Analysis and Expansion of the Role of the *Escherichia coli* Protein ProQ

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Abstract

The decrease in proline transport by the proline porter ProP in a ΔproQ strain has been well documented; however, the reason for this phenotype remains undefined. Previous studies have speculated that ProQ facilitates translation of *proP* mRNA. Here, we demonstrate that ProQ is enriched in the polysome fractions of sucrose gradient separations of *E. coli* lysates and the 3OS fractions of lysates separated under conditions causing ribosomal subunit dissociation. Thus, ProQ is a *bona fide* ribosome associated protein. Analysis of *proQ* constructs lacking predicted structural domains implicates the N-terminal domain in ribosome association. Association with the ribosome appears to be mediated by an interaction with the mRNA being translated, as limited treatment of lysates with Micrococcal Nuclease maintains ribosome integrity but disrupts ProQ localization with polysomes. ProQ also fails to robustly bind to mRNA-free 70S ribosomes *in vitro*. Interestingly, deletion of *proP* does not disrupt the localization of ProQ with translating ribosomes, and deletion of *proP* in combination with the *proQ* operon has no effect on ProQ localization. We also demonstrate that ProQ is necessary for robust biofilm formation, and this phenotype is independent of ProP. Binding studies were carried out using tryptophan fluorescence and *in vitro* transcribed *proP* mRNAs. *proP* is transcribed from two differentially regulated promoters, and ProQ interacts with *proP* mRNA transcribed from both promoters, as well as a control mRNA with similar affinities. In total, these data suggest that ProQ is positioned to function as a novel translational regulator, and its cellular role extends beyond its effects on proline uptake by ProP.

Introduction

The maintenance of osmotic balance is essential for the fitness and survival of bacteria. One mechanism used by prokaryotes to achieve this balance in hyperosmotic environments is the import of osmoprotectant molecules which balance internal and external osmolarity and prevent the flow of water out of the cell [for review see 1]. Several membrane bound transporters exist with a variety of specificities for different molecules. One such transporter, ProP, senses hyperosmotic stress, and responds by importing proline and glycine betaine [2-4]. Examination of the transcriptional regulation of *proP* has revealed a complex network of both growth-phase and osmolarity dependent control. Briefly, *proP* transcription can occur from a proximal (P2) or distal (P1) promoter [5]. Transcription from the P2 promoter occurs as cells transition from the logarithmic-growth phase into stationary phase and is dependent upon the stationary-phase sigma factor RpoS. Transcription from the P2 promoter is further enhanced by the nucleoid-associated factor Fis [6,7] and cyclic AMP receptor protein (CRP) [8]. The binding of Fis and CRP inhibit transcription from the P1 promoter [7,9]. The P1 promoter is activated after subculture into fresh media, and is responsible for responding to upshifts in media osmolarity [5,9,10].

Beyond transcription, ProP activity is modulated by the cytoplasmic effector ProQ [11]. ProQ is a 232-residue protein, predicted to contain two structural domains, tethered by an unstructured linker [12,13]. The N-terminal domain has been modeled on the structure of the RNA-binding, translational regulator FinO [12,14], and the C-terminal domain has been modeled on the RNA chaperone Hfq [15]. Biochemical studies have been performed to support the structural predictions. The FinO-like domain, as well as the full length protein, are capable of binding to a model dsRNA template. The FinO-like domain also facilitates strand exchange, and both domains promote duplexing between complimentary strands of RNA [15]. Thus, ProQ behaves as an RNA chaperone. The mechanism behind
ProQ regulation of ProP activity, however, remains largely unknown. Disruption of the proQ locus has no effect on the transcription of proP, but the proline uptake activity of ProP is significantly decreased in a ΔproQ strain [11,16]. A post-translational mechanism was initially proposed after ProP protein levels appeared unchanged in a ΔproQ strain [16]; however, a direct physical interaction between ProP and ProQ has not been found. Most recently it was shown that, at osmolalities lower than those previously examined, ProP levels are decreased in a proQ mutant [15]. Additionally, as cells enter stationary phase, there is a modest decrease in the level of ProP in a proQ mutant compared to wild type [15]. In light of these findings, and the homology models comparing the ProQ domains to known RNA-binding proteins, a post-transcriptional mode of regulation is likely.

It had been reported in a high throughput study that ProQ was associated with ribosomes [17]. This led to the hypothesis that ProQ regulates ProP activity at the level of translation. In this study, we verify that ProQ is associated with ribosomes in vivo, and we characterize this association under various conditions. We also determine which domains of ProQ are important for association. We demonstrate that ProQ binds tightly, but non-selectively to in vitro transcribed proP mRNA, and we report the Kd values for P1, P2, and an mRNA whose translation is not predicted to be dependent upon ProQ. Though we demonstrate that mRNA integrity is important for the association of ProQ with translating ribosomes, disruption of the proP locus, as well as the closely related proU operon, does not affect ProQ localization in polysome profiles. It had also been reported in a high throughput study that a proQ mutant exhibits decreased biofilm formation. Here we verify that result by complementing the phenotype and show that a proP mutant strain is not defective in biofilm formation. It follows that ProQ may act as a translation factor for a broader subset of mRNAs.

Materials and Methods

Bacterial strains and growth conditions

E. coli strains used in this study are listed in Table 1. Strains were grown at 37°C in Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter). Cultures of JM6733 containing plasmids were supplemented with 20 µg/mL chloramphenicol (BioExpress). JM6733 (ΔproQ::KAN), JM6753 (ΔproR::KAN), JM6754 (ΔproV::KAN), JM6755 (ΔproW::KAN), and JM6877 (ΔproX::KAN) were constructed by P1 transduction [18] of KEIO-collection mutants, JW5300, JW4072, JW2652, JW2653, and JW2654 respectively, into a clean BW25113 background [19]. JM6881 (ΔproVWX::KAN) strain was constructed by transformation of JM6753 (ΔaraD-araB)567, λlacZ4787::mB-3, lambda577, nhc-1, was transformed into BW25113, and subsequently, screening for sensitivity to kanamycin [21]. JM6906 (ΔproP::FRT, ΔproVWX::KAN) was constructed by transformation of JM6906 by phage grown on JM6881. Genotypes were verified using primers flanking the genomic regions of interest (Table 2). Culture growth was monitored by measuring the absorbance at 600 nm.

### Table 1. Strains and Plasmids.

| Strain       | Genotype Description                             | Origin          |
|--------------|--------------------------------------------------|-----------------|
| BW25113      | A(araD-araB)567, λlacZ4787::mB-3, lambda577, nhc-1 | [20]            |
| JM6733       | BW25113, ΔproQ::KAN                              | [19], This study|
| JM6753       | BW25113, ΔproR::KAN                              | [19], This study|
| JM6754       | BW25113, ΔproV::KAN                              | [19], This study|
| JM6755       | BW25113, ΔproW::KAN                              | [19], This study|
| JM6877       | BW25113, ΔproX::KAN                              | [19], This study|
| JM6881       | BW25113, ΔproVWX::KAN                            | This study      |
| JM6906       | BW25113, ΔproP::FRT                              | This study      |
| JM6926       | BW25113, ΔproP::FRT, ΔproVWX::KAN                | This study      |
| Plasmid      | Description                                     | Origin          |
| pCA24N-ProQ  | pCA24N-ProQ                                      | [23]            |
| pDS1         | pCA24N-ProQ-Gln115N                              | This study      |
| pDS2         | pCA24N-ProQ-Gln181C                              | This study      |
| pDS3         | pCA24N-ProQ-Gln124-180                          | This study      |
| pDS4         | pCA24N-ProQ-Gln168-180                          | This study      |
| pDS5         | pCA24N-ProQ-Gln123                              | This study      |
| pDS6         | pMCSG7-ProQ                                      | [24], This study|
| pMR20        | pMR20 empty vector                               | [27]            |
| pMR20-ProQ   | pMR20 containing proQ ORF plus 500 bp upstream   | This study      |
| pCP20        | Flip Recombinase containing vector               | [21]            |

Preparation of cell lysates for ribosomal fractionation

Isolation of ribosomal species was performed as previously described [22] with the following exceptions. Cultures of LB broth were inoculated with 1/200th volume of a stationary overnight culture. For strain JM6733 (ΔproQ::KAN) containing the indicated plasmids, Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 15 µM after the first 30 min of incubation to induce the expression from the lac promoter. Cell pellets were resuspended in 700 µL of lysis buffer containing 10 mM Tris pH 7.5, 10 mM MgCl2, and 60 mM NH4Cl per 100 mL of starting culture, flash frozen in liquid nitrogen, and stored at -80°C. 300 µm glass beads were added to frozen cells as they thawed. Cells were lysed by repeated vortexing for 1 min followed by 1 min on ice for a total time of 10 min. The lysate was cleared by centrifugation at 21,000 x g for 10 min at 4°C. For subunit dissociation experiments, cells were lysed without addition of chloramphenicol, in buffer containing 10 mM Tris pH 7.5, 1 mM MgCl2, and 60 mM NH4Cl and clarified as described. The absorbances of clarified lysates were measured at 260 nm.

MNase digestion of ribosomes

Lysates were prepared as previously described. 13 OD600 units of lysate were transferred to a clean, 1.5 mL microcentrifuge tube. CaCl2 was added to a final concentration of 5 mM. BSA (NEB) was added to a final concentration of 0.1 mg/mL. 4 µL of MNase (NEB) were added and the final reaction volume was brought up to 400 µL with lysis buffer. Digests were carried out at room temperature for 30 min.
| Name | Sequence (5’ - 3’) | Function |
|------|------------------|----------|
| DS001 | GAT CGA GCT CTC TGA CAT TAC AGC TCT GAC T | Construction of proQ124-180 |
| DS002 | GAT CGA GCT CTT CGC GGT TTT TCG CTT GCT G | Construction of proQ124-180 and proQ1-123 |
| DS003 | GAT CGA GCT CCC CCT ATG CGG CCG CTA A | Construction of proQ181C and proQ1-123 |
| DS004 | GAT CGA GCT CAA CCG GGG TGT GCT GTC T | Construction of proQ16-80 |
| DS005 | GAT CGA GCT CGA ACA GCA AGC GAA AAA ACG C | Construction of proQ115N and proQ181-222 |
| DS006 | GAT CGA GCT CCC TCA GGG CGG GAT CCG T | Construction of proQ116-180 |
| DS007 | GAT CGG TAC CCC CCT ATG CGG CCG CTA A | Construction of proQ116-180 |
| DS008 | GAT CGA GCT CAA CCG GGG TGT GCT GCT C | Construction of proQ116-180 |
| DS037 | pBluescript-popyS | pBluescript-popyS |
| DS038 | pBluescript-popyS | pBluescript-popyS |
| DS039 | pBluescript-popyS | pBluescript-popyS |
| rpoS-T7-forw. | CGG CGG CCG ACA ATT ACG | pBluescript-popyS |
| SacI | TAC TTG AGA CTG GGC | pBluescript-popyS |
| TTT CGT ATG GC | Construction of 6His-TEV-ProQ |
| DS046 | TTA TCC ACT CCT AAT GTT ATC AGA ACA CCA GGT TTG | Construction of 6His-TEV-ProQ |
| DS047 | CGC AGG ATA ATC AAC GGA TAA CG | proQ genotyping |
| DS048 | ATT TGA TCA GCA CGC GTG ATC | proQ genotyping |
| DS049 | CAG AGA TTG CAT CTT GCA ATT CCC | proP genotyping |
| DS054 | CTT GAT AGA ACA GCG TCA CAT CAG | proP genotyping |
| DS055 | CTT GAT AGA ACA GCG TCA CAT CAG | proP genotyping |
| DS056 | TAG CCA GAT AAG GAT AAC CTA TGC TGT CTT GCC | proW genotyping |
| DS057 | TAG CCA GAT AAG GAT AAC CTA TGC TGT CTT GCC | proW genotyping |
| DS058 | TAG CCA GAT AAG GAT AAC CTA TGC TGT CTT GCC | proW genotyping |
| DS059 | TAG CCA GAT AAG GAT AAC CTA TGC TGT CTT GCC | proW genotyping |
| DS060 | GGA CCT GGT CTG TGT GGT GGT | proX genotyping |
| DS061 | GAT GTC TTA GCA GAG TGG CAC | proX genotyping |
| DS062 | GAT GTC TTA GCA GAG TGG CAC | proW genotyping |
| DS063 | GAT GTC TTA GCA GAG TGG CAC | proW genotyping |
| DS064 | CGG GAT CCG TTA TAT GAG CGT C | proP and rpoS in vitro |
| DS065 | CGG GAT CCG TTA TAT GAG CGT C | rpoS in vitro |
| DS066 | CGG GAT CCG TTA TAT GAG CGT C | rpoS in vitro |

**Table 2 (continued).**

| Name | Sequence (5’ - 3’) | Function |
|------|------------------|----------|
| DS060 | GGA CCT GGT CTG TGT GGT GGT | proX genotyping |
| DS061 | GAT GTC TTA GCA GAG TGG CAC | proX genotyping |
| DS062 | GAT GTC TTA GCA GAG TGG CAC | proW genotyping |
| DS063 | GAT GTC TTA GCA GAG TGG CAC | proW genotyping |
| DS064 | CGG GAT CCG TTA TAT GAG CGT C | proP and rpoS in vitro |
| DS065 | CGG GAT CCG TTA TAT GAG CGT C | rpoS in vitro |
| DS066 | CGG GAT CCG TTA TAT GAG CGT C | rpoS in vitro |

Digests were set up as controls, containing all reagents except Mnase.

**In vitro binding of ProQ and 70S ribosomes**

70S ribosomes (NEB) were incubated with an equimolar ratio of purified ProQ in polysome lysis buffer, with or without equimolar P2 mRNA. Reactions were loaded onto sucrose gradients and separated as described.

**Ribosomal fractionation**

Ribosomal species were separated on sucrose gradients ranging from 10% to 47% sucrose in polyallomor ultracentrifuge tubes (Beckman). Sucrose was dissolved in the following buffer: 100 mM NaCl, 10 mM Tris pH 7.5, 10 mM MgCl₂. 550 µL of 47% sucrose was added into the bottom of the tube and placed at -80°C until frozen. Next, 42% sucrose was added and the tube was placed back at -80°C until frozen. This method was repeated until the gradient was complete, using the following concentrations of sucrose: 47%, 42%, 37%, 32%, 27%, 22%, 17%, 10%, 5%. Gradients were stored at -80°C. Gradients were allowed to thaw completely at room temperature. 13 OD₆₀₀ units of lysate were applied to the top of each gradient. Gradients were balanced and submitted to ultracentrifugation in an SW50.1 (Beckman) rotor at 41K RPM for 1.5 h at 4°C. After ultracentrifugation, fractionation was...
performed as previously described [22]. Separation of dissociated subunits was done by ultracentrifugation on 20% sucrose cushions in an SW40Ti (Beckman) rotor at 23 K RPM for 15 h at 4°C. Fractionation was performed as previously described.

**Mutant His-ProQ constructs**

Mutant constructs were made using pCA24N-ProQ from the ASKA collection [23] as a template for inside-out PCR. A SacI restriction endonuclease site was added to both primers (Table 2), allowing linear products from PCR amplification to be digested and self-ligated in frame to create pDS1-pDS5 (Table 1).

**ProQ purification and Antibody production**

A culture of JM6733 (ΔproQ::KAN), transformed with pCA24N-ProQ, was grown to late-logarithmic growth phase (OD$_{600}$ = 0.8) at 37°C in LB media. Production of a 6 histidine epitope-tagged ProQ was induced by addition of IPTG to a final concentration of 1mM, and the culture was shifted to ambient temperature with shaking. Cells were harvested 18 h post induction. Cells were resuspended in 8 mL of lysis buffer (10% glycerol, 20 mM Tris pH 8.0, 500 mM NaCl, 10 mM Imidazole, and 1 mM β-Mercaptoethanol) and complete EDTA-free protease inhibitors (Roche) per 1 g of wet pellet weight and disrupted by 5 passes through a french press. Insoluble debris was cleared via centrifugation at 15,000 RPM for 30 min at 4°C in an SA-600 rotor (Sorvall). Nickel affinity chromatography was performed using Ni-NTA Superflow resin (Qiagen) as per manufacturer’s directions with the following exceptions. After batch binding of 6His-ProQ the column was washed with 5 column volumes (CVs) of lysis buffer. Additional 5 CV washes were done with lysis buffer containing 1 M NaCl, as well as lysis buffer containing 20 mM and 30 mM Imidazole. 6His-ProQ was eluted from the column using 5 CVs of lysis buffer containing 500 mM Imidazole. Protein purity was monitored via SDS-PAGE, and the eluent was dialyzed overnight at 4°C in a low-salt buffer (10% glycerol, 20 mM Tris pH 8.0, 100 mM NaCl, 1 mM β-Mercaptoethanol). Cation exchange chromatography was performed using SP Sepharose Fast Flow resin (GE Healthcare). Cation exchange resin was equilibrated with 5 CVs of low salt buffer. Dialyzed 6His-ProQ was incubated with equilibrated resin for 30 min at room temperature with occasional agitation. After batch binding, the column was washed with 5 CVs of low salt buffer. 6His-ProQ was eluted from the column using a gradient of NaCl (100 mM to 1 M). 6His-ProQ purity was monitored via SDS-PAGE, and purified 6His-ProQ was again dialyzed against a low-salt buffer. After dialysis, 6His-ProQ was concentrated using a centrifugal filter with a 3 kDa molecular weight cutoff (Amicon), as per manufacturer’s directions. Purified 6His-ProQ was quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc.) and 600 µg was sent for antibody production in a rabbit host (Cocalico Biologicals, Inc).

**Western-blot analysis of ribosomal fractionations**

Denaturing SDS-PAGE loading buffer (50 mM Tris pH 6.8, 2% Sodium dodecyl sulfate (SDS), 10% glycerol, 1% β-

Mercaptoethanol, 13 mM EDTA, 0.02% Bromophenol blue) was added to trichloroacetic acid (TCA) precipitated ribosomal fractions. Briefly, TCA was added to polysome gradient fractions to a final concentration of 15% and incubated at 4°C for 30 min. The precipitant was pelleted in a microcentrifuge at maximum speed for 10 min. The supernatant was removed and the pellet was washed twice with ice cold 100% acetone. Proteins were separated via SDS-PAGE and transferred to nitrocellulose (GE Water & Process Technologies). Protein detection was carried out using antibodies against ProQ (1:5000 in 5% dry milk in PBS + 0.1% Tween20) and ribosomal proteins L3 (1:5000 in same) and S2 (1:5000 in same). Antibodies to L3 and S2 were a generous gift from Catherine Squires.

**Copy number calculation**

proQ was cloned into vector pMCSG7 as described [24] using DS046 and DS047 (Table 2) to make pDS6 (Table 1), which adds an N-terminal 6-Histidine epitope tag, followed by a TEV cleavage site. ProQ was purified as previously described with the following exceptions. After the first nickel affinity column, elution fractions were pooled and dialyzed overnight in the presence of 6His-TEV protease. A second nickel affinity column was used to remove TEV protease and the flow through was collected. Fractions were analyzed via SDS-PAGE and pooled for cation-exchange chromatography. Purified ProQ was quantified using a Bradford assay (Biorad). Known amounts of ProQ, as well as whole cell lysate from a known number of wild type BW25113 E. coli cells were loaded onto SDS-PAGE and transferred to nitrocellulose for western-blot analysis using anti-ProQ antibodies. Band intensities were quantified using ImageJ and cellular concentration was calculated.

**In vitro transcription**

The proP genomic region was cloned into pBluescriptKS+ in two forms, starting at either -182 (proP P1, DS037, DS039) or -95 (proP P2, DS038, DS039). The proS genomic region was cloned into pBluescriptKS+ (rpoS-T7-forward_SacII, rpoS-T7-reverse_PstI). PCR was performed to amplify the plasmid DNA for use as an in vitro transcription template. The forward primer maintained the integrity of the T7 polymerase dependent promoter (pBSIVT-forward), and the reverse primer added a stop codon after glycine 245 for proP P1 and P2 (DS076) and after tyrosine 147 for proS (pBSIVT-reverse), yielding mRNAs with sizes of 927 bp, 840 bp, and 1041 bp respectively. In vitro transcription was carried out as described previously [25], and the mRNAs were checked for quality using urea-acrylamide gel electrophoresis and TAE-agarose electrophoresis. The mRNA concentrations were determined by electrophoresis on a TAE-agarose gel and quantitative comparison to the RiboRuler High Range RNA Ladder (Thermo Scientific).

**Tryptophan fluorescence**

Purified ProQ was dialyzed into 10% glycerol, 20 mM HEPES pH 7.5, and 100 mM NaCl. The concentration was determined using Bradford assay. For binding experiments, ProQ was diluted to a final concentration of 20 nM in the same
buffer plus 0.005% Brij35. Temperature was held constant at 20°C and tryptophan was excited at 295 nm and fluorescence was monitored at 355 nm using a QuantaMaster4 (Photon Technology International). The excitation wavelength was chosen to avoid any possible inner-filter effect stemming from the use of nucleic acids as ligands [26]. Increasing amounts of in vitro transcribed mRNAs were added to the reaction and the change in fluorescence was observed.

Biofilm assays
Plasmid pMR20-ProQ was constructed by cloning the amplicon from primers DS083 and DS084 into pMR20 [27] cut with KpnI and SacI (Table 2). Biofilm assays were performed with modifications of previous protocols [28,29] as follows. Strains were grown overnight in LB + 12.5 µg/mL tetracycline. 30 µL of the saturated overnight was inoculated into 1 mL of LB without salt (10 g tryptone, 5 g yeast extract) plus 12.5 µg/mL tetracycline, in a previously unused borosilicate test tube. Tubes were incubated at 25°C without shaking for 6 d. After 6 d, the media and loose cells were removed and the OD$_{600}$ was measured. Tubes were gently rinsed by submerging in deionized water 5 times and allowed to air dry. Biofilms were stained using 1% crystal violet for 20 min. Tubes were again rinsed by submerging in deionized water to reduce background staining. Biofilms were resuspended completely in 0.5% SDS and the OD$_{600}$ was measured. Relative biofilm formation was calculated as described previously [28].

Results
ProQ associates with translating ribosomes
In order to verify the localization of ProQ on ribosomes, the soluble lysates from wild type E. coli (strain BW25113) were separated on sucrose density gradients (Figure 1A). Western-blot analysis of the resulting fractions revealed that ProQ predominantly associates with 70S and translating ribosomes, and to a lesser extent, at the top of the gradient and in fractions corresponding to the 30S ribosomal subunit. Antibodies against small and large ribosomal subunit proteins validated the assignment of the peaks in the UV-absorbance trace. Deletion of some ribosome-associated factors can lead to ribosomal defects and perturbation of the polysome profile [22]. Profiles from a ΔproQ strain were indistinguishable from wild type (data not shown), and therefore, ProQ is not likely a ribosome assembly factor, nor is it required for the translation of the majority of mRNAs in the cell.

Since the 30S and 50S ribosomal subunits work in tandem, but with separate roles during translation, identifying the subunit with which ProQ associates provides a clue as to the function of ProQ during translation. When isolated at lower concentrations of Mg$^{2+}$, 70S ribosomes dissociate into 30S and 50S subunits [30]. At 1 mM Mg$^{2+}$, the 70S and polysome species were predominantly dissociated, as revealed by the UV-absorbance trace and localization of S2 and L3 (Figure 1B). A large proportion of ProQ was found at the top of the

Figure 1. Ribosome association of ProQ under native and dissociating conditions. Polysome profiles (254 nm) from wild type cell extracts in (A) associating conditions (10 mM Mg$^{2+}$) and (B) dissociating conditions (1 mM Mg$^{2+}$) are shown. Western-blot analysis of TCA-precipitated fractions using antibodies to ProQ, small ribosomal subunit protein 2 (S2), and large ribosomal subunit protein 3 (L3) are shown and aligned to the UV-absorbance trace. Whole cell extracts from wild type (BW25113) and ΔproQ (JM6733) are included, as is the soluble lysate (Load). UV-absorbance peaks correspond to (L to R): Free RNA/protein, 30S, 50S, 70S, and polysomes.

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gradient, but ProQ that did migrate into the gradient peaked with the 30S subunit but not with the 50S subunit. These data are consistent with the localization under non-dissociating conditions (Figure 1A). Thus, ProQ appears to preferentially associate with the 30S subunit.

The N-terminus and linker regions are necessary for ribosome association

ProQ is predicted to contain two structural domains, tethered by a positively charged linker region. The N- and C-termini have been modeled on the RNA binding proteins FinO and Hfq respectively [12,15]. Using available bioinformatic tools to predict secondary structure, disordered regions, and nucleic acid binding propensity [31–33], we assigned the boundaries of the linker region as amino acids E116-V180. Assignment of the lower bound was the most difficult. The nucleic acid binding predictor, BindN, indicated the presence of an unstructured positively charged domain starting at E116. The secondary structure predictor, PSIPRED, indicated the presence of an alpha helix until A124. Domain constructs were made using the 6HIS-ProQ plasmid from the ASKA collection [23] as a template. A schematic of each construct is provided (Figure 2A). To determine the regions of ProQ that are necessary for its association with the ribosome, each ProQ construct was expressed in a ΔproQ background, such that it was the only copy of ProQ in the cell. We could not detect a ProQ variant expressing only the C-terminal domain (proQΔ181C) did not affect association with the ribosomes (Figure 2B), and therefore, this part of the protein is not required for ribosome localization. In contrast, both the N-terminus and linker regions are required for ribosome association, as the majority of proQΔ115N and proQΔ124-180 were found at the top of the gradient. Consistently, removal of the C-terminal and linker domains together (proQ2-123) caused ProQ to be found at the top of the gradient. Moreover, the linker domain (proQ116-180) was not sufficient for ribosome association. These data suggest that both the N-terminal FinO domain and the linker regions are required for ribosome association of ProQ.

ProQ interacts with the mRNA being translated

The comigration of ProQ into sucrose gradients with translating ribosomes could occur because of a physical interaction with the ribosome directly or because of an interaction with the mRNA bound to the N-terminal 6HIS-epitope tag. The high rate of speed and high G-forces produced during polysome fractionation can physically disrupt binding interactions, so addition of the epitope tag may cause dissociation of 6HIS-ProQ from ribosomes as it progresses through the gradient. Deletion of the C-terminal Hfq domain (proQΔ181C) did not affect association with the ribosomes (Figure 2B), and therefore, this part of the protein is not required for ribosome localization. In contrast, both the N-terminus and linker regions are required for ribosome association, as the majority of proQΔ115N and proQΔ124-180 were found at the top of the gradient. Consistently, removal of the C-terminal and linker domains together (proQ2-123) caused ProQ to be found at the top of the gradient. Moreover, the linker domain (proQ116-180) was not sufficient for ribosome association. These data suggest that both the N-terminal FinO domain and the linker regions are required for ribosome association of ProQ.

![Figure 2. Ribosome association of plasmid encoded proQ mutant constructs in polysome profiles.](image-url)

(A) Schematic representation of proQ mutant constructs expressed from the IPTG inducible plasmid pCA24N. The predicted RNA binding region is colored in black, and amino-acid boundaries of each construct are labeled. (B) Mutant constructs were expressed as the lone copy of proQ. Western-blot analysis of TCA-precipitated fractions from the separation of ribosomal moieties on sucrose density gradients was performed. Whole cell extracts of cells after induction were included (Induced) along with soluble lysate (Load). Position of the 30S, 50S, 70S, and Polysome species are indicated based on western-blot localization of S2 (data not shown).
not perturb the integrity of the highly structured, and relatively RNase insensitive, ribosome. As expected, the small ribosomal protein S2 was found associated with the remaining 70S and 2-mers, and no appreciable amount of S2 was found at the top of the gradient. This supports the claim that the integrity of these ribosomes was maintained. In mock treatments, mRNAs with up to 4 ribosomes are seen in sucrose gradients, and ProQ associates with all translating ribosomes (both the 70S and polysome forms) (Figure 3A). After treatment with MNase, the level of total polysomes was decreased concomitant with a large increase in free 70S ribosomes (Figure 3A) and consistent with cleavage of intra-ribosome mRNA. Strikingly, in contrast to a robust association of ProQ with 2-mer and 3-mer polysomes in untreated samples, ProQ was almost absent from these particles in the MNase treated samples. Additionally, a significant amount of ProQ was found at the top of the gradient, consistent with dissociation of ProQ from the ribosomes after disruption of mRNA. Thus, ribosome association of ProQ appears to be partially dependent on the mRNA being translated. In order to support this finding, we also performed in vitro binding experiments with mRNA-free 70S ribosomes. 70S ribosomes were incubated with equimolar purified ProQ, with or without proP P2 mRNA. After incubation, the reactions were applied to sucrose gradients and separated as before (Figure 3B). Though some ProQ is found in the gradient under the 70S peak, indicating a weak interaction with 70S ribosomes, the bulk of the ProQ is located in the fractions not corresponding to the 70S ribosomes. It is also worth noting that when proP mRNA was added, we consistently observed the concentration of ProQ in the gradient peaking around fraction 5. The most likely explanation for this result is that ProQ is binding to the proP mRNA and migrating to this region of the gradient.

**ProQ does not bind selectively to proP mRNA**

It has been demonstrated that ProQ can bind to a model RNA substrate in vitro [15]. However, the model double-stranded substrate used previously was relatively small (39 bp in duplex form) compared to the size of an mRNA. To further understand the RNA binding properties of ProQ, binding experiments were performed using purified ProQ and in vitro transcribed mRNA substrates (Figure 4A). One possibility is that ProQ acts as a proP mRNA-specific translation factor. If true, we would predict that ProQ should interact preferentially with proP mRNA in vitro. We tested the ability of ProQ to bind to mRNA made from the proP P1 and P2 promoters, as well as an unrelated mRNA, rpoS (see methods). Under the conditions

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**Figure 3. Ribosome association of ProQ after mRNA disruption.** (A) Cell lysates were left untreated (solid line) or treated with limited MNase (dashed line) and ribosomal species separated on sucrose gradients. The positions of ProQ and ribosomal protein S2 in the resulting TCA-precipitated fractions is determined by western-blot analysis. (B) mRNA free 70S ribosomes were mixed with equimolar amounts of purified ProQ without (foreground) or with (background) the addition of equimolar P2 mRNA before application to sucrose gradients. The position of ProQ and ribosomal protein S2 in the resulting TCA-precipitated fraction is determined by western-blot analysis.

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Ribosome association of ProQ is not dependent on proP or proU

We have shown that ProQ is found on translating ribosomes and this association appears to be mediated through mRNA. Because the only well characterized phenotype for a proQ mutant is a decrease in proline uptake, we posit that ProQ is involved in the translation of a subset of mRNAs in the cell, including one or more of the proline transporter mRNAs. We therefore asked whether the ribosome association of ProQ was dependent on these mRNA transcripts. We first examined the localization of ProQ in a ΔproP mutant, as proP mRNA is the predicted target of ProQ. We found, however, that the ribosome association of ProQ was similar in a ΔproP strain to that seen in the wild type strain (Figures 1 and 5). A genetic interaction has been reported between proQ and the proW and proX loci within the proU operon [36] and therefore, these genes may be regulated by ProQ. For this reason, we examined ProQ localization in strain backgrounds deleted for each member of the proU operon, as well as a deletion of the entire proU operon (proVWX), and deletion of both proVWX and proP together. In all backgrounds tested, ProQ is repeatedly found associated with 70S particles and polysomes (Figure 5). Thus, ProQ ribosome association is not absolutely dependent on any of these genes, and the ribosome association of ProQ may be due to one or more additional cellular mRNAs.

ProQ is necessary for biofilm formation, independent of ProP

Having shown that ProQ localization to the ribosome is independent of all known potential interactions involved in proline transport, we sought to expand the ProQ target list by looking for new ΔproQ phenotypes. A high throughput study implicated ProQ in biofilm formation; though, it only reported it as a hit and did not verify the result through plasmid-based complementation [28]. Under the conditions tested, a proQ mutant strain was found to be about 50% deficient in biofilm formation compared to wild type cells (Figure 6). This phenotype was complemented by transforming ΔproQ cells with a low-copy plasmid containing the proQ gene, plus 500 bp of sequence upstream of the translation start site. To test if this phenotype was independent of the role of ProQ in ProP regulation, we examined biofilm formation in a proP mutant strain. This strain was able to form biofilms as well as wild type. As a result, we conclude that the decrease in biofilm formation is unique to a proQ mutant and is independent of the ProQ-ProP interaction, though the reason for this deficiency remains unclear.

Assuming a cellular volume of 1.3 µm³ [34], the cellular concentration of ProQ is 2.43 ± 0.414 µM. This number is repeatedly found associated with 70S particles and polysomes predicted target of ProQ. We found, however, that the involved in the translation of a subset of mRNAs in the cell, cytosolic proteins [35], and at approximately 2,000 copies/cell, including one or more of the proline transporter mRNAs. We each member of the ProQ is necessary for biofilm formation, independent of ProP. For this reason, we looked for new ΔproQ strains with high-throughput proteomic analysis of E. coli cytosolic proteins [35], and at approximately 2,000 copies/cell, ProQ is in the top 25% in terms of protein abundance.

**Figure 4.** mRNA binding kinetics monitored by native tryptophan fluorescence. (A) 20 nM ProQ was titrated with increasing amounts of in vitro transcribed mRNAs (“●” proP P1, “▲” proP P2, “■” rpoS). Tryptophan was excited at 295 nm and the fluorescence was monitored at 355 nm. The y-axis is provided in terms of normalized fluorescence (FLF/FL0) by the fluorescence for 20nM protein only (FL0). DNA yielded no measurable fluorescence shift and is included as a negative control (“○” DNA) (B) Summary of binding affinities of ProQ for in vitro transcribed mRNAs.

- proP P1: 17.0 ± 6.1
- proP P2: 11.6 ± 2.7
- rpoS: 19.5 ± 7.8
- DNA: None Detected

Analysis of E. coli Protein ProQ
Discussion

It has been proposed that ProQ is a translational regulator of proP mRNA [15]. This hypothesis has evolved and emerged out of a sort of "process of elimination", whereby it has been shown that i) proP transcript levels are unchanged in a proQ mutant [11] ii) no physical interaction can be detected between ProQ and ProP [36], and recently iii) ProP expression levels are affected by deletion of proQ [15]. In this study, we present the first direct evidence of a ProQ-translation link by demonstrating the association of ProQ with 70S particles, translating ribosomes, and 30S particles (Figure 1), but we have not detected a specific interaction between ProQ and proP mRNA at the ribosome. Some proteins that associate with the ribosome are involved in ribosome maturation and assembly [22,37]. Deletion of these factors can result in defects in polysome profiles and decreased growth rates [for reviews see 38]. These phenotypes are not observed in a proQ mutant (data not shown), and because of the relative dearth of phenotypes associated with a proQ deletion, it is unlikely that ProQ is an assembly factor. Thus far, ProQ has only been implicated in proline uptake [11] and stimulation of biofilm formation [28 and this study]. For these reasons, we propose that ProQ has a very narrowly defined role in the translation of only a subset of mRNAs. This hypothesis is bolstered by the fact that the N-terminus of ProQ has been modeled on FinO, a highly specified translational regulator, which acts to specifically represses the translation of traJ mRNA by facilitating the interaction between traJ and the anti-sense RNA finP [39,40]. As for the identities of the specific mRNA targets of ProQ, proP remains a favored target, but it is unknown if this effect is direct or indirect. Additionally, one or more additional targets are predicted to exist, based on the ProP-independent biofilm defect in a proQ mutant.

It had previously been shown that ProQ could bind to a model RNA, and that ProQ exhibits RNA chaperone-like activity [15]. Here we sought to explore the interaction between ProQ and RNA in three distinct ways. First, we examined the consequences of nuclease treatment on the association of ProQ with the ribosome (Figure 1). Limited treatment of lysates with MNase caused a decrease in the number of polysomes observed after centrifugation. Specifically, the...
number of 3-mers and higher molecular weight species were significantly decreased. The loss of 3-mers is consistent with the increase in the number of 70S ribosomes observed. Though 3-mers were almost completely missing, a significant amount of ribosomes were still found to exist as 2-mers. MNase treatment had a large effect on the localization of ProQ, however, causing it to almost completely delocalize from 2-mers and 70S ribosomes. To further explore the requirement of mRNA for ProQ-ribosome association, we examined the ability of ProQ to bind to mRNA-free ribosomes in vitro. Not surprisingly, ProQ did not robustly associate with these ribosomes, and instead, seemed to prefer to bind to mRNA when present in the reactions (Figure 3B). For these reasons, we conclude that the mRNA being translated is very important for the comigration of ProQ with 70S and polysome species. Next, we used a quantitative in vitro binding assay to determine the affinity of ProQ for its predicted mRNA target, proP (Figure 5). The hypothesis that ProQ enhances proP translation, to the exclusion of other mRNAs, leads to the prediction that ProQ should selectively recognize proP. Under the conditions tested, ProQ binds tightly to proP mRNAs, but this binding is not selective, as ProQ binds with similar affinity to proS mRNA (Figure 4). Though we cannot rule out the possibility that proS is also a target of ProQ regulation, we believe this is unlikely since RpoS is a known regulator of proP transcription [6,7]. proS transcript levels are unchanged in a ProQ mutant [11], and a proQ deletion strain does not have the same lack of thermotolerance seen in an rpoS deletion strain [15]. If proP is a direct target of ProQ, some other unknown factor must confer specificity in vivo.

Little is known about ProQ, mechanistically. Previous studies have shown that exogenous expression of the N-terminal, FinO-like domain (residues 1-130) can partially complement the proline uptake deficiency of a proQ chromosomal deletion, and the N-terminus (1-130) is necessary and sufficient for binding to RNA [13,15]. Our study of the domains of ProQ involved in ribosome association is not entirely consistent with these previous results. For example, even though the first 130 residues could bind to a model RNA, we do not observe ribosome association of a similar construct to any appreciable degree (Figure 2B). In this study, we expand the linker by seven residues to include amino acids 124-180, thus shortening the N-terminal domain from 1-130 to 1-123 (Figure 2A). This difference could account for the lack of ribosome association if residues 124-130 are necessary for RNA binding. Another inconsistency is observed with the C-terminus. In this study we find that a construct lacking residues 181-232 is associated with the ribosome in a manner comparable to plasmid-expressed wild type ProQ (Figure 2B). However, in previous studies, this construct did not suppress the proline uptake deficiency as well as a construct containing the C-terminal domain [13]. It must be noted that our present study sought only to identify the domains of ProQ which are important for ribosome association, independent of proQ-proP genetic interactions. Based on our results, we conclude that only the C-terminus is dispensable for ribosome association. Though we would predict that ribosome association is necessary for suppression of the proline uptake phenotype, we do not observe a direct dependence. This further complicates the mechanism by which ProQ enhances proline uptake.

A deeper exploration of the mechanism of ProQ action will be predicated on the discovery of direct mRNA targets. To date, finding these targets has been challenging. To begin the process, here we verified that ProQ is involved in promoting biofilm formation [28]. More importantly, we show that this phenotype is independent of ProP, yielding the first ProP-independent ProQ phenotype (Figure 6). Further independence of ProQ from ProP is demonstrated by the fact that ProQ is still associated with ribosomes in the absence of proP and proU mRNAs (Figure 5).

Though further studies are needed to definitively determine the function of the ribosomal association of ProQ, it is not unreasonable to propose a role for ProQ in translation initiation. Under non-dissociating conditions, we observe the highest concentration of ProQ in fractions corresponding to 70S and polysome species, but there is a modest, yet consistent, increase in signal under the peak corresponding to 30S particles, the first particle to bind during initiation (Figure 1A). This 30S localization is confirmed under dissociating conditions (Figure 1B). We present data showing that ProQ can bind tightly to mRNA in vitro (Figure 4), and mRNA integrity is necessary for robust ribosome association (Figure 3). Thus, ProQ is ideally positioned to function in translation initiation of its mRNA targets, perhaps by facilitating the interaction between 30S particles and these as-yet unidentified mRNAs.

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Author Contributions

Conceived and designed the experiments: DTS RZ. Performed the experiments: DTS RZ. Analyzed the data: DTS. Contributed reagents/materials/analysis tools: DTS. Wrote the manuscript: DTS. Critical reading and approval of the final manuscript: DTS RZ.

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