential for the RbbA-promoted stimulation of the aminoacyl-tRNA to the A-site. Thus, the primary function of RbbA (and EF-3) to release [^{33}P]tRNA^{fMet} from the E-site after peptide-bond formation fits all of the reported observations. Since the release of [^{33}P]tRNA^{fMet} is part of the translocation process, it remains possible that the RbbA-stimulated ejection of the [^{33}P]tRNA^{fMet} is linked to the movement of mRNA during translocation. This could explain the protection by RbbA of bases that bind mRNA to the ribosome as well as the inhibition by hygromycin B which alters both fidelity and translocation.

An important clue to the function of the RbbA protein is that the RbbA-stimulated ribosome-dependent ATPase is specifically inhibited by hygromycin B, an antibiotic that impairs translational fidelity and translocation of mRNA on ribosomes (19). Single molecule fluorescence (FRET) studies have identified several intervening steps in the process of aminoacyl-tRNA selection that are targeted by different antibiotics (23). These steps are essential to the accurate decoding of aminoacyl-tRNA and include an accommodation of the ternary complex of EF-Tu•GDP•aminoacyl-tRNA followed by a marked rotation of the complex after codon–anticodon interaction. Several other intermediate steps precede the interaction of the complex with the GTPase center of the 50S subunit resulting in hydrolysis of GTP, ejection of EF-Tu•GDP and proofreading for non-cognate aminoacyl-tRNAs (23). It is possible that the initial accommodation of the ternary complex may indeed be a target for the action of RbbA. The ternary complex is weakly bound to the ribosome by interactions of EF-Tu with the L7/L12 protein and by interactions of the tRNA with the mRNA and with portions of the 30S subunit.

Residues A1492, A1493 and G530 mediate the early events involved in codon recognition. G530 binds EF-Tu whereas A1492 and A1493 are involved, with ribosomal protein S12, in recognition of the codon–anticodon complex and appear responsible for the induced fit or domain closure mechanism that participates in decoding and selection against non-cognate codons. A1494, which is the base protected by hygromycin B, a specific inhibitor of RbbA, occurs adjacent to the bases involved in codon–anticodon recognition (23). Thus, it may be that the RbbA protein acts to release the deacyl-tRNA from the E-site by interfering with the codon–anticodon interaction. A similar conclusion was deduced from related experiments carried out with EF-3 (8).

The hypotheses that RbbA accelerates the release of the deacyl-tRNA from the ribosome and the inhibition of this reaction by antibiotics that affect translational fidelity suggest that suitable mutants of RbbA ought to increase translational errors. This work also suggests that EF-3 and RbbA serve the same function in translation. The sequence homology and common reactions catalyzed by both proteins confirm this conclusion, as is the fact that most essential functions of the translation apparatus are highly conserved (27,28).

NOTE ADDED IN-PROOF

Two conformations of 70S ribosomes have been detected by X-ray crystallography. One of these has the deacyl-tRNA^{fMet} in the E-site while the other conformation has the tRNA^{fMet} in the pre-translocation, P/E, hybrid site (29). Thus, it is possible that the deacyl-tRNAfMet used in this study bound to the P/E hybrid state, underscoring its possible involvement in translocation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Yonath,A. and Bashan,A. (2004) Ribosomal crystallography: initiation, peptide bond formation, and amino acid polymerization are hampered by antibiotics. *Annu. Rev. Microbiol.*, **58**, 233–251.
2. Wilson,D.N. and Nierhaus,K.H. (2003) The ribosome through the looking glass. *Angew. Chem. Int. Ed. Engl.*, **42**, 3464–3486.
3. Liljas,A. (2004) Ribosomal sites and ribosomal states. The catalysts-translation factors. The process-translation (Chapters 8, 9, 11). *Structural Aspects of Protein Synthesis*. World Scientific Publishing Company, New Jersey, London, Singapore, Beijing, Shangai, Hong-Kong, Taipei, and Chennai, pp. 75–98, 99–158, 175–207.
4. Nissen,P.M., Kjeldgaard,M., Thirup,S., Polekhina,G., Reshetnikova,L., Clark,B.F. and Nyborg,J. (1995) Crystal structure of the ternary complex of Phe-tRNA^{Phe}, EF-Tu and a GTP analog. *Science*, **270**, 1464–1472.
5. Rodnina,M.V., Savelsbergh,A., Katunin,V.I. and Wintermeyer,W. (1997) Hydrolysis of GTP by elongation factor G drives tRNA movement on the ribosome. *Nature*, **385**, 37–41.
6. Wilson,K.S. and Noller,H.F. (1998) Molecular movement inside the translational engine. *Cell*, **92**, 337–349.
7. Andersen,C.F., Anand,M., Boesen,T., Bich Van,L., Kinzy,T.G. and Andersen,G.R. (2004) Purification and crystallization of the yeast translation elongation factor EF-3. *Acta. Crys.*, **D60**, 1304–1307.
8. Triana-Alonso,F.J., Chakraburtty,K. and Nierhaus,K.H. (1995) The elongation factor 3 unique in higher fungi and essential for protein

biosynthesis is an E site factor. *J. Biol. Chem.*, **270**, 20473–20478.
9. Ganoza,M.C., Cunningham,C. and Green,R.M. (1995) A new factor from *Escherichia coli* affects translocation of mRNA. *J. Biol. Chem.*, **270**, 26377–26381.
10. Kiel,M.C., Aoki,H. and Ganoza,M.C. (1999) Identification of a ribosomal ATPase in *Escherichia coli* cells. *Biochimie.*, **81**, 1097–1108.
11. Kiel,M.C. and Ganoza,M.C. (2001) Functional interactions of an *Escherichia coli* ribosomal ATPase. *Eur. J. Biochem.*, **268**, 278–286.
12. Chakraburtty,K. and Triana-Alonso,F.J. (1998) Yeast elongation factor 3: structure and function. *Biol. Chem.*, **379**, 831–840.
13. Green,R.H., Glick,B.R. and Ganoza,M.C. (1985) Requirements for *in vitro* reconstruction of protein synthesis. *Biochem. Biophys. Res. Commun.*, **126**, 792–798.
14. Nishizuka,Y. and Lipmann,F. (1966) Comparison of guanosine triphosphate split and polypeptide synthesis with a purified *E.coli* system. *Proc. Natl Acad. Sci. USA*, **55**, 212–219.
15. Xu,J., Golshani,A., Aoki,H., Remme,J., Chosay,J., Shinabarger,D.L. and Ganoza,M.C. (2005) Protected nucleotide G2608 in 23S rRNA confers resistance to oxazolidinones in *E.coli*. *Biochem. Biophys. Res. Commun.*, **328**, 471–476.
16. Sanger,F., Nicklen,S. and Coulson,A.R. (1997) DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
17. Ganoza,M.C., Aoki,H., Burkhardt,N. and Murphy,B.J. (1996) The ribosome as affinity matrix: efficient purification scheme for translation factors. *Biochimie.*, **78**, 51–61.
18. Burkhardt,N., Junemann,R., Spahn,C.H. and Nierhaus,K.H. (1998) Ribosomal tRNA binding sites: three site model of translation. *Crit. Rev. Biochem. Mol. Biol.*, **33**, 95–149.
19. Ganoza,M.C. and Kiel,M.C. (2001) A ribosomal ATPase is a target for hygromycin B inhibition on *Escherichia coli* ribosomes. *Antimicrob. Agents and Chemo.*, **45**, 2813–2819.
20. Yusupov,M.M., Yusupova,G.Z., Baucom,A., Lieberman,K., Earnest,T.N., Cate,J.H. and Noller,H.F. (2001) Crystal structure of the ribosome at 5.5 A resolution. *Science*, **292**, 883–896.
21. Davidson,A.L. and Chen,J. (2004) ATP-binding cassette transporters in bacteria. *Annu. Rev. Biochem.*, **73**, 241–268.
22. Czworkowski,J. and Moore,P.B. (1996) The elongation phase of protein synthesis. *Prog. Nucleic Acid. Res. Mol. Biol.*, **54**, 293–332.
23. Blanchard,S.C., Gonzalez,R.L.Jr, , Kim,H.D., Chu,S. and Puglisi,J.D. (2004) tRNA selection and kinetic proofreading in translation. *Nature Struct. Mol. Biol.*, **11**, 1008–1014.
24. Sergiev,P.V., Lavirk,I.N., Wlassoff,V.A., Dokudovskaya,S.S., Dontsova,O.A., Bogdanov,A.A. and Brimacombe,R. (1997) The path of the mRNA through the bacterial ribosome: a site-directed crosslinking study using new photoreactive derivatives of guanosine and uridine. *RNA*, **3**, 464–475.
25. Shatsky,I.N., Bakin,A.V., Bogdanov,A.A. and Vasiliev,V.D. (1991) How does mRNA pass through the ribosome? *Biochemie.*, **73**, 937–945.
26. Gulnara,Z., Yusupova,H., Yusopova,M.M., Cate,J.M.D. and Noller,H.F. (2001) The path of messenger RNA through the ribosome. *Cell*, **106**, 233–241.
27. Triana,F., Nierhaus,K.H., Ziehler,J. and Chakraburtty,K. (1993) Defining the function of EF-3, a unique elongation factor in low fungi. In Nierhaus,K.H., Franceschi,F., Subramanian,A., Erdmann,V. and Wittmann-Liebold,B. (eds), *The Translation Apparatus.* Plenum Publishing Corp., NY, pp. 327–338.
28. Ganoza,M.C., Kiel,M.C. and Aoki,H. (2002) Evolutionary conservation of reactions in translation. *Microbiol. Mol. Biol. Rev.*, **66**, 460–485.
29. Schawirth,B.S., Bovinskaya,M.A., Hau,C.W., Zhang,W., Vila-Sanjurjo,A., Holton,J.M. and Cate,J.H. (2005) Structure of the bacterial ribosome at the 3.5 A resolution. *Science*, **310**, 827–834.